

## ABSTRACT

LIU, ALICE. Assessing Sex as a Biological Variable in Oxytocin Receptor Activation and Neuroscience Research. (Under the direction of Dr. John Meitzen).

Research has widely demonstrated that sex differences biological exist at multiple levels between male and females. Thus, it is crucial to consider sex as a biological variable (SABV) in scientific investigation to produce representative results and advance unbiased scientific discovery. Here, SABV was investigated at a cellular level in our own experiment, and then more broadly by assessing how SABV is employed across neuroscience research studies. At the cellular level, SABV was examined in the context of oxytocin receptor (OXTR) activation of medium spiny neurons (MSNs) in the nucleus accumbens (NAc). This mechanism is fundamental in understanding how social play is regulated. Social play is a highly rewarding behavior with sex differences in phenotype and incidence. Social play is exhibited across many animal species, including humans and rodents, and is essential for the development of healthy relationships. Chronic social play deficits in the juvenile period have implications in neurodevelopmental disorders that show sex differences, reinforcing the importance of considering SABV. Two intersectional neural systems are key components in the mechanisms underlying social play behavior. The brain mesolimbic reward system contains a brain region called the NAc, which has discovered to regulate social play in juvenile male rat among other species, with females much less explored. Secondly, the neuropeptide oxytocin (OXT) is known to mediate social behavior in sex-specific ways. Thus, we hypothesized that OXT receptor (OXTR) activation sex-specifically modulates the electrophysiological properties of MSN in the NAc. To test this hypothesis, the response of resting membrane potential (RMP) to control or OXTR selective agonist [Thr4,Gly7]-oxytocin (TGOT)(0.2  $\mu$ M or 1.0  $\mu$ M) of NAc MSN were recorded from male and female pre-pubertal rats using whole-cell patch clamp in acute brain

slice preparation. Overall, OXTR activation demonstrated a depolarization in response to to 1.0  $\mu\text{M}$  TGOT that did not differ by sex, suggesting that this mechanism is not sex-specific. These results indicate that OXTR activation can regulate a fundamental electrophysiological property of NAc MSNs, providing important progression for understanding the implication of OXT in social play and related disorders. Importantly, this response did not differ by sex, invalidating this portion of our hypothesis and providing the valuable insight that sex differences must be generated via a different mechanism. Regarding SABV across neuroscience research, sex bias, the favoring of male research models over females, and sex omission, the lack of sex reporting, have repeatedly been demonstrated across time. This is problematic given established sex differences in the brain, behavior and neurological disorders. Here, we extended previous findings by analyzing sex bias and omission in research articles of six difference neuroscience journals in 2020. Our findings showed a complex presentation of SABV in neuroscience research. Compared to past analyses, sex omission declined but sex bias remained relatively constant. There is increased reporting of research model sex, however SABV was only employed in 10% of total articles. There findings are further complicated by the identification of 34 different research model types with varying degrees of sex bias, sex omission, and employment of SABV. A small proportion of articles employed more than one model type. These findings show that while sex bias and omission are improving over time, they are still persistent and continue to present a complex picture of SABV in neuroscience research. Together, the two chapters of this thesis demonstrate how to employ SABV in a cellular neuroscience experiment, and then places this experiment within a much broader context, especially given that the vast majority of neuroscience experiments do not employ SABV.

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Assessing Sex as a Biological Variable in Oxytocin Receptor Activation and Neuroscience  
Research

by  
Alice Liu

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## **DEDICATION**

This thesis is dedicated to my mom and dad, as their incredible sacrifices allowed me to pursue my passions in science. It is also dedicated to my two dogs, for never failing to make me smile.

## **BIOGRAPHY**

Alice Liu is a first generation Taiwanese-American student born in the town of Gastonia, North Carolina. She eventually moved to Apex, North Carolina, where she nurtured her love in music by playing clarinet and cello. She was an avid member of her high school marching band and travelled throughout the country to participate in competitions. Alice initially decided to pursue a music degree at North Carolina State University but eventually turned her interests towards science. While working in the Emergency Department as a medical scribe throughout the COVID-19 pandemic and seeing the lack of knowledge in this topic amongst patients and healthcare workers alike, Alice realized the importance of scientific research and educating the public on accurate scientific discovery. This not only motivated her to seek opportunities in research but secured her passion for medicine. Her opportunity came when she joined the Meitzen Laboratory and where she experienced much growth in her academic and personal life. In her free time, Alice likes being creative and pursues photography as well as videography. She enjoys spending time in the outdoors and hiking with her dogs.

## ACKNOWLEDGMENTS

First, I would like to acknowledge my advisor and mentor, Dr. Meitzen. Truthfully, I do not think I can thank you enough. Your wisdom and guidance has not only played a tremendous role in this working coming into fruition but also taught me how fortunate I am for having a mentor who is so invested in my academic and personal success. Your dedication and enthusiasm for science is so inspirational and motivates me to continue pursuing neuroscience in the future. There are no words to express how grateful I am for the patience, support and encouragement you've showed me all these years.

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## **CHAPTER 1**

### **Assessing oxytocin receptor activation of medium spiny neurons in the nucleus accumbens in male vs female rats**

Research in this chapter will be submitted to a peer-reviewed journal:

Liu, A., Meitzen, J. (2024): Assessing oxytocin receptor activation of medium spiny neurons in the nucleus accumbens in male vs female rats. *In progress.*

## **Abstract**

Social play in human, primate and rodent juveniles is a highly rewarding behavior which is critical for the development of healthy social skills. Two neural mechanisms that are crucial to regulating social behavior are oxytocin (OXT) and the brain mesolimbic reward circuitry, including a key region called the nucleus accumbens (NAc). Social play differs by sex. However, little is known about the integrated relationship between the NAc, the role of OXT, and potential sex differences. We hypothesized that oxytocin receptor (OXTR) activation influences the electrophysiological properties of neurons within the NAc and that this response would differ by sex. To test this hypothesis, NAc medium spiny neurons (MSNs) from male and female prepubertal Wistar rats were recorded using whole-cell patch clamp in acute brain slice preparation. Resting membrane potential (RMP) was recorded and analyzed in response to artificial cerebrospinal fluid (ACSF) control or OXTR selective agonist [Thr4,Gly7]-oxytocin (TGOT)(0.2  $\mu$ M or 1.0  $\mu$ M). Exposure to control or 0.2  $\mu$ M TGOT did not elicit a significant RMP response. OXTR activation in response to 1.0  $\mu$ M TGOT did display a depolarizing effect on RMP. These responses did not differ by sex, suggesting no sex difference was detected via our methodology. Overall, these findings provide a foundation for better understanding how OXT modulates the neurons implicated in social play behavior and related disorders, providing a crucial first step towards improved personalized treatment and novel therapeutic strategies.

## **Introduction**

Social play can be defined as a repeatedly expressed behavior containing elements that are aggressive, predatory, and/or sexual in nature, displayed in a modified or exaggerated form (Heintz et al., 2017; Vanderschuren et al., 2016; Vanderschuren & Trezza, 2014). Visual or auditory signals may accompany or precede these behaviors to signify it is playful in nature. Social play behavior is often performed by mammalian and bird species, however it is also suggested in reptilian, fish and amphibian species (Dinets, 2023). Social play is abundant from post-weaning to early/mid adolescence, and functionality of this behavior during the juvenile period is crucial for developing healthy social relationships in maturation (Nijhof & Bird, 2019; Sigman et al., 1999; Vanderschuren et al., 2016). Conclusively, early intervention is essential for the appropriate development of healthy social behaviors and this it is important to understand the neural basis of social play in juveniles before puberty.

The neural basis of social play has been a target of significant prior research. Previous studies demonstrate that social play is a rewarding behavior in rats (Trezza et al., 2010, 2011; Vanderschuren et al., 2016). The neural circuits regulating reward are well document, and include the mesolimbic reward pathway. One key component of this circuitry is the NAc. The NAc is a brain region widely involved in modulating rewarding effects, and it is well known to be involved in social play but there are limitations to this conclusion (Klawonn & Malenka, 2018; Manduca et al., 2016). For example, previous data reveals the NAc regulates social play in juvenile male rats, however it is unknown whether it exerts similar effects in juvenile female rats (Manduca et al., 2016). This lack of information is problematic given the strong evidence suggesting the presence of sex differences in social play and neurodevelopmental disorders (Marquardt et al., 2023; VanRyzin et al., 2020). For example, when engaging in social play,

juvenile males and female rats phenotypically express this behavior differently. Males are more likely to initiate play and display behavior described as wrestling, counterattacking, boxing and pinning more frequently than females (Vanderschuren et al., 2016; VanRyzin et al., 2020). Meanwhile, females are more likely to exhibit evasive maneuvers when playing (VanRyzin et al., 2020). Collectively, this diversity of phenotypic and incidence presentation in social play and neurodevelopmental disorders indicates that similarities across sex should not be assumed. Thus, studying the neural basis of social play in both sexes is imperative to understanding the underlying physiological mechanisms driving these sex differences.

One relevant target for investigating sex difference is OXT action in the NAc. OXT is a highly evolutionarily conserved neuropeptide known to regulate a wide spectrum of social behavior in sex-specific ways across various species, including humans and rats (Bredewold & Veenema, 2018; K. M. Dumais & Veenema, 2016; K. Dumais & Veenema, 2016). OXT-producing magnocellular neurons of the hypothalamic paraventricular nucleus have been demonstrated to project onto the NAc, where OXTRs are predominantly observed in D1 MSNs and interneurons (Luo et al., 2022). However, changes in the electrophysiological properties of MSNs in the NAc in response to OXTR activation and whether these include sex differences is still unknown.

To address this knowledge gap, here we test the hypothesis that OXTR activation modulates the electrophysiological properties of MSNs in the NAc differently in male vs female Wistar rats. We focus on RMP, a fundamental feature of neurons that is critically important for neuronal excitability and function. RMP is the electrical potential difference across the plasma membrane in a non-stimulated state due to the presence of leak sodium and potassium channels, among other more minor influences. RMP is a relatively easily testable, fundamental

characteristic that all neurons possess. This hypothesis is assessed by recording RMP in response to a control or the selective OXTR agonist [Thr4,Gly7]-oxytocin (TGOT) (0.2  $\mu$ M or 1.0  $\mu$ M). To account for possible sex differences, prepubertal male and female Wistar rats are utilized for this experiment and sex was treated as a biological variable.

## Methods and Materials

### *Animals*

All animal protocols were approved by the Institutional Animal Care and Use Committee at North Carolina State University. Wistar rats were born from timed-pregnant females purchased from Charles River Laboratories. Rats were housed with littermates and dam until weaning (P21). After weaning, animals were group housed in same-sex cages until experimental collection day (P30±1). 23 male and 22 female Wistar rats were utilized for this study. The Biological Resource Facility of North Carolina State University housed these animals in a temperature and light-controlled room (23 °C, 40% humidity, 12:12-hour light/dark cycle with lights on 7am-7pm). Cages are polysulfone Bisphenol A (BPA) free and filled with bedding produced from virgin hardwood chips (Beta chip, NEPCO, Warrensburg, NY). Animals were provided glass-bottle water and soy protein free rodent chow (2020X, Teklad, Madison, WI, USA) *ad libitum*.

### *Acute brain slice preparation*

Brain slices for electrophysiological recordings were prepared following previous protocols in our laboratory (Dorris et al., 2015). Rats were anesthetized with isoflurane gas and killed via rapid decapitation. The brain was dissected into chilled oxygenated sucrose artificial cerebrospinal fluid (s-ACSF), containing (in mM): 75 sucrose, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 3 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2.4 Na pyruvate, 1.3 ascorbic acid, 75 NaCl, 25 NaHCO<sub>3</sub>, 15 dextrose, 2 KCL. Osmolarity was between 290-313 mOsm, with a pH between 7.3-7.5. The brain was coronally sectioned at 300 µm using a vibratome (Leica), followed by separation of the left and right hemispheres. Slices were placed in ACSF containing (in mM): 126 NaCl, 26 Na HCO<sub>3</sub>, 10 dextrose, 3 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, for incubation. Osmolarity was between 290-

313 mOsm, with a pH between 7.3-7.5. Slices incubated for 20 minutes at 30-35°C and then allowed to slowly decrease to room temperature (22-23 °C) for a total resting time of approximately 1-5 hours prior to use.

### *Electrophysiological recording*

After resting for at least 1 hour, each slice was individually visualized using a Zeiss Axioscope equipped with IR-DIC optics, Dage IR-1000 video camera, and 10x and 40x lenses with optical zoom. The recording chamber received a continuously perfused flow of oxygenated ACSF between 25±0.4°C. Whole-cell patch clamp recording techniques were employed to record resting membrane potentials of MSNs of the nucleus accumbens core (NAcc). Recordings were localized within the NAcc, medial of the anterior commissure between Bregma ~2.28 mm and ~1.44 mm. Glass electrodes (8-19MΩ) contained an internal solution of (in mM): 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 MgCl<sub>2</sub>, 2 MgATP, 0.3 NaGTP, 10 phosphocreatine, 0.3% biocytin and 10 HEPES, 285 mOsm. pH 7.2-7.4. MultiClamp 700B amplifier attached to a Digidata 1550 system and personal computer using pClamp 10.7 software amplified, filtered (2 kHz) and digitized (10 kHz) signals. Once electrical access to the MSN was established, the MSN was acclimated for approximately 3 minutes to allow the resting membrane potential to stabilize (Mu et al., 2010). After this stabilization period, a series of positive, depolarizing currents were performed to assess action potential (AP) properties, followed by a series of negative, hyperpolarizing currents to assess passive and inward rectification properties. Assessment of these properties allowed for MSN verification using procedures previously described (Belleau & Warren, 2000; O'Donnell & Grace, 1993), in addition to the presence of a medium sized soma. RMP was then recorded continuously, and monitored in response to bath application of control solution (ACSF) for 1 minute or the selective oxytocin receptor agonist [Thr4,Gly7]-oxytocin

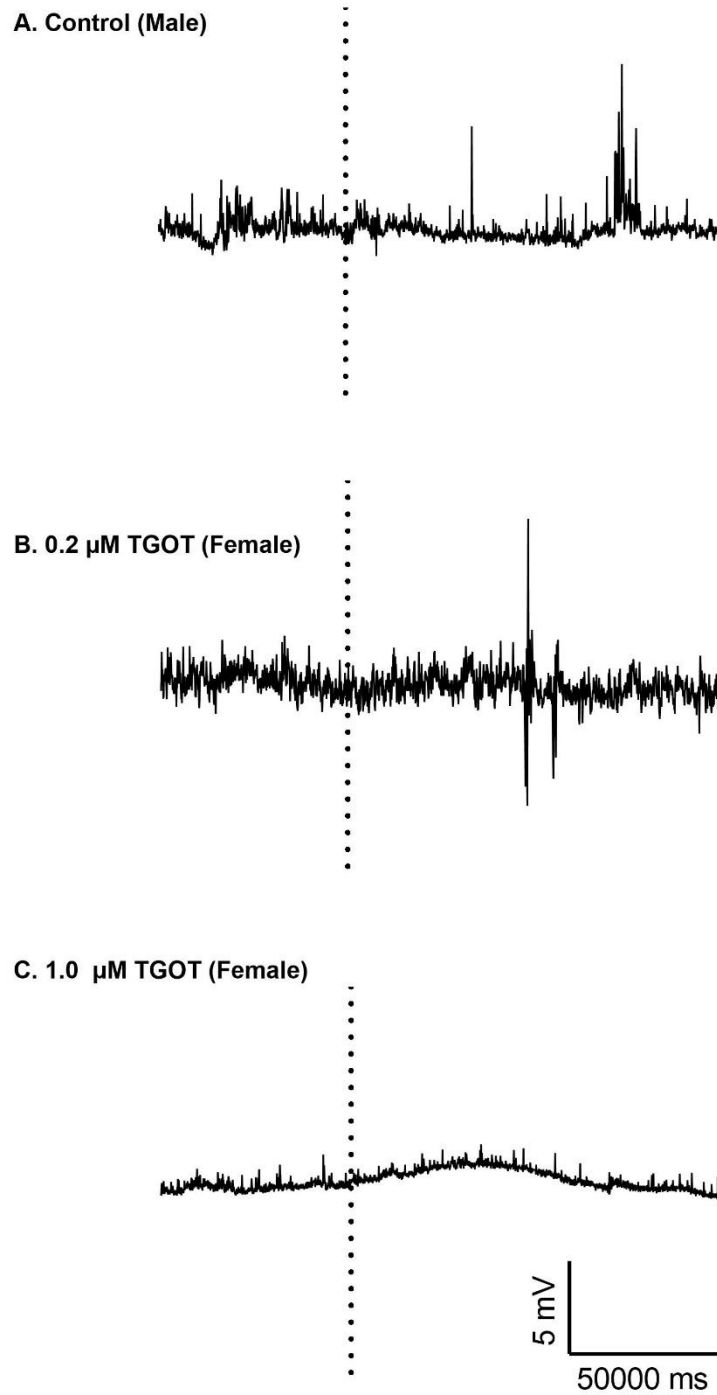
(TGOT, 0.2  $\mu$ M, 1  $\mu$ M) mixed in ACSF for 1 and 3 minutes, respectively. Electrical offset and confirmation of MSN location in the nucleus accumbens core were noted after recording.

Perfusion tubing was rinsed with ddH<sub>2</sub>O and 70% ethanol after use.

#### *Data analysis and statistics*

Peak, antipeak and mean of MSN RMP were analyzed in 30 second intervals using Clampfit 11.2 software. Peak is defined as the most positive voltage reached during the analysis period. Antipeak is defined as the most negative voltage reached during the analysis period. Mean is the average of all values recorded for the RMP during the analysis period. For population analyses, peak, antipeak and mean RMP values per cell were normalized as a percent changed compared with the baseline period. Following a previous manuscript (Krentzel et al., 2019), percent normalized change was calculated by averaging the values of the recording period before exposure, and then using that pre-exposure average to normalize each minutes of the recording [ percent change = (measured value – average of the baseline)/average of the baseline) x 100]. Experiments were analyzed with two-way repeated measures ANOVA (GraphPad Prism, version 9.5.1.). *P* values <0.05 were considered statistically significant. Data are means  $\pm$  SE.

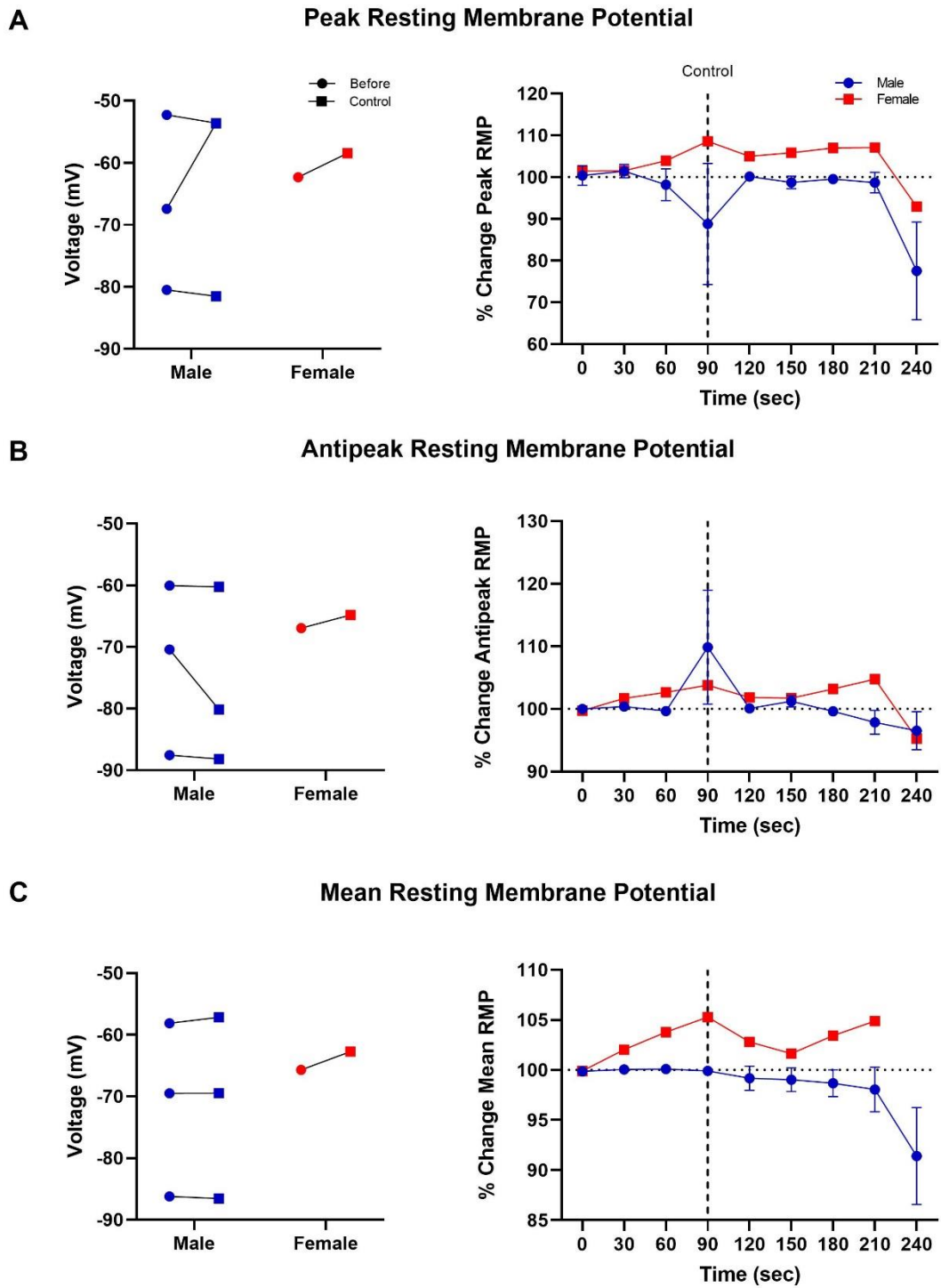
## Results



**Figure 1.1.** Raw traces of RMP data show different effects in response to control ACSF or TGOT in prepubertal male or female rats. Dotted line indicates time of control or drug exposure. A) Raw trace of RMP shows no response to ACSF control. B) Raw trace of RMP shows no response to 0.2  $\mu\text{M}$  TGOT. C.) Depolarizing effect is seen in RMP in response to 1.0  $\mu\text{M}$  TGOT.

Application of control ACSF did not affect peak, antipeak or mean RMP

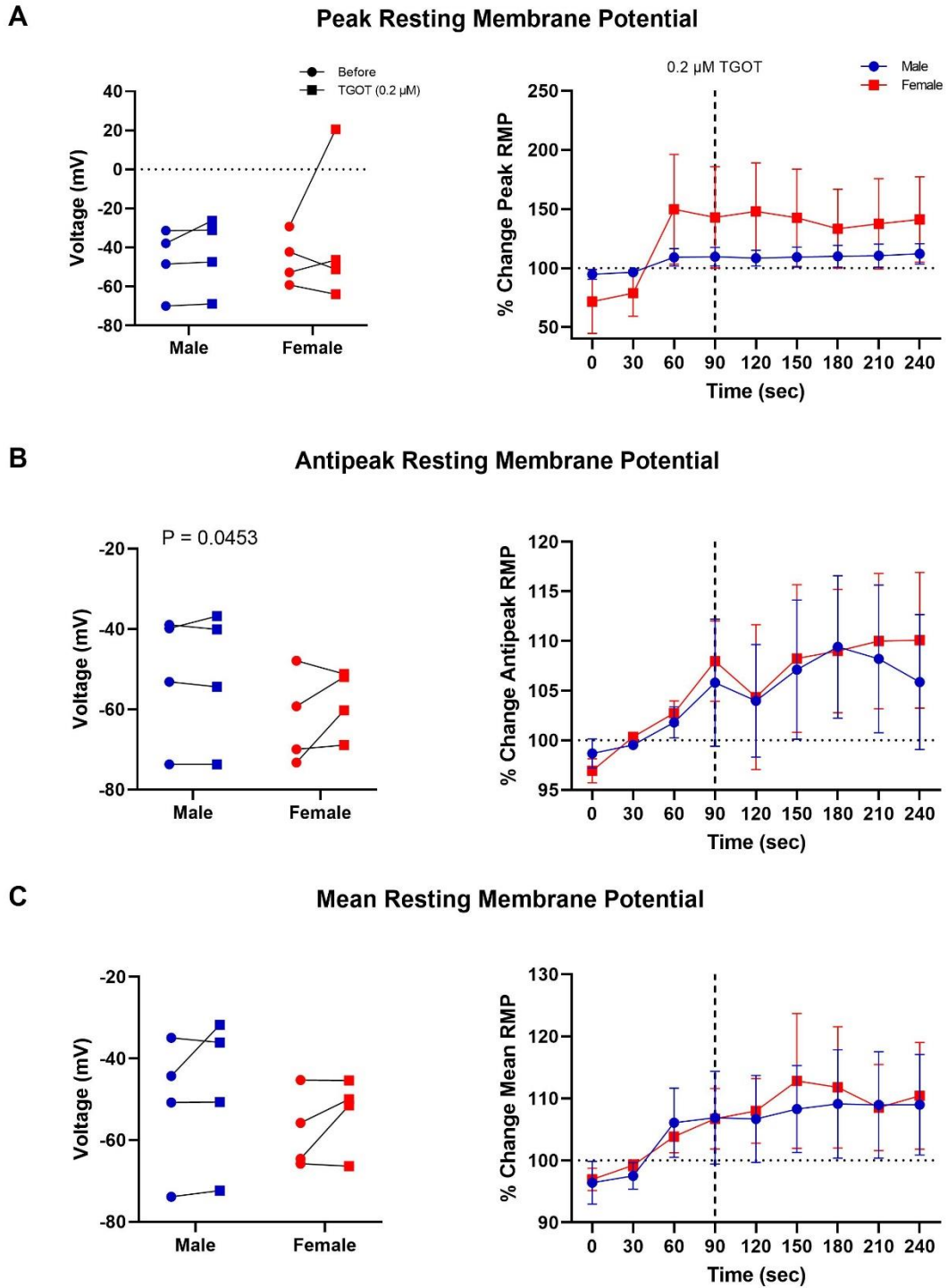
To establish a control for our experiment, MSN RMP was recorded continuously in response to ACSF application (Figure 1.1A). For all experiments, data was disaggregated between males and females to account for sex as a biological variable. For all experiments, the data were quantified using a non-normalized analysis and a normalized analysis to account for differences in starting RMP across MSNs. No differences in peak RMP was detected in response to control ACSF in both the non-normalized (Figure 1.2A, left; Interaction:  $F_{(1,2)}=0.00$ ,  $P=0.99$ ; Sex:  $F_{(1,2)}=0.07$ ,  $P=0.82$ ; Drug:  $F_{(1,2)}=0.60$ ,  $P=0.52$ ) or normalized analysis (Figure 1.2A, right; Interaction:  $F_{(8,6)}=0.37$ ,  $P=0.92$ ; Sex:  $F_{(2,3)}=0.59$ ,  $P=0.57$ ; Drug:  $F_{(1,2)}=0.00$ ,  $P=0.99$ ). No differences in antipeak RMP was detected in response to control ACSF in both the non-normalized (Figure 1.2B, left; Interaction:  $F_{(1,2)}=0.84$ ,  $P=0.46$ ; Sex:  $F_{(1,2)}=0.28$ ,  $P=0.65$ ; Drug:  $F_{(1,2)}=0.05$ ,  $P=0.85$ ) or normalized analysis (Figure 1.2B, right; Interaction:  $F_{(8,16)}=0.40$ ,  $P=0.90$ ; Sex:  $F_{(1,2)}=0.88$ ,  $P=0.54$ ; Drug:  $F_{(1,2)}=0.48$ ,  $P=0.59$ ). No differences in mean RMP was detected in response to control ACSF in both the non-normalized (Figure 1.2C, left; Interaction:  $F_{(1,2)}=12.44$ ,  $P=0.07$ ; Sex:  $F_{(1,2)}=0.17$ ,  $P=0.72$ ; Drug:  $F_{(1,2)}=16.09$ ,  $P=0.05$ ) or normalized analysis (Figure 1.2C, right; Interaction:  $F_{(8,16)}=0.65$ ,  $P=0.73$ ; Sex:  $F_{(1,2)}=0.15$ ,  $P=0.74$ ; Drug:  $F_{(1,2)}=1.81$ ,  $P=0.30$ ). These analyses collectively indicate there is no significant RMP response to the application of ACSF as a control variable, and that these results are not distinguished between sexes.



**Figure 1.2.** No response was detected in RMP when exposure to ACSF control. There is no sex-specific effect. A, left) Non-normalized peak RMP. A, right) Normalized peak RMP. B, left) Non-normalized antipeak RMP. B, right) Normalized antipeak RMP. C, left) Mean non-normalized peak RMP. C, right) Normalized mean RMP.

Application of 0.2  $\mu$ M TGOT did not affect on peak, antipeak or mean RMP: possible sex difference in antipeak?

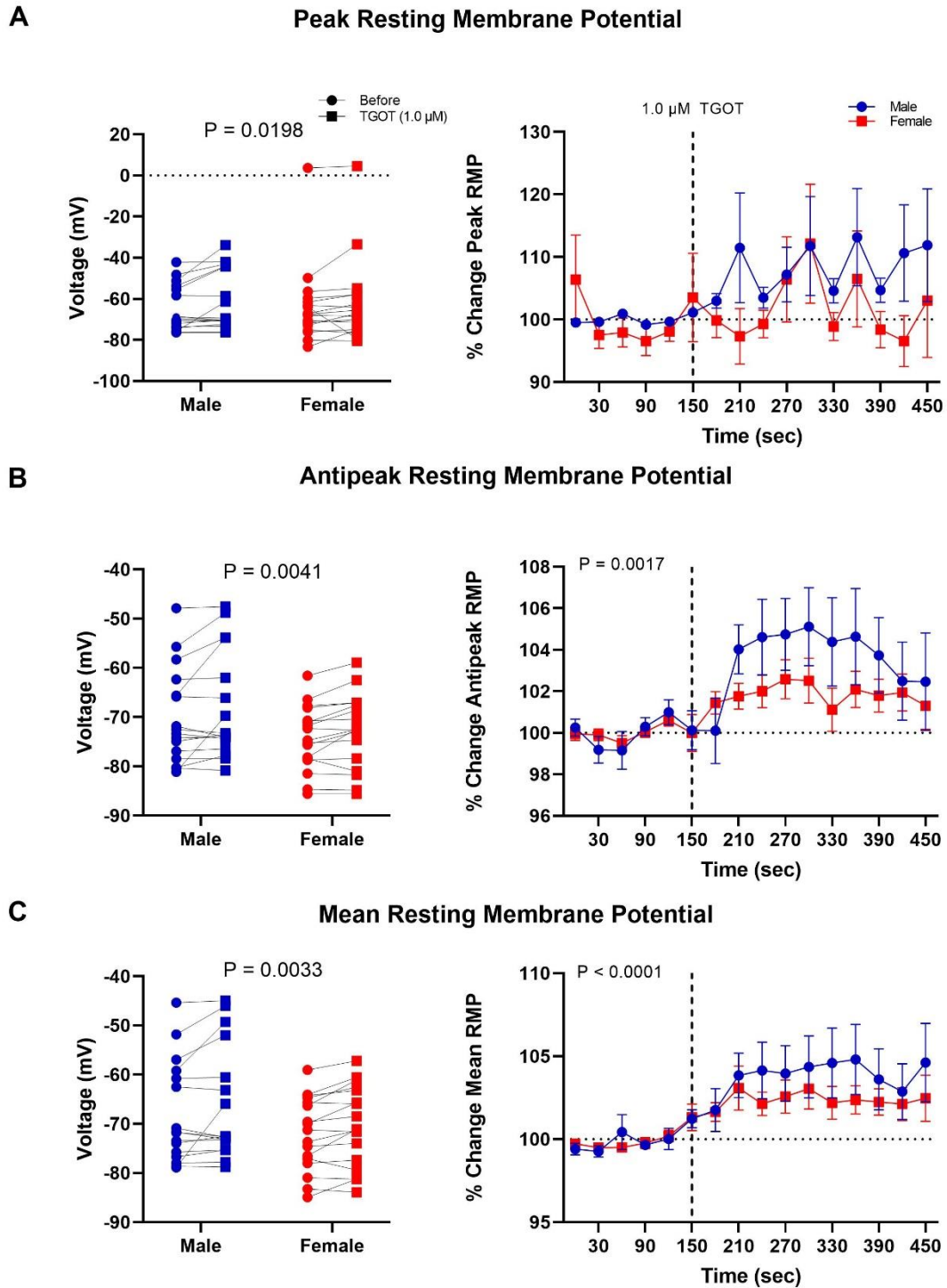
MSN RMP was recorded continuously in response to 0.2  $\mu$ M TGOT application (Figure 1.1B). No differences in peak RMP was detected in response to 0.2  $\mu$ M TGOT in both the non-normalized (Figure 1.3A, left; Interaction:  $F_{(1,6)}=0.27$ ,  $P=0.62$ ; Sex:  $F_{(1,6)}=0.09$ ,  $P=0.78$ ; Drug:  $F_{(1,6)}=1.07$ ,  $P=0.34$ ) or normalized analysis (Figure 1.3A, right; Interaction:  $F_{(8,48)}=0.69$ ,  $P=0.70$ ; Sex:  $F_{(1,6)}=0.61$ ,  $P=0.47$ ; Drug:  $F_{(1,6)}=1.59$ ,  $P=0.25$ ). No differences in antipeak RMP was detected in response to 0.2  $\mu$ M TGOT in both the non-normalized (Figure 1.3B, left; Interaction:  $F_{(1,6)}=0.37$ ,  $P=0.57$ ; Sex:  $F_{(1,6)}=6.35$ ,  $P=0.05$ ; Drug:  $F_{(1,6)}=0.07$ ,  $P=0.80$ ) or normalized analysis (Figure 1.3B, right; Interaction:  $F_{(8,48)}=0.11$ ,  $P=1.00$ ; Sex:  $F_{(1,6)}=0.03$ ,  $P=0.87$ ; Drug:  $F_{(1,8)}=2.75$ ,  $P=0.14$ ). Interestingly, a significance between sex was indicated in the non-normalized data analysis but not the normalized analysis. No differences in mean RMP was detected in response to 0.2  $\mu$ M TGOT in both the non-normalized (Figure 1.3C, left; Interaction:  $F_{(1,6)}=0.03$ ,  $P=0.88$ ; Sex:  $F_{(1,6)}=2.36$ ,  $P=0.18$ ; Drug:  $F_{(1,6)}=0.19$ ,  $P=0.68$ ) or normalized analysis (Figure 1.3C, right; Interaction:  $F_{(8,48)}=0.09$ ,  $P=1.00$ ; Sex:  $F_{(1,6)}=0.02$ ,  $P=0.89$ ; Drug:  $F_{(1,7)}=2.50$ ,  $P=0.16$ ). These analyses collectively indicate there is no significant RMP response to the application of 0.2  $\mu$ M TGOT, and that the results are not largely distinguished between sexes. There is a possibility of a sex difference in antipeak, however, the preponderance of data do not strongly support this specific conclusion.



**Figure 1.3.** No response was detected in RMP when exposed to 0.2 $\mu$ M TGOT. Interestingly, a sex difference was demonstrated in non-normalized antipeak RMP. A, left) Non-normalized peak RMP. A, right) Normalized peak RMP. B, left) A significance in sex was found in non-normalized antipeak RMP (Sex:  $F_{(1,6)}=6.35$ ,  $P=0.05$ ), but not in drug response. B, right) Non-normalized antipeak RMP. C, right) Non-normalized mean RMP. C, left) Normalized mean RMP.

TGOT (1.0  $\mu$ M) application depolarizes the RMP in both males and females

MSN RMP was recorded continuously in response to 1.0  $\mu$ M TGOT application (Figure 1.1C). A depolarization in peak RMP was detected in response to 1.0  $\mu$ M TGOT in the non-normalized (Figure 1.4A, left; Interaction:  $F_{(1,31)}=0.83$ ,  $P=0.37$ ; Sex:  $F_{(1,31)}=0.00$ ,  $P=1.00$ ; Drug:  $F_{(1,31)}=6.04$ ,  $P=0.02$ ) but not the normalized analysis (Figure 1.4A, right; Interaction:  $F_{(15,465)}=0.66$ ,  $P=0.82$ ; Sex:  $F_{(1,31)}=1.52$ ,  $P=0.23$ ; Drug:  $F_{(4,111)}=1.42$ ,  $P=0.22$ ). A depolarization in antipeak RMP was detected in response to 1.0  $\mu$ M TGOT in both the non-normalized (Figure 1.4B, left; Interaction:  $F_{(1,31)}=0.51$ ,  $P=0.48$ ; Sex:  $F_{(1,31)}=2.39$ ,  $P=0.13$ ; Drug:  $F_{(1,31)}=9.58$ ,  $P=0.00$ ) and the normalized analysis (Figure 1.4B, right; Interaction:  $F_{(15,465)}=1.11$ ,  $P=0.35$ ; Sex:  $F_{(1,31)}=0.97$ ,  $P=0.33$ ; Drug:  $F_{(3,88)}=5.68$ ,  $P=0.00$ ). A depolarization in resting RMP was detected in response to 1.0  $\mu$ M TGOT in both the non-normalized (Figure 1.4C, left; Interaction:  $F_{(1,31)}=0.13$ ,  $P=0.72$ ; Sex:  $F_{(1,31)}=1.73$ ,  $P=0.20$ ; Drug:  $F_{(1,31)}=10.12$ ,  $P=0.00$ ) and the normalized analysis (Figure 1.4C, right; Interaction:  $F_{(15,465)}=0.60$ ,  $P=0.88$ ; Sex:  $F_{(1,31)}=0.64$ ,  $P=0.43$ ; Drug:  $F_{(15,465)}=6.89$ ,  $P<0.00$ ). Overall, these analyses indicate that there is a depolarizing response in the RMP in response to 1.0  $\mu$ M TGOT in both males and females.



**Figure 1.4.** Depolarizing change in RMP was detected in response to TGOT (1.0  $\mu\text{M}$ ). A, left) Non-normalized peak RMP (Drug:  $F_{(1,31)}=6.04$ ,  $P=0.02$ ). A, right) Normalized peak RMP. B, left) Non-normalized antipeak RMP (Drug:  $F_{(1,31)}=9.58$ ,  $P=0.00$ ). B, right) Non-normalized antipeak RMP (Drug:  $F_{(3,88)}=5.68$ ,  $P=0.00$ ). C, right) Non-normalized mean RMP (Drug:  $F_{(1,31)}=10.12$ ,  $P=0.00$ ). C, left) Normalized mean RMP (Drug:  $F_{(15,465)}=6.89$ ,  $P<0.00$ ).

## Discussion

The data obtained conveyed two key findings. MSNs in the NAc displayed a depolarizing change in RMP in response to OXTR activation following exposure to 1.0  $\mu\text{M}$  OXTR-agonist, TGOT. This is an important finding given that it is the first to show how OXTR activation can regulate a fundamental electrophysiological property of NAc MSNs. No significant changes in RMP were seen in response to control ACSF or a lower dose of TGOT (0.2  $\mu\text{M}$ ), showing a dose dependent effect. Interestingly, a possible sex difference was indicated in the non-normalized antipeak RMP analysis of 0.2  $\mu\text{M}$  TGOT, but not in the normalized analysis. A significant difference between sex was not detected elsewhere in any of our analyses. Thus, the preponderance of evidence suggests that RMP is not regulated by OXT in a sex-specific fashion, largely suggesting that the sexually differential behavior exhibited in social play is not modulated in this specific mechanism but exists elsewhere. Overall, the data provided validates one area of our hypothesis in which OXTR activation of MSNs in the NAc modulate electrophysiological properties by demonstrating a depolarizing response. However this response does not differ by sex, which invalidates part of our hypothesis, but provides valuable insight.

Our experiment was designed to test the influence of OXTR activation on the RMP. OXT is structurally similar to vasopressin (AVP) and can potentially bind and activate vasopressin receptors (AVPR) (Anacker et al., 2016; Bous et al., 2023; Chini et al., 2008; Rae et al., 2022; Sala et al., 2011; Song et al., 2014). Therefore, the OXTR selective agonist TGOT was used in our experiment instead of OXT to avoid any possible confounding effects. Other studies investigating similar electrophysiological properties have demonstrated an increase in neuronal excitability in response to TGOT and/or OXT in other brain regions, including the neurons in the endopiriform nucleus (EPN) and CA2 hippocampal neurons in male and female mice (Biggs &

Hammock, 2022; Tirko et al., 2018). There are several explanations for this mechanism. For example, in gonadotropin-releasing hormone (GnRH)-positive immortalized GN11 murine cells, OXTR activation is associated with an inhibition of inward rectifying potassium channels, which lead to depolarization and decreased potassium conductance across the membrane (Gravati et al., 2010). Thus, one possibility of OXTR electrophysiological modulation of MSN RMP is through the inhibition of a membrane spanning potassium ion channel, such as an inward rectifying potassium channel. Leak sodium channels also can contribute to membrane potential. In the CA2 pyramidal neurons of male and female mice, an inward tetrodotoxin-resistant (TTX-R) sodium current was identified and found to be activated by OXTR activation, promoting neuron excitability (Liu et al., 2022). Thus, it is likely an ionic mechanism is influencing changes in RMP excitability in response to OXTR activation, given that RMP is maintained by sodium and potassium leak channels (Chrysafides et al., 2024). These two hypotheses could be tested by directly targeting these ion channels via voltage clamp coupled with the appropriate agonists and/or antagonists. For example, OXTR activation may decrease the magnitude of potassium currents and/or conversely increase the magnitude of sodium currents. Aside from ions, neurotransmitters such as glutamate can also influence excitability. Malenka and colleagues examined the effect of OXTR activation in the NAc in socially conditioned vs socially isolated male mice by measuring excitatory post synaptic currents (EPSCs) in the context of social reward. When glutamate is bound to  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, an influx of sodium is sent into the synapse, influencing neuronal excitability. It was discovered that long term depression (LTD) of EPSCs was induced via OXTR activation when bound to OXT (Dölen et al., 2013). EPSC magnitude was decreased in slices from socially conditioned mice vs isolated mice, demonstrating mice with social

experience influence the generation of OXT-LTD. Glutamatergic circuits and OXT neuron firing have also been studied in association with the neural mechanisms underlying lactation (Leithead et al., 2021; Stern et al., 2000), revealing an increase of OXT release dependent upon glutamatergic transmission. Stern and colleagues investigated this further by investigating the EPSCs from identified OXT neurons in the supraoptic nuclei (SON) from adult virgin and lactating rats. They found that the frequency of MAPA-mediated miniature EPSCs (mEPSCs) more than doubled during lactation, suggesting an increase in membrane potential driven by glutamatergic inputs in response to OXT release. Thus, glutamatergic inputs and their close association with OXT firing could also be a likely mechanism for increasing excitability. We also note that NAc MSNs receive tonic GABAergic input (Ade et al., 2008), which could be another avenue by which OXTR activation could regulate RMP.

Our experiment was also designed to investigate SABV to account for potential sex differences seen in social play. Our analyses did not detect a difference in sex, excluding analysis of non-normalized antipeak RMP in response to 0.2  $\mu$ M TGOT application exposure. Given that this response was not seen elsewhere in our analyses, the preponderance of data points to no sex difference. Although no sex difference was uncovered using this particular methodology, it would be inaccurate to conclude that there is no sex difference in social play behavior at all. Rather, this result suggests the mechanism modulating sexually differentiated behavior in social play is elsewhere. This could mean a different neuromodulator, a different electrophysiological mechanism or in a different part of the relevant circuit. Regarding neuromodulators, a likely possibility would be AVP. AVP activates AVP and OXT receptors, and is heavily implicated in social behaviors (Bredewold & Veenema, 2018; Dumais & Veenema, 2016; Rae et al., 2022; Rigney et al., 2023). Regarding electrophysiological mechanisms, one likely target for

investigation could be excitatory glutamatergic input, perhaps by assessing spontaneous excitatory postsynaptic currents. Regarding other brain regions, one possibility is the lateral septum (LS). For example, in another study conducted on juvenile rats and social play behavior, OXT was injected into the lateral septum (LS) of the brain, which resulted in decreased social play behavior in females but not males (Bredewold et al., 2014). Other studies in rodents have also reported similar results, suggesting a reduction in pro-social behavior and an increase in anxiety is mediated by OXTRs in the LS (Beery et al., 2008; Beery & Zucker, 2010; Guzmán et al., 2013; Olazábal & Young, 2006). This demonstrates the complexity of the OXT system on sex-specific social behaviors, indicating that brain-region specific effects may be at play.

Several limitations are presented in the data above. Our experiment only utilized 4 controls with more males than females, and it would be important to increase this number and employ a balanced approach towards sex. Also, our experiments only employed a relatively restricted prepubertal age range. While this age range is justified due to the exhibition of abundant and sex-specific social play behaviors, it is possible that sex differences or other OXTR activation processes may differ across the prepubertal period or emerge post-puberty. One study in mice found exposure to OXT influenced the frequency of miniature excitatory postsynaptic currents (mEPSCs) in pyramidal neurons at different pre-pubertal age ranges (Zhang et al., 2021). mEPSCs were found to be significantly increased around P10 and P14, however reduced at ages P22 and P28. So it may be plausible that the RMP response to OXT could differ by age even within the pre-puberty period. Additionally, our methodology only tested the effect of TGOT on OXTR to avoid possible confounding effects, however OXT itself was not utilized. Although exposure to both TGOT and OXT in other studies have demonstrated similar depolarizing effects (Biggs & Hammock, 2022; Liu et al., 2022; Tirko et al., 2018), this has not

been investigated in the MSN of the NAc of rats and should be tested to accurately reflect physiological processes, even if it stimulates both AVP and OXT receptors. OXT signaling involves several brain regions and OXTRs have been found on other neurons expressing other neurotransmitters, for example, serotonergic neurons in the raphe nucleus (Biggs & Hammock, 2022; Oubrain et al., 2023) Thus, indirect effects from other neurotransmitters should be considered in future experimentation.

Ultimately, OXTR activation of MSNs in the NAc modulates electrophysiological properties of MSNs in the NAc, demonstrated by a depolarization in RMP in response to 1  $\mu$ M TGOT. It is a fundamental first step to understanding how OXT regulates social behavior via the brain mesolimbic reward circuitry during the juvenile period. While this finding was not sex specific, it establishes the fact that the sex difference seen in social play is not found within our investigated mechanism and allows the potential exploration of another pathway. These crucial findings are the foundations for understanding how neurodevelopmental disorders are affected by social play behavior and expose the potential for more effective targeted therapeutic strategies.

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## CHAPTER 2

### **The complex presentation of sex bias and omission in neuroscience research**

Research in this chapter will be submitted to a peer-reviewed journal:

Liu, A., Vainorius, G., Hedrick, M., Dorris, D., Beeson, A.L.S., Fletcher, S., Crawford, A.,

Meitzen, J. (2024): The complex presentation of sex bias and omission in neuroscience research.

*In progress.*

## **Abstract**

Despite known sex differences in the brain, behavior, and neurological disorders, neuroscience preclinical research has historically exhibited a sex bias, defined as male research models being favored over their female counterparts. In addition, neuroscience research has historically demonstrated sex omission, the lack of sex reporting. Here we extended previous studies by analyzing sex bias and omission in research articles published across six neuroscience journals in 2020. Regarding sex omission, 14% of articles did not report sex. Compared to previous years, sex omission appears to be declining. Regarding sex bias, 23% of articles used only males, while 5% utilized only females. Though 57% of the articles utilized both sexes, only 17% of these considered sex as a biological variable (SABV). Compared to previous years, sex bias is moderating yet remains persistent. Sex bias and omission varied widely across research models. 34 different research model types were identified in the neuroscience literature. Most studies employed one type of research model, however 6.67% of articles employed more than one. The percentage of articles exhibiting multiple research models correlated strongly with the impact factor of the originating journal. Overall, these findings illustrate the complex and changing picture of sex bias and omission in neuroscience research. While sex bias and omission are declining compared to previous years, they remain persistent.

## **Introduction**

The underreporting of sex in neuroscience research has demonstrated a historical pattern of repetition in which sex bias and omission persist in literature. Sex bias is defined as a favoritism of one sex over another, a concept which neuroscience literature has continuously exhibited by utilizing male models over females (Beery & Zucker, 2011; Berkley, 1992; Mamlouk et al., 2020; Mogil & Chanda, 2005; Shansky & Woolley, 2016; Will et al., 2017; Woolley, 2021). Sex omission is defined as the absence of reporting research model sex when applicable. These concepts are not limited to neuroscience and extend into other biomedical fields (Kim et al., 2021; McCarthy, 2015; Potluri et al., 2017; Woitowich et al., 2020). Although single sex use may be justified in appropriate circumstances, the lack of justification in scientific literature has provoked significant discussion across various biomedical disciplines. As a result, new journal regulatory policies have been created to better support the scientific advancements of men and women (Clayton, 2018; Clayton & Collins, 2014).

Understanding the changing complexity of sex bias and omission in neuroscience literature is crucial. Research articles are emphasized as they are essential as both an accumulation of previous findings and a foundation for new research and discovery. An initial comprehensive study of neuroscience literature examined articles published between 2010 and 2014 utilizing only mice and rats (Will et al., 2017). In this analysis, sex omission decreased while sex bias remained persistent. While an increased amount of articles did report sex of models, sex was seldom considered an experimental variable. The most recent comprehensive analysis of neuroscience research articles examined studies published in 2017 (Mamlouk et al., 2020). On January 25 2016, the implementation of the National Institute of Health (NIH) Sex as a Biological Variable (SABV) (NOT-OD-15-102) regulatory policy was placed (Clayton, 2018;

Clayton & Collins, 2014). Thus, NIH funded and non-NIH funded studies were analyzed to assess if sex bias and omission differed by funding status. Little evidence was found that NIH funding status influenced sex bias and omission. In this study, all research models utilized were considered for analysis. It was found sex bias and omission varied across research models. Across journals, sex omission varied though sex bias levels appeared similar. Although more than half of analyzed articles utilized both male and female models, only a comparatively few considered SABV.

The persistence of sex omission and bias across journals even after the advent of regulatory policies addresses a challenge for sex-specific scientific and biomedical progression. As the most recent data available in regards to sex bias and omission in neuroscientific literature dates back to 2017, there has since been a lack of data. Here we expand on this knowledge by assessing sex bias and omission in neuroscience research articles published in 2020 across six journals: Journal of Neuroscience, Journal of Neurophysiology, Science, Neuron, Nature and Nature Neuroscience. These journals were selected given their significance within the neuroscience field and to align with previous studies (Mamlouk et al., 2020; Will et al., 2017). Articles with multiple models were analyzed against journal impact factor. Sex bias and omission were assessed in the context of research model and journal.

## **Methods and Materials**

### *Article inclusion criteria and analysis*

Methods for research article inclusion and analysis follow those previously published (Mamlouk et al., 2020; Will et al., 2017). Briefly, research articles published in the year 2020 were analyzed from the following journals: Journal of Neuroscience, Journal of Neurophysiology, Science, Neuron, Nature, and Nature Neuroscience. 8 trained curators (5 female, 3 male) analyzed all published research articles in these journals within 2020 to minimize sampling bias. Trained curators were employed because the divergent and extensive vocabulary used to describe animal sex and its treatment as an experimental variable make automated text mining approaches challenging. Inter-curator reliability was evaluated as in previous studies (Will et al., 2017; Mamlouk et al. 2020). Curators first determined whether an article was a primary research article. Reviews, editorials and non-primary research articles were excluded from further analysis. Research articles were then assessed for neuroscience relevance using a broad inclusion criterion. Articles originating from the Journal of Neurophysiology, Journal of Neuroscience, Neuron and Nature Neuroscience were automatically accepted as neuroscience relevant. Articles from Nature and Science were accepted if the article topic encompassed any aspect of the nervous system, ranging from the molecular to behavioral level of analysis.

### *Research model coding*

Articles were then coded for species or research model. Research models represented in the overall data set were categorized as follows: amphibia (including *X. laevis*, salamanders, other frogs), aplysia, *C. elegans*, cats, crabs, drosophila, ferrets, fish: other (including goldfish, electric fish, lampreys, other fish), gerbils, guinea pigs, humans, immortalized cell lines, in silico

models, insects: other (including bumblebees, cockroaches, locusts, crickets, mosquitos), invertebrates: other (including leeches, oysters, lobsters), mammals: other (including dogs, pigs, bats), mice, non-human primates (including marmosets, chimpanzees, monkeys), non-oscine birds (including chickens, owls, pigeons), oscine birds (including finches, sparrows, crows), rabbits, rats, reptilia (including turtles, bearded dragons), rodents: other (including ferrets, chinchillas), zebrafish. “Other” categories were implemented with the purpose of grouping species with little representation. Articles using primary cell cultures or embryonic animals were categorized with species of origin, following previous studies (Will et al., 2017; Mamlouk et al. 2020). Articles using immortalized cell lines were coded as an individual research model, immortalized cell lines, following previous studies. Immortalized cell lines were categorized as a unique model system given that this research model exhibits common aspects that are shared across origin species. Articles using purely computational and/or mathematical models with no originative use of model organisms were categorized as *in silico*. Multiple species used within one article were each analyzed individually. This protocol thus organized the articles into a pool of 2,167 entries categorized by research model.

#### *Article entry analysis*

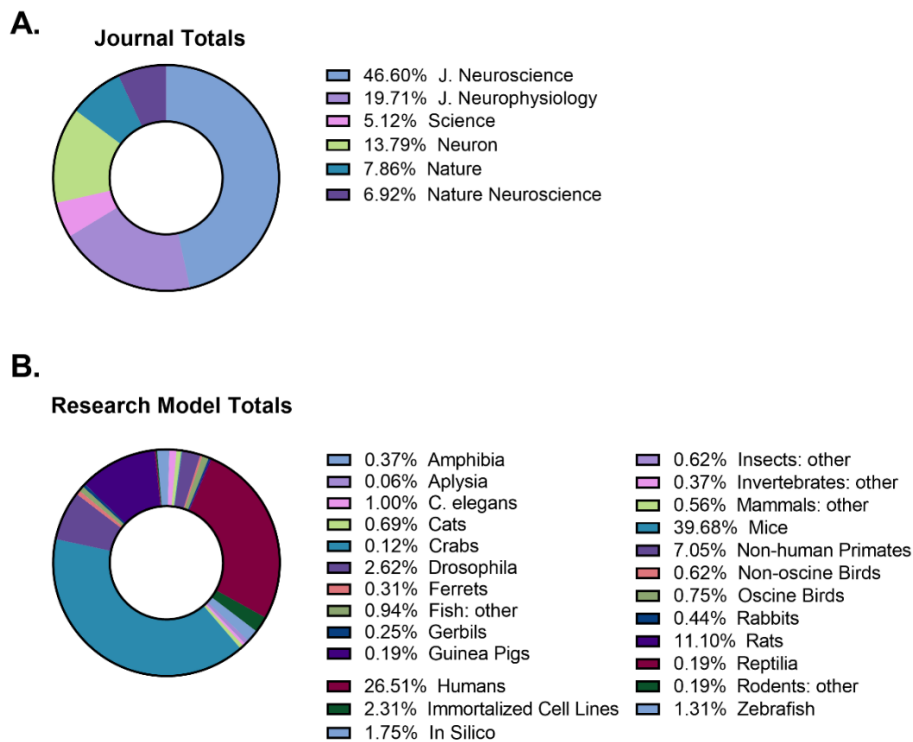
Article entries were then analyzed for biological sex. Sex categories included: male, female, male and female wherein biological sex was considered an experimental variable, male and female wherein biological was not considered an experiment variable, sex not reported and hermaphrodite. Following a broad inclusion paradigm, articles were considered to have addressed SABV if any formal statistical comparison of assertion of such a comparison of males and females was performed, including the use of sex as a covariate, whether the data or analysis was shown or not shown, and also including whether sex differences were detected or not. No

articles reported data disaggregated by sex but did not perform or assert to have performed a statistical comparison. When distinct experiments within an article differentially reported sex in the same research model, articles were coded using the most descriptive experiment. For example, if one experiment did not report sex in mice, while another experiment reported using combined male and female mice, then the article was coded as “mice: male and female wherein biological was not considered an experiment variable.” When distinct experiment within an article solely employed different sexes, then articles were coded male/female, biological sex not considered, following previous studies (Beery and Zucker, 2011; Will et al., 2017; Mamlouk et al. 2020). Data was analyzed via linear regression and chi-squared test (Prism version 9.5.1, Graphpad Software). P values <0.05 were considered as significant.

## Results

### Qualities of neuroscience articles published in 2020

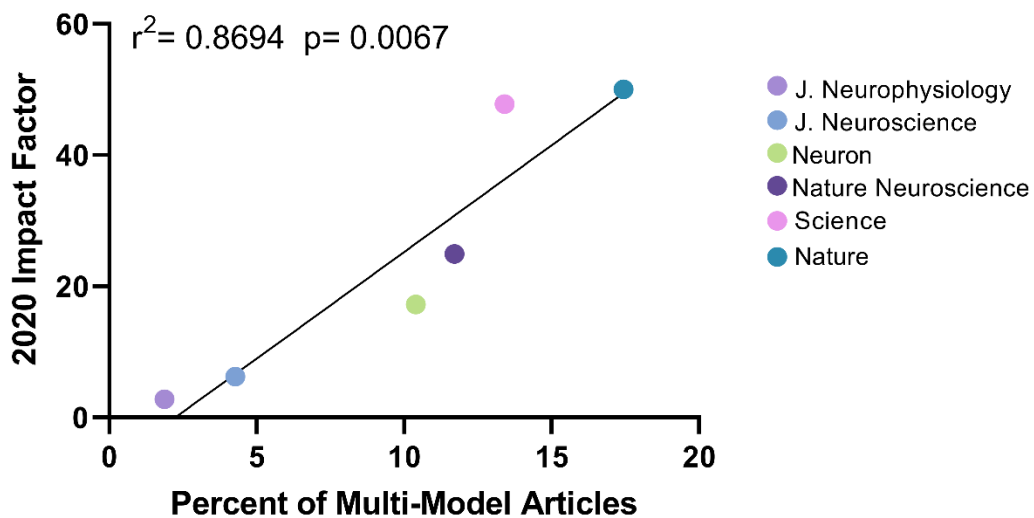
Articles were obtained from six neuroscience journals of varying impact factor: The Journal of Neuroscience, Neuron, Nature, Nature Neuroscience, Science, and The Journal of Neurophysiology. Each journal contributed a different number of articles to the analyzed pool, with The Journal of Neuroscience contributing the greatest (46.60%), subordinated by The Journal of Neurophysiology (19.71%), Neuron (13.729%), Nature (7.86%), Nature Neuroscience (6.92%) and Science (5.12%) (Figure 2.1A). A wide range of research models were employed across all research articles (Figure 2.1B). Mice were the most prevalent model (39.68%), followed by humans (26.51%), rats (11.10%), and non-human primates (7.05%). The remaining 15.66 % of articles spanned 21 other research model classifications



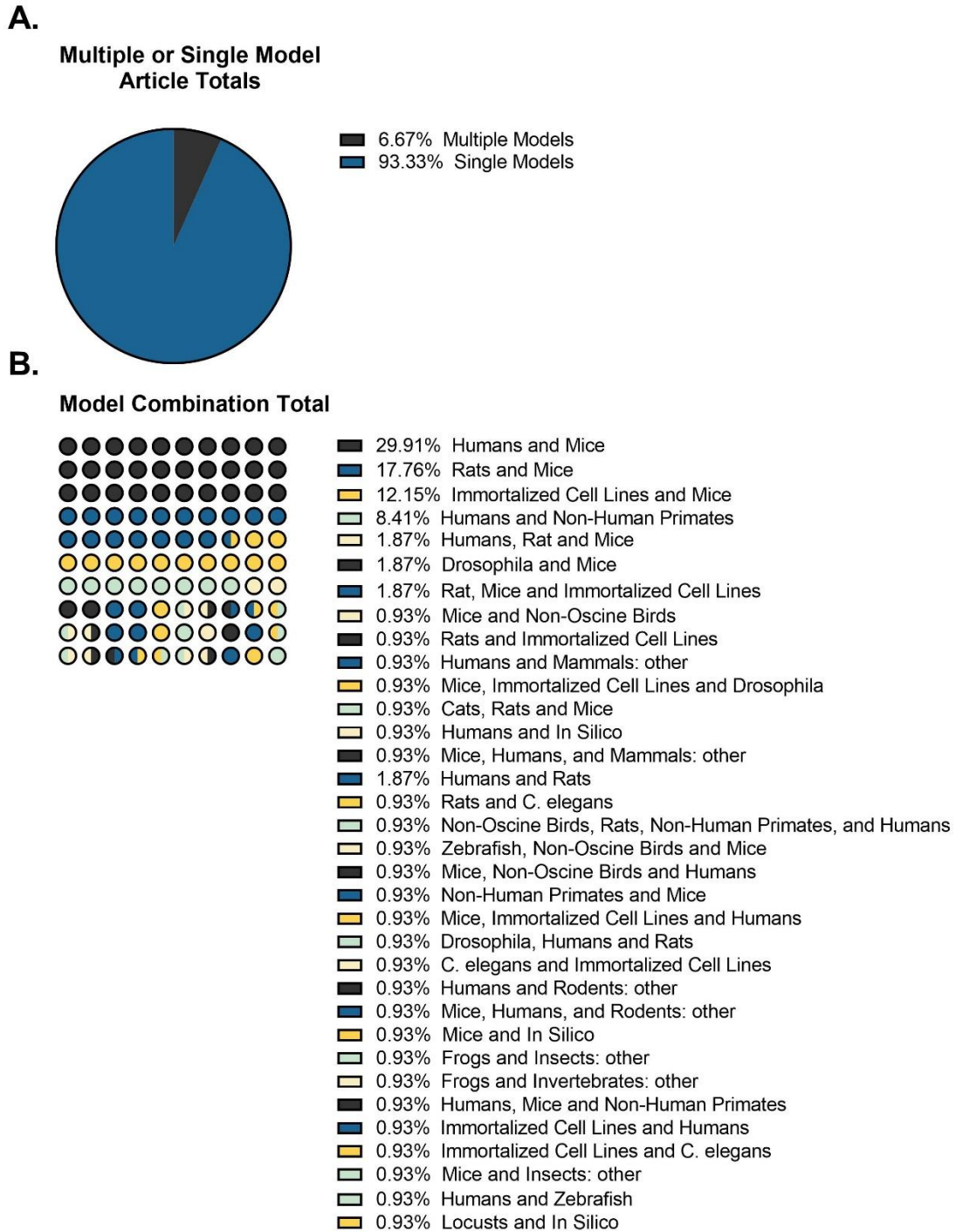
**Figure 2.1.** Journal article total and research model in journals published in 2020. A) Journal article total. J. Neuroscience contributes the largest proportions of articles, while science contributes the least. B) Research model total. Mice, rats and humans made up the top 3 models employed.

Select articles employed more than one model classification

6.67% of articles employed more than one model classification (Figure 2.3A). These articles featured numerous combinations of models (Figure 2.3B). The most common model combinations were humans and mice (29.91%), followed by rats and mice (17.76%), and immortalized cell lines and mice (12.15%). The percentage of articles employing more than one model correlated significantly with the 2020 journal impact factor (Figure 2.2)( $r^2= 0.8694$ ,  $p= 0.0067$ ), with Nature (impact factor 49.96, 17.46% employing more than one model) and Science (impact factor 47.72, 13.41% employing more than one model) showing the highest percentage of multi-model articles.



**Figure 2.2.** Percent of multi-model neuroscience articles published in 2020 correlated significantly with 2020 impact factor. Nature and Science had the most amount of multi-model use.



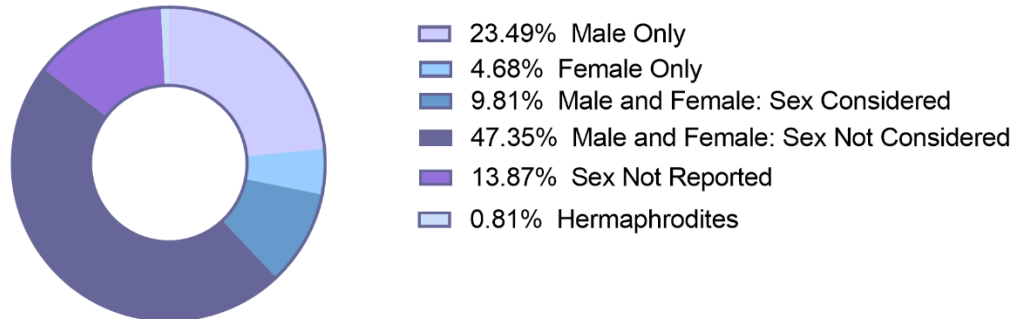
**Figure 2.3.** Some journal articles employed more than one model in their study. A) Single vs multiple model article totals. Most articles in 2020 employed only one model in their research. B) Model combinations across articles that employed multiple models. Humans and mice were the most popular combination utilized.

*Sex bias and omission continue to decline but are still evident in neuroscience studies*

Articles were classified as employing only males, only females, males and females without consideration of sex as an experimental variable, males and females with consideration of sex as an experimental variable, hermaphrodites, or not reporting sex (Figure 2.4A). Articles utilizing males and females without consideration of sex as an experimental variable represented 47.35% of total articles, followed by male only (23.49%), sex not reported (13.87%), males and females with sex considered as an experimental variable (9.81%), female only (4.68%) and hermaphrodites (0.81%). Compared to articles published in 2017, the percentage of articles employing males and females without consideration of SABV continues to climb from ~44% to ~47% and remains the largest proportion of the neuroscience literature. Sex bias remained present, with more articles featuring only males compared to only females, similar to 2017 (male only ~26%, female only ~5%). Overall sex omission declined compared to 2017, dropping from ~16% to ~14%. While sex appears to be widely acknowledged given the dataset, the number of articles considering sex as an experimental variable is relatively small (9.81%), though increasing from 2017 (8%). This lack of employing sex as an experimental variable is highlighted when directly comparing articles employing male and females. Of these articles, approximately 83% did not include sex as an experimental variable when compared to the ~17% of articles that did incorporate sex as an experimental variable (Figure 2.4B). These analyses indicate that overall sex bias and omission continues to decline, while still remaining persistent and complex.

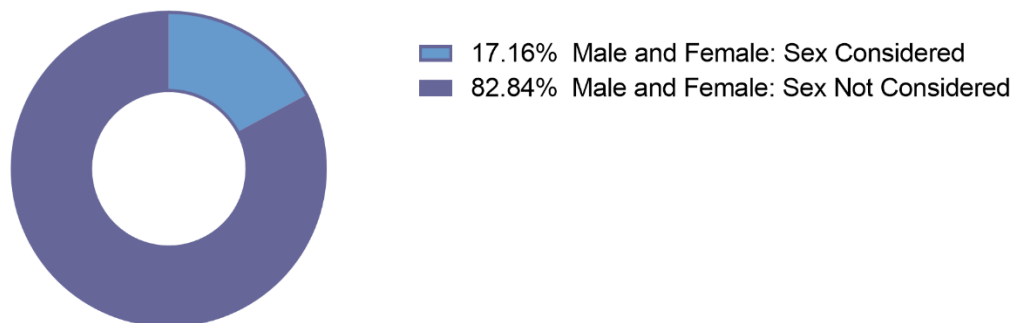
**A.**

**Percent of total articles**



**B.**

**Percent of articles with males and females**



**Figure 2.4.** Distribution of sex bias and omission in neuroscience articles published in 2020. A) The majority of studies reported sex in their methodology, however sex is largely not considered. Male models are employed in frequently than females. B) Within the proportion of articles that employed male and female models, SABV is not widely demonstrated.

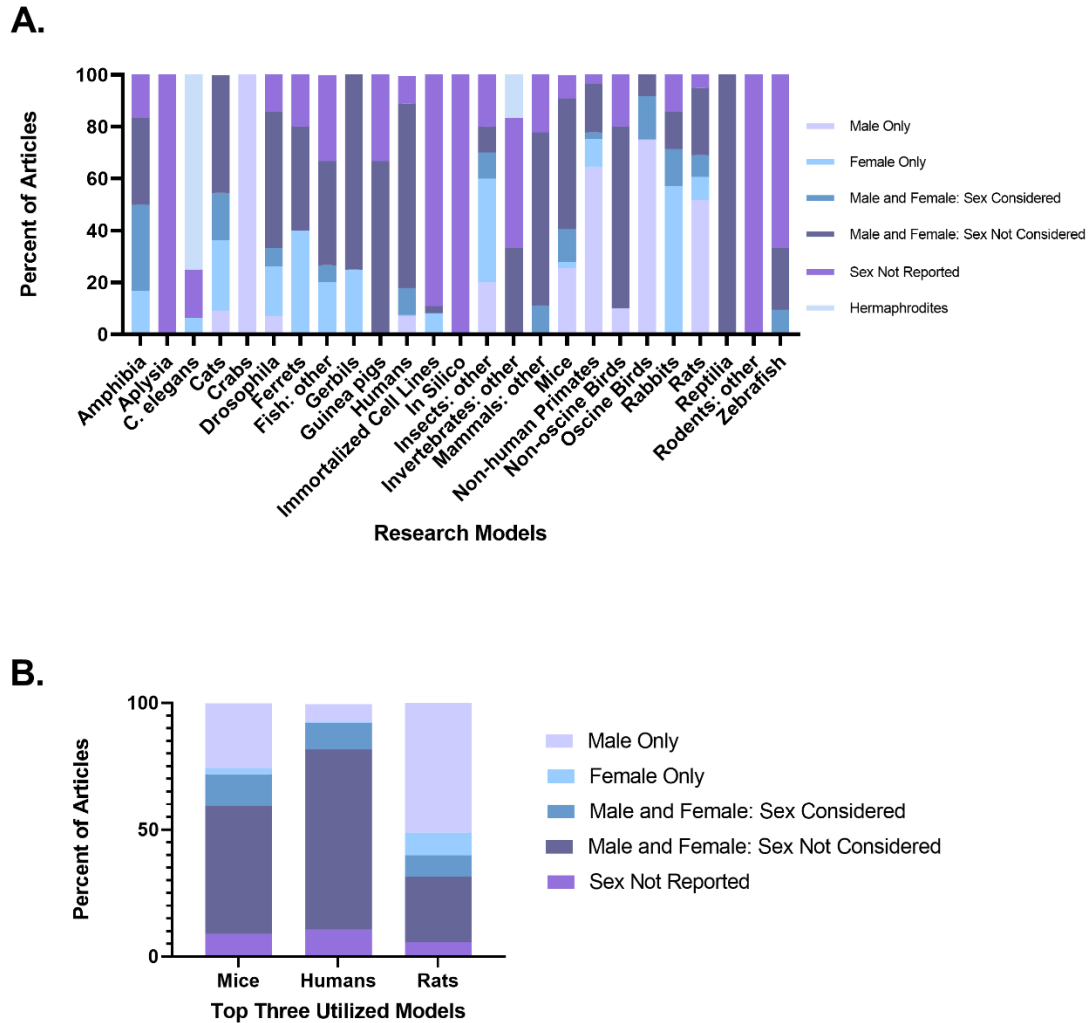
Research model influences sex bias and omission

Research models varied in every aspect of sex bias and omission (Figure 2.5), including the representation of articles which utilized only male models ( $\chi^2= 1227, p < 0.001$ ), only female

models ( $\chi^2 = 543.7$ ,  $p < 0.00$ ), hermaphrodites ( $\chi^2 = 1395$ ,  $p < 0.001$ ), the proportion which did not consider sex as an experimental variable ( $\chi^2 = 601.1$ ,  $p < 0.001$ ), did consider sex as an experimental variable ( $\chi^2 = 271.2$ ,  $p < 0.001$ ) and did not report sex at all ( $\chi^2 = 922.9$ ,  $p < 0.001$ ) (Figure 2.5A). When examining the top three most commonly employed research models, this diversity is emphasized (Figure 2.5B). Mice were the most commonly used species, with approximately 40% of articles utilizing this model. Of these, only 13% considered sex as an experimental variable when using male and females while 50% did not consider sex as an experimental variable when using male and females. Approximately 26% used only males, 2% used only females and 9% did not report sex. Following mice were humans, comprising about 27% of articles. When employing both male and females, 71% of human articles did not consider sex as an experimental variable while 10% did consider sex as an experimental variable. 7% used males only, 0.5% used females only and 11% did not report sex at all. Rounding out the top three research models were rats, being employed by 11% of articles. Rat articles exhibited a different pattern, with 26% of articles using both male and female rats did not consider sex as an experimental variable and 8% of rat articles using both sexes did consider sex as an experimental variable. 52% used males only, 9% females only, and 6% did not report sex.

Beyond the most commonly used research models, sex bias and omission widely diverged. In regards to sex bias, one extreme highlights the favoritism of one sex over another. For example, crabs feature 100% use of males, followed by oscine birds (75% of males). The opposite extreme is also present, with animals such as ferrets and rabbits featuring increased female use compared to other research models (57% and 40% female respectively). Importantly, scientific justifications in usage of one sex or another can be provided regarding certain models and experimental goals. In the most commonly employed oscine songbird model, the zebra finch,

only males typically exhibit song behavior and the associated neural substrate, and thus these studies normally employ male only models (Nixdorf-Bergweiler, 2001; Wade, 2001; Wade & Arnold, 2004). In genetic disorders, males may be utilized to study X-linked disorders due to only possessing one X chromosome, unlike females (Gilbert et al., 2020). On the other hand, it would be logical to use only female models to assess processes such as reproduction (Proaño & Meitzen, 2020; F. Wang et al., 2020). Between these extremes are research models featuring widespread integration of male and female usage, such as humans (71%), and gerbils (75%). With regards to sex omission, select research models largely fail or neglect to report sex, including immortalized cell lines (89%), aplysia (100%) and reptilia (100%). Meanwhile, other research models such as cats, non-human primates, and amphibia exhibited little to no sex omission. Taken in total, this analysis indicates that variation was wide in regards to sex bias and omission across research models, consistent with previous analyses of prior literature (Beery and Zucker, 2011; Mamlouk et al., 2020, Will et al., 2017).

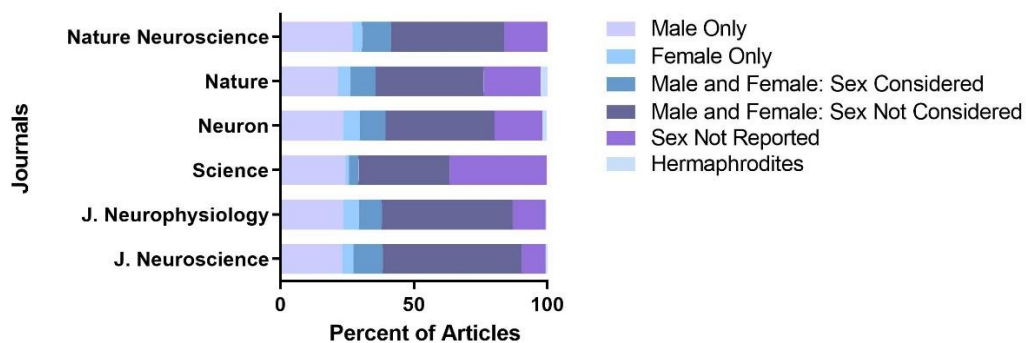


**Figure 2.5.** Sex bias and omission vary greatly across research models. A) All research models. Models were coded into appropriate model categories, showing great variation in employment in regards to sex. B) Top Three Utilized Models. This including mice, humans and rats. Sex reporting across top 3 models also vary.

*Sex omission but not bias varies by journal*

Thus far, sex bias and omission analyses have been analyzed independent of article journals. Previous studies of sex bias and omission have documented differences in sex bias and omission between scientific journals (Beery & Zucker, 2011; Mamlouk et al., 2020; Potluri et al., 2017; Will et al., 2017; Woitowich et al., 2020). Therefore, articles were analyzed by journal to assess whether variability in sex reporting continued across journals (Figure 2.6). When reporting sex bias, journals varied in the proportion of articles that reported males and females

without considering sex an experimental variable ( $\chi^2 = 12.51$ ,  $p = 0.0284$ ). The Journal of Neuroscience reported the highest percentage of male and female employment with sex not considered as an experimental variable (52%), followed by Journal of Neurophysiology (49%), Neuron (41%), Nature Neuroscience (41%), Nature (39%) and Science (23%). There were no differences between the proportion of articles that utilized male and female models and considered sex as an experimental variable ( $\chi^2 = 6.500$ ,  $p = 0.2606$ ), and the usage of males only ( $\chi^2 = 1.864$ ,  $p = 0.8677$ ), females only ( $\chi^2 = 6.250$ ,  $p = 0.2826$ ), or hermaphrodites ( $\chi^2 = 8.000$ ,  $p = 0.1562$ ). Sex bias thus appeared similar across all journals, with an average of 24% favoring male models over females. In regards to sex omission, the proportion of articles that did not report sex varied by journal ( $\chi^2 = 14.42$ ,  $p = 0.0131$ ). Science exhibited the highest levels of sex omission (37%), followed by Nature (21%), Neuron (18%), Nature Neuroscience (16%), Journal of Neurophysiology (12.34%) and Journal of Neuroscience (9%). Sex omission does not correlate with the number of articles published by that journal in 2020 ( $r^2 = 0.4601$ ,  $p = 0.3186$ ).



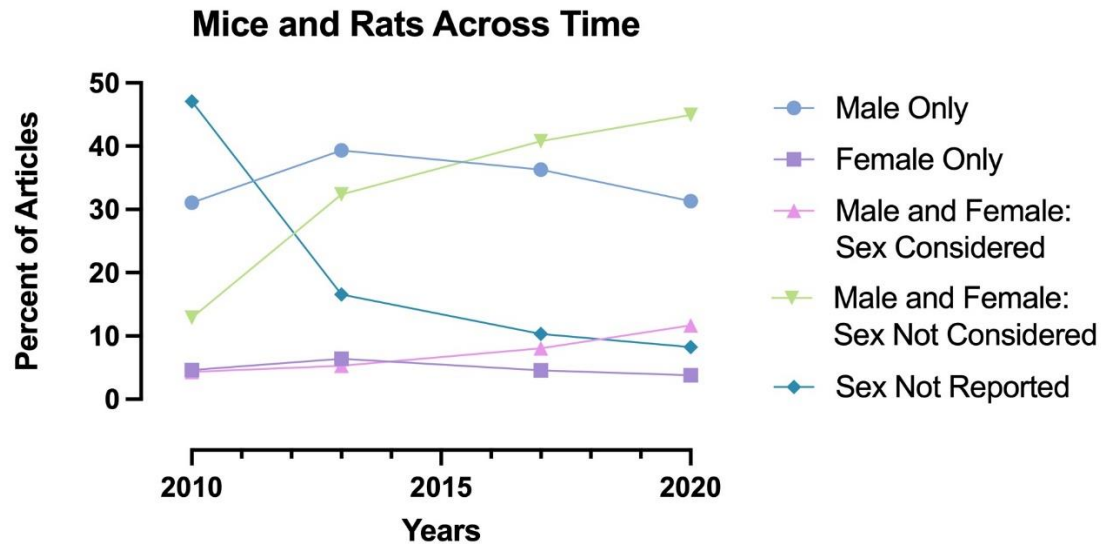
**Figure 2.6.** Sex omission but not bias varies by journal. Sex reporting varies between journals. Across all journals, male models are always employed more than their female counterparts.

## Discussion

The key finding of this study is that while sex bias and omission is declining in neuroscience research literature, it continues to persist. However, there is no simple generalization that can easily illustrate the complex presentation of sex bias and omission within this literature review. While a little over half of total articles (57.08%) utilized both sexes in their experiments, only about 10% considered SABV. This finding reveals that while a majority of neuroscience articles are employing both female and male research models, SABV is still not widely considered. Meanwhile, 14% of articles did not report the sex of their research model. This is further complicated by the variation of sex bias and omission between journal and research model. Additionally, some articles utilized more than one research model. The correlation between multi-model species articles and journal impact factor note the elaborate relationship between model utilization and journal.

Approximately 14% of neuroscience research articles published in 2020 did not report research model sex. However a comparison across time is crucial to fully assess the rate and intensity of sex omission in neuroscience research literature. Previous studies executed by our research group with nearly identical methodology investigated SABV in neuroscience research articles within the same 6 journals between 2010 and 2017 (Mamlouk et al., 2020; Will et al., 2017). The difference in methodology between the studies is that Will and colleagues limited their analysis to assessing only mice and rats. However, it is still critical to assess sex omission across these models and combining this previous data with the current study allows for a thorough understanding of sex omission overtime (Figure 2.7). Will and colleagues found about 47.1% of assessed articles did not report sex in 2010, similar to other investigations of neuroscience literature within that general timeframe (Beery & Zucker, 2011; Shansky &

Woolley, 2016). The rate of sex omission eventually decreased to 16.6% in 2013, 10.3% in 2017 and 8.2% in 2020 from our current study. Thus, while sex omission is improving, it still persists.



**Figure 2.7.** Analysis of mice and rats across time, from 2010 – 2020. Sex bias remains relatively the same since 2010, but sex omission improves across time.

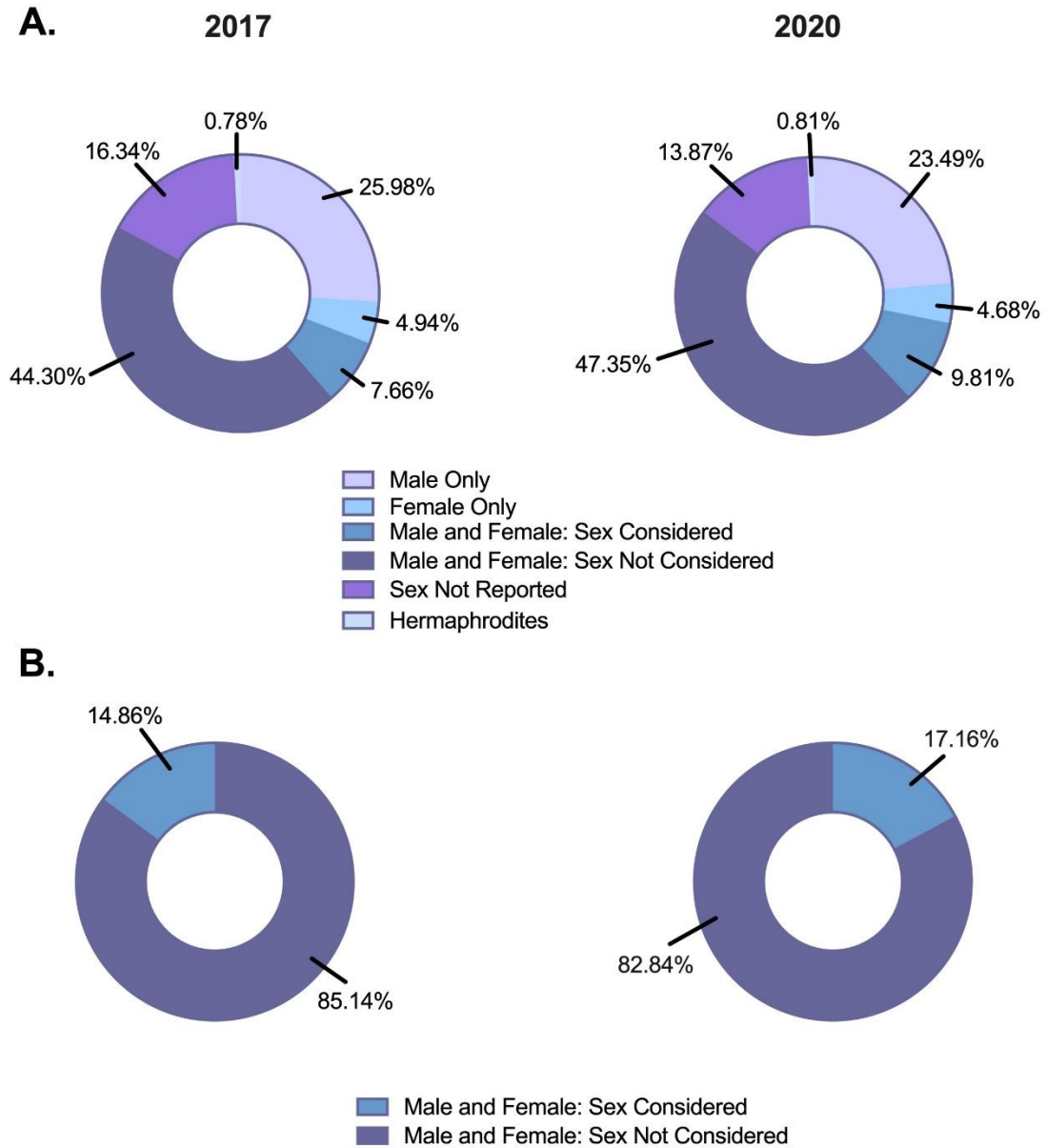
A more recent study from our research group utilizing identical methodology to this current study assessed SABV in neuroscience research articles published in 2017 within the same 6 journals in all research models (Mamlouk et al., 2020). When analyzing across all research models, in 2017, it was found that 16.3% of articles did not report research model sex. In 2020, sex omission decreased to about 13.9%, revealing a continuing marginal improvement in sex reporting (Figure 2.8). Thus, overtime, sex omission appeared to decrease, which is consistent with the finding of another study that employed a different methodology (Woitowich et al., 2020). This attenuation of sex omission appears encouraging. However, there is a major point of concern. Our analysis revealed that sex omission can vary widely among research models. For some models, it seems reasonable that sex would not be as widely reported, such as studies that work with juvenile zebrafish where sex is not readily assessed across the sometimes hundreds of subjects (Harmon et al., 2020; Liew & Orbán, 2014; Ma et al., 2020; E. T. Smith et

al., 2020; Wilson et al., 2014). For other models, it is easier to report sex and it is surprising that sex omission remains high. For example, our study found that articles using cell lines or primary cell cultures as their research model widely do not report sex, which is consistent with previous work from multiple groups (Kim et al., 2021; Mamlouk et al., 2020; Shah et al., 2014).

Interestingly, sex omission is widespread in computational neuroscience projects classified by this study as *in silico*. At first glance, it perhaps seems rational that these studies would not report sex. However, depending upon how the studies are designed and how the source data is collected, processed and labeled, omission could inadvertently introduce significant consequences. The lack of sex reporting in these types of models contribute to the persistence of sex omission, attenuating the progress made in this domain.

Sex bias remained persistent in 2020, with the utilization of male research models more than triple the percentage of their female counterparts across all research models (Figure 2.8). By employing data from previous studies focused upon mice and rats, we can draw conclusions regarding the persistence of sex bias across time, similar to our approach regarding sex omission. In mice and rats, sex bias has remained largely constant since 2010 (Figure 2.7). In 2010, approximately 31.1% of articles only studied males and this percentage increased to 39.3% in 2013. This proportion later decreased to 36.3% in 2017 and then to 31.3% in 2020. The proportion of articles exclusively utilizing female mice and rats also fluctuated in that time period. 4.6% of studies used females only in 2010. This proportion increased to 6.4% in 2013 however decreased in 2017 to 4.6% and continued to decline to 3.8% in 2020. In all research models, the exclusive utilization of only male models decreased from 2017 to 2020, from 26% to 23.5% respectively. However, exclusive utilization of female only models was relatively the same. In 2017, 4.9% of articles used only females and this percentage very slightly decreased to

4.7% in 2020. Complementing these data, the proportion of articles that employed both males and females regardless of SABV grew from 17.2% in 2010, to 37.7% in 2013, to 48.8% in 2017 and 56.6% in 2020 (Figure 2.8).



**Figure 2.8.** Sex bias and omission across all research models in 2017 vs. 2020. Sex bias and omission marginally improve. Since 2017, the proportion of articles that consider SABV has also improved.

The presentation of sex bias across the years despite policies implemented in 2016 by the National Institute of Health (NIH) requiring sex to be considered a biological variable is

disconcerting (Clayton, 2018; Clayton & Collins, 2014). With the intentions of equal representation for both male and female models in vertebrate research, there would be expectations of sex bias significantly declining with the inclusion of sex based analyses. However, this is not the case. The proportions of male and female only models employed for experimentation have remained relatively stagnant overtime. The exception to this would be single sex studies with proper justification. However, the inclusion of a justification is not regulated and is up to the discretion of the author. The lack of a standard justification protocol allows for inappropriate variability in sex reporting and representation, such as excluding the usage of female models due to sex differences in behavior, anatomy and physiology (Bramson et al., 2020; Shimojo et al., 2020; G. Smith et al., 2020; B.A. Wang et al., 2020). This undermines the importance of SABV in regards to scientific progression given the substantiated sex differences between male and female biological and molecular processes. The underrepresentation of females in research is a problem, as half of the population is not accounted for. This lack of representation may not accurately reflect scientific findings as they would not account for possible sex differences. Additionally, only studying male models and comparing females to that standard neglects female specific processes such as reproduction, pregnancy and other hormonal related processes such as hormonal contraception and menopause (Galea et al., 2023; Puri et al., 2023).

This temporal analysis presents a complex picture of sex bias. One piece of this picture is the evident greater frequency in which males are utilized over females across all years sampled. Thus it would be easy to conclude that sex bias persists in neuroscience preclinical research. Countering this simple explanation is the increase in manuscripts employing both males and females, which has substantially increased across all years sampled. This finding

argues that sex bias is diminishing, as both females and males are increasingly employed in research studies. This is an encouraging finding, however, it masks a somewhat surprising complication: that sex is widely neglected as an experimental variable, even in studies that utilize both males and females.

Again examining the available longitudinal data that focuses on mice and rats (Figure 2.7), the percentage of articles using male and females without considering SABV increased from 12.9% in 2010 to 32.4% in 2013. This further increased to 40.8% in 2017 and reached 44.9% in 2020. When considering all research models, there is an increase from 44.3% in 2017 to 47.4% in 2020. Thus, by 2020, roughly half of the sampled research studies employed both males and females but did not test whether sex impacted the gathered data (Figure 2.8).

For our studies, a broad definition of employment SABV was employed, and we are thus presenting the most optimistic representation possible. Any assessment of sex, whether a sex difference was detected or not, was considered as incorporating SABV. Under these methodological considerations, we detected a relatively small but growing population of articles evaluating SABV. SABV in rats and mice showed an upward trend overtime from 4.3% in 2010 to 5.3% in 2013. This trend persists with an increase from 8.1% in 2017 to 11.7% in 2020. Considering all research models, a similar marginal increase is observed, with a percentage of 7.7% in 2017 rising to 9.9% in 2020.

Overall, these findings are encouraging, since more studies are employing males and females and incorporating sex as part of their data analytic pipeline. However, we must acknowledge that a great potential for growth remains. Despite the increase of articles using male and female models in research, sex-based analyses are still being overlooked. The exclusion of SABV applies to a diverse variety of biomedical fields and is not just a concept

localized to neuroscience (Beery, 2018; Yoon et al., 2014). This is evident with specialties such as immunology, physiology, and endocrinology lacking a significant increase in sex-based assessments since previous analyses in 2009 (Woitowich et al., 2020). This overlook assumes that SABV is the bare minimum and is not worth pursuing further, which is far from the truth. The discovery of sex differences for a given trait allows for the optimal development of biomedical advancements, drug therapies, or other health and medical benefits of both sexes (Yang et al., 2019). Simply reporting for sex does not allow the uncovering of sex differences and further prompts the reader to possibly incorrectly assume there were none or that these differences have yet to be found.

The data presented in this study can be perceived as optimistic given the decline in sex omission alongside the increased inclusion of both male and female models in neuroscience research. Both of these findings have continued to trend upward since our first comparative analysis in 2010 and again in 2017, indicating more researchers are considering to report sex or utilize it as a biological variable (Mamlouk et al., 2020; Will et al., 2017). However, this optimism is tempered by several points.

First, the amelioration in regards to sex omission and SABV are marginal at best, consistent with other studies that report similar results (Rechlin et al., 2021). Since our last full research model assessment in 2017, sex omission has only decreased approximately 3%. Although more researchers are incorporating both male and females in their studies, there has only been an approximate 2% increase since 2017 in regards to sex being considered as a biological variable. This highlights the second point of concern, in which sex is still widely not being addressed as a biological variable despite the increased utilization of both sexes for neuroscience research purposes. In 2017, 44% of studies employing both male and females did

not consider SABV and this increased to 47% in 2020. Lastly, sex bias has continued to persist with only 4% of articles using female only models since 2017. While there is a 2% decrease in male only model incorporation since 2017, this decline is quite unimpressive.

The findings above are problematic and can be remedied in several ways. While sex does not have to be assessed in every study, researchers must incorporate rationale to justify their methodology in regards to sex omission, same sex reporting or the exclusion of sex-based analysis. Doing so allows the furthering of scientific discovery in several ways. At the very least, the reporting of sex provides a method for reproducibility of a scientific finding and thus should always be reported if applicable. If only one sex is utilized for sex-specific behaviors, physiological processes, or anatomical features, appropriate justification must be provided. Some studies have reported research articles that study one sex to limit experimental variability (Beery, 2018; Prendergast et al., 2014). This bluntly can be considered negligent and unwise, as this reasoning straightforwardly ignores the diversity of male and female processes. Otherwise, a lack of justification would give the assumption that the research methods and findings may be applicable to both sexes, which may not be accurate or appropriate as it disregards possible sex differences. Additionally, to ignore SABV is to discount established sex differences and the potential to uncover new sex difference findings. Across the nervous system, sex differences have been studied and suspected to influence multiple disorders, including neurodevelopmental, neurodegenerative, autoimmune and affective disorders (Bangasser & Valentino, 2014; Gold et al., 2019; Zagni et al., 2016). These disorders are known to affect males and females disproportionately and possibilities for optimal health preventatives or therapeutic strategies are limited without first investigating SABV.

Even if no sex difference is detected, the knowledge obtained is crucial and can be used to further improve hypotheses, adjust methodologies or better distribute resources. An example of this is presented in the first chapter of my thesis, where it was discovered there is no sex difference regarding the change in resting membrane potential in response to oxytocin receptor activation. This finding indicates the sexually differentiated behavior seen in social play is influenced by another biological property, allowing the invalidation of our initial hypothesis. This discovery informs us that there needs to be further investigation done to better understand social play behavior and the associated sexually differentiated behavior. Therefore, properly assessing SABV facilitates efficient scientific advancement, allowing appropriate measures to be taken to further optimize the understanding and preservation of our health.

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## APPENDICES

## Appendix A

### Relevant Protocols

Data analysis protocol for electrophysiology, including assessing resting membrane potential.

This is the standard protocol employed by the Meitzen laboratory. It is not original to me and has been developed by many individuals over the years. I include it in my thesis so that the protocol is archived and to best document the data analysis methods.

### Data Analysis Software:



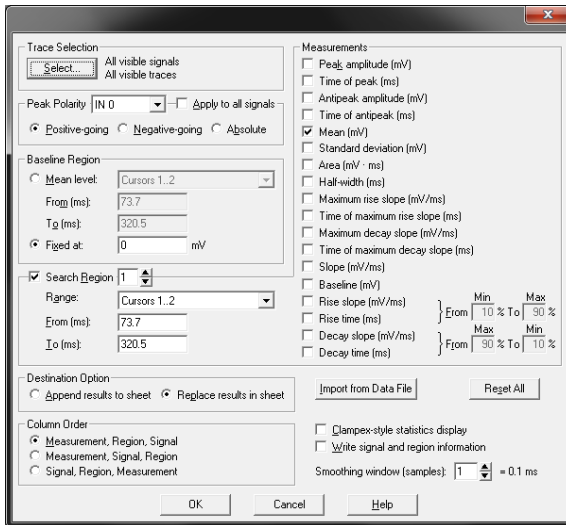
Clampfit Download: [Clampfit 10.6.2](#) or [pClamp Website](#)



MiniAnalysis Download: [Demo Form](#) or [Demo Download](#)

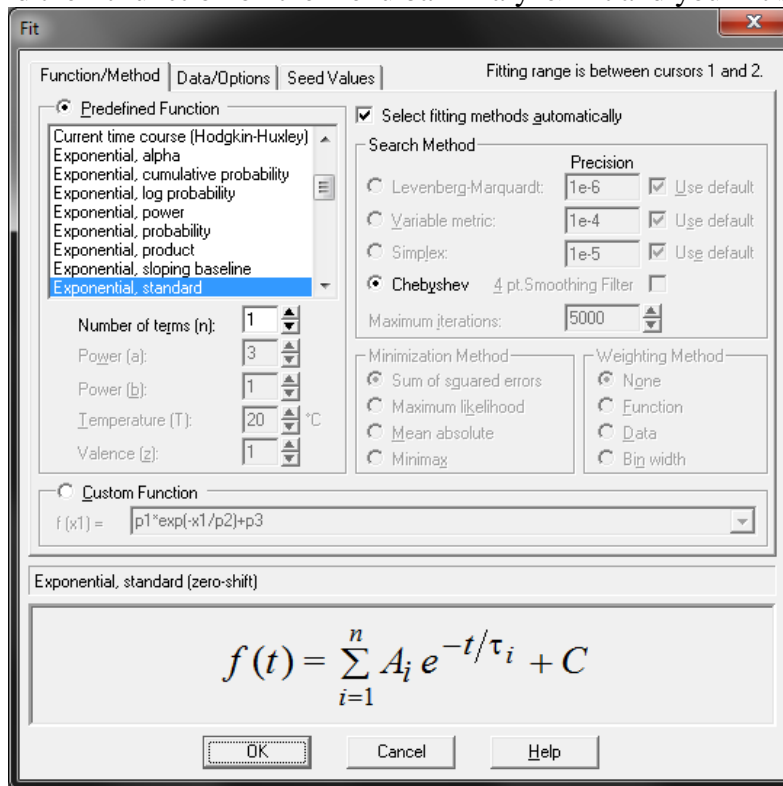
### Data Analysis Protocol:

1. Start analyzing data by first analyzing the **negative current step files**. Open the corresponding negative step pClamp files in Clampfit.
2. Average the files together by using the average files function in Clampfit. On the menu bar select Analyze>Average Files. Select all of the negative step files and hit OK. This will generate a new average file.
3. Close the negative step files but make sure to leave open the average file that you just created.



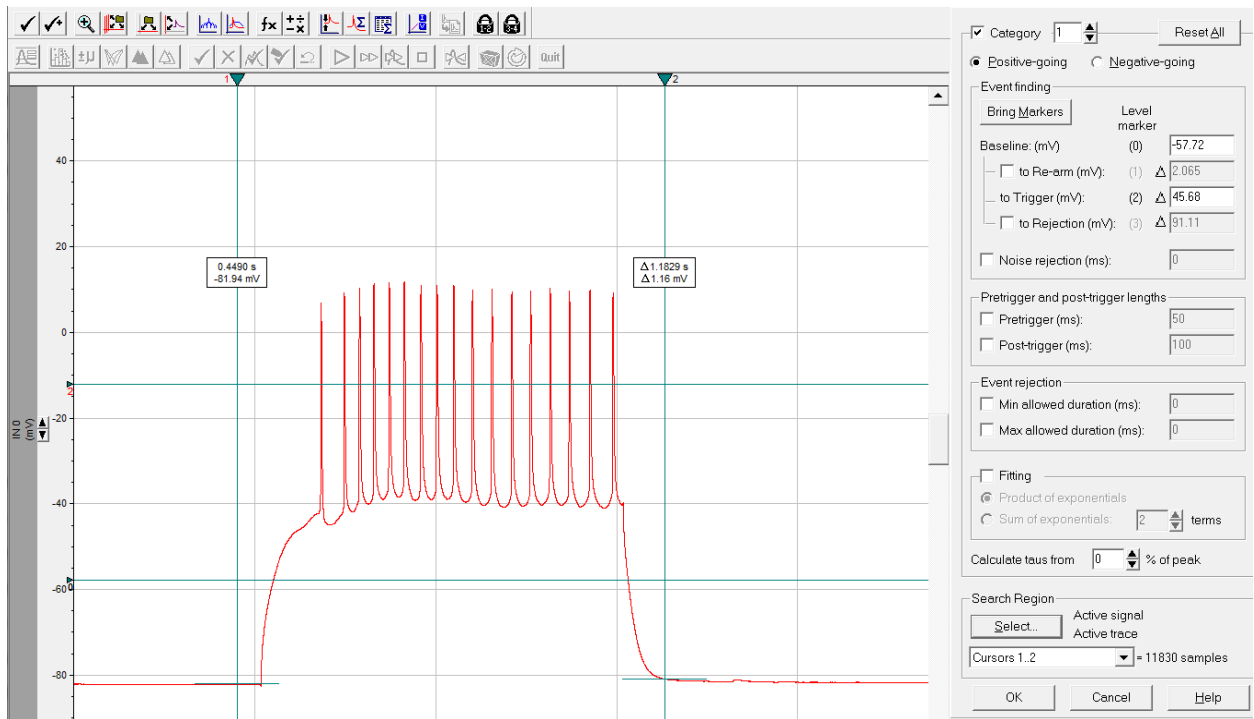
4. In the average file, we will start our analysis with first finding the baseline **RIS1 mean** in mV. Place cursor 1 near the beginning of the traces and then place cursor 2 at least 0.2 s away from cursor 1, but before the trace starts to deflect due to injected current. Run a statistical mean of all the traces between cursor 1 and 2 by using the statistical analysis in Clampfit. It is found on the menu bar by selecting Analyze>Statistics. The Set-up of the analysis should resemble above.
5. After hitting OK in the window from above, the results will be in the Results sheet in Clampfit. Copy out the RIS1 Mean column and paste the results into the Baseline RIS1 column in the neuron's spreadsheet.
  - a. To clear the Results sheet: Select Edit>Clear Sheet>OK.
6. To measure the **steady state RIS1 mean**, move the cursors to span a range within the step function. Place the cursors at least 0.2 s apart so that they rest on a steady portion of the traces and are before the input current is turned off. Try to find a region in which the -0.02 nA current trace is the most stable.
7. Repeat the statistical analysis from the step 4 and copy out the Mean RIS1 column and paste the results into the Baseline Steady State RIS1 column in the neuron's sheet. If the equations from the previous neuron were successfully copied over then the **Input Resistance (IR)** should have been automatically calculated. If not copy over the equations from another sheet.
8. Find the **Lowest Steady RIS1** by selecting the final trace recorded using the > key. The trace should now be highlighted in red. Drag cursor 1 over the initial deflection due to injected current and determine the lowest voltage achieved within 0.2 sec of current injection. Record this value as the Lowest Steady RIS1 in the neuron's sheet. The sheet will automatically calculate the **Sag Index**.
9. Now it is time to find **Tau**. Place cursor 1 at the point where the trace starts to exponentially deflect back to the baseline. Place cursor 2 at a distance 0.1 sec to the right of cursor 1. Run an exponential line fit between the two cursors for all the traces. You

will find the fit function on the menu bar Analyze>Fit and your fit should resemble

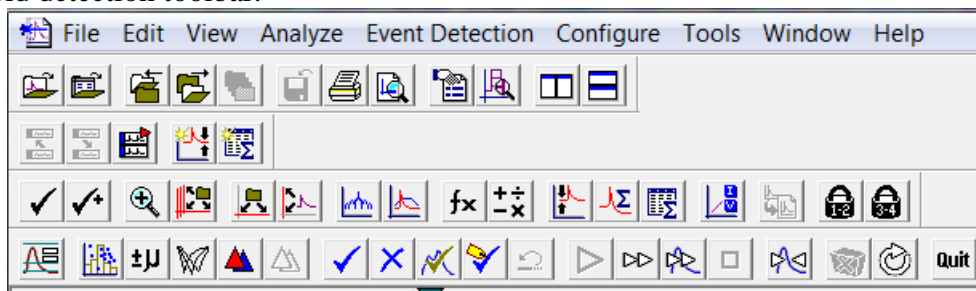




below.

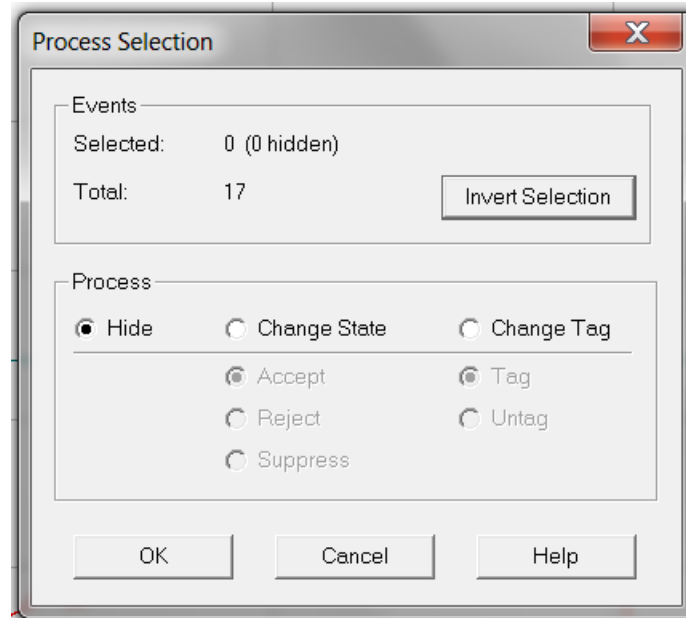
10. In the Results window in Clampfit copy out the Tau column and paste it in the Tau column in the neuron's sheet. The sheet will automatically calculate the **Capacitance** for the input resistance of .01 and 0.2 nA. If not you can copy the equations over from another sheet.
11. The sheet should automatically calculate out the **RRIR, Inward Rectification** and **% Inward Rectification**.
12. Open the **positive current step files** in Clampfit. For each file you will need to fill in the data corresponding to the frequency curves in the neuron's excel spreadsheet. Visualize individual traces by menu bar View>Select Sweeps>Select Sweep #1>OK. Click through sweeps using < / > keys until the sweep containing the first spike is located. Enter 0 in the excel spreadsheet for the number of spikes for all previous sweeps. Move cursor 1 just to the left of the initial deflection. Move cursor 2 just to the right of the deflection back to baseline.
13. In the top menu bar, click on "Event Detection." Then select "Threshold Search." Move the Baseline cursor (0) to some point below threshold. Move the Trigger cursor (2) to some point after threshold. Select positive-going traces. De-select Pretrigger and Post-trigger lengths if selected. Select active signal, active trace, and cursors 1..2 search regions. Click OK.




14. Close the Event Viewer window that opens. You should now have a new threshold detection toolbar.



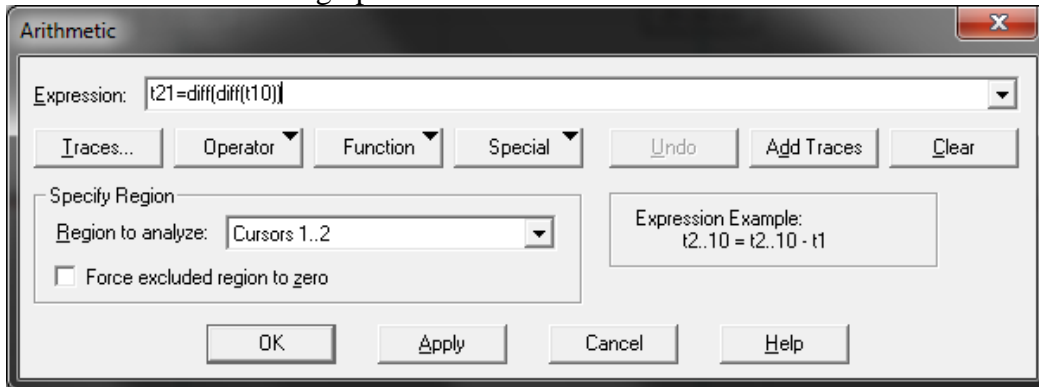
15. Click the “Non-stop”  button. Then click the “Process selection”  button. A window should pop up containing the number of action potentials present in the sweep. Enter this number as the number of spikes in the excel spreadsheet. Click OK.



16. Click to the next sweep using the > key. Repeat the analysis by first clicking the “Restart”  button and selecting “Yes” to “Delete existing data before restarting?” Then, repeat steps 15-16 until all sweeps have been quantified.
17. After that visually count the number of **Spikes** at each input current step and place the counts in the neuron’s sheet. Scroll through sweeps using the > key. You can double check the input waveform and make sure that your input current steps are correct by menu bar View>Stimulus Waveform Display. Zoom in on the waveform and you can verify the pA steps. A 10 pA step corresponds to a .01 nA change in the input na column in the sheet.
18. For each of the **FI Slope** calculations, change the equation so that it is on the linear range of the slope. The green and red boxes should correspond to the current and frequency which elicited the first spike(s). The purple and blue boxes should correspond to the current and frequency at which the number of spikes begins to repeat.
19. For the **Rheobase** calculation, move the boxes so that the equation calculates the average of the input current which elicited the first spike(s) for each file.
20. Now we characterize the first action potential of each positive step file.
21. Isolate the first sweep with action potentials using the < / > keys.
22. To find the **Threshold** of the first Action Potential bring cursor 1 and 2 and put them on either side of the AP respectively. In the trace window hit the arithmetic button

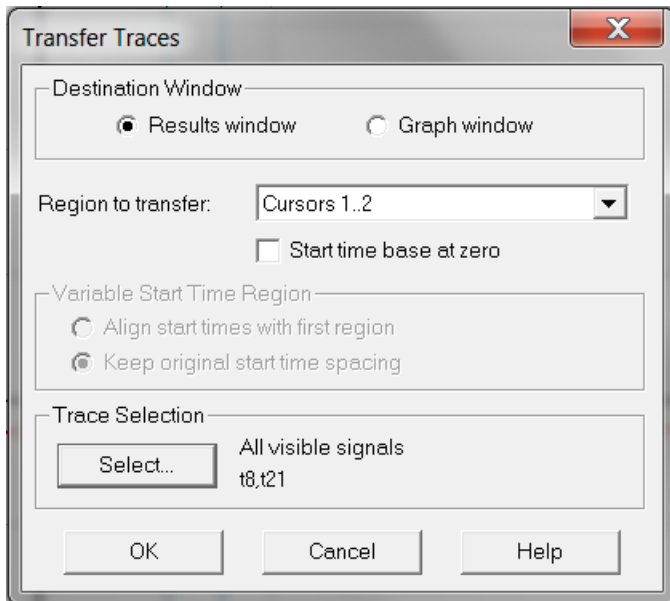


and it should bring up a window as below.



23. Hit the Add Traces button to add one trace. Modify the equation in the Expression field to include the number of the trace you just added at the beginning and the number of the trace with the AP inside the ( ). Make sure the region to analyze is set to Cursors 1 and 2. Hit OK and the program will revert to all the traces visible again. Select the sweep with the first APs and the sweep that you added using View>Select Sweeps. Use the Ctrl key to select both at once. Bring cursors 1 and 2 closer in toward the AP then export the data using on the menu bar Edit>Transfer Traces and the window below will open.

24. Hit the Select button and select all visible signals and then select the two traces you have been working with from the list.

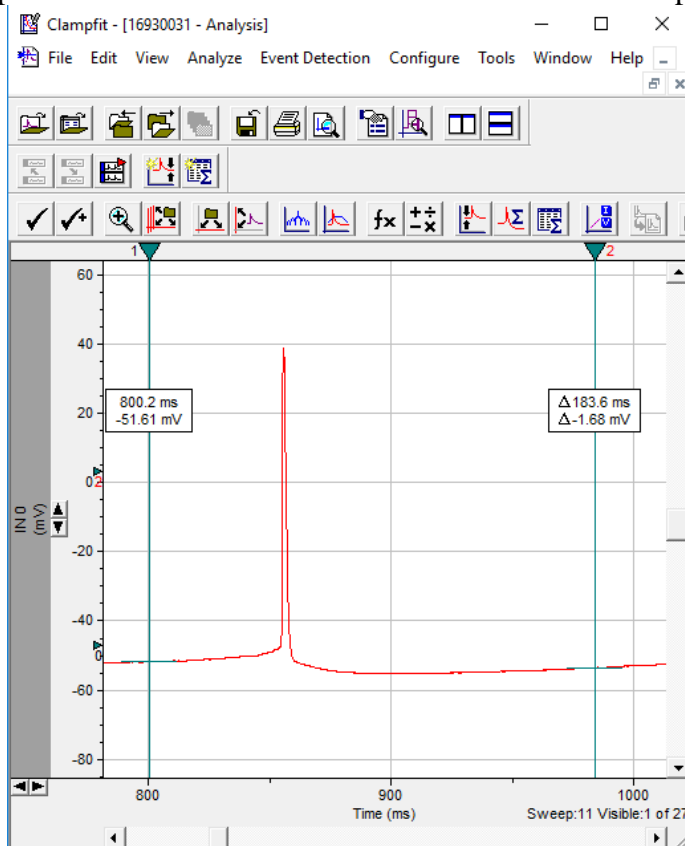


25. Hit OK in both windows then go to the Results window. You will see three columns one labeled Time and the others labeled with the number of the traces you have been working with. Look in the column corresponding to the added trace where you ran the double differential. Find the point in the column where the value is equal to three times the

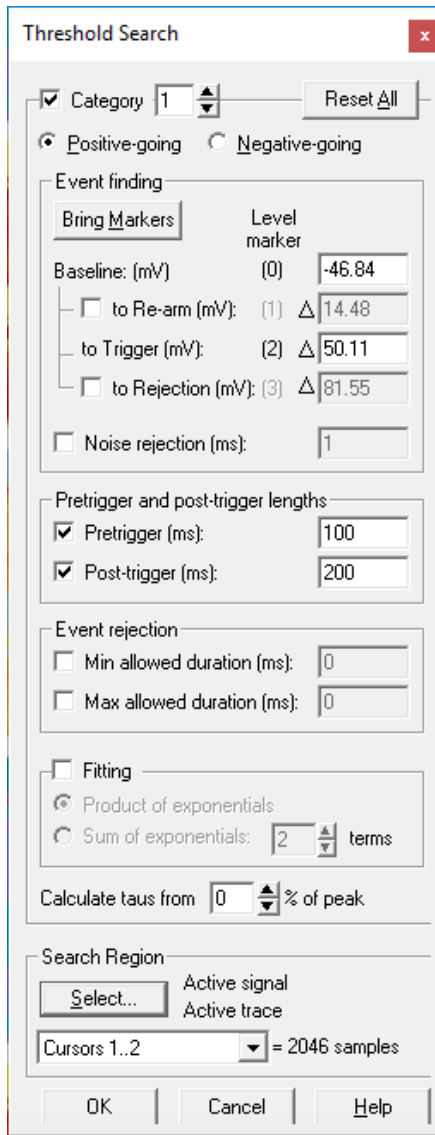
standard deviation with a visual search. Once you have found that point, copy the corresponding point from the second column where values for the other trace are listed. That value represents the threshold for that AP in mV. Place that value in the neuron's sheet in the appropriate column in the threshold analysis section of the sheet.


26. Find the sweep with the AP being analyzed. Right click "Time (ms)" on the bottom of the screen and click "Properties." Then in the drop down menu, select "milliseconds."

27. Place cursor 1 before the first action potential and cursor 2 after the first action potential such that the cursors contain the full action potential, including the AHP.



28. In the top menu bar, click on "Event Detection" and then "Threshold Search." In the "Level marker (0)" field, input the threshold value that you determined in step 25. Check the "Pretrigger" field and input 100 ms. Check the "Post-trigger" field and input 200 ms. Under "Search Region" click the "Select Button" and click "Active Signal" and "Active Trace." Move the Trigger cursor (2) to some point after the threshold. Then click "OK" and close the "Event Viewer" window that opens."



29. In the event threshold toolbar, click the “Non-stop”  button. Then go to the results page. Record the following values into the spreadsheet:
- AP Amplitude** is listed as “Peak Amp.”
  - AP half-width** is listed as “Half-width.”
  - AHP Peak** is listed as “Antipeak A.”
30. Note the “Time of Antipeak” and “Quit” the Event Threshold toolbar.
31. To find the **AHP Time to Peak**, place cursor 1 at threshold value *after* the AP peak. Then right click on cursor 2 and select “Cursor Properties.” In the time field, input the value from “Time of Antipeak” in the results sheet and click “OK.” Then return to the action potential, look at cursor 2 and record the delta time value in mSec as **AHP Time to Peak** in the spreadsheet.
- 3.2 Repeat all steps pertaining to AP analysis for the first Action Potential elicited in each of the positive step files.

**33. Delay to first spike** is only measured for files in which the only one or two AP's are elicited in the sweep. To find that value, place cursor 1 at the threshold of that AP, then place cursor 2 (to the left of cursor 1) at the initial deflection of the trace where the input current was turned on. The delta in time of cursor 2 is the Time to initial spike and is recorded as positive.

### Literature Review Protocol for Chapter 2

*This protocol was originally developed by Tyler Will, adapted by Garbriella Mamlouk, and adapted again by me. It is included so that it is archived and also to best document the employed methods.*

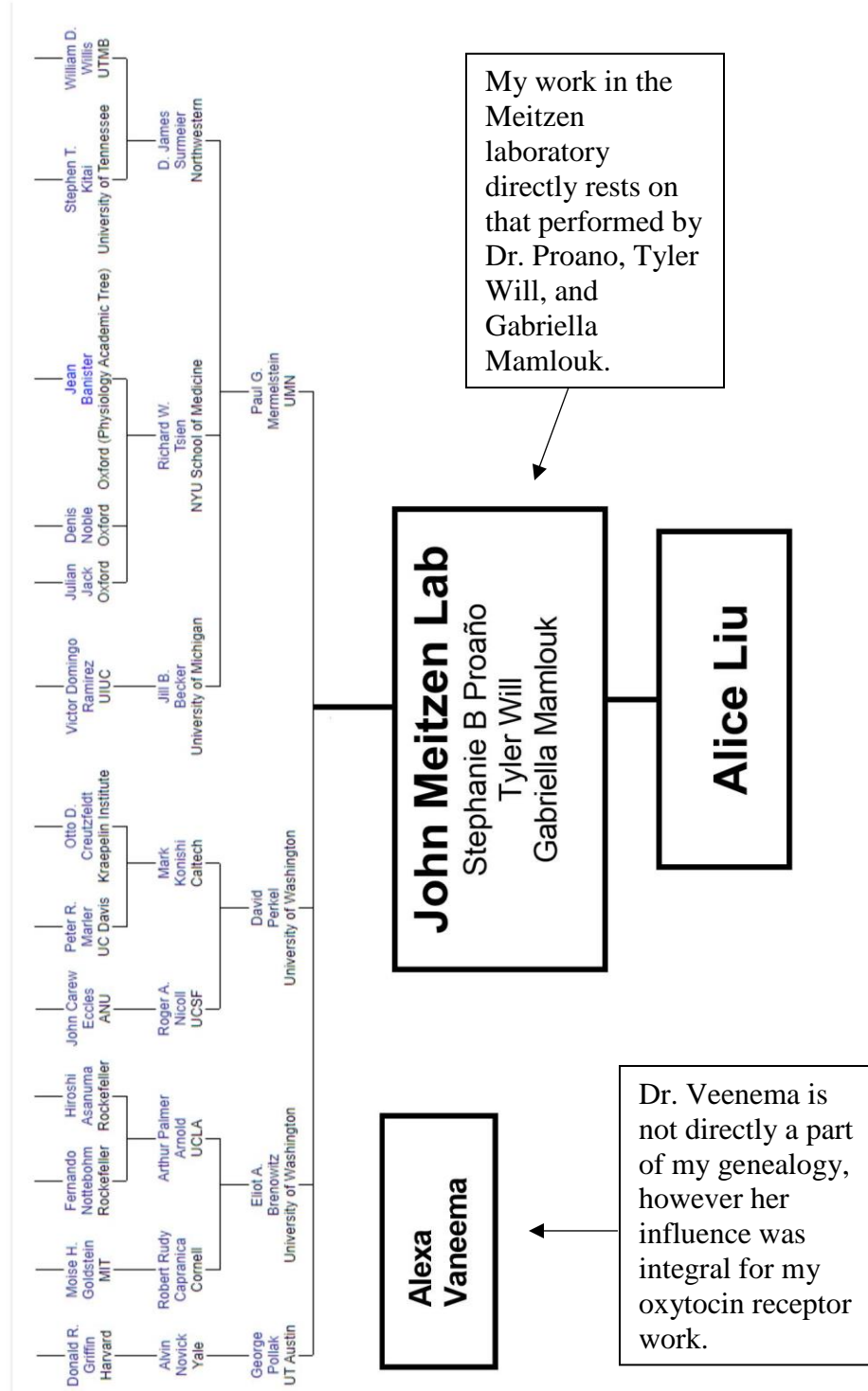
Open up the journal you were given and go to the specific year you are working on. You may need to open the journal through the library database.

1. Once you have the journal open, go to January of 2010 (or whatever month and year you are on) and tally up all of the articles that were submitted in in that month (there should be two issues per month). Exclude all editorials, reviews, methodology, etc. Once you have counted all of the articles, indicate that number in the spreadsheet on the "Total Articles" column of 2010 tab, making sure to add it to the correct month. You don't need to open up the articles for this, just simply count them.
2. Now you will open up each individual article one at a time.
3. Once you have an article opened, scroll down to the methods section, or where they explain their procedures. You will read this section and find out what animal they used (if any, at all). If it is anything other than rats or mice, discard the article and move on to the next one! If you cannot figure out the animal they used, do a search (command F) and type in rats and mice/mouse and see if it is in the article at all. If not, they did not use those animals and you can discard it.
4. When you are reading the methods section and it says they used rats or mice, first check what sex they used. Here are the definitions of our variables:
  - a. Male only: explicitly stated they used only male rats/mice
  - b. Female only: explicitly stated they used only female rats/mice
  - c. Male/Female Distinction: This means that they used BOTH males and females, AND they showed them as two separate groups at some point in the article. You will have to look through the results/graphs to see if they had separate ones for males and females. An article will fall under this section also if they state in the article that they found no sex differences, thus the data was pooled. I will typically do a search (command F) and type in words such as sex, pooled, combined, compiled, and grouped to find them easier and faster.
  - d. Male/Female No Distinction: This means that they simply used both sexes but did not pay any attention to any sex differences throughout the article.
  - e. No sex reported: This simply means that they stated that they used rats or mice, but they did not indicate what sex they used. I typically will do a search on the page (command F) and type in words such as female, male, sex, or sexes and if none of those words are in the article, then I put it in this group.

5. Once you figure out what group your article will go in, you will add a number in the column that it goes under at the top of the excel sheet.
6. Next, you will scroll to the bottom of the excel sheet and add a number in the correct column for either rats or mice (if they used mice in their article, add it to mice and do not change anything about the rats only one). This column will be the same one as the one indicated in step 5.
7. Now that you have determined the animal they used and the sex of that animal, you will scroll up to the top of the article and look at the first author and the last author.
8. If you can clearly tell that they are a man or woman, then that is great, but most of the time you cannot tell because they could be from a different country. Thus, you will have to copy their name and do a Google search of them and try to find a picture or anything that will tell you they are a man or woman. I typically will type in the university they are affiliated with, or use Google scholar, or use LinkedIn. If worse comes to worst and you cannot figure out their gender, that is fine and you will add a tally in the “unidentified” section
9. Once you figure out the first and last authors gender, you will scroll to the middle two sections of the excel sheet (first and last authors) and add a number to the specific row (the rows are for gender) and specific column (this should be the same as column in step 5). Pay close attention to the row you are adding a number in!! If no gender could be found, add it to unidentified row.
10. Now you have successfully finished one article and can move on to the next one. Once you start a month, I would finish it right then and there (don't stop for the day in the middle of a month because you could easily lose your place and have to start that month over).
11. Things on the excel sheet you will **not** need to add numbers to: Total rat/mice articles, totals for gender, total rat articles, and total mice articles. These are already set to be added up as you go along with adding numbers in the other columns.

## Appendix B

Academic Genealogy, adapted from <https://neurotree.org>



My work in the Meitzen laboratory directly rests on that performed by Dr. Proano, Tyler Will, and Gabriella Mamlouk.

**John Meitzen Lab**  
Stephanie B Proaño  
Tyler Will  
Gabriella Mamlouk

**Alice Liu**

Dr. Veneema is not directly a part of my genealogy, however her influence was integral for my oxytocin receptor work.

**Alexa Vaneema**