Sensory Representation of Social Stimuli in Aromatase Expressing Neurons in the Medial Amygdala

Charles J. Gualtieri
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Sensory Representation of Social Stimuli in Aromatase Expressing Neurons in the Medial Amygdala

A Master’s Thesis Presented

By

CHARLES J. GUALTIERI

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Neuroscience and Behavior Graduate Program
Sensory Representation of Social Stimuli in Aromatase Expressing Neurons in
the Medial Amygdala

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ABSTRACT

SENSORY REPRESENTATION OF SOCIAL STIMULI IN AROMATASE EXPRESSING NEURONS IN THE MEDIAL AMYGDALA

MAY 2021

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Directed by: Professor Joseph F. Bergan

The ability of animals to sense, interpret, and respond appropriately to social stimuli in their environment is essential for identifying and distinguishing between members of their own species. In mammals, social interactions both within and across species play a key role in determining if an animal will live to pass on its genes to the next generation or else be removed from the gene pool. The result of this selection pressure can be observed in specialized neural circuits that respond to social stimuli and orchestrate appropriate behavioral responses. This highly conserved network of brain structures is often referred to as the Social Behavior Network (SBN). The medial amygdala (MeA) is a central node in the SBN and has been shown to be involved in transforming information from olfactory sensory systems into social and defensive behavioral responses. Previous research has shown that individual neurons in the MeA of anesthetized mice respond selectively to different chemosensory social cues, a characteristic not observed in its upstream relay, the accessory olfactory bulb (AOB). However, the cause of this stimulus selectivity in the MeA is not yet understood. Here, I hypothesize that a subpopulation of neurons in the MeA that express the enzyme aromatase are involved in the sensory representation of social stimuli in awake, behaving animals. To test this hypothesis, I designed and built a novel behavioral apparatus that allows for discrete presentations of social stimuli in a
highly controllable and reproducible environment. I then injected the adeno-associated virus (AAV) AAV-Syn-Flex-GCaMP6s into the MeA of Aromatase:Cre transgenic mice and implanted a fiber optic cannula slightly above the injection site. The combination of this transgenic mouse line and conditional AAV caused GCaMP6s expression to be exclusive to aromatase-expressing neurons. By coupling my novel behavioral apparatus to a fiber photometry system, I successfully recorded the moment-to-moment activity of aromatase neurons in the MeA of awake, behaving animals as they investigated various social stimuli. Aromatase neurons in the MeA of adult male mice respond strongly to conspecific social stimuli, including live adult mice, mouse pups, and mouse urine samples. Sniffing and investigative behaviors correlated strongly with increased GCaMP6s signal in aromatase neurons, reflecting increases in their neural activity. Interestingly, after repeated investigations of the same stimuli the activity of aromatase neurons gradually diminished. Presenting a novel stimulus following repeated investigations of a familiar stimulus reinstated some, but not all of the initial GCaMP6s signal. This points to the potential role that aromatase neurons may play in the habituation to social stimuli that are consistently present in their environment. Investigations of predator stimuli did not evoke significant responses from aromatase neurons, nor did investigations of non-social stimuli. These results demonstrate that aromatase expressing neurons in the MeA of awake, behaving animals encode the sensory representation of conspecific social stimuli, and their responses are highly selective to the type of stimulus presented.
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Introduction

Behavioral and Endocrine Responses to Social Stimuli

The ability to interpret social information and transform it into an appropriate behavioral response is found throughout the animal kingdom and is critical for the propagation of species (Darwin, 1871). Evolution and sexual selection have favored a wide variety of animal-animal signaling mechanisms that allow organisms to identify and distinguish between members of their own species. Male songbirds sing elaborate melodies to attract potential mates, coleoid cephalopods use pigmented chromatophore organs in their skin to change color during courtship and mating behaviors, and rodents signal their social status to others using pheromones and chemical cues (Brainard and Doupe, 2002; Allen et al., 2017; Dulac and Kimchi, 2007). These signaling mechanisms have resulted in the evolution of neural circuits which mediate instinctive behaviors such as mating, parenting, territory defense, and predator avoidance (Tinbergen, 1952). Sensory cues in an animal’s environment are processed by these neural circuits to provide context about the sex, endocrine, and developmental status of another individual (Insel and Fernald, 2004). In many species, pheromones are the primary mechanism by which information about social and sexual status is exchanged between individuals (Dulac and Torello, 2003). However, mechanisms by which these sensory cues are represented in neural subpopulations of the brain structures involved in social behavior are not fully understood.

Highly reproducible and species-specific responses to pheromones have allowed neuroscientists to uncover the neural mechanisms of innate social behaviors across many species. Studies of the pheromone-behavior relationship in insects have shown that territory marking, colony identification, social hierarchy, reproductive status, and mating rituals are all behaviors tightly linked to chemical communication and pheromone signaling (Regnier and Wilson, 1971;
Sorensen, Christensen, and Stacey, 1998). Ants living in complex social hierarchies within a colony have been found to release pheromones in blends, which is thought to increase the specificity of recognition and allow communication of more complex messages to other individuals (Hölldobler and Wilson, 1990). For example, ants of the *Formica sanguinea* group produce a cocktail of multiple chemical compounds that create an `alarm pheromone` used to alert other members of the colony to a potential threat, and to induce behaviors aimed at protecting their young and attacking an intruder (Regnier and Wilson, 1971). Using chemicals for communication and behavioral state changes has also been observed in animals living in aquatic environments (Sorensen et al., 1988), as water is an effective medium for transmitting chemosensory cues over long distances. Terrestrial vertebrates have evolved two functionally and anatomically distinct olfactory systems to detect chemosignals (Dulac and Torello, 2003), further demonstrating the evolutionary advantage of pheromone signaling.

In addition to the immediate effects pheromone signaling can have on an animals’ behavior, they can also produce long lasting changes to an animals’ physiological and hormonal states (Wilson, 1963). When male pheromones are detected by a young female mouse, the onset of puberty will be advanced, the oestrous cycle will begin, and a current pregnancy may be terminated (Vandenbergh, Whitsett, and Lombardi, 1975; Novotny et al., 1999). These effects are the result of modifications to the hypothalamic-pituitary axis (HPA) which regulates the release of two hormones, luteinizing hormone (LH) and prolactin (PRL) (Halpern, 1987). Responses to male pheromones typically result in an increase of LH production and a decrease in PRL, while responses to female pheromones are associated with an increase in PRL (Keverne and de la Riva, 1982). In mammals, the HPA axis serves as a key modulator of long-term physiological and endocrine changes and links the sensory detection of chemical cues in the environment to lasting effects.
The Vomeronasal Pathway

Terrestrial vertebrates have evolved two anatomically and functionally separate olfactory systems used to respond to chemosensory input and to orchestrate behavioral responses (Dulac and Wagner, 2006; Dulac and Kimchi, 2007). The main olfactory system and the vomeronasal pathway work together to sense, interpret, and respond appropriately to chemosensory inputs. Each system is classified by the kinds of chemical cues that they detect. Volatile compounds are detected in the main olfactory system by the main olfactory epithelium (MOE), which projects to brain regions involved in cognitive odor processing (Sosulski et al., 2011). Non-volatile compounds are detected in the vomeronasal pathway by the vomeronasal organ (VNO), which sends projections to the accessory olfactory bulb (AOB) that then sends axons to the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) (Morris et al., 2008). My study focuses on the vomeronasal pathway and MeA and the role they play in the sensory representation of social stimuli.

The VNO is a bilaterally symmetrical tubular structure divided by the nasal septum with a crescent shaped lumen and in rodents is located at the base of the nasal cavity (Keverne, 1999). It is enclosed in a bony capsule, and opens to the base of the nasal canal through a duct (Keverne, 1999). The lumen of the VNO is lined with two families of G-protein-coupled receptors (GPCRs), termed V1Rs and V2Rs (Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Dulac and Torello, 2003). The expression pattern of these receptors is restricted such that each receptor gene is only expressed by a small subset of neurons in the VNO epithelium (Dulac and Axel, 1995). These distinct populations of VNO receptor cells each
respond to different chemosignals with high specificity, highlighting the role that the VNO plays in beginning the segregation of behaviorally relevant information (Holy, Dulac, and Meister, 2000; Leinders-Zufall et al., 2000; Nodari et al., 2008; Isogai et al., 2011; Turaga and Holy, 2012).

The accessory olfactory bulb (AOB) is the main recipient of VNO projections (Keverne, 1999) and is divided into two divisions, the anterior AOB and posterior AOB. Neurons in the anterior AOB receive input from V1R receptor expressing cells in the VNO, while neurons in the posterior AOB get input from V2R receptor expressing cells (Billing et al., 2020). Recordings from individual neurons in the AOB showed complex response patterns, suggesting that it is here where sensory information across distinct vomeronasal receptors is integrated (Wagner et al., 2006; Bergan, Ben-Shaul, and Dulac, 2014).

The medial amygdala (MeA) is the primary recipient of AOB projections. While it receives input from both divisions of the AOB, a subpopulation of cells in the MeA that express the enzyme aromatase receives a significantly higher number of projections from the anterior AOB (Billing et al., 2020). Individual neurons in the MeA have been shown to respond selectively to different social cues (Bergan, Ben-Shaul, and Dulac, 2014), indicating the role it plays in transforming vomeronasal sensory information into both sex specific and species specific responses. Outputs from the MeA project to nuclei in the hypothalamus involved in reproductive and aggression motor responses (Petrovich, 2001; Choi et al., 2005).

**The Medial Amygdala and the Social Behavior Network**

The medial amygdala (MeA) is known to play a central role in identifying social cues and generating innate social behaviors (Newman, 1999; Yao et al., 2017; Bergan et al., 2014; Unger et al., 2015). In mice, the MeA is important for behaviors such as aggression, reproduction,
social recognition, and anxiety (Yao et al., 2017; Unger et al., 2015; Ferguson et al., 2001; Hong et al., 2014). If MeA signaling is disrupted, mice show significant declines in both social and predator recognition (Ferguson et al., 2001).

Different subregions of the MeA are tightly linked to different behaviors. In particular, the posteroverentral MeA (MeA_pv) is critical for defensive behavior (Choi et al., 2005), and the posterodorsal MeA (MeApd) is critical for social recognition and reproductive behaviors (Meredith, 2004). The MeA processes social information differently in males and females. Recording from individual neurons in the MeA of anesthetized males and females during VNO stimulation with conspecific cues showed that neurons respond more frequently to opposite sex stimuli than same-sex stimuli (Bergan, Ben-Shaul, and Dulac, 2014).

MeA anatomy can differ across the sexes. In rodents, the MeApd of females is smaller than in males (Cooke, 1999), and the neuronal soma cell size is larger in males compared to females (Morris et al., 2008). Additionally, the density of nerve fibers projecting from the AOB to aromatase-expressing neurons in the MeA is denser in males than females (Billing et al. 2020). These properties make the MeA an intriguing region to target and record from to further understand its role in sex-specific encoding of social stimuli.

**Sex-Specific Processing of Social Cues**

The medial amygdala has been previously shown to display sexually dimorphic sensory representations of social stimuli (Bergan, Ben-Shaul, and Dulac, 2014), unlike its upstream relay stations (AOB and VNO). In anesthetized animals, extracellular recordings showed that neurons in the MeA respond selectively when the VNO is stimulated with urine samples from
predators, male, and female mice (Bergan, Ben-Shaul, and Dulac, 2014). Neurons that responded to conspecific social stimuli were located more dorsally than were neurons that responded to predator stimuli (Bergan, Ben-Shaul, and Dulac, 2014). Neurons in the MeA have also been shown to respond significantly more frequently to opposite-sex stimuli, with 82% of male MeA neurons responding more strongly to female stimuli, and 83% of female MeA units responding stronger to male stimuli (Bergan, Ben-Shaul, and Dulac, 2014). This sexual dimorphism was not observed in recordings from juvenile mice, where MeA neurons were shown to be significantly less selective in their responses to social stimuli. This result indicates that sexually dimorphic sensory representation of social stimuli in the MeA emerges after puberty.

The synaptic connections found in the MeA have also been shown to be sex specific (Billing et al., 2020). AOB inputs to aromatase expressing neurons in the MeA are different between males and females, with males showing a higher density of anterior AOB axonal projections into the MeA (Billing et al., 2020). This difference in synaptic connectivity between males and females is a likely mechanism that directs sex differences in social behaviors that are thought to originate in the MeA.

**The Aromatase Enzyme**

Aromatization, the metabolism of androgens into estrogens, is a fundamental process for both endocrine control and development and has been shown to occur in many species (Callard, Petro, and Ryan, 1978; Knapstein et al., 1968; Naftolin et al., 1975). Aromatase, the enzyme responsible for this process, has been linked to the regulation of neuroendocrine events and reproductive behaviors (Naftolin and MacLusky, 1982; Garcia-Segura, 2008). Recent work has also shown a broader scope for aromatase function including roles in synaptic plasticity,
neurogenesis, mood, and cognition (Garcia-Segura, 2008). By regulating local levels of estrogens, aromatase also plays a role in the sexual differentiation of brain regions associated with gonadotrophin release and sexual behavior (MacLusky and Naftolin, 1981; Naftolin, 1994; Lephart, 1996).

In the mouse brain, the MeA contains the highest population of aromatase-expressing neurons (Balthazart et al., 1990; Yao et al., 2017). The MeA also has a high density of both androgen and estrogen receptors (Sheridan, 1979; Yokosuka et al., 1997; Cooke, 1999), suggesting the local activity of aromatase is linked to MeA neuron function. Much of testosterone’s effect on the brain comes after its conversion to estradiol and subsequent activation of estrogen receptors (Bakker et al., 2003; Sholl et al., 1989; Lephart, 1996; Phoenix et al., 1959). If the aromatase gene is knocked out in male mice they show reduced preference towards female mice when compared to male wild type mice (Fisher et al., 1998). The sexually dimorphic responses in the MeA are not observed in juvenile mice or in mice lacking aromatase (Bergan, Ben-Shaul, and Dulac, 2014), consistent with the hypothesis that steroid hormone activity is activated at puberty. The localization of aromatase in the MeA and the corresponding neural circuitry of the vomeronasal system makes aromatase-expressing neurons an interesting target to investigate their role in social recognition and sensory representation.
Hypothesis & Specific Aims

The main question that I set out to answer at the start of this project was: How do aromatase-expressing neurons in the MeA of awake, behaving mice respond to social stimuli on a moment-to-moment basis? This population of neurons has never been previously recorded from in awake, behaving animals, but previous studies led me to hypothesize that aromatase expressing neurons in the MeA of awake, behaving mice are responsible for the sensory representation of social stimuli, and the activity of these neurons is strongly correlated with the onset of an investigation. To test this hypothesis, I set out to accomplish three specific aims which I have outlined below:

Specific Aim #1
Build a novel behavioral apparatus capable of presenting social stimuli to live animals in a controlled and reproducible environment, while also permitting in situ recordings from the brain.

Specific Aim #2
Record the moment-to-moment activity of aromatase expressing neurons in the medial amygdala of awake, behaving mice in response to social stimuli.

Specific Aim #3
Investigate the role of aromatase neurons in responding to social stimuli, governing sex recognition, and generating social behaviors.

Each specific aim listed above played a role in my overall approach to test my hypothesis. I conducted three different behavioral experiments to investigate the role of aromatase-expressing neurons in responding to social stimuli. The behavioral apparatus described in Specific Aim #1 allowed me to conduct two of these, which involved presenting an awake, behaving mouse with up to four live animal stimuli, or four urine sample stimuli. The third behavioral experiment involved putting a mouse in a clean cage and manually placing and
removing social stimuli (live animals or urine samples) into the cage. Each experimental design was paired with a fiber photometry system, outlined in Specific Aim #2, which I developed a working protocol for. This technique was new to our lab at the start of my project but is now fully operational and proven to be efficient at capturing the activity of a genetically defined population of neurons in awake, behaving mice. These three experimental designs allowed me to ask another question: Do aromatase-expressing neurons respond differently based on the type of social stimuli presented, and do different methods of stimuli presentation alter this response? For this question, I hypothesized that aromatase-expressing neurons will respond stronger to live animal stimuli then urine sample stimuli, and unrestricted social interactions with live animal stimuli will drive aromatase neuron activity more than restricted social interactions.

Based on previous studies, I predict that aromatase-expressing neurons will respond to social stimuli from males, females, pups, and predators, and that the activity of these neurons will be directly linked to investigative social behaviors. My project used only male mice as test animals and based on previous research I predict that aromatase neurons in males will respond stronger to female stimuli than to both male and predator stimuli. By comparing results from the three experimental designs I have laid out, I predict that a response selectivity pattern will emerge showing that aromatase neuron response strength is dictated by stimuli salience and presentation style.
Materials and Methods

Fiber Photometry

A 2-channel demodulating fiber photometry system configuration (Doric Lenses, Quebec, QC) was used to measure the change in fluorescence over time of two different fluorescent protein markers present in the MeA of freely moving animals. We used a fiber photometry console (Doric Lenses, FPC) and a 2-channel LED driver (Doric Lenses, LEDRVP_2CH_1A – US) to modulate the wavelengths emitted from two independent LED sources. The first LED emitted blue light with wavelength of 465nm (Doric Lenses, LEDC1B_FC), and the second LED emitted green-yellow light with a wavelength of 560nm (Doric Lenses, LEDC1-560_FC). Both LEDs, the fiber photometry console, and the LED driver were positioned upstream of a five port Integrated Fluorescence Mini Cube (Doric Lenses, FMC5_E1(450-490)_F1(500-540)_E2(550-580)_F2(600-680)_S). Two excitation input ports on the mini cube connected to the LEDs via fiber optic cables allowed the light from each LED to enter the mini cube. The two excitation wavelengths were then combined in the mini cube and channeled into a 2-meter low autofluorescence mono-fiberoptic patch cord with a 400um core diameter (Doric Lenses, MFP_400/430/1100-0.48_2m_FCM-MF1.25). The distal end of the patch cable was connected to a metal ferrule adhered to the skull of the subject animal that had received a unilateral pressure injection of AAV-Syn-Flex-GCAM6s in the MeA and then been implanted with a 7mm fiber optic cannula (Doric Lenses, MFC_400/430 0.66_7mm_MF1.25_FLT).

Following a calcium influx into a neuron infected with GCAMP6s, a conformational change occurs as the M13 subunit of the GCaMP protein binds to a calmodulin subunit. While in its active conformation, the cpEGFP protein absorbs the 465nm light from the LED and emits
green light with a wavelength of 525nm. Our control signal, the fluorescent marker tdTomato, is not calcium dependent and is excited by the second LED emitting light at 560nm wavelength. These cells emit red light with a wavelength of 600nm. The GCaMP6s (525nm) and tdTomato (600nm) emission wavelengths then combine to form one beam of light which re-enters the patch cord and travels back into the Mini Cube where it is split back into two independent streams by dichromic mirrors. Two fluorescence emission ports on the mini cube, each equipped with a fluorescence detector head (Doric Lenses, FDH), capture the split light beams and send them to their respective fluorescence detector amplifiers (Doric Lenses, FDA) via fiber optic connector cables. Here, each beam of light is amplified and returned back to the fiber photometry console where the data is synchronized and sent to a computer running Doric Neuroscience Studio software. This software allows for real time data visualization, and all files were saved in .csv format and exported for subsequent analysis.

**Animals**

All mice used in this study were bred in house and maintained in a temperature (22°C) and light (12hr light:12hr dark) controlled vivarium with ad libitum access to food and water. Experimental animals between 2-6 months old were used for fiber photometry surgeries and experiments. Only male mice were used as experimental animals in this study, as they have a larger population of aromatase-expressing neurons in the MeA than females (Billing et al., 2020). Surgeries conducted on two female test animals were unsuccessful. Future studies will aim to investigate the possible sex difference in aromatase-expressing neuron responses. Stimulus animals used for experiments ranged between 9 days and 9 months old. Animals were
group housed in single-sex cages prior to surgery, after which they were placed in single-housing for recovery and subsequent experimental use and euthanasia. All experiments were performed in compliance with National Institute of Health guidelines. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

Transgenic Animals & Adeno-Associated Viruses

The Cyp19a1-Cre transgenic line was generated through BAC (bacterial artificial chromosome) recombination as reported previously in Yao et al. (2017). The Cre coding sequence was inserted in front of the start codon of the Cyp19a1 gene, which encodes aromatase. The ATG codon of the Cyp19a1 gene in the BAC was mutated into a TTG stop codon. This resulted in the expression of Cre in all aromatase-expressing cells in the brain.

A Lox-STOP-Lox-tdTomato reporter line with a LoxP-flanked STOP was used to express tdTomato in Cre+ cells (Ai9, The Jackson Laboratory, Bar Harbor, Maine; Madisen et al., 2010). Crossing homozygous Cyp19a1-Cre mice with homozygous Rosa26-lsl-tdTomato reporter line resulted in a double transgenic mouse line that expressed bright tdTomato fluorescence in aromatase expressing neurons. Expression of both Cre and tdTomato was observed for multiple generations, and the tdTomato expression pattern mirrored endogenous aromatase expression throughout the brain. Transgenic animal behavior and development were consistent with that of wild type mice. Reliable genetic access to aromatase neurons using this strategy was previously described and verified in the study by Yao et al. (2017). Genotyping of tissue samples (ear snips) provided verification of both Cre and tdTomato expression in MeA neurons throughout multiple generations (Transnetyx, Inc., Cordova, TN).
An adeno-associated virus (AAV) was used to target and infect aromatase cells in the MeA with the Cre recombinase-activated ultrasensitive protein calcium sensor GCaMP6s. AAV-Syn-Flex-GCaMP6s-WPRE-SV40 was purchased from Addgene (Catalog #:100845) and stored in a -80 °C freezer. This AAV contains the coding sequence of GCaMP6s flipped upside down and in the reverse orientation, and two flanking Lox sites, LoxP and Lox511. When injected into the brain of a Cyp19a1-Cre animal this virus will infect all cells in the target region, but GCaMP will only be expressed in neurons that express Cre. After entering a Cre+ neuron, the lox sites are cut by Cre but cannot recombine with each other. This results in the coding sequence of GCaMP being flipped into the correct orientation for transcription and translation. Cre+ neurons infected with AAV-Syn-Flex-GCaMP6s will continuously express GCaMP6s until the host animal is euthanized. Neurons that do not express Cre will not express GCaMP6s, as they do not have the ability to flip the coding sequence into the appropriate configuration. The effectiveness of the GCaMP6s protein for recording neural activity was originally demonstrated by Chen et al. (2013).

**Surgical Procedure for Fiber Optic Cannula Implant**

Animals were initially anesthetized using an isoflurane incubation chamber and then moved to a stereotaxic surgery rig where anesthesia was maintained with 1-2% isoflurane and oxygen flowing at 1.5 lpm. Aseptic conditions were maintained throughout the surgery. Mice were secured to the surgery rig using a bite bar and two head screws secured anterior to each ear for stabilization. Hair was removed from the scalp using surgical scissors, and Nair was applied to expose the skin. Iodine was spread around the exposed skin and washed away with sterile
An incision was made along the midline of the scalp beginning posterior to both ears and extending approximately 1cm anterior. Excess skin was cut away to reveal the top of the skull and to allow sufficient surface area for adhesive to bind. Hydrogen peroxide was applied to the skull to remove connective tissue and expose Bregma. A small ink dot was placed over the coordinates to target the MeA, and a small craniotomy was preformed to expose the brain.

Each animal received 5-7 unilateral pressure injections (50.6nL each) of the Cre-dependent calcium sensor AAV-Syn-flex-GCaMP6s (Addgene, ID#100845) in the posterior dorsal MeA (Bregma -1.8, Lateral 1.8, Depth 4.5 to 5.5) using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA). Glass pipettes used for injections were backfilled with mineral oil prior to being loaded with the virus. Injections were spaced 10 minutes apart to allow sufficient local viral infection. A second craniotomy was then performed on the contralateral side of the skull, approximately 0.5cm anterior to the original craniotomy, where a bone screw was inserted. A small space was left between the head of the screw and the base of the skull to serve as an anchor for the adhesive. Immediately following viral injection and bone screw placement, mice were implanted with a fiber optic cannula (Doric Lenses, MFC_400/430-0.66-7mm) 0.5mm dorsal to the viral injection site in the MeA. Fiber optic cannulas were secured to the skull using adhesive dental cement (Metabond, Parkell Inc.) and approximately 15 minutes were allowed for this to dry.

Immediately following surgery mice were placed in a clean cage, single-housed, and set on a heating pad overnight with ad libitum access to food and water. The first dose of pain medication (Meloxicam, 2ml/dose, 1:10 dilution in PBS) was then administered. The following morning, mice were assessed for activity, pain, and the incision was checked for bleeding and infection. Mice were then moved back to the vivarium where they received pain medication once daily for three days. To allow sufficient viral expression, 10-14 days were allowed before fiber
photometry experiments began. All surgeries were documented and logged according to lab protocols.

**D.R.O.P. Box**

A novel behavioral apparatus, the D.R.O.P. box (Delivers Rodents, Odors, & Pheromones), was built to allow precise control of social stimuli presentations to awake, behaving animals. A 24”x24” acrylic box was outfitted with four pulleys, a video camera, and hardware to allow compatibility with our Doric fiber photometry system. The acrylic box was raised 29” above the floor with t-slotted framing (McMaster Carr, Elmhurst, IL) providing structural support. Additional t-slotted framing was secured above the acrylic box and used to mount four stepper motors (Sparkfun, ID#ROB-09238), one above each of the four corners. This t-slotted framing extended 42” above the acrylic box and was secured to the box at each corner. Stepper motors were mounted 36” above the base of the acrylic box and were programed using a TeensyLC 32-bit microcontroller board (Sparkfun, ID#DEV-13305) running custom code written using Arduino software. Each motor was driven by a Big Easy Driver (Sparkfun, ID#ROB-12859). Custom spools and gears were modeled using OpenSCAD software and 3D printed by the Advanced Digital Design and Fabrication (ADDfab) core facility at UMass Amherst. Each stepper motor was outfitted with a gear, spool, 3 meters of fishing wire, metal clamp attachments, and weights. To provide underlighting, a custom 24”x36” sheet of styrofoam (FloraCraft) was cut to fit the base of the acrylic box, and infrared LED strips were secured underneath. A clear piece of acrylic was cut to size and placed over the styrofoam/LED complex allowing visualization of the entire behavioral arena. Custom MATLAB (Mathworks, Natick,
scripts allowed the user full control over each of the four motors, the LED underlighting, and timing parameters specific to the experimental design.

The ability to present multiple types of social stimuli in a randomized sequence was central to the design of this system. For the presentation of live animal stimuli, four metal wire cups were customized with an acrylic bottom attached to a hinge that allowed the experimenter to place a stimulus animal inside and secure the floor underneath them. Metal O-rings were fastened to the tops of each cup that allowed them to be attached to the pulley string. Four PVC pipes, 12” long and 3” wide, were mounted to the t-slotted framing 22” above the surface of the box. When the pulleys were in the UP position the metal wire cups would fit into the PVC pipe, enclosing the social stimulus and removing it from the behavioral arena. When the pulleys were in the DOWN position the cups would drop directly underneath the PVC pipe and rest on the surface of the box, where the experimental animal was then able to investigate the social stimuli inside.

For the presentation of urine sample stimuli, slight modifications were made to each pulley. The Arduino code that ran each stepper motor was changed to account for the reduced weight, as the metal wire cups were not used for this configuration. Each stepper motor now spun approximately twice as fast as it did when the cups were attached. This decreased the time between the stepper motor beginning to spin and the start of the presentation period. An 8oz fishing weight was attached to a metal O-ring tied to the bottom of each pulley string to provide a small amount of tension to the lines. Urine samples (~2ml) (See ‘Social Stimuli’ below for collection method) were placed on cotton nestlet squares (2”x2”) which then were secured to the O-rings at the bottom of each pulley line. The four PVC pipes described previously were left in the same position and enclosed each stimulus when the pulleys were in the UP position. When
the pulleys were in the DOWN position, the nestlet and metal fishing weight rested on the surface of the box where it was able to be investigated by the experimental animal.

Social Stimuli

The D.R.O.P. box described above was built to be able to present multiple types of social stimuli to an experimental animal. For this thesis I have focused on two kinds of social stimuli, live mice and urine samples.

Male and female mice between 3-9 months old from our colony in vivarium were used as stimulus animals for experiments involving animal-animal interactions. Pups between 9-14 days old were used only for clean-cage intruder trials. No pups were placed in metal wire cages attached to pulley systems (No pups were harmed in the making of this thesis!). All stimulus animals were transgenic, coming from either our Aromatase:Cre or Aromatase:Cre x tdTomato lines. Neither transgenic line has previously shown irregularities in behavior, reproduction, or development, justifying their use as stimuli in social behavior experiments (Yao et al., 2017). A detailed log was kept regarding which stimulus animals were used on a given day for trials. The same stimulus animals were used for more than one trial, but never on back-to-back days. Pups were only used one time each in an effort to reduce stress. Before a trial, all stimulus animals were acclimated to the behavior room for 30 minutes with access to food and water. If metal cups were being used to present animals, the stimulus animals were placed inside for 10 minutes for acclimation and then removed and put back in their home cage until the experiment began. After a trial, animals were placed back in their home cage and returned to the vivarium.
Urine samples were obtained from our adult male and female mice in vivarium. Mice were placed on a clear, flat plastic sheet on a sterilized surface in a fume hood. A clear, hollow acrylic cylinder approximately 1 foot tall and 6 inches wide was placed over the animal. Nose holes were drilled around the bottom of the cylinder to allow for ventilation. The animal was left in this cylinder for 10-15 minutes. After a urination, the cylinder was moved with the animal still inside and the sample was collected using a plastic bulb pipette and stored in a 50ml plastic tube. Urine from multiple animals of the same sex was pooled to make a stock solution which was stored in a -4°C refrigerator. For predator urine samples, wolf urine was purchased from Maine Outdoor Solutions, LLC (Hermon, ME) and stored in a -4°C refrigerator. Before an experiment, urine stock solutions were removed from the refrigerator and allowed to thaw to room temperature for 10 minutes. Next, 2ml of urine was collected in a plastic bulb pipette and smeared onto a cotton nestlet which was then placed on an O-ring clamp at the end of a pulley string. After an experiment nestlets were disposed of and urine stock solutions were placed back into the -4°C refrigerator.

**Stimulus Presentations**

Custom MATLAB scripts were written to control the D.R.O.P. box that allowed stimulus presentations to be randomized, reproduced, and easily manipulated to fit different experimental designs. Each of the four stepper motors were randomly assigned to deliver one type of stimulus (male, female, predator scent, control) and the corresponding pulley was loaded with the appropriate social stimuli. Each motor had three time parameters that were set prior to each experiment. In my experiments, stimuli were presented to the experimental animal for 60 seconds at a time. Each stimulus presentation was flanked by a 30 second ‘pre-presentation’ and
a 30 second ‘post-presentation’ time window. One ‘set’ of stimulus presentations was defined as the total time it took for each stimulus to be presented once in a random order. The D.R.O.P. box was then programmed to cycle through 8 ‘sets’, the order of which was randomly generated by custom MATLAB scripts (4 stimuli x 8 sets = 32 total stimulus presentations). A five-minute recording period before the first stimulus was presented, and a five-minute recording period after the last stimuli was raised were used to get pre- and post-baseline fiber photometry data. This trial structure was consistent for all experiments using the D.R.O.P. box, regardless of the type of social stimuli being presented.

For clean-cage intruder experiments, the experimental animal was placed in a clean, empty cage with the top removed. A ten-minute baseline fiber photometry recording was done. After this, a social stimulus (adult male, adult female, pups, wolf urine) was manually placed in the center of the cage. The social stimulus was left in the cage for a maximum of 10 minutes, and immediately removed if aggression or distress was observed by either animal. After 10 minutes the social stimulus was removed, and a five-minute post baseline fiber photometry recording period began. If multiple clean-cage intruder trials were run on the same day, a fresh cage would replace the cage used in the previous experiment.

**Tissue Collection and Imaging**

Animals were deeply anesthetized with isoflurane and exsanguinated with 50ml cold PBS followed by 25ml cold PFA. The brain was extracted, and fiber optic cannula removed. The brains were fixed in 25ml hydrogel and stored at -4°C for 48 hours. After 48 hours, excess hydrogel was removed, and tissue was washed with PBS overnight in a 37°C water bath with
gentle rocking. Brains were next transferred to a clearing solution and passively incubated for two days before active clearing. After passive clearing brains were actively cleared using a magnetohydrodynamic (MHD) clearing device (Dwyer, unpublished observations). In brief, this process removes unbound lipids from the tissue using a strong magnetic field, turning the sample from opaque to translucent. This process takes approximately 48 hours to complete. Following tissue clearing, the brain was placed in OptiView imaging solution and images of the MeA were acquired using a Zeiss Z.1 Lightsheet microscope (Carl Zeiss, Jena, Germany). Removal of the fiber optic cannula left a tract of displaced tissue which could be seen in the reconstructed image of the brain. This fiber tract was used to confirm correct cannula placement above the MeA injection site.

**Data Processing and Analysis**

All experiments were recorded using a NightVision DVR infrared camera system and video files were saved and exported in .avi format. Fiber photometry data files were saved and exported in .csv format. For experiments that used the D.R.O.P. box, a MATLAB file containing the names, times, and order of stimulus presentations was generated and saved in .mat format. All files were backed up to a secure server and stored on an analysis computer.

Custom MATLAB analysis scripts were written to work through each step of data analysis. They are as follows:

**Step 1: Load data into a user interface**

An Excel file was made that contained the file path names for each video, fiber photometry, and MATLAB file generated during experiments. Each trial was given a unique name that stated the date, test animal ID, and trial number. A custom GUI (graphical user interface)
interface) was then started in MATLAB and the Excel file was loaded in. A window in the GUI displayed each unique trial name. Selecting a trial would allow MATLAB to access the original data files associated with that trial and use them for analysis.

**Step 2: Time-synch all data files**

To time-synch the video recordings and photometry data, an LED was placed in the video frame during every trial and connected to the fiber photometry system via an analog output cable such that it would turn on when data acquisition began and turn off when fiber photometry recording stopped. To time-synch the video file and MATLAB output, the D.R.O.P. box was outfitted with infrared LED light strips beneath the surface which came on when the trial started and turned off when the trial was complete. The first step in analyzing these data was inputting the start and end times of the trial and fiber photometry recording into a pop-up window in the GUI.

**Step 3: Load, filter, and process fiber photometry data**

Each fiber photometry data file contained columns for time, GCaMP signal, and control signal. After being loaded into the GUI from the Excel file, the fiber photometry data were filtered using a running average filter. To smooth the fiber photometry signal each second of the data was filtered to produce the average signal per second. A new MATLAB file was then created and saved that contained the filtered data which was used for the remaining analysis steps. After filtering, four different processing options were able to be selected from in a dropdown menu in the GUI: ‘Regression Normalization’, ‘Regression Normalization (manual)’, ‘Regression Standard Deviation Restricted’, and ‘Plot Time Bins’.
Selecting ‘Regression Normalization’ would produce three plots. The first plot showed the filtered photometry data as a regression, with GCaMP signal on the Y-axis and control signal on the X-axis. Each data point plotted on the regression represented the signal of GCaMP and control at a single time period. A curve of best fit for the regression was generated using a polynomial equation that predicted the GCaMP signal from the control signal. To prevent overfitting or under-fitting the data, the user could input which degree polynomial they wished to use to fit the data best. On average, 4\textsuperscript{th} and 5\textsuperscript{th} degree polynomials produced the most accurate curves of best fit for the data. The second plot showed both the GCaMP signal and the control signal strength on the Y-axis and time on the X-axis. This plot allowed the user to see where in the dataset the GCaMP signal significantly deviated from the control signal. When no signal was present, these two lines would fluctuate in synchrony with each other. However, when there was a change in GCaMP signal and no change in control signal, the GCaMP line would show large spikes above the control signal line. This plot also showed the effect of photobleaching during a trial. Photobleaching is the gradual, but persistent, decrease in fluorescence signal that occurs over the course of fiber photometry data collection. It appears in both channels and is most noticeable early in data collection before gradually tapering off to a steady level. Despite the effects of photobleaching, GCaMP signal remained constant in its deviation from the control signal throughout each trial. The third plot showed the normalized GCaMP signal, with signal strength on the Y-axis and time on the X-axis. Subtracting the control signal from the predicted GCaMP values allowed spikes in GCaMP activity over the course of a trial to be visualized and removed the effect of photobleaching. All three plots were saved in a folder corresponding to the trial from which they were generated.

Selecting ‘Regression Normalization (manual)’ would load a window containing a
regression plot of the fiber photometry data without a curve of best fit. The user could then input which degree polynomial they wished to use to fit the data, and the number of data points they would later manually place on the regression plot. If the user chose to fit the data with a $5^{th}$ degree polynomial and 13 manual inputs, they would then be prompted to place 13 points on the regression plot in a way that reflected how they thought best fit the data. These points would then be used in a $5^{th}$ degree polynomial equation to produce a curve of best fit. While this approach may seem subjective, comparing these plots with those generated using the original regression strategy showed minimal differences and even reduced instances of over-fitting the data.

Selecting ‘Regression Standard Deviation Restricted’ would prompt the user to input the type of polynomial they wished to use to fit the data, as well as the number of standard deviations from the mean they wished to visualize. A regression plot showing data points in two different colors, red and cyan, fit to the polynomial degree inputted by the user would then be generated. Data points in cyan represented all data points that fell within one standard deviation of the mean, while data points in red represented those outside that range. This plot provided a way to easily visualize how much GCaMP signal was present in a dataset and how significant the response was.

Selecting ‘Plot Time Bins’ would prompt the user to input the number of bins they wished to group the fiber photometry data into. For example, inputting 10 would break up the fiber photometry data from a trial into 10 bins. A regression plot would then be generated, again with red and cyan colored data points. The most cyan colored points represented data points from early in a trial, and the reddest data points represented data points from the end of a trial. Shaded
points in between represented data points in the middle of a trial and were grouped by bin. This plot added the ability to see the transition of signal over time in a regression plot.

**Step 4: Score behavior**

Mouse behaviors were manually scored for each trial using video recordings and custom MATLAB scripts which allowed the user to place time stamped markers at multiple points in the video that contained behaviors of interest. Both D.R.O.P. box and clean-cage intruder experiments were scored using this method, with slight modifications based on the experimental design.

When scoring D.R.O.P. box experiments, the MATLAB file generated during a trial was loaded into a window along with the video and photometry data files. The video could then be played while a time-synched trace of the photometry signal containing events corresponding to stimulus presentations (pre-presentation, presentation, post-presentation) ran along the bottom of the video frame. This photometry trace could also be hidden to allow blind, unbiased video scoring. While the video was being played, the user was able to annotate time stamps down linking mouse behaviors to one of four behavioral state variables. ‘Base State’, ‘Start Sniff’, ‘Stop Sniff’, and ‘Grooming’ were the behavioral state variables chosen to be scored for all experiments. Each video file was scored from beginning to end such that every frame of the video was assigned to a behavioral state. Time stamps placed within the window of a stimulus presentation were automatically linked to that stimulus. The user also had the ability to remove time stamps that were placed incorrectly, move between stimulus presentation events quickly, and change the speed of video playback. When video scoring was complete, a new MATLAB file was generated and saved that contained the newly created time stamped behavioral state variables.
When scoring clean-cage intruder experiments the same process described above was used, but the MATLAB scripts were modified to account for the absence of the MATLAB file containing information about D.R.O.P. box stimulus presentations. A drop-down menu allowed the user to select which type of experiment they wished to score, and from there they were directed to the correct video playback window.

All trials were scored by two independent parties to reduce inter-observer bias in the video scoring process. With the help of an undergraduate researcher, I was able to generate two fully scored videos for each trial.

**Step 5: Plot results**

Combining the scored video file with filtered and processed fiber photometry data allowed the visualization of responses to each social stimulus presented during a trial. A set of custom MATLAB scripts were written that allowed the user to select which stimulus and behavioral state they wished to plot. For example, the user could select ‘male + start sniff’ to produce a graph showing the fiber photometry data that corresponded with each time an experimental animal began investigating a male stimulus during a trial. The user could also select to plot either z-score or fiber photometry signal on the Y-axis, as well as the amount of time both prior to and post investigation on the X-axis. Additional filtering, scaling, and baseline subtraction parameters were able to be modified. Three plot types were available to be chosen from in a drop-down box: ‘Mean and SEM’, ‘SNIPS’, and ‘Raster’. Selecting ‘Mean and SEM’ would produce a plot that collapsed all investigations of a particular stimulus into a solid black line indicating the mean response, and a shaded grey border indicating the SEM. Selecting ‘SNIPS’ would produce a plot showing the signal traces of all investigations towards a particular
stimulus, with a different colored line for each one. Selecting ‘Raster’ would produce a heatmap with each row on the Y-axis indicating a single investigation event, time on the X-axis, and a legend showing the color relationship to either z-score or photometry signal. All plots were automatically saved to a folder that contained the original data files generated from the trial.

**Step 6: Population analysis**

To produce plots combining data from multiple trials, a checkbox was put into the GUI that allowed the user to select which trials they wished to include for population analysis. Selection criteria for this step involved selecting all trials of the same structure from the three available experimental designs (D.R.O.P. box with live animal stimuli, D.R.O.P. box with urine stimuli, clean-cage intruder). After selecting the desired trials, clicking a button named ‘Population Analysis’ would open a new GUI that displayed a drop-down menu showing each social stimulus. The user was then able to choose which stimulus they wanted to perform a population analysis on and generate a plot showing the average response to that stimuli over the number of trials that had been previously selected. These plots had either z-score or photometry signal on the Y-axis, and time on the X-axis. The user could produce plots showing either the mean and SEM of all responses to a stimulus, or SNIPS from all investigations. Trials for each cycle of population analysis were selected based on experimental design. D.R.O.P. box experiments with live stimulus animals, D.R.O.P. box experiments with urine stimuli, and clean cage intruder experiments were all analyzed separately to produce plots showing the differences in responses for each experimental design.
Results

Fiber Photometry Signal Acquisition and Extraction

Injecting AAV-flex-GCaMP6s into the MeA of Aromatase:Cre transgenic animals allowed aromatase expressing neurons to transcribe the calcium sensitive fluorescent protein GCaMP6s (Figure 1A). Following injection, a fiber optic cannula implanted above the injection site was secured to the skull and 14 days were allowed for sufficient viral infection and healing. Connecting the fiber optic cannula to a patch cable running from our Doric fiber photometry system allowed the fluorescence of GCaMP6s to be captured and measured (Figure 1B). This system allowed for the simultaneous recording of two fluorescent channels, green (GCaMP6s) and red (control/tdTomato). To visualize GCaMP6s signal, data from both channels was plotted using a regression model (Figure 1C). Periods of increased GCaMP6s activity can be seen as deviations from the regression curve of best fit (Figure 1C, arrows). Plotting both the GCaMP6s and tdTomato fluorescence traces against time allowed individual GCaMP6s spikes to be seen (Figure 1D, arrows). Periods of no signal can also be seen in this plot during periods when both fluorescent traces deviate in synch with each other (Figure 1D, asterisk). The gradual decrease in fluorescence of both channels seen in Figure 1D is the result of photobleaching. Despite this, GCaMP6s signal strength remained consistent throughout the course of experiments. Fiber photometry recordings lasting 60+ minutes were able to consistently produce significant GCaMP6s signal, which was measured in z-score and shown in Figure 1E.
**Figure 1:** Fiber photometry signal acquisition and extraction. (A) Strategy to express GCaMP6s in aromatase neurons in the MeA and fiber optic cannula placement. Aromatase-expressing neurons in Arom:Cre transgenic mice express the enzyme Cre, which flips the GCaMP6s gene in the correct orientation for transcription and translation by an infected neuron. Implanting a fiber optic cannula above the MeA injection site allows fluctuations in the light emitted by GCaMP6s to be recorded. (B) Fiber photometry setup to record GCaMP6s activity in a freely behaving mouse. Two excitation wavelengths (560nm and 465nm) emitted by separate LEDs are combined by dichroic mirrors in a MiniCube and sent down a fiber optic patch cable and through
Figure 1 (cont.): a fiber optic implant above brain tissue infected with GCaMP6s. Fluorophores excited by these two wavelengths (600nm and 525nm) travel back up the patch cable where they are separated in the MiniCube by dichroic mirrors. The two response wavelengths are then sent to their respective detectors which amplify the fluorescent signal and send it to a computer running Doric Neuroscience Studio software for visualization and analysis. Lightbulb shown on mouse cage is used to synchronize the video recording to the fiber photometry data stream. (C) Regression plot showing GCaMP6s fluorescence on the Y-axis and tdTomato fluorescence on the X-axis. Arrows indicate periods of increased GCaMP6s activity. (D) GCaMP6s and tdTomato signal traces over the course of an experiment (~60min). Inset box shows a 10-minute time window where arrows indicate three GCaMP6s spikes. Asterisk indicates a fluctuation in both GCaMP6s and tdTomato fluorescence, marking a time when no GCaMP6s signal is present. (E) Z-score of GCaMP6s signal during the same experiment shown in C and D. Black horizontal line indicates a z-score of 0.

D.R.O.P. Box Experiments

A novel behavioral apparatus, the D.R.O.P. box (Delivers Rodents Odors & Pheromones) was designed to present social stimuli to awake, behaving animals and coupled directly to our fiber photometry system. Four pulleys located in each corner of the box were programed to lower social stimuli into a behavioral arena for a brief time period, and then raise stimuli out and where they were concealed in a plastic PVC tube. (Figure 2A). This apparatus allowed social interactions and stimulus presentations to be highly controllable and reproducible, while providing an open arena for a test animal to freely explore. Test animals repeatedly investigated presented stimuli (Figure 2B) and spent more time in the area of a presented stimuli compared to areas not containing stimuli. The D.R.O.P. box was designed to present two kinds
Figure 2: D.R.O.P. box design, configurations, and trial structure. (A) D.R.O.P. box frontal view. (B) Snapshot from a video recording an awake, behaving mouse in the D.R.O.P. box tethered to a fiber photometry system investigating a social stimulus (Stimulus is a live mouse in a metal wire cup). (C) Two configurations of the D.R.O.P. box, designed to present either live animals or urine samples to an experimental animal connected to a fiber photometry system. (D) Trial structure for D.R.O.P. box experiments. Stimuli were grouped in sets of 3 or 4 (depending on the experimental design) and each set was cycled through 8 times. Stimulus presentation order for each set was randomly generated. Asterisks indicate stimulus investigations by the test animal.
of social stimuli, urine samples and live animals (Figure 2C). Adult male and female animals were placed in metal wire cups attached to each pulley system. Each cup was outfitted with an acrylic floor attached to a locking mechanism to allow stimulus animals inside to move freely. Urine samples (2ml) were placed on cotton nestlets and attached to each pulley system along with a metal weight to provide the tension necessary for raising and lowering the stimuli. The D.R.O.P. box could be switched between these two experimental configurations in approximately 5 minutes. Each D.R.O.P box trial began with a 5-minute baseline fiber photometry recording period (Figure 2D). This was followed by 8 rounds of stimulus presentations where stimuli were grouped in sets of 3 or 4 and a unique presentation order was randomly generated for each set. One set of presentations was completed after each stimulus had been presented once. Each individual presentation event was flanked by both a pre-presentation and post-presentation period of 30 seconds, resulting in one minute between each presentation. Each stimulus was presented for 60 seconds at a time, during which the test animal was free to investigate (Figure 2D, asterisks). After all rounds of stimulus presentation were complete, a 5-minute post-baseline fiber photometry recording period occurred before the conclusion of the trial.

The GCaMP6s signal strength, corresponding animal position, and three time periods during a D.R.O.P. box trial can be seen in Figure 3A. During the baseline recording period (Figure 3A-1) no social stimuli were presented, but some significant GCaMP6s activity was recorded. This has been attributed to lingering scents in the room used for behavioral experiments, and the constant sniffing behavior exhibited by our test animals. During a stimulus presentation (Figure 3A-2), increased GCaMP6s activity strongly correlated with the animal’s
Figure 3: GCaMP6s signal trace, animal position, and SNIP data from a single D.R.O.P. box experiment. (A) Z-score plot of GCaMP6s signal for the course of a D.R.O.P box experiment where live animals were presented. Vertical lines indicate stimulus investigations. Numbers 1-3 indicate pre-baseline recording period, stimulus presentation, and post-baseline recording period, respectively. (A1) Animal position during pre-baseline fiber photometry recording period. (A2) Test animal investigation of a presented stimulus. (A3) Animal position during post-baseline fiber photometry recording period. (B) Z-score plot showing one investigation of a male stimulus
Figure 3 (cont.): during an experiment (Time indicated by asterisk in A). Solid red line indicates start of investigation. T=0 marks the onset of investigation. (C) Raster plot showing fiber photometry response (as z-score) to 8 individual investigations of a social stimuli from the same experiment. Yellow shift indicates higher z-score, blue shift indicates lower z-score. T=0 marks the onset of an investigation.

Investigation of a presented stimuli. Vertical lines in Figure 3A show additional investigations of the same social stimuli during the trial. Z-scores of photometry signal between 2-5 were consistently recorded during investigations of social stimuli. During the post-baseline recording period, GCaMP6s activity decreased as no stimuli were presented during this time (Figure 3A3). Manual scoring of test animal behavior for each trial allowed individual investigations towards each stimulus to be recorded. The GCaMP6s signal associated with investigations of each stimuli could then be plotted, as shown in Figure 3B. These SNIPS of photometry data spanned 20 seconds prior to an investigation, and 40 seconds following an investigation. Each colored line in Figure 3B represents one investigation of the same social stimuli during a trial. Time zero (time re onset) marks the start of an investigation. To observe the change in the strength of response towards a stimulus over the course of an experiment, a raster plot (Figure 3C) was generated. Throughout the course of a trial, GCaMP6s signal decreased during repeated investigations of the same stimulus. While this phenomenon was not observed in every experiment, it points to the potential role of aromatase neurons in the habituation and adaptation to sensory cues in the environment.

A population analysis of all D.R.O.P. box trials that used live animals as social stimuli showed that aromatase neurons in the MeA of awake, behaving animals respond consistently and
**Figure 4:** Live animal stimuli evoke strong responses in arom+ neurons. Z-score indicates strength of response. (Black line indicates mean response, grey shaded region indicates SEM, T=0 indicates onset of investigation. (A) Response to investigations of live female stimuli (B) Response to investigations of live male stimuli (C) Response to investigation of control stimuli (empty metal wire cup). (N=4 trials)
significantly to both male and female stimuli (**Figure 4**). Male animals showed significant responses to both male and female stimuli, but not to our control stimuli. Male stimuli produced the highest responses (z-score = 0.8 +/- 0.2, n=4 trials), while responses to female stimuli were slightly lower (z-score 0.6 +/- 0.2, n=4 trials) (**Figure 4A-B**). However, responses to female stimuli lasted longer than responses to male stimuli (average female response = 30 seconds; average male response = 15 seconds). Investigations of the control stimuli did not show significant responses (z-score = 0.1 +/- 0.1, n=4 trials). (**Figure 4C**).

A population analysis of all D.R.O.P. box trials using urine samples as social stimuli showed that aromatase neurons in the MeA of awake, behaving animals also respond consistently and significantly to both male and female stimuli, but not to predator or control stimuli (**Figure 5**). However, responses to urine samples were, on average, lower than responses to live animals. Of all responses to urine samples, female urine samples produced the highest responses (z-score = 0.4 +/- 0.2, n=4 trials), while responses to male stimuli were slightly lower (z-score 0.25 +/- 0.15, n=4 trials) (**Figure 5A-B**). Responses to male urine samples lasted longer than responses to female urine samples (average male response = 25 seconds; average female response = 10 seconds). Interestingly, investigations of wolf urine did not cause neural activity to elevate in any statistically significant way above baseline conditions (z-score = -0.2 +/- 0.2, n = 4 trials) (**Figure 5C**). Investigations of the control stimuli also did not show significant responses (z-score = -0.5 +/- 0.2, n= 4 trials). (**Figure 5D**).
**Figure 5:** Urine sample stimuli evoke smaller responses in arom+ neurons than do live stimuli. Z-score indicates strength of response. (Refer to Figure 4 for comparison. Axes are standardized) (Black line indicates mean response, grey shaded region indicates SEM, T=0 indicates onset of investigation. (A) Response to investigations of female urine sample. (B) Response to investigations of male urine sample. (C) Response to investigations of wolf urine sample (D) Response to investigations of control stimuli (cotton nestlet with no urine applied). (N=4 trials)
Clean-cage intruder Experiments

Aromatase neurons in the MeA showed strong responses to conspecific social stimuli, but not to predator stimuli, in clean-cage intruder experiments (Figure 6). Test animals were placed in a clean cage, and a 10-minute baseline fiber photometry recording period occurred before social stimuli were manually placed in the cage. Stimuli were left in the cage for 10 minutes, after which they were removed and a 10-minute post-baseline recording period began. Investigations of stimuli were manually scored using the same methods as D.R.O.P. box experiments. Clean-cage intruder experiments showed higher responses to conspecific social stimuli than were observed in the D.R.O.P. box experiments. Investigations of both male and female clean-cage intruders showed significant responses (z-score = 2.5 +/- 0.5, and z-score = 1.5 +/- 0.5, respectively) (Figure 6A-B). When 9-day old pups were placed in a cage with a test animal, responses tied to investigations were higher than the responses seen in response to both male and female stimuli (z-score = 3.0 +/- 0.5). (Figure 6C). Consistent with previous results, responses to investigations of wolf urine samples did not produce significant responses (z-score = 0.4 +/- 0.2) (Figure 6D). This set of clean-cage intruder trials shows that while D.R.O.P. box experiments are effective in presenting social stimuli and generating aromatase neuron responses, more naturalistic behavioral interactions may be more effective in promoting aromatase neuron activity.
Figure 6: Clean-cage intruder experiments evoke strong responses in arom+ neurons. Z-score indicates strength of response. (Refer to Figure 4 and Figure 5 for comparison) (Black line indicates mean response, grey shaded region indicates SEM, T=0 indicates onset of investigation). (A) Response to investigations of a male clean-cage intruder. (B) Response to investigations of a female clean-cage intruder. (C) Response to investigations of a pup clean-cage intruder. (D) Response to investigations of wolf urine sample clean-cage intruder. (N=1 trial)
Discussion and Future Directions

Aromatase and social recognition

Results from these experiments show that aromatase neurons in the MeA of awake, behaving male animals respond robustly to conspecific social stimuli, but do not respond to predator stimuli or non-social stimuli. The D.R.O.P. box experiments showed that this apparatus is effective in delivering social stimuli to awake, behaving animals in a controllable and reproducible manner. The results also indicate that responses differ based on both the type of stimuli presented and the mode of presentation. D.R.O.P. box experiments using urine sample stimuli produced the weakest responses, followed by D.R.O.P. box experiments using live animals, with clean-cage intruder experiments producing the strongest responses. This indicates that social interactions which allow for full animal-animal contact and are non-restrictive are instances where aromatase neuron activity is at its highest. From a naturalistic standpoint this is to be expected, as animals in their natural environment are not restricted in their social interactions and often use multiple social behaviors in the process of investigating another individual.

Wolf urine stimuli did not produce responses in aromatase neurons, indicating that these cells are not responsible for the sensory representation of predator stimuli. Previous recordings have shown that neurons in the MeA do respond to predator stimuli (Bergan, Ben-Shaul, and Dulac, 2014), but these recordings were not done in exclusively aromatase expressing cells and spanned the entitle length of the MeA. Recordings for the experiments in this thesis targeted one region of the MeA, the MeApd, which has been linked to social and reproductive behaviors.
Another region of the MeA, the MeApv, is primarily involved in defensive behaviors and was not the primary target for these experiments. This explains the minimal responses to predator stimuli.

**Habituation and motivation**

Multiple trials showed the gradual decrease in aromatase neuron activity following multiple investigations of the same stimuli during an experiment. This has led to discussion about the potential role of these neurons in habituation and social homeostasis. Again, when thinking in terms of a naturalistic environment, the ability of an animal to remember a previous social interaction is beneficial and can allow for the formation of more complex social hierarchies within a species. The ability to remember specific social stimuli can also allow species such as the monogamous prairie vole (*Microtus ochogaster*) to remember their previous mating partner and form strong social bonds with them (Amadei et al., 2017). Future experiments will provide further insight into this phenomenon. An experiment designed to repeatedly present the same social stimuli for a period of time, and then switch and present a different social stimulus of the same sex may produce results that will allow us to determine if the individual stimulus is the cause for this habituation, or if the decline in response remains consistent toward stimuli of the same sex over time.

Another future experiment will involve making modifications to the D.R.O.P. box such that the experimental animal will have access to either a lever or nose-poke hole that when activated will activate a pulley system and present social stimuli. This configuration will allow the role of aromatase neurons in social motivation to be investigated, as test animals will be able to actively initiate social interactions as opposed to simply being presented with social stimuli.
Similar experimental designs have been used to study the generation of aggression in the ventromedial hypothalamus (Hashikawa et al., 2017). Our current system allows for a similar experimental design to be built and a behavioral paradigm created to study the neural responses to social motivation in awake, behaving animals.

**Ultrasonic vocalizations**

We have successfully recorded ultrasonic vocalizations from mice using specialized microphones during both D.R.O.P. box and clean-cage intruder trials. While we are in the early stages of this process, we have observed that mice vocalize during investigations of live social stimuli but not to urine samples. Vocalizations were consistently captured when test animals had a prolonged interaction with a stimulus, and the strongest vocalizations were captured from a male mouse investigation 9-day old mouse pups. Our goal is to tie in this vocalization data into our fiber photometry data analysis pipeline and investigate the possible link between aromatase neuron activity and the production of these ultrasonic vocalizations.

**Effects of oxytocin and vasopressin on aromatase neurons**

The neuropeptides oxytocin and vasopressin, which evolved from the same progenitor peptide, have been shown to be important in social behavior across many species (Yao et al., 2017; Ferguson et al., 2001; Boender and Young, 2020; Bosch and Young, 2018). These two peptides are secreted in by cells in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON), which are both brain regions that send projections to the MeA. Our lab has recently planned experiments aimed at studying the neuromodulatory effects of these
neuropeptides on aromatase neurons in the MeA and the effects they have on social behavior and social interactions. Combining our fiber photometry system with the behavioral paradigms outlined in this paper will allow us to conduct a range of experiments investigating the relationship between oxytocin, vasopressin, aromatase, and social behavior. Briefly, we have plans to use both excitatory and inhibitory DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) to control the activity of oxytocin and vasopressin in the MeA. We also have plans to modify our fiber photometry system to record two calcium dependent wavelengths from the same brain region, allowing the activity of two neural subpopulations to be observed simultaneously. The projects shown in this thesis have provided a baseline dataset for aromatase neuron function in the absence of neuromodulation. Comparing data from these experiments to data collected from our planned neuromodulatory experiments will allow us to further explore the role of aromatase neurons in the sensory representation of social stimuli.
Supplementary Figure 1: Multiple regression plot types show same fit for fiber photometry data. (A) Regression plot showing standard deviation restricted model. Cyan points fall within one standard deviation of the regression curve. Red points fall outside one standard deviation of...
**Supplementary Figure 1 (cont.):** the regression curve. Solid red line indicates curve of best fit using a 2\textsuperscript{nd} degree polynomial. (B) Regression plot showing time bin restricted model. Cyan points indicate data collected early in an experiment. Red points indicate data collected later in an experiment. (C) Signal trace of GCaMP6s and tdTomato showing GCaMP6s signal and photobleaching effects. Fit using manual regression plotting shown in (D). (D) Manual regression plot using 13 inputs and a 2\textsuperscript{nd} degree polynomial fit. Cyan points indicate data collected early in an experiment. Red points indicate data collected later in an experiment. Red circles indicate manual inputs. Solid blue line indicates curve of best fit using a 2\textsuperscript{nd} degree polynomial.
References


