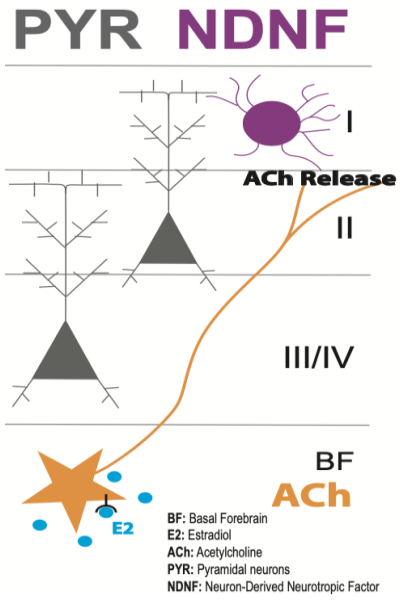
When Sound Matters: State-Dependent Processing of Vocalizations in Mouse Auditory Cortex

Introduction

You know that song, the one that simply gets you crying. For me, that's Olivia Rodrigo's recent album. Music is powerful, able to elicit behavioral responses linked to emotions in humans, such as pupil dilation and heart rate changes. As a musician and a listener, I've pondered how complex sounds can elicit such behavioral reactions. To create such behavioral responses, emotional cues must be extracted from the sound stimuli. Furthermore, the effect of sound on emotional state may not solely depend on the acoustic components. The processing of sound stimuli may also depend on our internal state, such as hormones.



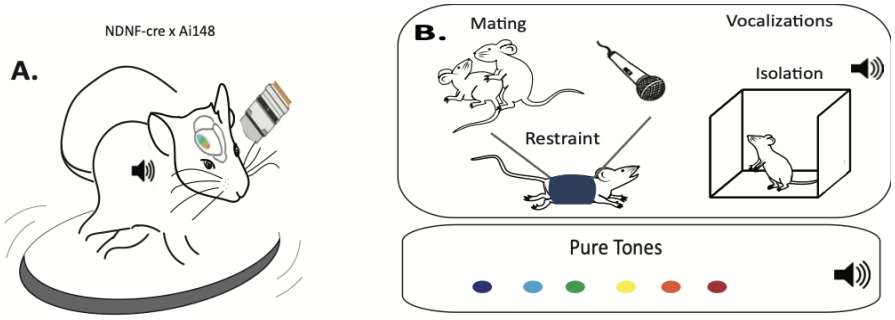
The superficial layers of the auditory cortex process the acoustic features of complex sounds while integrating top-down signals that shape their interpretation Interneurons in these layers expressing Neuron-Derived Neurotrophic Factor (NDNF) are well positioned to encode both the features of sensory stimuli and the emotional salience of these stimuli. In turn, these NDNF interneurons modulate the activity of both excitatory and other inhibitory cortical cell types. By integrating extensive neuromodulatory inputs, these neurons may shape auditory processing and influence behavioral responses. However, how internal states affect the responses of these interneurons to salient sensory stimuli remains unknown.

My project in the Takesian Lab has focused on how the hormonal cycle in female mice may affect how NDNF interneurons in the mouse auditory cortex encodee salient vocalizations. We used vocalizations with positive (mating), negative (restraint), and neutral (isolation) valence as a model for understanding how internal state influences auditory processing and shapes behavior. We analyzed NDNF interneuron responses longitudinally under two photon imaging as awake, head-fixed mice listened to these salient vocalizations. We hypothesized that estrous state may change NDNF interneurons responses. NDNF interneurons respond robustly to acetylcholine, which influences processing of sounds. Cholinergic neurons in the basal forebrain have estrogen receptors, and the released estrogen may therefore result in increased acetylcholine release in response to salient vocalizations. An increase in cholinergic-evoked activation of NDNF interneurons may result in state-dependent activity of these powerful interneurons. We predicted that cholinergic neurons are more activated during estrus, leading to increased NDNF interneuron activity. Therefore, our first step was testing to see if NDNF interneuron activity to vocalizations is impacted by the estrous cycle.

Data Collection

This project uses two-photon imaging and vaginal cytology data previously collected by my mentor, Dr. Zahra Ghasemahmad.

To examine NDNF interneurons activity, transgenic mouse lines expressing genetically encoded calcium indicators (GCaMP) in NDNF neurons were used for two photon imaging. Calcium indicators show changes in fluorescence when bound to free calcium; therefore, GCaMP activity detects action potentials that cause a rise in calcium. To visualize these changes in fluorescence, these transgenic mice were implanted with a small 3mm cranial window over the auditory cortex and a headplate. After recovery and acclimation, the calcium activity of cortical layer 1 (<100µm in depth from the pial surface) NDNF interneurons were monitored using a two-photon microscope, while awake, head-fixed mice listened to vocalizations (Figure A). Mice freely ran on a circular treadmill during recordings. In addition to calcium activity, the behaviors of the mice were monitored. Locomotion was measured using a rotary encoder on the treadmill, while pupil diameter and whisking were monitored using a high-speed camera. To track the estrous cycle, a cytological sample was collected from female mice via vaginal lavage and analyzed through staining techniques.

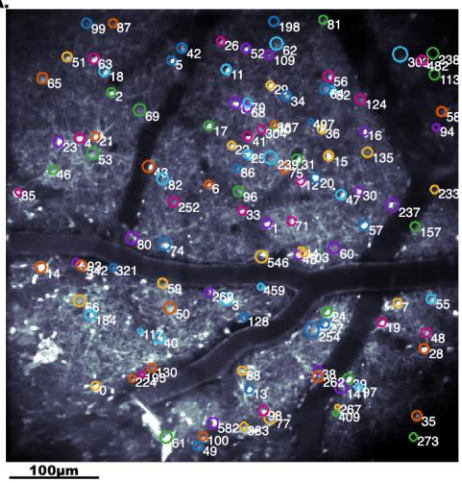


The vocalizations used as stimuli were recorded by Dr. Ghasemahmad in Dr. Jeff Wenstrup's laboratory during various behavioral contexts (Figure B). During my summer project, I investigated the neural responses to high intensity mating vocalizations emitted by both male and female mice, recorded during high-arousal copulation phases of mating, and to low frequency harmonics emitted by female mice during high-intensity mating interactions. We focused on the mating vocalizations for this project, with plans to investigate the processing of negatively salient vocalizations, such as responses emitted during restraint or isolation.

Data Refinement

After setting up a computer and workspace in the lab, I started the journey of refining our imaging data. Considering that two-photon experiments contain a large amount of data, there were multiple pipelines needed to extract neural fluorescence into quantifiable measurements and further statistical measurements. Data refinement occurred in several steps: imaging data were registered to correct for 2-D motion, regions of interest (ROI) corresponding to individual neurons were identified, and cellular fluorescence responses were extracted from the identified ROIs via Suite2p; these neurons were tracked over multiple recording sessions via ROICaT; and finally, statistical measurements of neuron activity were computed via a custom MATLAB script created by the Takesian Lab. For this preliminary analysis, we used data collected from two female mice and one male mouse.

2A.



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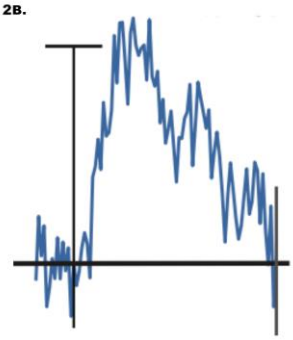
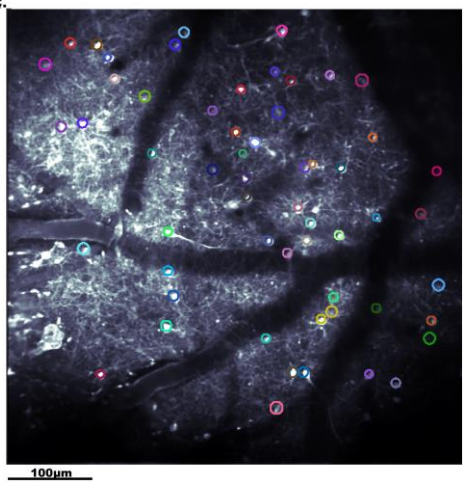


To utilize the Suite2P imaging processing pipeline, I was tasked with setting up an Anaconda environment. As someone with limited coding history, this was my first experience setting up an isolated environment and therefore I encountered many obstacles installing an older Suite2P version compatible with our data. Once Suite2P was successfully installed, I then processed the images via Suite2P. After Suite2P registered and extracted the fluorescent signals, I then confirmed the quality of movement stabilization and sorted ROIs into cells or non-cells. ROIs were determined to be cells based on clear, circular borders (Figure A, FOV with all cells in female case 2, with matched cells circled in red), and transient signals (Figure 2B, example z-score transient signal from female case 2) extracted by Suite2P.

2C.

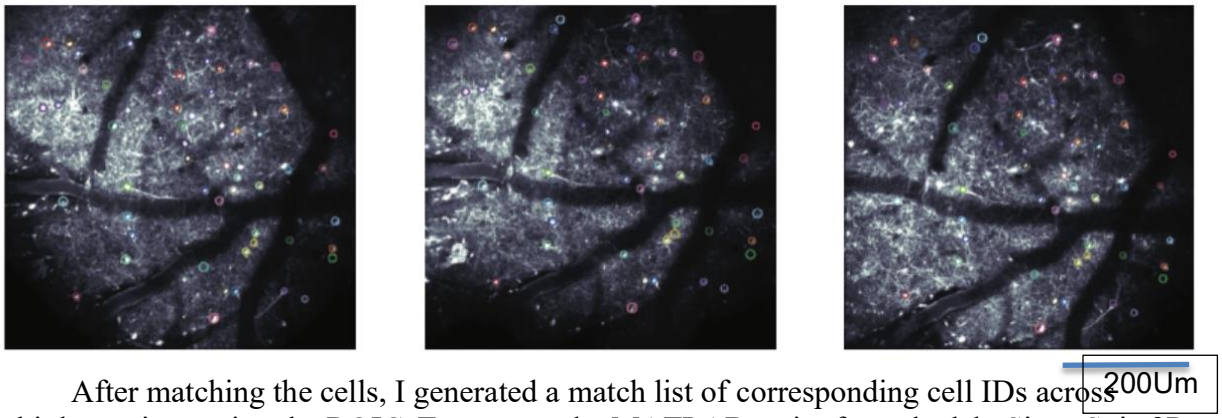


For a longitudinal study over multiple days, FOVs needed to be properly aligned to allow identification of the same cell over multiple days. Using ROICaT developed in the Sabatini laboratory, cells were then matched across sessions (Figure 2C, FOV of female case 2 limited to matched cells only). ROICaT has many different interfaces for its pipeline. For this project, I used the interactive notebook on Jupiter Notebooks. Jupiter Notebook was friendly for a first-time user and allowed me to change parameters easily. The most challenging part of this step was optimizing the number of cells tracked across all sessions. Collaborating with other members of the Takesian Lab, especially Dr. Victor Adenis, I determined that tracking the images sequentially – aligning the images to neighbor images instead of aligning all images to one mean image – worked best for our large datasets. There were other parameters that I adjusted, such as increasing the amount of blurring the ROIs and therefore tracking ROIs that drifted between sessions.

Since the ROICaT pipeline is very extensive, there's more parameters that may increase the number of matched neurons across sessions. Across the vocalization stimuli sessions, our male mouse had 48 matched cells; our female mouse case 1 had 78 matched cells; and our female mouse case 2 had 57 matched cells (Figure 2D, example of matched cells across all three

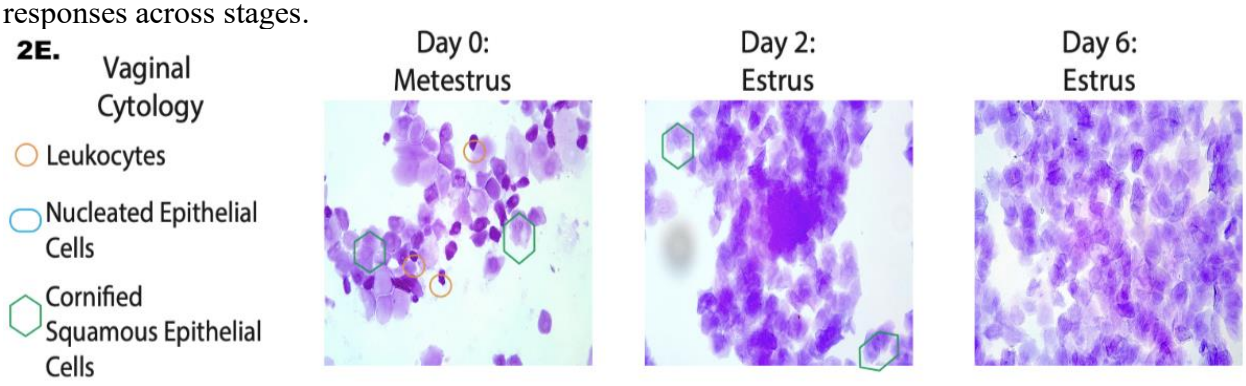
sessions seen in female case 2).

2D.



After matching the cells, I generated a match list of corresponding cell IDs across multiple sessions using the ROICaT output and a MATLAB script from the lab. Since Suite2P assigns cell identification numbers based on the cell's fluorescence during one session, IDs across sessions are not consistent. After formatting a list for each matched cell's ID per session, I fed this information and Suite2P data into custom MATLAB scripts that compile all experimental data together, such as timing of stimuli and neural responses, and then extracts the data in statistical measurements per cell.

With our two female mice, I examined the vaginal cytology under multiple different microscopes and identified the estrus stage of the mice based on these samples (Figure E, example of vaginal cytology under microscope seen in female case 1). Mice undergo four stages during the estrous cycle: proestrus, estrus, metestrus, and diestrus. After searching through the samples and identifying cell types, we determined that one recording day in the female mouse corresponded to metestrus and two days corresponded to estrus. In metestrus, there are two cell types clearly seen: the first is leukocytes, observed as small,round dots, and the second is cornified squamous epithelial cells. In estrus, cornified squamous epithelial cells are the dominant cell type; these cells are often clumped together with non-round borders. The inclusion of multiple estrous stages in each female mouse's imaging data was important to answer the our question of whether neural responses during the same stage are more consistent than neural responses across stages.



Investigating through Statistics

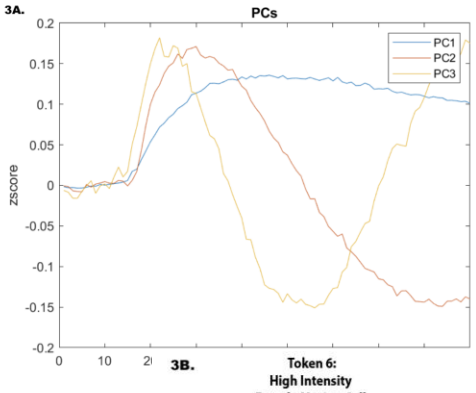
Using the extracted data, I was able to construct preliminary graphs to visualize each matched cell's response to different stimuli over multiple days. Using knowledge acquired in a Harvard statistics class, I analyzed each mouse's neural responses to different vocalization

stimuli using RStudio and MATLAB. I explored how different computational measurements of neural activity impact statistical and graphical analyses. I calculated the difference in the peak of the calcium response to various vocalizations across multiple days. Given that the female mice were recorded during two estrous days and one non-estrous day, I compared the results of two calculations: 1) the differences in neuronal responses across dissimilar stages (estrous day minus non-estrous day) and 2) differences across similar stages (later estrous day - earlier estrous day). Using these measurements, I determined if the difference in neural responses to stimuli was more similar between two estrous days than between the estrous and non-estrous days. Although the results were inconclusive and showed more complicated, cell and vocalization-dependent shifts over time, this was my first simple test to investigate clear stage-based neural responses.

I was able to plot various statistics graphs, including boxplot distribution graphs displaying the difference in neural responses between days, distribution charts of neural responses per stimuli, line graphs of matched neuron's difference in responses across days, per stimuli, and more.

Visualizing Neural Patterns

To better understand the shifts in NDNF interneuron activity across recording days and estrous stages, we clustered the neurons using Principal Component Analysis (PCA) and K-Means clustering. PCA reduces complex neural data into a linear combination of definable patterns (Figure 3A, example of principal components). Clustering therefore groups neurons based on these similar response patterns.



In our raster plots (Figure 3B, example of craster plot showing each neuron's activity, grouped by clusterlustering), neurons are clustered on day 0 and bound by such clusters in following days. These graphs display mean calcium activity of NDNF interneurons, with one row representing one matched cell over multiple sessions. The stimuli are played at 0.5 seconds, markedt by the grey line. We observed that some clusters of NDNF interneurons exhibited clear sound-evoked responses. The coloring of these graphs represents neuron activation via red, suppression via blue, and magnitude by shade of red and blue.

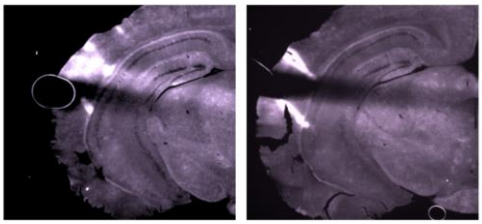
Sectioning and Mounting

To confirm the imaging sites in these mice, a fluorescent dye was injected into the cranial window after concluding recordings. During this summer, I cut the brain tissue into 40µm slices using a vibratome and then mounted the slices onto a microscope slide. Since this was my first time using a vibratome, I was trained on vibratome and mounting procedures for a few weeks

using brain practice. After this experience, I discovered that histology is hard but extremely rewarding.

Using a confocal microscope, Zahra and I examined the left cerebral hemisphere for fluorescence dye. With this, we determined the location of recording for our male mouse case to be in the secondary auditory cortex (Figure 4A, male mouse brain sectioning with fluorescent dye shown through sharp, bright lines) by overlaying the section tissue with the Allen Mouse Atlas tissue.

4A.



Preliminary Conclusions

With this project, we have established a protocol to evaluate the responses of NDNF interneurons to vocalizations across the estrous cycle. In this preliminary analysis, we found that some subnetworks fo NDNF interneurons exhibit changes in response to specific vocalizations and changes across estrous stages; these subnetworks also seem to respond differently to stage changes, with some showing activation while others showing suppression.

Future work will focus on expanding this dataset, recording from mice over multiple estrus cycles to determine if neuron patterns “reset” during each cycle. We are also interested in investigating the mechanism of estrous modulation of neural activity by monitoring acetylcholine levels in the auditory cortex across the estrous cycle.

Transforming into a Laidlaw Scientist

The skill section on my resume has doubled. I learned more than I thought was possible: mouse brain slicing, operating a confocal microscope, Principal Component Analysis and K-Means Clustering, MATLAB coding, scientific poster formatting, scientific figure creation, and much more. In conducting the data analysis portion of a two-photon experiment, I operated computational programs necessary to analyze this large dataset. With this new longitudinal experiment, I explored various methods to analyze our estrous dataset. Most importantly, I continued to train on presenting complicated data through discussions with my mentor, my PI, and the whole lab. As a result of this summer’s science project, I feel competent in curating, analyzing, and presenting two-photon imaging data.

Spending 10 weeks in the lab expanded more than my skillset: these hours surrounded by researchers and medical professionals expanded my understanding of perspective disparities between medical professionals, hearing parents of deaf children, and the Deaf community. My identity as a Child of Deaf Adults (CODA) contributes to my “why” in auditory neuroscience. Growing up surrounded by the Deaf community, I saw a resilient community bonded through their fight against society’s barriers. In that resilience, people and their brains adapted. Entering the auditory neuroscience field, I am fueled by the question of how an area can adapt to understand such complex sounds, such as music, and adapt to understand no sound. During a late-night dive into recent auditory neuroscience advancements, I stumbled upon articles discussing the recent FDA approval of OTARMENI – a gene therapy that promises improved hearing for two years in congenitally deaf youth possessing the OTOF gene mutation, utilizing

the adeno-associated virus as a vector. The science itself is brilliant, but the phrasing of these studies often misrepresents what a Deaf life looks like in the US. For example, a Harvard article reflecting on this new gene therapy states, "Babies with the *OTOF* mutation are completely deaf at birth, which affects speech acquisition and can hinder cognitive development unless children are given a cochlear implant at a young age." Language deprivation hinders cognitive development; congenital deafness does not. Cognitive development of deaf or hard-of-hearing youth parallels hearing counterparts when early sign language education is provided and supported. Despite decades of research proving sign language's equivalence to spoken languages in child development milestones, this article and many others, including published papers, reflect an oralist perspective that spoken language is superior to signed language. In neuroscience though, signed languages and spoken languages are processed through similar language circuitry.

Discovering this sector of auditory neuroscience and its clear lack of sensitivity in their publications angered me. At the core of this issue, I questioned whether a solution to societal hindrance, not medical hindrance, is truly necessary. Deafness does not create decreased quality of life due to medical issues; the documented decreased quality of life is rooted in societal issues.

Approaching this as an opportunity to learn both the medical perspective and the Deaf community's perspective, I reached out to a lab manager who works with otoferlin research. After a thorough discussion over lunch, I gathered three main points from the medical perspective: hearing parents fear the decreased quality of life due to societal barriers, medical treatment is an alternative to the impossible task of making society fully accessible, and ultimately, the insensitivity of publications can be most easily addressed through journal policies. While much of the medical community presents gene therapy as though deafness inherently causes developmental delay, the Deaf community's perspective on gene therapy is unclear. Discussions with my parents revealed the Deaf community is disparate on whether deafness is an identity or a disability. Specifically, whether a deaf individual would sacrifice their deaf identity to live a life with less societal barriers as hearing truly depends on the individual. As a witness to deaf oppression but not someone who experiences it myself, I never considered deafness a disability but rather an identity. On a surface level, identities that have previously limited one's mobility in society, such as sexuality or gender, are being embraced in modern times. One hundred years ago, my identity as a queer woman would have prevented me from becoming a Harvard scientist and getting married. With this perspective, is deafness an identity or a disability in today's world?

As I continue my research in the Takesian lab, expanding our research on the effects of female hormonal cycle on auditory neurons and beginning a new project investigating experience-related plasticity, I'm excited to grow my research into something meaningful for the field of neuroscience. While I'm investigating the biology of neural plasticity, I will continue to expand my "why" in auditory neuroscience. As Director of Initiatives in Harvard's Deaf Advocacy Coalition, I look forward to organizing events with Boston's Deaf community and challenging society's perspectives on Deaf lives. One step at a time, I'm integrating my passions, making a positive impact on the lives of others, and contributing meaningfully to the fields of neuroscience and Deaf advocacy.

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