Changes in Social Motivation Following Oxytocin Receptor Inhibition

By: Rija Asim

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Thesis Committee:

Tiffany Rogers, Thesis Director

John Vile, Thesis Committee Chair

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APPROVED:

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Tiffany Rogers, Thesis Director
Assistant Professor, Psychology

_____
John Vile, Thesis Committee Chair
Dean, University Honors College

Abstract

The purpose of our study was to adapt a social behavior arena for mice and to test the correlation between oxytocin receptor activation/inhibition and social motivation since no current measurement for social motivation exists. The long-term impact of this research was to identify a standardized way of measuring social motivation so that social disorders like autism can be better characterized. Our research was conducted with 90 mice across both sexes. The levels of social motivation were pharmacologically manipulated with oxytocin, atosiban, or saline. We were able to successfully validate a social reward chamber. A sex by drug interaction indicated a differential effect exists between atosiban and oxytocin depending on sex. We found that atosiban treated males and oxytocin treated females were more socially motivated than other groups. The understanding of these factors will aid us in better developing treatment targets and pharmacological improvements for disorders with social symptoms like autism.

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CHAPTER I: INTRODUCTION

Animal behavior is a dynamic area in biomedical research, and it can be studied empirically through observation and experimentation in the laboratory. Animal studies are essential for research that seeks to understand complexity of disease progression, genetics, assessment of pharmaceutical effectiveness and an alternative method for testing on human subjects. Animals promote high internal validity for experimental research designs as they allow the study to control the environment, maximize genetical identicality, and utilize invasive techniques. Specifically, mice have served as the ideal species for animal models in biomedical research due to their anatomical, physiological, and genetic similarity to humans. Mice and humans each have about 30,000 genes of which approximately 95% of those genes are shared by both species (Bryda, 2013). Mice and humans have evolutionarily conserved brains indicating similar brain structure and connectivity which is highly beneficial in conducting animal model research for brain and behavior (Semple, 2013).

Mice are keen, sociable, and curious animals, and multiple studies have been done to showcase these elaborate social behaviors. They have a natural tendency to inhabit territories, form groups, and then form complex social organizations, including dominance hierarchies, within these groups (Williamson, 2016). Mice also present specific maternal and paternal behaviors that are often critical for the survival and development of their offspring. The neural circuitry that orchestrates parental behavior has been researched and shows the social behavior differences between the parental mice (Kohl, 2018). One of the aspects to mice sociability and communication is their capability to emit vocalizations across a broad range of frequencies under a variety of

behavioral contexts. Audible squeaks are produced by mice to reveal emotion under stressful situations, aggressive encounters, and mating (Lahvis, 2011).

Multiple standard measurements of social behavior and general sociability exist for a social stimulus (another mouse) that requires no effort to access. One example is the three-chambered sociability task which has been widely employed as a standard test for assessing social approach in mice. It is a three-chambered apparatus designed to quantify direct social behaviors when a subject mouse is presented with the choice between either a novel mouse or a novel object. Sociability is operationally defined as the subject mouse spending more time in the chamber with the novel mouse than in the chamber containing the novel object (Yang, 2011). Another standardized test is the tube dominance test which is utilized as a reliable measure of social hierarchy in mice. Subject mice are released into opposite ends of a narrow tube through which the animals are unable to pass each other. The more dominant animal proceeds forward and forces its opponent to back out of the tube. This tube test can be used to identify deficits in social interactions in different strains of mice and evaluate chemical entities for their effect on cognition and social behavior (Fan, 2019). Additionally, the ultrasonic vocalization test is a method to quantitatively assess social communication of mice by recording and measuring the number, frequency, amplitude, and duration of ultrasonic calls (Ferhat, 2016).

Despite mice being a well-used animal model to investigate social behavior and the common use of many social behavior measurement in these animals, no standard to measure specific aspects of social behavior such as social motivation exists. Social motivation can be defined as the willingness to exert effort to interact with a social stimulus. In an effort to create a standardized method, Borland and colleagues initially

described a weighted doors task and a social reward chamber (Borland, 2017). They aimed to measure social reward and social motivation in hamsters, and they developed the social reward chamber as a means to effectively allow hamsters to choose between two stimuli and to exert effort to enter the chamber of their choice. In this way both the choice to explore social stimuli and the amount of effort expended to do so can be measured. However, Borland et al. tested only hamsters in the test and did not attempt to alter motivation to validate the arena. The chamber has not been used since its original description despite being a simple and well-designed way to measure social motivation.

In the spring of 2021, we gathered preliminary data based on the this previously described arena. We adjusted the model and methodology of Borland and colleagues to make an appropriate measurement of social motivation to suit mice. The experiment was to test social motivation between conspecifics, defined as organisms belonging to the same species, which are age- and sex-matched. In our experiment, we did not use opposite sex partners to avoid confounds of sexual behavior. Also, previous research has focused more on reproductive aspect of social behavior rather than social interactions between conspecifics. We created a social motivation behavior arena with a social reward task designed to understand social motivation in mice. The arena's structure was divided into one large chamber and two small chambers that are separated by weighted one-way vertical swing doors (reference appendix A for visual of arena). The one large chamber held the experimental mouse, and the two smaller chambers contained either a social stimulus or remained empty. Weights were added to increase the degree of effort required by the mice for the stimuli in the smaller chambers. This arena tested for the direct investigation of the effect of the stimuli on the mice's motivation to access it. However,

these initial only included male mice and changes in their social motivation following oxytocin administration or saline.

However, we found limitations to this preliminary research, and improved them. One of the limitations was the lack of data for the amount of time that the mice were attempting to spend to access the social stimuli chamber. Since both chambers, social and non-social, were available there was no measurement for the effort for only the social chamber. We also had low power in the preliminary study since there were too few mice per group and only male mice were tested. Another limitation relating to our data was the discovery of a ceiling affect with the highest weight, which refers to the weight beyond which there is no additional effect on the mice. The last limitation found revolved around the concept of serial reversal learning at which mice are incompetent. Serial reversal learning requires the subjects to adjust their behavior or choices when the reward-related contingencies that they previously learned are reversed. In our case, we switched the side that the stimuli mouse would be located in each trial, and this introduced discrepancy in our data. In our extension to this study, we adjusted the procedure to make an appropriate measurement of social motivation with improvements of these limitations.

Oxytocin was used in this study to further validate the social reward test. Oxytocin is a neuropeptide that is produced in the hypothalamus and stored in the pituitary gland. Oxytocin plays a crucial role in childbirth and breastfeeding, but more importantly it has been associated with social behaviors in both humans and mice (Lukas, 2011). The oxytocinergic system has shown to be a prime target for treating diseases that involve aberrant social behavior such as autism (Fineberg, 2017). Oxytocin appears to impact dopaminergic activity which is affiliated with reward and motivated behaviors.

Dopamine, a neurotransmitter, influences motivational salience, impacting the drive toward certain rewards rather than the pleasure derived from the reward itself. Since dopamine is well known for motivated behaviors and oxytocin interacts with dopamine, this evidence suggests a linked dopamine and oxytocinergic functioning towards social motivation (Love, 2014). This connection allows our research to validate that our behavioral test does measure social motivation and to further investigate the role oxytocin may have on social motivation.

Since oxytocin is a hormone associated with prosocial behaviors, we initially hypothesized that oxytocin administration would increase the amount of social motivation. Contrary to our preliminary hypothesis, our preliminary data suggested that oxytocin served to reduce social motivation instead of increasing it. This difference in oxytocin found in our preliminary data encouraged us to further explore this unexpected result. We did not believe the changes observed in oxytocin were affected by the limitations in the study. However, improving the study enhanced our ability to detect differences in social motivation following the manipulation of oxytocin levels.

As the field remained without a standard way to measure social motivation, our project revisited this chamber and attempted to pharmacologically manipulate levels of social motivation. Our goal was to both validate and explore differences in social motivation by using oxytocin. We further supported our findings by administering an oxytocin receptor antagonist, Atosiban, in separate groups of mice. By applying a drug that inhibits oxytocin receptors instead of activating them, we further supported our behavioral data by showing both an increase and decrease of social motivation.

CHAPTER II: THESIS STATEMENT

The goal of this study was to adapt a social behavior arena for mice and test the correlation between oxytocin receptor activation and social motivation. We updated our previous methodology and arena to accommodate for the limitations found in our preliminary research. This study incorporated two phases to improve our limitations. Phase 1 consisted of one of the chambers blocked off to measure the effort required to access only the social door. Phase 2 had both weighted doors accessible to the mice and a choice given between the two chambers: a chamber with the social stimulus (another mouse) or the one that remains empty. Our study included a larger population of mice across both sexes (90 mice) to improve the study's power. In order to prevent a ceiling effect, we increased the weights by 0.25oz compared to the increments of 0.5oz from the previous study and lowered our highest weight. We also contained the social stimuli mice on the same side of the arena since our preliminary research found that the mice were not cognitively flexible to the reversal.

The overall significance of this project was to help establish a standard way of measuring social motivation in mice since there is no current standard. The long- term impact of this research was to identify a standardized way of measuring social motivation so that the social behavior of animal models of clinical disorders, such as autism mouse models, can be better characterized. Instead of measuring general sociability in these models only, we believed that measuring specific aspects of social behavior allows for better behavioral descriptions of these models. Increased specificity in behavioral characterization may then lead to discovering more coherent patterns in neural activation associated with changes in social behavior.

Two aims were addressed in our study. First, we evaluated sex-differences between male and female mice by female subjects because there are known expression differences in oxytocin receptors across the two sexes (Lukas, 2011). The second aim was to manipulate the mouse's level of social motivation pharmacologically by administering the three drug categories of oxytocin, an oxytocin antagonist, and saline. Oxytocin was used based on the differences between groups in our preliminary research. An oxytocin antagonist (atosiban) was incorporated since it offers an opposite effect to oxytocin. Saline is the control group for the experiment. We anticipated that we would be able to demonstrate the reverse of our current oxytocin results by inhibiting oxytocin receptors. Together these aims demonstrated: 1) sex-differences between males and females in a social motivation task, and 2) provided more confidence that activity levels at oxytocin receptors is the causal factor in the observed changes in social motivation. This study tested the hypotheses that:

1) distinct behavioral patterns exist across males and females in terms of social motivation and in oxytocin-related changes to social motivation, and

2) oxytocin receptor inhibition utilizing an oxytocin antagonist will increase social motivation in males.

CHAPTER III: METHODS

This research was conducted with 15 mice in each of six groups (90 mice total; see table 1). Groups of males and females were administered with an intraperitoneal injection of oxytocin, Atosiban (an oxytocin receptor antagonist), or saline. Oxytocin requires chronic administration of 14 days before behavioral tests can be conducted. In order to match oxytocin's dosing schedule and limit dosing variations, the oxytocin

antagonist and saline was also administered chronically over 14 days before behavioral tests were conducted.

This experimental study contained three behavioral phases: training, phase 1 and phase 2. The training phase was conducted on a day before the two behavioral phases and phase 1 preceded phase 2 on the same day. The training phase allowed the experimental mice to habituate to the weighted door arena (reference appendix A for visual of arena). Each experimental mouse was placed in the larger chamber and given the opportunity to assess the arena with both smaller chamber doors open, then chamber doors propped open, and then chamber doors closed.

Phase 1 consisted of one of the chambers blocked off to assay the time and effort that is required from the mice to access only the social door. Experimental mice were inserted into the one large chamber and the one smaller chamber with weighted doors contained a stimulus mouse. In phase 2 the experimental mice were inserted into the one large chamber and both two smaller chambers with weighted doors were accessible. The experimental mouse chose between the two chambers, the one with a social stimulus (another mouse) or the one that remains empty. The effort exerted by the mouse on the weighted door, the time spent to enter a chamber, and the choice of the chamber were measured as its social motivation. Each experimental mouse received three minutes to enter a chamber, given three trials in which a new stimulus mouse is introduced per trial, and the weights increased by 0.25 oz. across three days.

The experiment was video recorded. The data such as the weight pushed by the mice, the time spent pushing, and the chamber selected was obtained through hand coding of the video recordings. Finally, data analysis was conducted through SPSS. A

chi-square test analysis was completed to determine the effect of sex (male and female) and pharmacological treatment (oxytocin, atosiban and saline) on door choice (social, empty, neither). A multivariant ANOVA was used to compare multiple dependent variables such as the latency to enter a chamber or the time spent attempting to open a chamber door.

Table 1: Experimental Groups in the Study

Sex	Drug	Number of Mice
Male	Oxytocin	15
Male	Atosiban	15
Male	Saline	15
Female	Oxytocin	15
Female	Atosiban	15
Female	Saline	15

CHAPTER IV: RESULTS

The multivariant ANOVA was used to assess the impact of the independent variable (weight) on the dependent variables (phase 1 latency time, phase 2 latency time, social door push time, and door choice). The test indicated that the time to choose a door increased, time spent pushing the social door decreased, and more subjects chose no door as the weights of the door increased ($p < 0.05$). This served as a manipulation check validating the efficacy of the weights on the doors towards the subject's willingness to choose or access the door. There was no sex by weight interaction indicating that the

weight of the doors did not differentially affect the two sexes eliminating confound variables such as sex and size difference ($p > 0.05$).

The chi square test was used to assess the effects of our independent's variables (drug and sex) on the dependent variable (door choice). Neither sex nor drug had a significant effect on door choice at any of the weights used ($p > 0.05$). The multivariant ANOVA was used to assess the impact of the independent variables (sex and drug) on the dependent variables (phase 1 latency time, phase 2 latency time, social door push time, and empty door push time at each weight). The multivariate ANOVA indicated a main effect for sex (F (12, 73) = 2.042, p = 0.032), drug (F (24, 148) = 2.043, p = 0.005), and a sex by drug interaction (F (24, 148) = 4.598, p = 0.000).

The significant between-subject effects are demonstrated in tables 2-4. There is a sex by drug interaction in which a differential effect exists between atosiban and oxytocin depending on sex seen in figures 1 & 2. These figures show the opposing interaction that occurs between the males and females for atosiban and oxytocin prominently shown in graphs A, B, D, E, F and G. Almost all of the significant effects indicated that the latency time and time pushing chamber doors was increased by atosiban for male subjects and oxytocin for female subjects shown on figures 3 & 4.

As shown in graphs B, D, E, and G of figure 3, the male subjects treated with atosiban have the highest latency time to choose a chamber compared to the other drug groups even as the weights of the door increased. On the other hand, graphs B, D, E, G, and H of figure 4 showed that the females treated with oxytocin had the highest latency time in choosing a chamber compared to the other two drug groups. The atosiban-treated males and oxytocin-treated females spent the most time pushing a social door represented

in graphs C & I of figure 3 and graphs C & F of figure 4 respectively. There is also an interesting trailing trend that occurs between the two sexes and the drug categories in graphs B, C, D & E of both figures 3 & 4. Males administered with atosiban are the highest group for time with saline as second and oxytocin as the lowest group; females show an opposite trend with oxytocin as the highest time, saline as second and atosiban as the lowest time.

Table 2: Tests of Between-Subject Effects for Sex (red text indicates significance of effects)

sex	W0.5_P1_Time	0.740
	W0.5_P2_Time	0.858
	W0.5_SocialPush	0.001
	W0.5_EmptyPush	0.895
	W1.0_P1_Time	0.464
	W1.0_P2_Time	0.315
	W1.0_SocialPush	0.259
	W1.0_EmptyPush	0.278
	W1.5_P1_Time	0.087
	W1.5_P2_Time	0.019
	W1.5_SocialPush	0.221

	W1.5_EmptyPush	0.085

Table 3: Tests of Between-Subject Effects for Drug (red text indicates significance of effects)

drug	W0.5_P1_Time	0.297
	W0.5_P2_Time	0.463
	W0.5_SocialPush	0.016
	W0.5_EmptyPush	0.012
	W1.0_P1_Time	0.720
	W1.0_P2_Time	0.073
	W1.0_SocialPush	0.042
	W1.0_EmptyPush	0.782
	W1.5_P1_Time	0.036
	W1.5_P2_Time	0.012
	W1.5_SocialPush	0.997
	W1.5_EmptyPush	0.870

Table 4: Tests of Between-Subject Effects for Sex*Drug (red text indicates significance of effects)

sex * drug	W0.5_P1_Time	0.703
	W0.5_P2_Time	0.005
	W0.5_SocialPush	0.012
	W0.5_EmptyPush	0.001
	W1.0_P1_Time	0.009
	W1.0_P2_Time	0.000
	W1.0_SocialPush	0.066
	W1.0_EmptyPush	0.002
	W1.5_P1_Time	0.004
	W1.5_P2_Time	0.000
	W1.5_SocialPush	0.053
	W1.5_EmptyPush	0.302

Figure 1: Bar Graphs Comparing Latency Time. Latency time for both sexes and each drug category (oxytocin, atosiban, and saline across each weight 0.5oz (A, B, C), 1.0oz (D, E, F) and 1.5oz (G, H, I).

Figure 2: Line Graphs Comparing Latency Time. Latency time for both sexes for oxytocin and atosiban across each weight 0.5oz (A, B, C), 1.0oz (D, E, F) and 1.5oz (G, H, I)

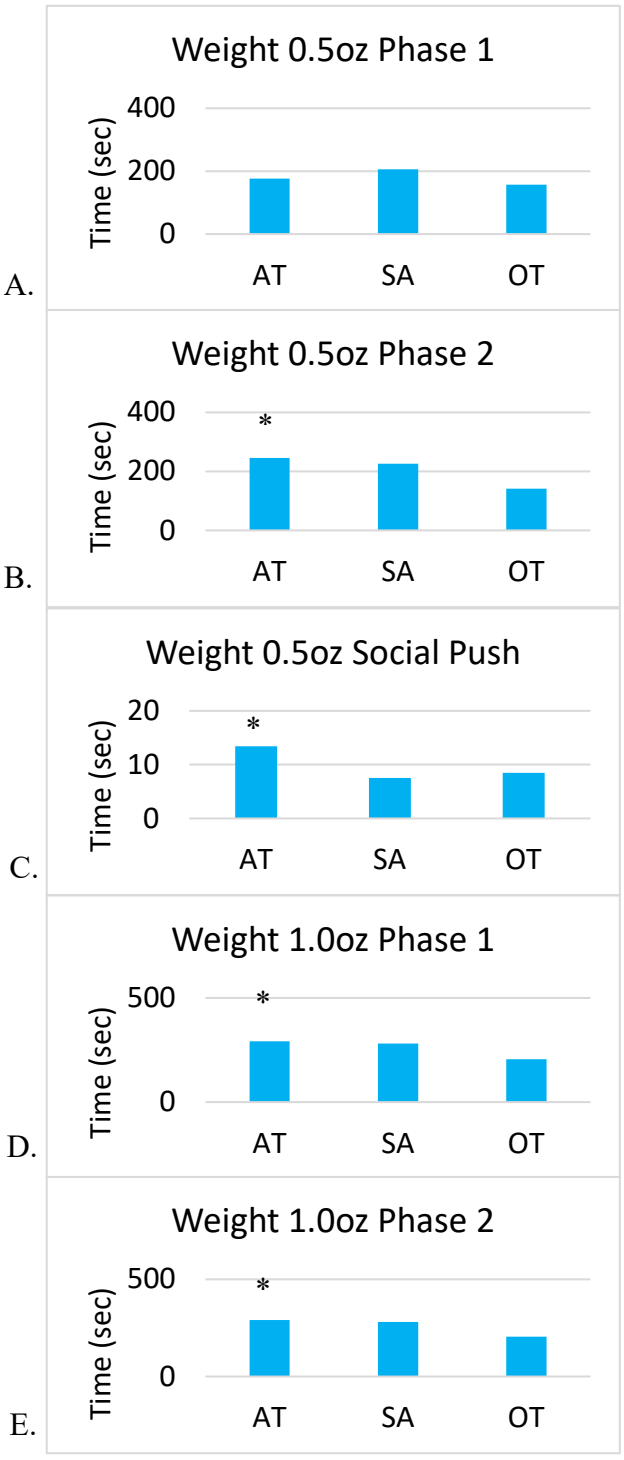


Figure 3: Male Bar Graphs. Comparison of latency time for males across the 3 drug categories for each weight 0.5oz (A, B, C), 1.0oz (D, E, F), 1.5oz (G, H, I)

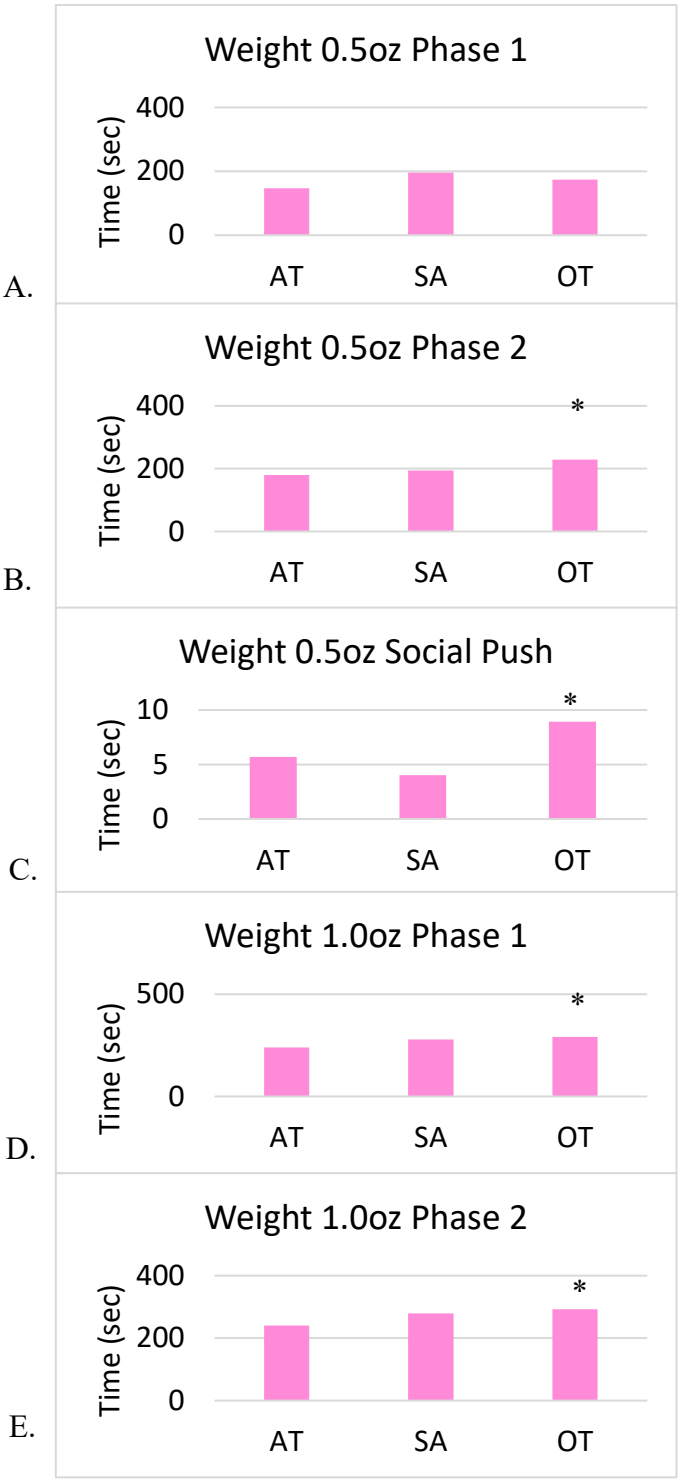
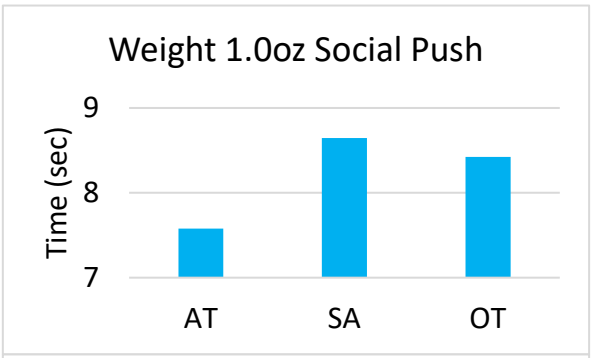
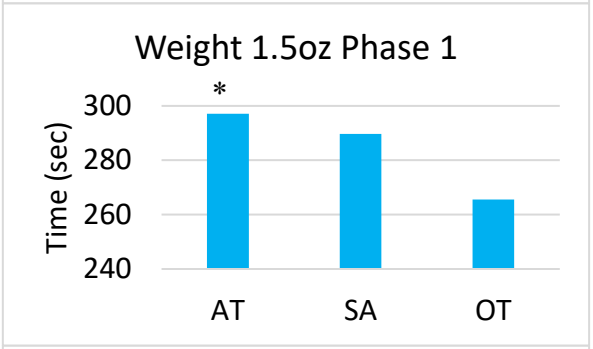


Figure 4: Female Bar Graphs. Comparison of latency time for females across the 3 drug categories for each weight 0.5oz (A, B, C), 1.0oz (D, E, F), 1.5oz (G, H, I)

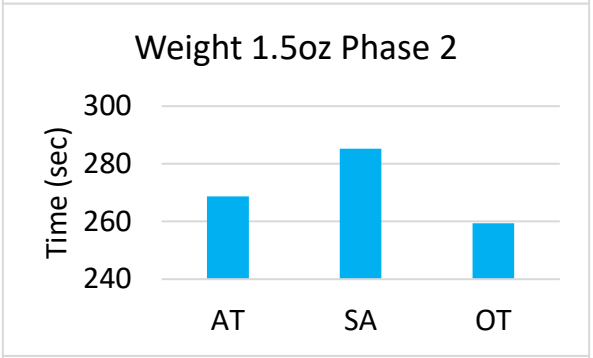
F.



G.



H.



I.

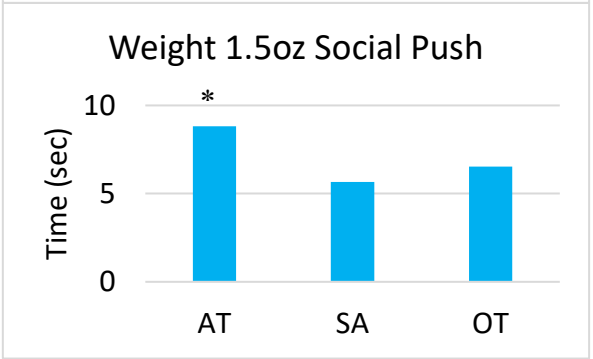
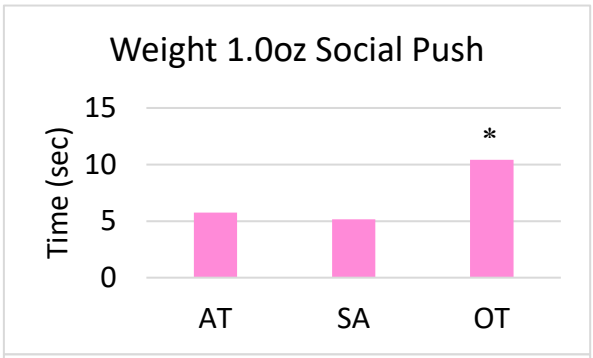
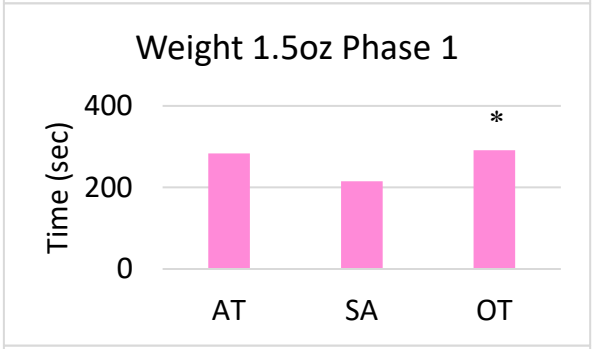


Figure 3: Male Bar Graphs. Comparison of latency time for males across the 3 drug categories for each weight 0.5oz (A, B, C), 1.0oz (D, E, F), 1.5oz (G, H, I)

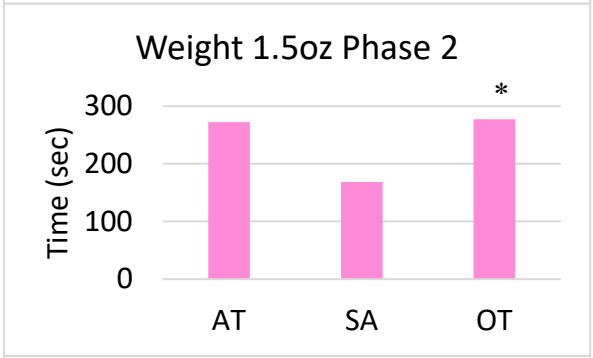
F.



G.



H.



I.

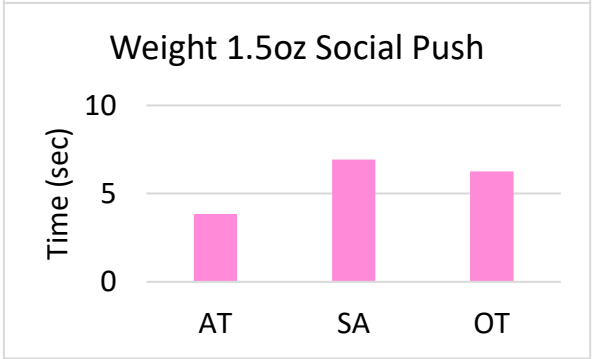


Figure 4: Female Bar Graphs. Comparison of latency time for females across the 3 drug categories for each weight 0.5oz (A, B, C), 1.0oz (D, E, F), 1.5oz (G, H, I)

CHAPTER V: DISCUSSION

The purpose of this study was to adapt a social behavior arena for mice and test the correlation between oxytocin receptor activation and social motivation. Two aims were addressed in our study. First, we evaluated sex-differences between male and female mice by adding trials with female mice because there are known expression differences in oxytocin receptors across the two sexes (Lukas, 2011). The second aim was to manipulate the mouse's level of social motivation pharmacologically by administering the three drug categories of oxytocin, an oxytocin antagonist, and saline. Together these aims demonstrated that a sex by drug interaction does exist as a causal factor in the observed changes in social motivation. We found that atosiban-treated males and oxytocin-treated females had a higher latency time choosing and pushing on a door compared to the other drug groups thus representing higher social motivation.

Borland and colleagues initially described a weighted doors task aimed to measure social motivation in hamster. However, Borland et al. used hamsters in the test and did not attempt to alter motivation to validate the arena. We utilized their research to successfully develop a protocol and validate an arena to measure social motivation in mice but found limitations to it. In our current project, we expanded our preliminary research by updating the previous methodology and arena to accommodate for these limitations. We increased the power of the study by adding female subjects and increasing the group size, adjusted the arena to accurately measure social motivation, and added an oxytocin inhibitor to pharmacologically manipulate social motivation. We were able to successfully utilize our social reward arena with male and female mice across different drug categories. This led us to find sex and drug effects in our study which was

an important contribution to this research from before. Our study confirmed that distinct behavioral patterns do exist across sex and different drug categories allowing us to establish a measurement for social motivation in mice.

One of the most consistent findings in our study was oxytocin mediating social interactions among the female subjects. This oxytocinergic effect on female social bonding can be described through literature. Oxytocin is most well-known for its role in parturition and lactation but is also critical for the regulation of social behavior and bonding across mammals (Lukas, 2011). Furthermore, oxytocin function is dependent on steroid hormones and sex (Lee, 2010). There is research that shows that estrogen increases social behaviors and central oxytocin receptors in female rodents (Kent, 2013). In particular, estrogen also enhances oxytocin receptor affinity (Marazziti, 2019). Therefore, there is a prosocial connection in which estrogen appears to regulate oxytocin effects on social recognition in females. Also, the oxytocin system shows sex-related differences depending on the brain regions and species. In rats, the density of oxytocin immunoreactive axons rend to be higher in females (Marazziti, 2019). It was also observed that oxytocin receptor binding seems to increase by 40% by maternal licking and grooming in females (Marazziti, 2019).

The other finding was the correlation between atosiban and social motivation in the male subjects. Atosiban was originally meant to serve as an oxytocin antagonist in this study, however, it also has receptor binding for arginine-vasopressin. Arginine vasopressin/ antidiuretic hormone is a neuropeptide synthesized in the hypothalamus and secreted from the pituitary gland. It is known to play essential roles in the control of the body's osmotic balance, blood pressure regulation and homeostasis (Cuzzo, 2021).

Alongside these functions, vasopressin also mediates complex mammalian social behaviors such as pair bonding, social recognition, and aggression (Young, 2012). Atosiban has been tested in vitro for its affinity on vasopressin/oxytocin receptors using uterine preparations from different animal species (Bossmar, 1998). The overall results demonstrated that atosiban is a high-affinity, competitive antagonist for both oxytocin and vasopressin receptors which are distinct, though structurally related (Bossmar, 1998). Sex differences are commonly seen in studies of actions for oxytocin and vasopressin. There were findings that showed males are less sensitive than females to exogenous oxytocin administration (Kent, 2013). Most of these studies suggest that males either have more vasopressin or are more sensitive to the effects of vasopressin (Carter, 2017). Male rodents seem to be more dependent on vasopressin acting at the level of the lateral septum for social recognition compared to female rodents (Gabor 2012). Results of various studies suggest that testosterone and its metabolites influence social recognition in males primarily though vasopressin receptors (Gabor, 2012).

The results of different studies carried out on several different animal species resulted in more prominent effects of oxytocin in females and more significant effects of vasopressin in males. A study on prairie voles described this significant difference between oxytocin and vasopressin aimed to reveal mechanisms in which these hormones act on pair-bonding behavior. It was supported that the vasopressin receptor is necessary for both the formation and maintenance of pair-bonds in male prairie voles, suggesting vasopressin has a significant role in social bonding behavior of males (*Categories*, 2020). Female prairie voles showed that vasopressin inhibition appeared to have little effect on altering pair-bonding behaviors. Instead, the use of an oxytocin receptor antagonist

resulted in inhibition of partner preference formation demonstrating the crucial role of oxytocin on females (*Categories*, 2020).

Although we have expanded the current knowledge and measurement for social motivation, there still were limitations in our research. There was no drug or sex effect seen on door choice in our study. This is an important and interesting factor that should be explored in future studies by manipulating the methodology, arena, or independent variables. Another limitation is that only one oxytocin antagonist was used. In terms of future directions, it would be important to study other oxytocin antagonists and their effects on social motivation. It would also be worthwhile to test the direct effects of vasopressin on social motivation. Also, the drugs were administered systemically which can disadvantageous due to limited targeting of specific tissue, variable drug absorption, less bioavailability of drug, and decreased efficacy. Future studies should adjust the research for site specific infusions to determine specific brain areas that are mediating these social interactions.

This study emphasized the importance of social motivation compared to sociability. The overall significance of this project was to help establish a standard way of measuring social motivation in mice since there is no current standard. The correlation between oxytocin receptor activation/inhibition and social motivation was tested in which the variability of oxytocin and related hormones such as vasopressin across male and female mice were addressed. The understanding of these factors will aid us in better developing treatment targets and pharmacological improvements for disorders like autism with social symptoms. Instead of measuring general sociability in these models only, we believe that measuring specific aspects of social behavior will allow for better behavioral

descriptions of these models. Increased specificity in behavioral characterization may then lead to discovering more coherent patterns in neural activation associated with changes in social behavior.

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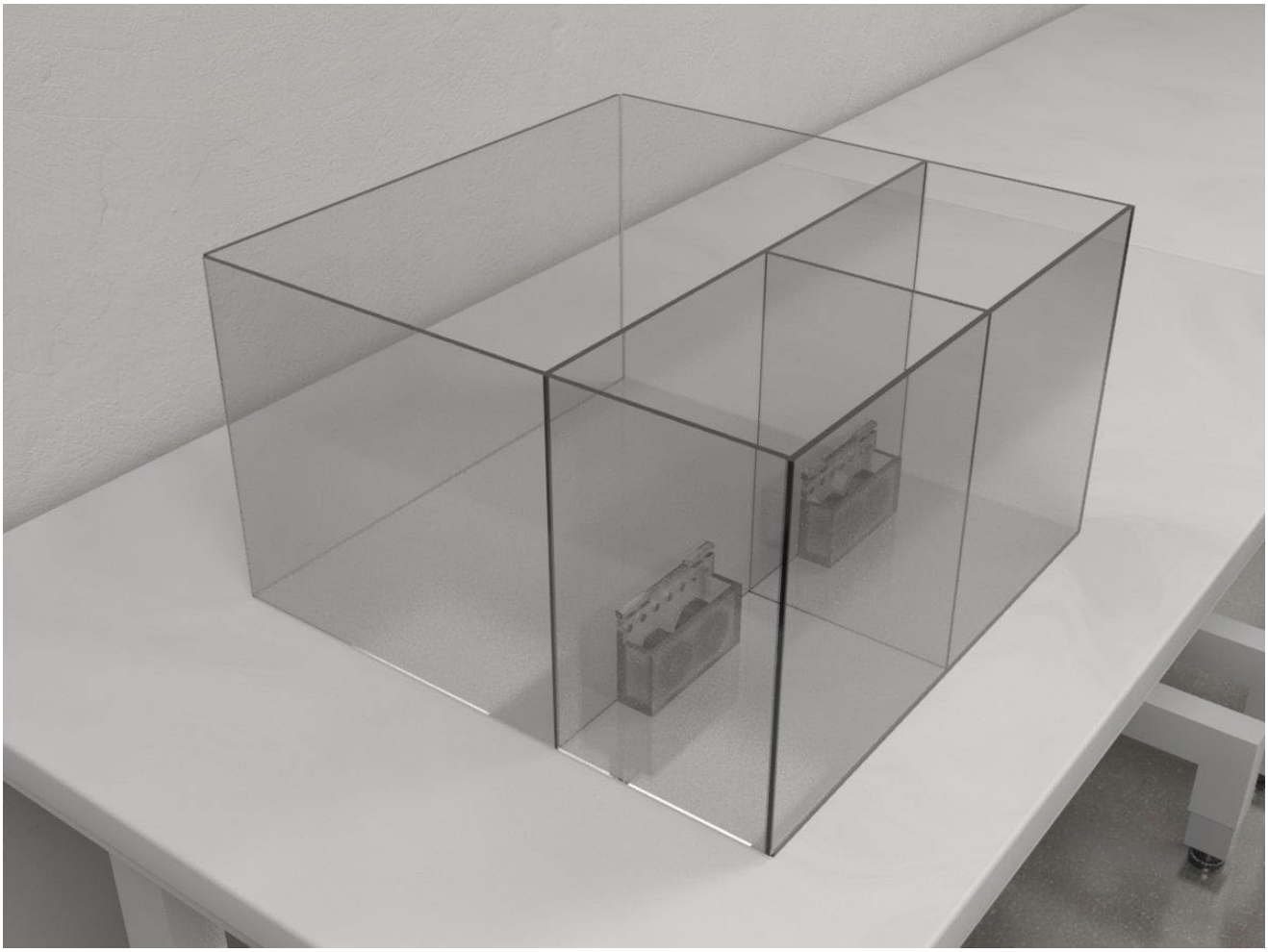
Appendix A: Weighted Doors Arena



Appendix B: IACUC Approval

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

MIDDLE TENNESSEE STATE UNIVERSITY

IACUCN006: FCR PROTOCOL APPROVAL NOTICE

Tuesday, June 15, 2021

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 from the date of this notification
Date of Expiration	5/31/2022
Number of Animals	192 (ONE HUNDRED and NINETY TWO)
Approved Species	C57BL/6J Mice (Jackson Laboratories)
Category Subclassifications	☐ Teaching ☐ Research ☐ Classroom ☐ Laboratory ☐ Field Research ☐ Field Study ☐ Laboratory ☐ Handling/Manipulation ☐ Observation Comment: NONE
Approved Site(s)	MTSU Vivarium: Rooms SCI 1170L (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	This protocol is on CR and it can be continued while awaiting IACUC decision

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 5/31/2022 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	CR request received on 05/05/2021 Status: FCR Pending IACUC Decision: The protocol is approved for extension (FCR 05/27/2021)
Second year report	4/30/2022	NONE
Final report	4/30/2023	NONE

Post-approval Actions:

Date	*Amendment*	*IACUC Notes*
NONE	NONE	NONE

Post-approval Amendments:

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	
10/02/2020	The following students have been approved by the IACUC to serve as co-investigators in this protocol: Kara Oberstaedt (kao2w), Jillian Gulledge (jgg2y), Rija Asim (ra6b), and Hannah Hudson (hrh3f).	Initial FCR approval
11/02/2020	The following student workers have been approved: Erica Burton (ebb3n) and Ana Sofia Magledo (asm3t)	Minor amendment (DMR)
11/16/2020	Amendments to inject drugs are approved	Minor Amendment (DMR)
11/17/2020	Student worker Veyan Guly (vg3b) is approved	Significant Amendment (FCR 11/16/2020)
11/25/2020	Student worker Yesilna Lamci (yml2h) is approved	Minor Amendment (DMR)
12/09/2020	Student worker Isabela Ramos (gi2a) is approved	Minor Amendment (DMR)
12/21/2020	Student worker Sam Mahin Aeen (smfkr) is approved	Minor Amendment (DMR)
02/02/2021	Student workers Matthew Martin (mam2hf), Mary Henson (mdh8e) and Amber Baker (ab2ac) are added to the protocol	Minor Amendment (DMR) Minor Amendment (DMR)
03/05/2021	FCR Review Comments: 1. How many mice will be subjected to the acute ip injections of each of the 5 drugs you list? 2. Are these ip injections performed on independent groups or is it a repeated-measures design? 3. What is your sample size for the chronic ip injection	Minor Amendment (DMR) FCR approved on 05/27/2021

	study? What is the control and control sample size? 4. Similarly, what is your sample size for the intranasal OT administration? 5. In general, please address how these treatments integrate into your previously approved protocol, especially regarding how they do not require an increase in animal numbers.	after reviewing the revisions
03/08/2021	1. Student workers Kara Oberstaedt (kao2w) and Erica Burton (ebb3n) are removed from this protocol. 2. Reeana Marie Paul (rmp4r) is approved.	Minor Amendment DMR
05/05/2021	John Griner (jxg2bq) and Catriona DeYamon-Lungrchapen (cxd3y) are added to the protocol	Minor Amendment DMR
05/12/2021	Reagan Canon (rec5q) is added to the protocol.	Minor Amendment DMR
05/20/2021	Britney Mounnaly (tzn6w) is added to the protocol.	Minor Amendment DMR
06/15/2021	1. Logan Galleth (lbg3t) and Alyson Campbell (ajc7f) are added to the protocol. 2. Jillian Gulledge has been removed from the protocol.	Minor Amendment DMR

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)