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Optogenetic Control of Social Communication

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OPTOGENETIC CONTROL OF SOCIAL COMMUNICATION

by

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Under the Direction of Aras Petrulis, PhD

ABSTRACT

With the rise in diagnoses of social deficit disorders comes an increased demand in elucidating the neural mechanisms that underlie social behavior. In the central nervous system arginine-vasopressin (AVP) has been shown to effect social communication, such as aggression, pair bonding, and maternal behavior, and many AVP cell bodies and fibers are distributed in a sexually dimorphic fashion. One such area is the bed nucleus of the stria terminalis (BNST), with males having more AVP cells than females, thus making it a likely candidate in the control of male specific social behavior. We found that activation of this specific cell population using optogenetics in socially-naïve males does not induce a place preference, affect male territorial aggression, or investigation towards females.

INDEX WORDS: Social behavior, Bed nucleus of the stria terminalis, Vasopressin, Optogenetics

OPTOGENETIC CONTROL OF SOCIAL BEHAVIOR

by

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DEDICATION

First and foremost, I dedicate this manuscript to my wonderful family. My parents, Bonnie and Larry Bowden, and my sister, Sarah Bowden, stood by me for my almost decade at Georgia State, never wavering in support and never doubting I could make it this far. I don't believe I can ever thank you enough. Secondly, this is for my friends. Specifically David Duplechain, Jenna Webb, Clayton Cowen, Mihaela Tufkova, Ben Barlament, and the countless others who have graciously lent me their ear and time to complain- and occasionally rejoice- during the duration of this project. You all have been my rock, and I could not have done this without your love.

Finally, I dedicate this to Dr. Aras Petrulis, Niko Rigney, and the rest of the Petrulis lab. I thank you for giving me a chance six years ago, and for helping me grow into a scientist who absolutely loves what she does. Without your guidance, support, and tough love, I would have given up on this project long ago.

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LIST OF ABBREVIATIONS

AAV- Adeno-associated virus

AVP- Arginine-Vasopressin

AVT- Arginine-Vasotocin

BNST- Bed nucleus of the stria terminalis

ChR2- Channelrhodopsin

eYFP- Enhanced yellow fluorescent protein

GDX- Gonadectomized

GDX + T- Gonadectomized with testosterone

GFP- Green fluorescent protein

LS- Lateral septum

MeA- Medial amygdala

OVX + E- Ovariectomized with estrogen

UM- Urine marking

USVs- Ultrasonic vocalizations

1 INTRODUCTION

As diagnoses of social deficit and communication disorders continue to rise, there is an increased need to study the neurobiology underlying these conditions. As with many diseases, social deficit disorders often arise in a sex-specific manner, as seen in autism (Lai et al., 2014) and social anxiety disorder (Asher et al., 2017). One explanation of this pattern is that sex differences in the brain contribute to sex differences in social and emotional behavior.

For decades, arginine-vasopressin (AVP), and its non-mammalian homolog arginine-vasotocin (AVT), has been known to influence social behavior. For example, AVT increases male calling behavior in several species including frogs (Miranda et al., 2015) and zebra finch (Goodson and Adkins-Regan, 1999), along with modulation of aggression and anxiety-like behavior in birds (Kuenzel et al., 2013). In mammals, AVP influences both affiliative and agonistic behaviors in several species. In monogamous vole species, brain AVP is critical for pair bonding and selective aggression (Winslow et al., 1993; Donaldson et al., 2010; Tabbaa et al., 2017) and in other species it affects agonistic behavior (Ho et al., 2014), juvenile play fighting (Veenema et al., 2013), as well as modulating anxiety-like behaviors (Wigger et al., 2004). There has also been links to the behavioral effects of AVP in humans and non-human primates. For example, intranasal AVP increases risky cooperative behavior in humans (Brunnlied et al., 2016) and intra-female aggression in macaques (Jiang and Platt, 2018).

These AVP effects on behavior are often sexually dimorphic, as are some of the cell populations in the brain. Though most of these AVP cells are in the hypothalamus, there are distinct populations in the bed nucleus of the stria terminalis (BNST) and the medial

amygdala (MeA)(deVries and Panzica, 2006). AVP fibers are more widely distributed throughout the brain than the cell bodies and innervate much of the social behavior neural network, such as the amygdala, lateral septum (LS), and preoptic area (Rood and DeVries, 2011). These AVP cells and their fibers are not only sexually dimorphic with males having a larger population than females, but are also androgen dependent. De Vries et al. (1983) showed that early castration of male rat pups eliminated the sex difference in AVP fiber density to the LS, and likewise, early administration of testosterone to females increases AVP fiber density to male-typical levels. Tracing studies have shown that much of this LS innervation is from the cells of the BNST, as lesioning the BNST greatly reduced the sexually dimorphic fiber density in the LS of male rats (DeVries and Buijs, 1983).

Lesions of the BNST as a whole have profound behavioral effects as well. Lesions of the BNST decrease male-typical sexual behavior in rats (Emery and Sach, 1976), and increase latency for males to mount, intromit, and ejaculate in sexually naïve male hamsters (Been and Petrulis, 2010), while injections of AVP increases male flank marking (Irvin et al., 1990). More specifically the AVP cells of the pBNST are activated during copulatory and social investigation in male mice, as shown by co-localization of fos and AVP in this region (Ho et al., 2010), while there is a decrease in AVP immuno-reactive cells in this region following social defeat (Steinman et al., 2014). Given this data, the sexually dimorphic population of AVP cells in the BNST is therefore a likely regulator of sex-different social behavior.

1.1 Purpose of the Study

Although there have been numerous studies on the lesioning of the entire BNST and a recent study by Rigney et al. (2019) that looked at deletion of the BNST-AVP neurons, there is no current information on how selective activation of this cell population effects social behavior. This project explores how activation of the BNST-AVP cells effect communication in awake, behaving animals. Here we use optogenetics, a technique that uses specific light frequencies to activate or inactivate genetically modified cells *in vivo*, to stimulate BNST-AVP cells in real-time. Male AVP-iCre mice were used in this experiment to take advantage of the combinatorial possibilities afforded by the cre/lox system to study how activation of AVP cells effects the investigation of male and female conspecifics, urine marking (UM), and ultrasonic vocalizations (USVs).

1.2 Expected Results

A recent paper by Rigney et al. (2019) demonstrated that specific ablation of the AVP cells in the BNST of male, but not female, mice decreased their investigation of male conspecifics as well as altering aspects of social communication. Given this data, we predicted that activating of AVP cells in the pBNST of male mice will increase the investigation of other male conspecifics, while maintaining normal preference to females as compared to the control.

2 EXPERIMENT

2.1 Animals and Husbandry

All animals were housed in ventilated cages on a 12/12 hour reverse light cycle with food and water available *ad libitum*. Animals were also given corncob bedding, a nestlet, and small shelter in each cage. All animal procedures were performed in accordance with Georgia State University Institutional Animal Care and Use Committee regulations and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.1 Subject Animals

The AVP-iCre mouse line was acquired from Dr. Michihiro Mieda (Kanazawa University, Japan). This line was generated using a bacterial artificial chromosome (BAC) such that codon-improved Cre recombinase (Shimshank et al., 2002) is expressed under the control of the AVP promotor (Mieda et al., 2015). This allowed for us to target specific AVP cell populations with minimal chance of over-expression of cre. AVP-iCre mice subject animals were generated from C57Bl/6J wild-type and heterozygous AVP-iCre+ breeding pairs. All AVP-iCre animals were genotyped using polymerase chain reaction (PCR) by Transnetyx. Both AVP-iCre+ and AVP-iCre- animals were utilized in this study, with iCre- animals serving as controls. All subjects were sexually-naïve, gonad-intact and group-housed with same-sex littermates until implant surgery. Post-surgery, all subjects were returned to the housing room for recovery and were singly-housed for the remainder of the experiment.

2.1.2 Stimulus Animals

Both male and female CD1 (Charles River Laboratories) mice served as stimulus animals for behavioral testing. Two different groups of stimulus males were used. The first group of males were gonadectomized and subcutaneously implanted with a testosterone capsule (GDX +

T). These served as aggressive and territorial male stimuli, here we term “dominant”. They were singly-housed and received a minimum of two sexual and two aggressive experiences prior to the onset of testing. These males were used as stimuli during the three-chamber test and as urine donors during aggression testing. The second group of males were gonadectomized (GDX) without testosterone replacement, group housed, and served as intruder stimuli during the home-cage aggression test.

Female stimuli animals were ovariectomized and subcutaneously implanted with estradiol (OVX + E) and were given sexual experience prior to testing. Before use in the three-chamber test, the females were subcutaneously injected with 500mg progesterone dissolved in 0.1 ml sesame oil (Sigma) four hours prior to testing to induce sexual receptivity.

2.2 Surgery

All surgeries were performed using 1.5-3% isoflurane anesthesia in 100% oxygen. Saline (0.2ml) and carprofen (3 mg/kg) were subcutaneously administered prior to surgery as an analgesia.

2.2.1 Virus and Implants

Expression of channelrhodopsin (ChR2), a membrane-bound light-sensitive ion channel, in cre-expressing AVP neurons of the BNST was produced using stereotaxic injections of an adeno-associated virus (AAV) (AAV5.EF1a-DiO-ChR2(H134)-eYFP; University of North Carolina Chapel Hill Viral Vector Core) into the BNST of iCre+ and iCre- control littermates. In only iCre+ cells, this virus expresses ChR2 and the fluorescent marking enhanced yellow fluorescent protein (eYFP) (Zeng and Madisen, 2012). Immediately after the virus injection, subjects had a fiber optic implant placed above the injection site. The implants were custom-made and trimmed to 4.6mm from the base of the ferrule (adapted from the protocol of Sparta et

al., 2011). This length was chosen to sit approximately 0.3-0.5mm dorsal to the AVP cells of the BNST.

2.2.2 Stereotaxic Injections

The subject was placed in a stereotaxic apparatus (Kopf Instruments) such that the skull was flat. A small hole was drilled to reach the dural surface of the brain into which was fitted a 000 screw (Antrin Miniature Specialities Inc., California, USA) to serve as an anchor for the implant. A second hole was drilled above the BNST (coordinates from bregma: AP -0.15, ML +/-0.8, DV -4.55; Paxinos and Franklin, 2012) and 250nl of viral vector (AAV5.EF1a-Dio-ChR2(H134)-eYFP) was delivered at 100 nl/min. After viral injection, the needle was left in place for 10 minutes before being withdrawn.

Immediately following viral injection, a 4.6mm fiber optic stub was implanted in the same drill site (coordinates from bregma: AP -0.3, ML +/-0.8, Paxinos and Franklin, 2012). The implant was lowered at 1.5mm/minute to reduce tissue damage. Once the implant was flush with the skull, it was attached to the skull with 3-5 layers of dental cement. After drying, the skin was sutured, and the animals returned to a clean cage for recovery.

2.2.3 Gonadectomy

All stimulus males had testes removed via cauterization at the ductus deferens (gonadectomy; GDX). The dominant stimulus males were then subcutaneously implanted with a SILASTIC capsule (1.5-cm active length, 1.02-mm inner diameter, 2.16-mm outer diameter; Dow Corning Corporation) (GDX + T) filled with crystalline testosterone (T) (Sigma) to maintain physiological levels of T (Barkley and Goldman, 1977). Subordinate males (GDX) did not receive T implants in order to reduce aggression (Beeman, 1947).

All females had their ovaries removed via cauterization at the uterine horn and were subcutaneously implanted with a SILASTIC capsule (0.7-cm active length; 1.02-mm inner diameter, 2.16 outer diameter; Dow Corning Corporation) filled with estradiol benzoate (E; diluted 1:1 with cholesterol) (GDX + E).

2.3 Behavioral Tests

Prior to surgery, all subject animals were socially and sexually naïve and group-housed with same sex littermates. Two weeks post-surgery subjects were handled by the researcher daily to acclimate them to attachment to the optogenetic cable. After a three-week viral incubation period, the subject animals then began behavioral testing with tests performed within the first five hours of the subject's dark cycle. Subjects first underwent a real-time place preference test, followed by 2 three-chamber social tests, and a home-cage aggression test. At the conclusion of testing, subjects were attached to the fiber optic cable and constantly stimulated for 5 minutes in their home cage in order to assess Fos expression in the BNST. As Fos is a marker of neuronal activation, it was used as a proxy measurement for whether experimenter-induced light activated the AVP-BNST cells. All animals were perfused, and brains were collected and sectioned. Viral spread, implant location, and co-localization of AVP/Fos were analyzed. All tests were video recorded using CinePlex software and scored offline.

2.3.1 Stimulation Parameters

Neurons were excited using pulses (5 ms; 20 Hz) of 10-mW 465 nm blue light from the tip of the stub via high-intensity LED (Plexon Inc, Dallas TX, USA). The power at the site of the cells was between 8-15 mW/mm^2 depending on the implant location (Predicted irradiance values, Deisseroth lab, Stanford University). These stimulation parameters were chosen because

they elicit depolarization of BNST-AVP neurons and generate action potentials with high fidelity *in vitro* (preliminary data).

2.3.2 Real-Time Place Preference

In order to determine if BNST-AVP cell stimulation was inherently rewarding or aversive, subjects were tested to see if this stimulation generated a real-time place preference (RTPP). First, subjects were tethered and habituated to a clear plastic box with two equal sized chambers (50cm x 50cm x 25cm) and were allowed to explore freely for 15 minutes with no light stimulation. Time spent in each chamber was recorded, and the preferred side (the side in which the subject spent the most time) was noted. The following day, subjects were once again tethered and placed in the apparatus on their preferred side. Subjects were allowed to freely explore for 15 minutes, receiving constant light stimulation upon entry to the non-preferred side and no light stimulation in the previously-preferred chamber. Time spent in each chamber was recorded, and a preference score generated to determine if stimulation changed the amount of time spent in each chamber (Stamatakis and Stuber, 2012).

2.3.3 Three-Chamber Test

On three consecutive testing days, the subjects were placed in a three-chamber apparatus (Harvard Apparatus; dimensions 20.3 x 42 x 22 cm) with an absorbent paper floor (Nalgene Versi-dry paper, Thermo Fisher Scientific) so that urine marking (UM) could be measured. Subjects were habituated to the apparatus on the first day while tethered to the optogenetic cable, without stimulation or the presence of a stimulus animal. The next day, subjects were exposed to both a clean cage and a caged stimulus animal (male/female) cage (8 cm D, 18 cm H; 3 mm diameter steel bars, 7.4 mm spacing) placed on opposite sides of the apparatus in the outermost chambers. Animals were tethered to the cable but did not receive stimulation. On the third day,

the subjects were again placed in the three-chamber apparatus containing a clean cage and caged stimulus animal (male/female) while receiving light stimulation in two minute on/off epochs. Time spent investigating the clean and stimulus cage was noted, along with urine marking and ultrasonic vocalizations, for each day of testing. This test was repeated the following week with all conditions counterbalanced, including stimulus animal sex and epoch.

2.3.4 Resident-Intruder Assay

Subjects were tethered to the optogenetic cable in their home cage and a GDX CD1 male stimulus mouse was placed in the cage. This stimulus male was painted with a mixture of dominant male urine on their lower backs to promote territorial aggression by the subject towards the intruder but without the stimulus animal initiating aggression. Urine was pooled from 3-5 different singly-house dominant male stimulus animals and mixed together before application to the CD1 stimulus male, and urine was collected no more than one hour prior to testing. Subjects received light stimulation when they investigated the stimulus animal (nose oriented and within 2 cm of the stimulus animal). Testing lasted for ten minutes, or until a fight occurred. Latency to approach the stimulus, time spent investigating, and latency to fight were noted.

2.4 Fos Induction

At the conclusion of all behavioral testing, subject animals were placed in their home cage with constant light stimulation for 5 minutes to determine if the BNST-AVP cells were activated by the light stimulation. As Fos is a marker of neuronal activation, we used this as a proxy measurement. After light stimulation, animals were anesthetized and perfused 70 minutes later. Brains were removed and placed in 4% paraformaldehyde for 24 hours before being placed in 30% sucrose until sectioning and analysis.

2.5 Analysis

All behavioral videos were recorded using CinePlex (Plexon Inc, Dallas TX, USA), and hand scored by a researcher blind to the experimental conditions. Statistical analysis was done using SPSS software (IBM) using parametric tests whenever possible. All means are given as mean ± standard error of the mean (S.E.M.)

All tissue analysis was performed by a researcher blind to the experimental conditions. Brain tissue was sectioned on a cryostat into cryoprotectant in 30 µm sections. Tissue from the posterior lateral septum to the posterior medial amygdala were stained for GFP (Primary: Chicken anti-GFP 1:5K, Abcam; Secondary: AlexaFluor 488 Donkey anti-Chicken 1:600, Sigma) and Fos (Primary: Rabbit anti-Fos 1:1K; Santa Cruz; Secondary: Goat anti-Rabbit 1:600, Sigma). Sections were mounted onto slides and allowed to dry overnight before cover slipping with Prolong Gold (Sigma) to preserve immunofluorescence. Tissue was imaged using a Zeiss fluorescent microscope (Zeiss Inc, Germany) and StereoInvestigator (BMF BioScience, VT USA). Images were analyzed for viral spread, placement of fiber stub, and cell counts of Fos, GFP, and co-localization of Fos/GFP using ImageJ.

3 RESULTS

3.1 Histology

Only animals that had viral expression contained within the BNST and stub placement 0.3-0.5mm dorsal to the BNST were included in the analysis (iCre+ n=5, iCre- n=7, figure 1).

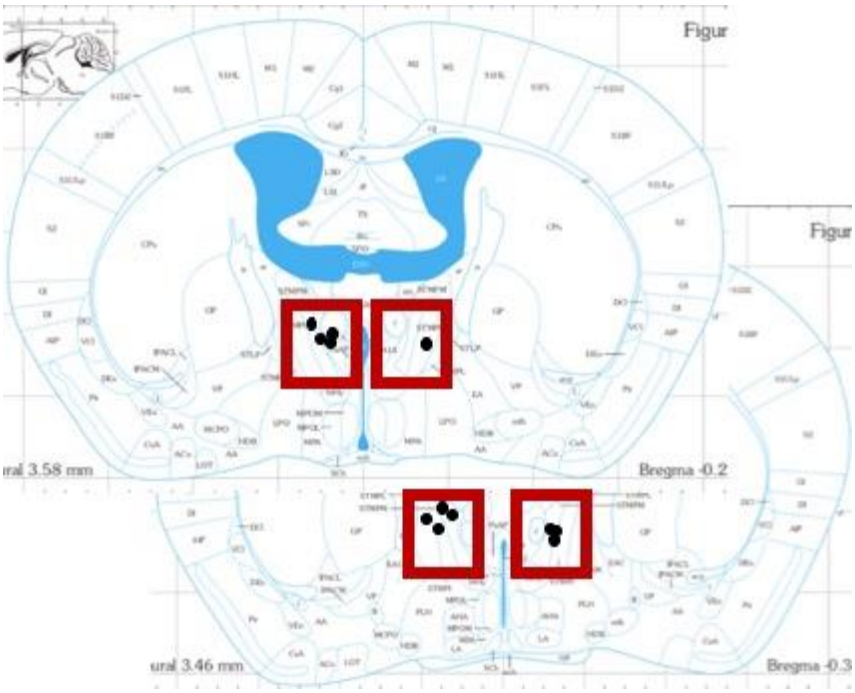

Figure 1 Fiber Stub Placement
Location of tips of fiber optic implants for all animals included in the behavioral analysis.

3.1.1 GFP/Fos Imaging

Injection of the Cre-dependent channelrhodopsin virus specifically targeted only cells of the BNST that expressed cre-recombinase, with no GFP-positive cells observed in sections of iCre- mice (Figure 2a). Because the data violated parametric assumptions, we used a Mann-Whitney U test which revealed there was significantly more Fos+ and GFP/Fos+ co-localized cells in iCre+ males over iCre- (Independent Samples Mann-Whitney *U*; GFP+ p=0.004; Fos+ p= 0.030; Co-localized p= 0.004, figure 2b). AVP-BNST cells were 75.9% colocalized with both GFP and Fos+ cells.

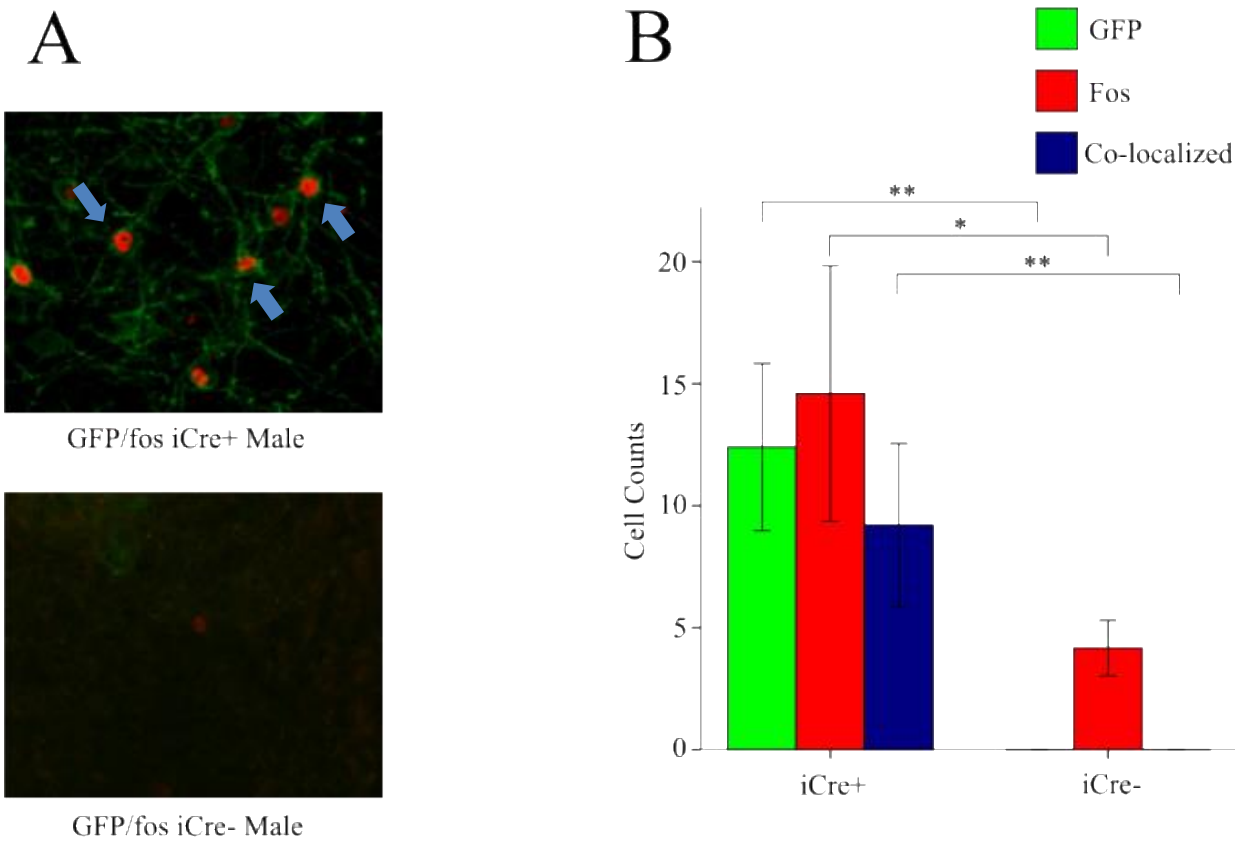

Figure 2: GFP/fos Co-localization
AVP-iCre+ males had significantly more GFP+, fos+ and GFP/fos co-localized cells in comparison to iCre- males. (A) Example of iCre+ (top) and iCre- (bottom) staining of GFP/fos. GFP shown in green, fos shown in red, arrows point to co-localized cells. (B) Number of cell counts for GFP, fos, and co-localization of both for iCre+ (n=5) and iCre- (n=7) in the BNST (+/- 1 SEM). * indicates a value of $p<0.05$, ** indicates a value of $p<0.01$.

3.2 Behavioral Data

All behavioral data was analyzed by a researcher blind to the experimental conditions and recorded using CinePlex software. Investigation and line crosses were scored later by hand. Statistics were all analyzed using parametric tests when possible, and non-parametric Mann-Whitney U tests when assumptions were violated.

3.2.1 Real-Time Place Preference

Neither iCre+ or iCre- animals exhibited a preference for the side of the chamber paired with light stimulation, indicating that activation of the BNST-AVP neurons is not inherently rewarding or aversive ($F_{(13)}$= 0.054, p= 0.820, figure 3). There was also no change in locomotion between groups in number of line crosses between the two chambers.

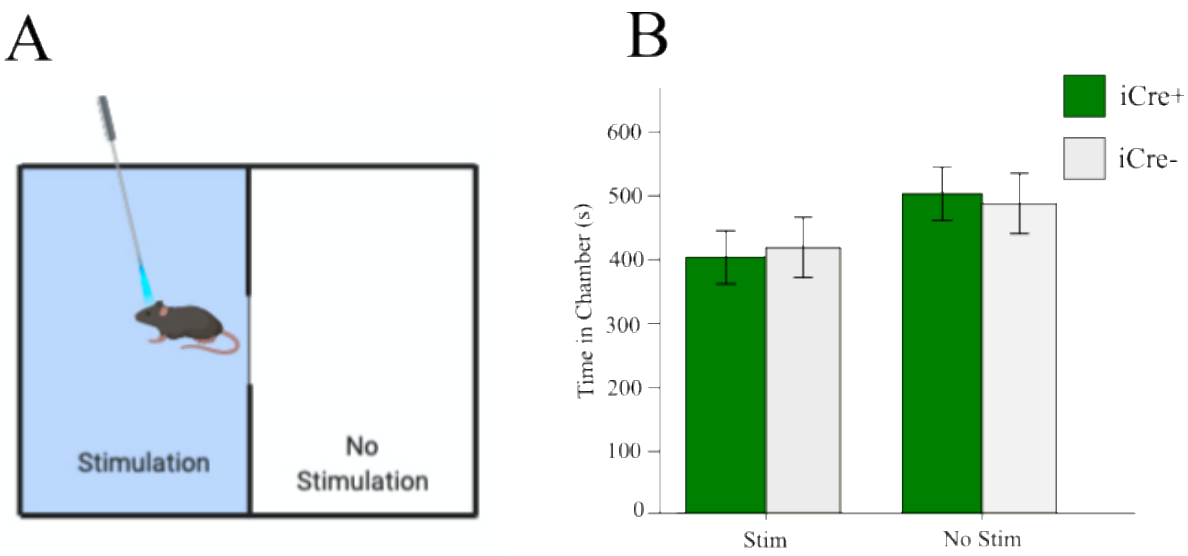

Figure 3: Real-Time Place Preference

Schematics and data for the real-time place preference (RTPP) test. (A) Visual representation of the RTPP 2-chamber apparatus. Subjects only received stimulation in one chamber of the box. (B) Mean +/- S.E.M. for time in seconds spent in each chamber. There was no difference in time spent in each chamber between iCre+ (n=6) and iCre- (n=8).

3.2.2 3-Chamber Test

For the 3-chamber test, we looked at differences in investigation towards stimulus animals between a test given with no light stimulation and a test given the following day accompanied with light stimulation in two minute epochs. There was no significant interaction of genotype and investigation time towards females ($F_{(3)}$=0.185, p=0.908) or males ($F_{(3)}$=1.905, p=0.150)(Figure 4). There was also no effect of stimulation epoch on investigation time $F_{(3)}$=0.284, p=0.835). Though we planned to analyze UM, the nature in which we attach the fiber

optic cable to the subjects head causes urination immediately prior to the onset of testing which led to little to no UM during testing. Also over the course of 50 3-Chamber trials only 272 total USVs were recorded, as such this data was not further analyzed (See appendix A). Stimulation also did not affect locomotion, ($F_{(11)}$=0.002, p=0.964) as measured by number of line crosses during testing.

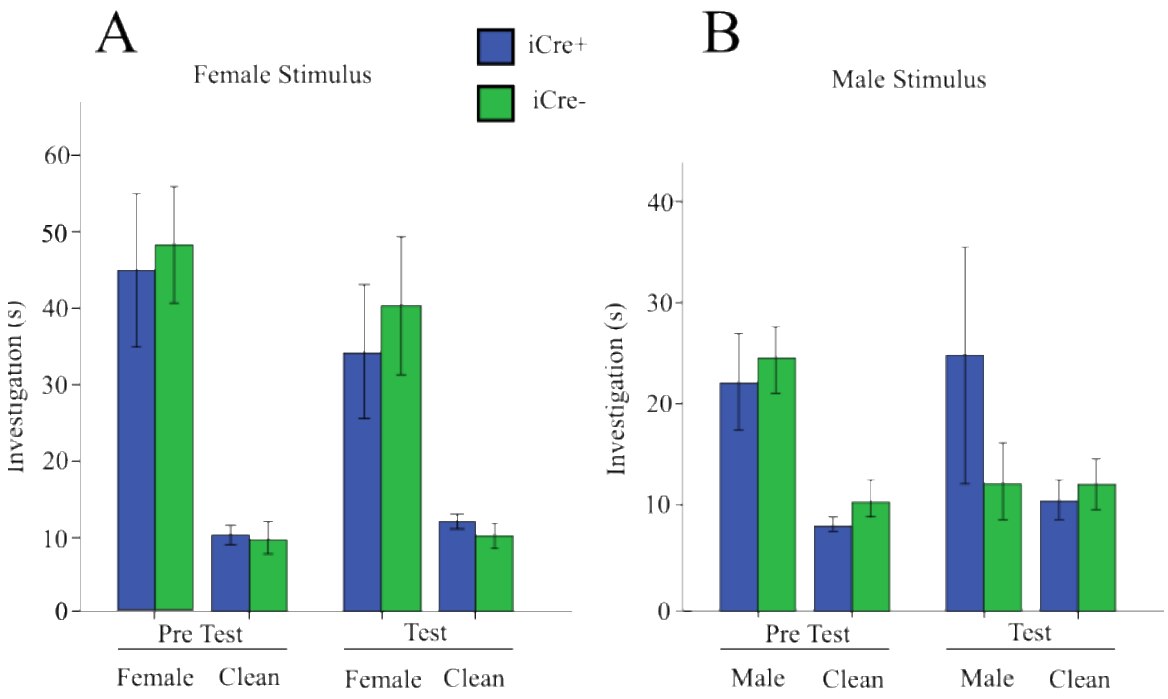

Figure 4: 3- Chamber Test
Light stimulation of BNST-AVP neurons did not have an effect on investigation of either female or male stimulus animals. (A) Female stimulus iCre+ (n=6) and iCre- (n=7) and (B) male stimulus iCre+ (n=5) and iCre- (n=7) did not differ from one another on either test day.

3.2.3 Resident-Intruder Assay

Over the course of a 10-minute test, subject animals received stimulation when investigating the stimulus male within 2cm. Only one animal had an aggressive bout, and was therefore excluded from the analysis of investigation. We found no difference between

investigation in iCre+ (124.39 ± 14.25 s) and iCre- (147.62 ± 25.19 s) towards a subordinate stimulus male ($t_{(8)}$= -0.695, p=0.507)(Figure 5). As only one fight occurred, no analysis was performed on aggressive behavior.

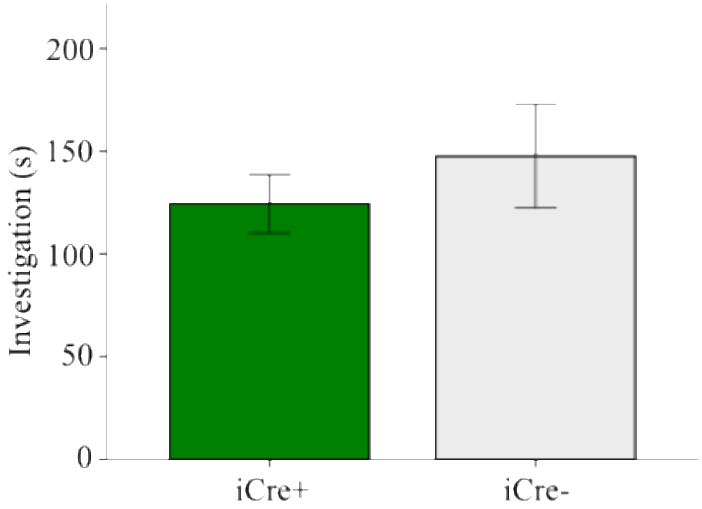

Figure 5: Resident-Intruder
No difference was found in investigation of an intruder when placed in the subjects home cage. Both iCre+ (n=4) and iCre- (n=6) animals investigated a subordinate male stimulus animal approximately the same amount.

4 DISSCUSSION

Social deficit disorders can be debilitating and costly to society, which is why there is an increased need to understand the underlying neurological causes and develop treatments. Both rates of diagnosis and treatment plans for diseases such as social anxiety disorder and autism vary by sex (Asher et al., 2017; Liu et al., 2014). Because the rates and effectiveness of treatment seem to be sex-dependent, one logical explanation is that sex differences in the brain contribute to sex differences in disease prevalence and treatment. One of the most well-known neuromodulators of social behavior is arginine-vasopressin (AVP), which contributes to anxiety-like behavior and sociosexual behavior differently in male and female rodents (Bredewold, R. and Veenema, AH., 2018). Because of its implications in a wide variety of social behaviors, this study looked at the activation of BNST-AVP cells to determine how this specific cell population may affect social and communicative behaviors in male mice.

Our first test was to determine if stimulation of the BNST-AVP cells was innately rewarding or aversive using a real-time place preference (RTPP) paradigm. A major caveat of optogenetic experiments is that stimulation of a cell population could produce behavioral effects in the absence of context. Here we found that both iCre+ and iCre- animals explored the stimulated and non-stimulated sides equally, and thus activation of these cells in the absence of a social stimulus did not have an innate behavioral effect.

Next we looked at activation of these BNST-AVP cells possible effects on investigation of a male or female stimulus animal. Subjects received stimulation in 2-minute epochs, alternating on/off, over the course of an 8-minute test. We found no difference in investigation of the stimulus regardless of sex and no difference in investigation during the light on/off epochs. We also found no difference in the number of line crosses between the groups, demonstrating

that stimulation of this cell population does not effect locomotion. These findings were interesting, as deletion of this cell population in males decreased investigation towards male conspecifics (Rigney et al., 2019). We were unable to assess UM as the subjects urinated when being attached to the cable, which is an issue we are trying to address for future studies. We also did not include a full analysis of USVs as the overall syllable number was so low. A few things may have contributed to this low number. During analysis, there was excessive noise found on all the files, which may have caused actual USVs to be thrown out with the noise. Also, lack of experience could have influenced the number of USVs as all subjects were socially- and sexually-naïve at the start of this experiment.

Surprisingly, activation of this cell group had no effect on aggression in the resident-intruder assay. It has been shown that the BNST-AVP cells are a significant source of AVP fiber innervation in the LS (De Vries and Buijs, 1983; De Vries et al., 1984), and that AVP release in the LS increases following a resident-intruder test in highly-aggressive males (Veenema et al., 2010). Given this, we expected the activation of the BNST-AVP cells to increase aggression, but we found no increase in aggressive bouts or differences in investigation time between the two groups. This result could be attributed to the lack of social or sexual experience given to the subject animals. Veenema and colleagues (2010) did find an increase in LS AVP release in highly-aggressive male rats, but infusion of AVP into the LS of low-aggression males had no effects on attack latency or the amount of time spent in an aggressive bout. As the animals used in this study had no prior social experience, they may be in this ‘low-aggression’ group, and therefore AVP release in the LS does not have an effect. In addition, co-localization of AVP cells and Fos in the BNST following an aggressive interaction in male mice increased across all groups, including submissive males (Ho et al., 2010). This may mean that activation of these

cells is not necessarily linked to aggression, just social investigation generally. There was an increase in colocalization in the BNST following a copulatory experience (Ho et al., 2010), which may be a behavior to investigate in future experiments.

As of now, these BNST-AVP neurons are not well categorized. Though we were able to activate this cell population, there was no way for us to show there was actual vasopressin or other neurotransmitter release from these neurons terminals. Future studies could include micro-dialysis in known projection sites to verify vasopressin release. In addition, it is currently unknown if these connections are excitatory or inhibitory. Further understanding of the BNST-AVP cell population is needed to fully understand this circuit and how it effects aspect of social behavior.

Though we did not find any behavioral effects of the activation of the BNST-AVP cells, we were able to conclude that we did in fact activate these neurons as planned. We found no eYFP staining in the iCre- animals and a significant increase in Fos expression and GFP-Fos co-localization in the BNST of iCre+ animals, with AVP-BNST cells at 75.9% co-localized.

In future optogenetic studies, several changes to the experimental design will be made. For the next line of experiments, all subjects will be socially- and sexually-experienced before the start of the study. Additionally, we will change the stimulation time frame for experiments. For the 3-chamber test and resident-intruder assay, testing will last for five minutes and animals will receive stimulation for the entirety of the testing period. We will also be working on a different way in which to attach the animals to the cable to hopefully measure UM in the future.

Currently, this is the first known study to directly activate the BNST-AVP cell bodies of mice. Though we did not find behavioral effects here, we believe that correcting the issues

described above will provide us with a more complete understanding of this cell population and its effects on social communication.

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APPENDICES

Appendix A: USVs

USV syllable repertoire and total counts over all 3-Chamber tests for all subjects. With less than 300 total counts over 48 trails, no further analysis was performed.