Introduction: A fundamental goal of neuroscience is to understand how neural circuits, assembled through genetic programs, can give rise to complex behavior. Through evolution, species display a wide range of behaviors, some of which have been mapped to specific genetic variations[1]. Genes that mediate complex behavior must act via neural circuits, yet little is known about these intermediate changes. ***In this proposal, I will bridge this knowledge-gap by investigating neural circuit differences that determine vocal communication behaviors in two closely-related rodent species.***

Background and Rationale: Using sounds to communicate is widespread in nature — from croaking frogs, duetting birds, to us, humans, engaged in conversation. Our lab has recently discovered a rodent species (Alston's singing mice) that engages in similar fast vocal interactions. Singing mice breed in captivity, can be maintained in a colony, and show stereotyped vocal behaviors even in laboratory settings. Additionally, we have already established the use of viral tools for mapping, manipulating, and measuring neural circuits[2].

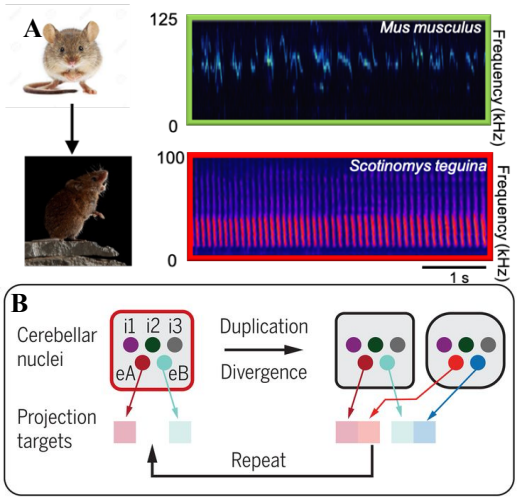

FIG. 1: (A) *Phenotypic variation in vocalizations of the lab mouse and Alston's singing mouse. (Spectrograms from the Phelps lab, U.T. Austin)* ***(B)*** *Divergence and duplication model as observed in cerebellar nuclei[8].*

Singing mice *(Scotinomys teguina)* and lab mice (*Mus musculus*) are separated by a few million years of evolution (Steve Phelps lab, unpublished), are roughly the same size, and brain slices are largely indistinguishable between the species. Yet, there are key differences in their vocal repertoires; Lab mice produce only short, variable ultrasonic vocalizations (USVs), while *S. teguina* produce both USVs and human-audible 'songs' (Figure 1A). Crucially, unlike singing mice, lab mice do not participate in vocal turn-taking. Thus, we ask: What are the neural circuit differences underlying this behavioral distinction?

Though traditionally thought to be unique to the primate lineage, our lab recently demonstrated robust motor cortical control of vocal behavior in the singing mice. Using four complementary lines of evidence (intracortical micro-stimulation, stimulation induced vocal arrest, focal cooling and pharmacological silencing), we defined a region of orofacial motor cortex (OMC) that mediates flexible vocal behaviors in the singing mice[2]. In contrast, lab mice born without the entire cortex (including OMC) can still produce USVs[3]. ***Therefore, we predict that differences in the motor cortical circuitry between the lab mice and singing mice underlie differences in their vocal behaviors.*** We hypothesize that motor cortical control over vocalization in the singing mice evolved from the ancestral orofacial control neural circuits via a duplication of OMC followed by cell-type divergence (Figure 1B). This duplication-divergence model predicts the existence of a dedicated group of song-specific neurons in the singing mouse OMC with specific projection patterns to downstream vocal pattern generators in the midbrain and the brainstem. ***Using novel spatial transcriptomics and barcoded projection mapping methods developed in Tony Zador's (my co-advisor) lab, I will determine the diversity of cell-types in the motor cortex and their downstream projection patterns in both the singing mice and the lab mice.***

Aim 1: Do motor cortical cell types differ between lab mice and singing mice? The duplication and divergence model suggests that neural cell types in the OMC of singing mice evolved in a spatially segregated manner. First, to determine differences in cell types, I will perform single cell RNA sequencing (scRNAseq) in the OMC of lab and singing mice. Analysis of scRNAseq data requires aligning sequenced reads to a genome, publicly available for the lab mouse and recently generated by our collaborators for the singing mouse (unpublished, Steve Phelps). Cell types will be identified using known marker genes found in the literature. We will identify potentially novel cell types as those which have no assigned identities based on canonical marker genes.

While scRNAseq will allow us to quantify differences in neural cell types through in-depth transcriptomics, we lose spatial information. To determine spatial location of neuronal cell types, we will

use a spatial transcriptomic method, BARseq, developed in the Zador lab[4]. This technique uses hybridized probes and *in situ* sequencing to determine spatially resolved expression data for hundreds of genes in parallel[4]. I am confident that I can perform this experiment as the Zador lab has a dedicated pipeline to complete this experiment and regularly performs spatial transcriptomic experiments.

Aim 2: Do projection patterns of motor cortical neurons differ between lab and singing mice? To determine OMC projection patterns, I will first perform viral tracing experiments. I will inject AAV vector that expresses GFP into the OMC of both species and image the brains using confocal microscopy. While viral tracing can detect bulk anatomical differences, this method lacks accurate quantification of projections and cannot distinguish changes that occur on the single cell level. To address these inadequacies, we will also be performing MAPseq, a method for single cell tracing developed by the Zador lab. MAPseq is a method that uses virus to infect neurons with a DNA barcode that is expressed in the cell body and axon of the neuron[5,6]. Through dissection and sequencing, we can recover the projection patterns of thousands of individual cells (Figure 2). I am confident that I can perform these experiments as I have already generated preliminary results for the lab mouse OMC. Furthermore, we can combine MAPseq with our spatial transcriptomic method to correlate projection patterns and cell types[4,7].

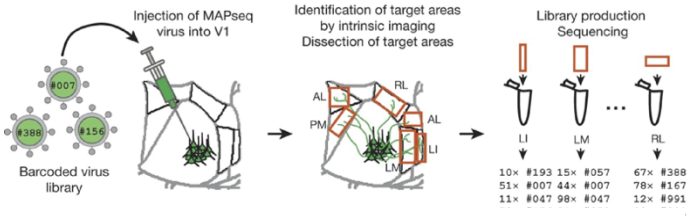

FIG. 2: *MAPseq protocol involves injecting barcodes into target area and sequencing barcodes expressed in neural projections in downstream areas[5].*

Anticipated results: If the duplication-divergence model of the singing mouse OMC holds true, I would expect to observe the following results: **(1)** novel cell types in the OMC of singing mouse **(2)** the spatial location of these novel cell types to be located in a spatially distinct area, and **(3)** novel projection patterns, perhaps to brainstem pattern generators, correlated with these novel cell types. ***In summary, the duplication-divergence model predicts correlated changes in cell transcriptomes and their projection patterns***. Of course, another possibility is that cell type and projection pattern differences occur independently. Even so, I will be able to distinguish independent changes due to the resolution of the outlined experimental design. ***Thus, we have designed experiments that will produce results whether or not our expected model (duplication and divergence) is true.***

Intellectual Merit: I anticipate three major contributions to neurobiological methods, as well as our understanding of the evolution of neural circuits. First, this study can identify distinct neural populations based on projection patterns and/or genetic markers. Identifying neural populations in this manner allows scientists to target these neural population for further functional validation and experimentation. Second, our results could identify genes underlying neural circuits in vocal communication, findings which could contribute to the development of better molecular tools for manipulating vocal circuits. Lastly, this study would provide insight into the evolutionary underpinnings and biological basis of vocal communication.

Broader Impact: I plan to make code and data available on open-source websites including GitHub. During my time at NIH, I created a RNAseq tutorial and shared resources on my GitHub page in addition to uploading code I wrote for analyzing RNAseq data[9]. I plan to maintain my GitHub page and upload code developed for analyzing data collected through this project for other scientists to consult and use. In addition, I plan to create an online tutorial geared toward high school and/or college students that have little coding experience or exposure to bioinformatics. I also plan to publish our results in open access journals including uploading early drafts of the manuscript to bioRxiv to facilitate timely advancement of scientific knowledge.

References: **[1]** Metz et al. 2017. *Current Biology.* **[2]** Okobi, Banerjee et al. 2019. *Science.* **[3]** Hammerschmidt et al., 2015. *Scientific Reports.* **[4]** Chen et al., 2019. *Cell.* **[5]** Kebschull et al. 2016. *Neuron.* **[6]** Han, Kebschull, Campbell et al., 2018. *Nature.* **[7]** Sun, Chen et al., 2021. *Nature Neuroscience.* **[8]** Kebschull et al. 2020. *Science.* **[9]** https://github.com/eisko/RNAseq/