

Neural Circuits Underlying Motivation for Singing in Zebra Finches (*Taeniopygia guttata*)

Shuoyi Li
shuoyi.li@duke.edu

Mooney Lab
Department of Neurobiology
Duke University, NC, Durham

November 29, 2023

Abstract

Learning and maintaining complex motor skills, such as singing or playing a violin, requires prolonged and intensive practice often without any external reward or punishment. Once mastered, these skills are performed in front of an audience for social rewards. What are the neural circuits that motivate intensive practice, and are these circuits distinct from those that motivate us to perform these skills for reward? The brain of a male zebra finch provides an exceptional system in which to explore the neural circuits that motivate socially isolated practice and social performance of a complex motor skill. A male zebra finch sings thousands of times a day without any external reward to learn and maintain its complex song. Once perfected, the male uses his song to attract a nearby female, which is a socially rewarding experience. The medial preoptic area (POM) of the hypothalamus is a key regulator of social and sexual motivation in all vertebrates. Prior studies suggest that the pathways from POM to a midbrain dopaminergic cell group A11 and ventral tegmental area (VTA) regulate different types of singing behaviors in birds. Here, I propose to test the hypothesis that distinct subtypes of POM neurons in the male finch provide motivational signals for socially isolated practice and social performance, with neurons that project to the VTA driving undirected song and neurons projecting to A11 driving directed singing. I will use retrograde, anterograde, and intersectional viral tracing methods to map the anatomical connectivity of the POM, and perform single-cell RNA sequencing to categorize the POM cell types that are activated during directed and undirected singing. Additionally, I will use optogenetic and chemogenetic methods to determine whether POM cell types motivate singing. Lastly, I will use miniscope imaging to monitor POM activity during singing. I expect that completion of Aim 1 will show that distinct subpopulations of POM neurons project to A11 versus VTA, and that these POM subtypes exhibit distinct mRNA expression profiles. Completion of Aim 2 will elucidate that the POM to A11 cell type drives song performance to a female, whereas the POM to VTA projection promotes socially isolated song practice.

Introduction to the revised proposal

I would like to thank the reviewers for their constructive comments on my proposal. I am encouraged by the reviewers' positive feedback and will address each of the major critiques below. I have also made major and minor revisions to the proposal.

1. How to distinguish “the motivation to sing” from “the ability to sing”

Reviewers raised the concern that if the finch stops singing after POM manipulations, it is unclear whether the finches are no longer motivated to sing or whether they are not able to sing. Indeed, their motivation and ability to sing can be differentiated by the singing-accompanied movements and behaviors. During undirected and directed singing, male finches also adopt a range of singing displays, including upright postures and erected plumage. During directed singing, male finches would twist their head and tail towards the female and pivot their head from side to side. I expect that if the finch is motivated but unable to sing, he will still display such singing-accompanied movements without vocal outputs. Specifically, with a female presents, he would still display the hop-pivots dance and face towards the female. Without a female, he would adopt different undirected singing postures. However, if the finch is able to sing but lacks motivation, he would not display any of these singing-accompanied behaviors. As I described in the approach section, I will measure the effects of POM manipulations on non-vocal behaviors, including postural changes and head gestures, using a supervised learning algorithm (DeepLabCut)¹¹, which allows me to distinguish motivation from ability to sing.

2. Uncertainty about extrinsic and intrinsic motivation and the relationship between motivation and reward

In my first submission, I proposed to relate extrinsic and intrinsic motivation to directed and undirected singing. However, this parallel was not fully defined, and the relationship between being “motivated” and being “rewarded” was not discussed. In the revision, I proposed to understand undirected singing as the socially isolated practice of a complex motor skill and directed singing as the social performance of the skill in front of an audience for social rewards. Please see the revised proposal for more details.

3. Concerns with sensory inputs and non-POM pathways that innervate the song circuit

In this proposal, I mainly focus on POM pathways and POM efferent projections that innervate the song circuit. I propose to examine how different POM pathways regulate the motivation to maintain or perform a motor skill. Regarding sensory inputs, visual stimuli of females are essential for triggering directed singing in male zebra finches. It would be a great future direction to examine how different sensory inputs trigger different types of motivation to sing through POM and non-POM pathways.

4. Lack of the comparison between the finch POM and the mouse POM

To address this concern, I included more literature on the mouse POM in specific aims and significance sections and emphasized the highly heterogeneous cell types in the mouse POM. Although the finch POM architecture and molecular profile are unknown, the evidence of the mouse POM and their evolutionarily conserved functions in social and sexual motivation has prompted me to investigate the functions of POM cell types and pathways in the motivation of different types of singing.

5. Lack of details for the behavioral assays before single-cell RNA sequencing

Reviewers raised multiple concerns about the behavioral assays before single-cell RNA sequencing. To address the concerns, I made major revisions to this part of the approach section. Firstly, I will start monitoring the undirected singing of male zebra finches at the onset of the singing behaviors of the test day, instead of at the time when lights are turned on. Secondly, 45 minutes after the onset of singing, I will sacrifice the animals via rapid decapitation and quickly excise the brains from the skulls on ice. The time window of 45 minutes after the onset of singing behaviors is determined because previous studies in both mice and finches have shown that the mRNA expression for many genes including immediate early genes activated by a behavior usually takes ~45 minutes to reach their peak level^{4,15,17}. It is unknown how much singing is sufficient for me to see transcriptional differences based on current literature, the duration of singing that I monitor might need to be adjusted based on my preliminary results. Additionally, I increased the sample size from 5 birds per group to 10 birds per group. However, the actual number of healthy cells I can dissociate from the finch POM is unknown, and the number of birds per group may need to be adjusted to collect enough cells for sequencing. Finally, I agree with the reviewers' concern that it is unclear whether darkness or the diurnal clock influences POM gene expressions. Thus, for group 3's “no singing” finches, instead of keeping the light off to prevent them from singing as I previously proposed, I will keep the lights on and all other environmental conditions the same as other groups and choose the finches that do not sing in the first 45 minutes.

6. Concerns with the feasibility of developing CellREADR constructs and applying them in finches

There are ongoing collaborations between the Huang and Mooney Laboratories on developing CellREADR constructs for finches. The CellREADR viral constructs have been shown to be expressed in multiple brain regions in finches, and more versions are being developed to enhance the expression efficiency and specificity.

Specific Aims: Learning and maintaining complex motor skills, such as singing or playing the violin, requires prolonged practice often without any external reward. Once mastered, these skills are performed in front of an audience for social rewards. What are the neural circuits that motivate intensive practice, and are these circuits distinct from those that motivate us to perform these skills for social reward? The brain of a male zebra finch provides an exceptional system in which to explore the neural circuits that motivate socially isolated practice and social performance of a complex motor skill. A male zebra finch sings thousands of times a day without any external reward to learn and maintain its complex song. Once perfected, the male uses his song to attract a nearby female, which is a socially rewarding experience. The neural circuits that motivate song practice and performance (i.e., undirected versus directed singing) are poorly understood, although the hypothalamus is a likely source of song motivation signals.

The medial preoptic area (POM) of the hypothalamus is an evolutionarily conserved structure in birds and mammals that regulates sexual and social motivation. Prior studies indicate that the POM indirectly innervates the forebrain circuits important to song production through a midbrain dopaminergic cell group (A11) and circuits important to song learning and maintenance through the ventral tegmental area (VTA)². Lesions in A11 disrupt female-directed singing but preserve undirected singing³, suggesting that the POM to A11 pathway is responsible for motivating song performance. While the pathway that motivates song practice is less clear, immediate early gene expression in the POM scales with the amount of undirected singing⁴, raising the possibility that the POM motivates song practice through other targets, such as the VTA.

The POM is anatomically and functionally heterogeneous. Single-cell RNA sequencing identifies ~70 different neuronal subtypes in the mouse POA, and mating behaviors activate distinct neuronal subtypes in a sexually dimorphic manner¹⁵. More specifically, activity of a POM neuronal type that expresses the tachykinin receptor 1 (Tacr1) is both necessary and sufficient to drive male mating behaviors but not other social behaviors¹⁶. Furthermore, the axonal projections that POM^{Tacr1} neurons make to VTA and A11 regulate different aspects of motor output and reward linked to mating in mice¹⁶. Since the POM is highly conserved in birds and mammals, I hypothesize that distinct subtypes of POM neurons in the male finch provide motivational signals for song practice and performance, with VTA-projecting POM neurons driving undirected song and A11-projecting POM neurons driving directed singing. Here, I will systematically characterize POM cell types using RNA sequencing and viral tracing methods. Then I will use novel mRNA-based cell targeting methods to examine how specific POM cell types contribute to song practice and performance.

Aim 1. Characterize POM neurons based on their axonal projections and RNA expression profiles.

Based on the neuronal and functional heterogeneity of the mouse POA, I hypothesize that the finch POA contains many distinct neuronal cell types that serve different functions in courtship and reproduction. Thus, I will use retrograde, anterograde, and intersectional methods to map the connectivity of POM neuronal cell types in the male finch. To further characterize these POM subpopulations, I will perform single-cell RNA sequencing to identify different putative POM neuron types based on their mRNA expression profiles and to determine which of these cell types are activated during undirected and directed singing. I will leverage this knowledge by using CellREADR¹⁸, an RNA-based cell monitoring and manipulation technology, to gain genetic access to different, molecularly-defined POM cell types. These approaches will allow me to test the hypothesis that distinct subpopulations of POM neurons project to A11 versus VTA, and that these POM subtypes exhibit distinct mRNA expression profiles.

Aim 2. Determine how different POM cell types contribute to song practice and performance.

To test the necessity and sufficiency of the POM activity for singing, I will apply chemogenetic and optogenetic methods to suppress or stimulate the POM neurons and measure directed and undirected singing. To differentiate the roles of the POM cell types in regulating motivational signals, I will exploit CellREADR constructs to manipulate the activity of specific POM subpopulations and examine their behavioral effects. I will perform miniscope imaging to visualize the activity of POM cell types at single-cell resolution and relate neural ensemble dynamics to singing behaviors. I hypothesize that activity in the POM to A11 cell type drives song performance to a female, whereas the POM to VTA projection promotes socially isolated song practice.

Significance

We are not only motivated to perform a skilled complex motor behavior, such as singing or playing a violin, in front of an audience for social rewards but also motivated to practice to maintain the skill without immediate external reinforcers. However, the neural circuits that regulate the motivational signals for performance and practice of a complex motor skill are still unclear.

The brain of a male zebra finch provides an exceptional system in which to explore the neural circuits that motivate socially isolated practice and social performance of a complex motor skill. With female conspecifics present, male finches are motivated to perform courtship singing, or directed singing. More intriguingly, without immediate rewards or punishments, males practice and maintain undirected singing thousands of times every day. Directed song is a more intensive performance than undirected song²¹. Although directed and undirected song motifs consist of the same syllables, directed song has less spectral variability, more introductory elements, more song motifs per bout, and faster speed²⁰.

Additionally, the behavioral components differ between the two types of song. Birds that sing undirected song display a range of postures from weak to intense, but those that sing directed song only adopt the highly intense display with upright posture and erected plumage. The directed singing will also be accompanied by hop-pivots dancing toward the female²¹. These singing behaviors provide me with a tractable system that allows me to quantitatively examine the effects of manipulating POM cell types in directed and undirected singing.

The medial preoptic nucleus (POM) of the hypothalamus is a main source of social and sexual motivational signals across all vertebrates. Balthazart and colleagues showed that the metabolism of testosterone into estradiol in the avian POM is both required and sufficient for activating male courtship behaviors including singing⁶. Studies from our laboratory suggest that the POM projects to a midbrain dopaminergic cell group A11, which responds to social cues and regulates courtship singing by releasing dopamine into the vocal premotor regions (HVC)^{3,7}. We also studied the motivational circuits for courtship vocalizations in mice and revealed that the POM to A11 pathway might be evolutionarily conservative between finches and mice^{8,9}. Furthermore, Morishita and colleagues showed that in mice, POM GABAergic neurons regulate dopamine release in the ventral tegmental area (VTA) and modulate motivation¹⁰. In finches, the VTA to basal ganglia pathway is sufficient to guide song learning¹⁹. Here, I propose to test my hypothesis that distinct POM cell types motivate directed courtship singing through the A11 pathway and motivate undirected singing and song learning through VTA in male zebra finches (Figure 1).

Recent studies have revealed that the mouse POM consists of diverse transcriptionally defined neuronal cell types, each of which plays a different functional role. Moffitt et al profiled ~1 million cells and categorized ~70 neuronal subtypes in the preoptic area in mice¹⁵. They also showed that distinct neuronal subpopulations become activated during different social behaviors¹⁵. Furthermore, Bayless et al showed that a specific POM cell type that expresses tachykinin receptor 1 (Tacr1) is sufficient and necessary in driving male mating behaviors but not other social behaviors¹⁶. POM^{Tacr1} projections to VTA and A11 regulate different aspects of motor output and reward linked to mating in mice¹⁶. Since the POM is an evolutionarily conserved structure, the finch POM may also preserve highly heterogeneous neuronal cell types. During motivated behaviors, some POM clusters may become activated, while others may become inhibited. Without the information about the molecular identities of these neuronal subpopulations, it would be extremely challenging to study the role of the POM in motivated behaviors. Thus, I propose to transcriptionally characterize POM subpopulations and study the role of cell type and projection-specific neurons in motivating directed and undirected singing. Considering directed singing as a part of the finch mating behaviors and the role of POM^{Tacr1} in regulating mouse mating behaviors, POM^{Tacr1} is a candidate cell type that motivates finches to sing.

Systematically cataloging the POM neural cell types in zebra finches using single-cell RNA sequencing would constitute a significant advance. I will be able to identify the active cells in the POM during singing, not based on the expression of a single immediate early gene, but by comparing the expression levels of hundreds of immediate early genes. I will obtain information about thousands of genes that are upregulated or downregulated by undirected and directed singing in the active POM neurons. Based on gene markers, I will be able to unbiasedly catalog the neuronal cell types in the finch POM, which provides a foundation for

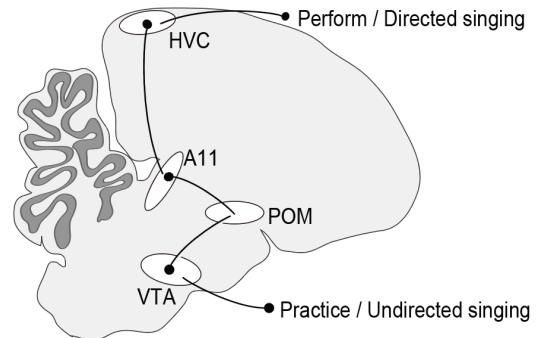


Figure 1. Schematic of projections from the POM that may motivate song practice or performance.

studying their functional roles. It will also be valuable from an evolutionary perspective to compare the hypothalamic cell types and their functional roles in birds and rodents in future studies.

While RNA sequencing can provide candidate cell types, testing the function of these cell types requires genetic methods to label and manipulate them. Different from mice, zebra finches do not have transgenic lines. Although the expression of viruses under specific promoters allows researchers to target some neuronal subpopulations such as inhibitory neurons, the lack of transgenic finches still makes it challenging to modify gene expression in specific cell types. The CellREADR technique developed by Dr. Josh Huang's laboratory provides access to detect the presence of a cellular RNA and triggers the translation of a designed effector protein to monitor and manipulate the cell in zebra finches. By designing CellREADR constructs that target specific RNA sequences, I will be able to express effector proteins such as Channelrhodopsin-2, Archaerhodopsin, or GCaMP in specific POM cell types and examine their contributions to singing. The application of CellREADR in zebra finches along with other modern techniques, including optogenetics, chemogenetics, and miniscope imaging, would be a powerful advance.

Innovation

Technical innovation:

- Using intersectional viral approaches to examine the anatomical organization of the POM;
- Using single-cell RNA sequencing to characterize the finch POM neuronal cell types;
- Developing CellREADR using single-cell RNA sequencing data of the finch hypothalamus to manipulate specific POM cell types;

Conceptual innovation:

- Studying motivational signals using the natural singing behaviors of male zebra finches;
- Determining the finch hypothalamus organization with cell type specificity for the first time, which is key to systematically teasing apart the relationships among motivation, reward, practice, and learning;
- Examining the functions of specific POM cell types in regulating motivation for different types of singing.

Approach

Aim 1. Characterize the finch POM neural cell types and their anatomical projections crucial for singing

1.1. Rationale

Prior studies suggest that sex steroids, including testosterone and estradiol, in the POM are crucial for regulating male courtship behaviors including singing in birds. To further explore the cellular mechanisms underlying how POM regulates singing, in my preliminary study, I performed *in situ* hybridization chain reaction to examine the identity of the POM neurons that express aromatase, an enzyme that converts testosterone into estradiol. The results showed that ~80% of aromatase-positive POM neurons express vesicular GABA transporter (VGAT), a marker for inhibitory neurons, while ~20% of the POM neurons that express aromatase also express vesicular glutamate transporter (VGLUT), a marker for excitatory neurons (Figure 2). Although further quantifications need to be performed to measure the percentage of POM neurons that express aromatase, this preliminary result is consistent with the cellular composition of the POM in rodents, in which ~80% of the POM neurons are inhibitory, and ~20% are excitatory. More experiments need to be performed to investigate the efferent projections of the POM neurons and their molecular identity.

Here, I seek to systematically document the diverse neuronal cell types and their projections within the zebra finch POM. This provides a foundation for studying the functional roles of POM cell types and associated circuits in regulating the motivation to sing. To determine the POM connectivity, I will use an adeno-associated virus to anterogradely trace the the brain regions that the POM projects to. I will apply an intersectional viral approach to examine whether distinct POM subpopulations project to different brain regions or whether the same POM neurons innervate multiple regions. To reveal the molecular profile of the POM neurons with

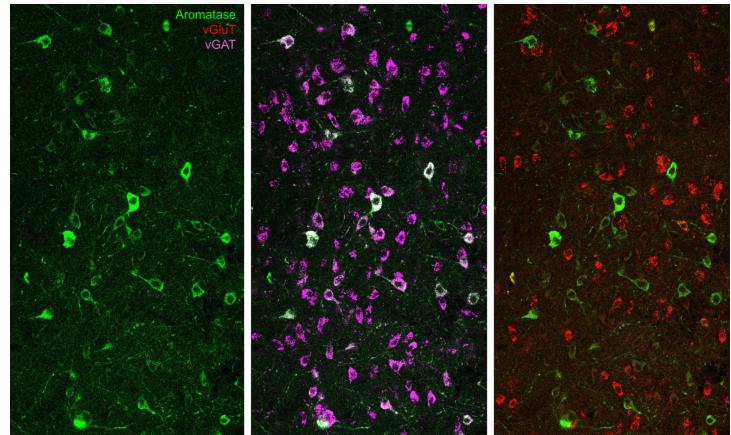


Figure 2. Overlap between aromatase (green) with VGAT (magenta) and VGLUT (red) in the POM

specific efferent projections, I will collaborate with Dr. Dmitry Velmeshev at Duke and perform single-cell RNA sequencing to compare the gene expression profiles for male finches that perform directed singing, undirected singing, or no singing behaviors. I will analyze the sequencing data to identify the highly expressed genes for different singing behaviors and categorize neuronal cell types in the POM. Using the sequencing results, I will collaborate with Dr. Josh Huang at Duke to develop CellREADR constructs that target specific neural subpopulations, which provide access to modify the gene expression of POM cell types in finches. I **hypothesize** that distinct subpopulations of POM neurons project to A11 versus VTA, and that these POM subtypes express distinct mRNAs.

1.2. Experimental Design and Methods

Characterize anatomical connectivity of the finch POM:

One week after I injected fluorescent retrobeads into VTA, a POM-projected brain region, I performed immunohistochemistry to verify the retrograde labeling in the POM. My preliminary result showed that the fluorescent retrobeads which I injected into VTA retrogradely labeled the cell bodies in the POM (Figure 4), confirming that some POM neurons directly project into VTA. However, it is important to map all the projected brain regions from the POM, not only focusing on VTA. Thus, to label all the projected brain regions from the POM, I will inject AAV-2/9-CAG-EGFP or AAV2/9-hsyn-axon-GCaMP8s into the POM. Three weeks later, I will collect the brain and perform immunohistochemistry to verify the viral injection site and identify all the brain regions that contain green fluorescence introduced by POM neuronal projections. Next, I will use an intersectional approach to examine whether the same POM neurons have efferent projections to different brain regions such as VTA and A11, or whether different POM neural subpopulations project to different regions. This step is important for understanding if the same POM neurons are regulating multiple brain regions and potentially have multiple effects on behaviors. I will inject scAAV-DJ/9/2-hCMV-Cre in POM-projected brain regions, such as VTA, to retrogradely transport Cre recombinase into the POM and inject AAV2/9-CAG-flex-EGFP in POM to express EGFP in POM neurons. Three weeks later, I will collect the brain and perform immunohistochemistry to verify the fluorescent marker in the other POM projected regions.

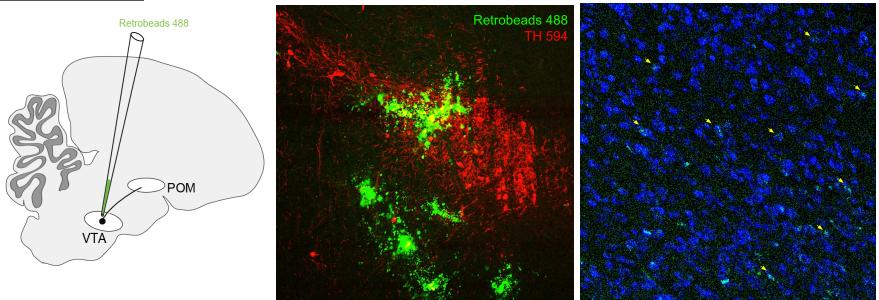


Figure 4. Left: retrobeads injection in the VTA. Middle: position of retrobeads (green) in the VTA, labeled by TH+ cells (red). Right: Retrobeads labeled cells (green) in the POM, NeuroTrace (blue).

Characterize POM cell types activated for undirected and directed singing: I will perform single-cell RNA sequencing by collaborating with Dr. Dmitry Velmeshev at Duke. 10 male zebra finches at the age of ~120 dph will be used in each group. Only finches displaying the corresponding behaviors were selected for further processing.

On the test day, I will place each male zebra finch in a soundproof box. In group 1, I will start monitoring the undirected singing of male zebra finches at the onset of the singing behaviors of the test day, which usually occurs 10 minutes after the lights are on. Previous studies in both mice and finches have shown that the mRNA expression for many genes including immediate early genes activated by a behavior usually takes ~45 minutes to reach their peak level^{4,15,17}. Thus, 45 minutes after the onset of undirected singing, I will sacrifice the animals via rapid decapitation and quickly excise the brains from the skulls on ice. I will dissect the POM regions from all 10 animals and perform tissue dissociation following the Worthington single-cell dissociation protocol. To minimize artificially induced transcriptional perturbations, I will add actinomycin D (actD), a transcription inhibitor of eukaryotic RNA polymerases, during the dissociation process. After obtaining single-cell suspension, I will use the 10X Chromium Single Cell Controller and single-cell reagent kits from 10X Genomics to generate nanoliter-scale Gel Beads-in-emulsion (GEMs) that contain single neurons (Figure 3). The RNA transcripts will undergo reverse transcription, and the cDNA will be barcoded. The barcoded cDNA will be amplified and the gene expression library will be constructed. Finally, the gene expression library will be sequenced. In group 2, instead of collecting the brain after undirected singing behaviors, I will expose each male zebra finch to a female zebra finch in their soundproof box to induce directed singing. All the other experimental conditions will be kept the same as for group 1. 45 minutes after the onset of directed singing, I

will sacrifice the male finches. In group 3, each male zebra finch will be placed in a soundproof box. Some finches tend to sing less frequently and may not sing in the first 45 minutes after the lights are on. I will sacrifice such male finches 45 minutes after the lights are on. All other treatments for group 3 will be the same as for groups 1 and 2.

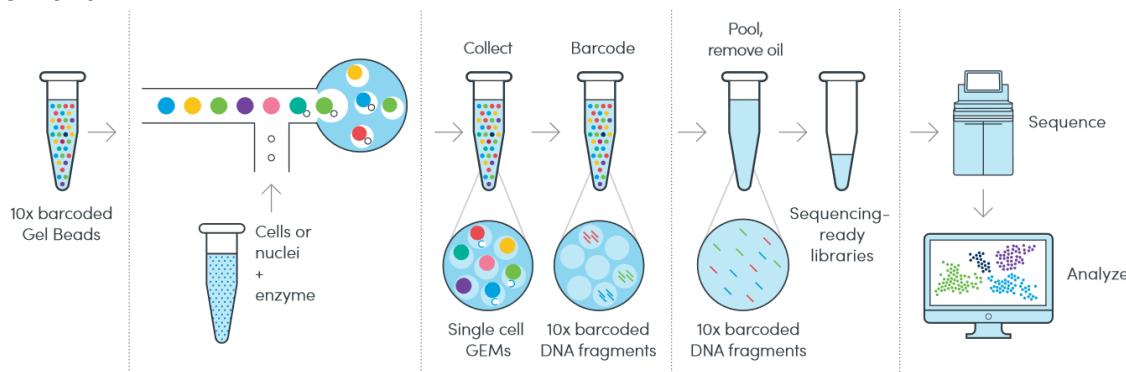


Figure 3. Single-cell encapsulation and library preparation workflow. *Image source: 10X Genomics*

I will use the expression level of immediate early gene markers to identify active cells in group 1 finches after undirected singing or group 2 finches after directed singing. I will identify differentially expressed genes in active cells after undirected versus directed singing and characterize different POM cell types. Using the sequencing results, I will collaborate with Dr. Josh Huang to develop CellREADR constructs that target specific neural subpopulations, which provide access to modify the gene expression of POM cell types in finches.

1.3. Expected Outcomes

I anticipate that three weeks after the viral injection of EGFP or GCaMP in the POM, green fluorescent labeling will be found in multiple brain regions including but not limited to VTA, A11, locus coeruleus, and dorsomedial nucleus of the nucleus intercollicularis¹⁴. For the intersectional approach, if I find EGFP not only in the viral injection sites but also in other POM-projected regions, I conclude that the same population of POM neurons project to multiple brain regions. If I only find EGFP in the viral injection sites, I conclude that the same population of POM neurons only projects to one destined region.

I expect that based on single-cell RNA sequencing data, I will identify multiple gene markers that are upregulated or downregulated for both undirected and directed singing conditions compared to no singing condition. I also expect to find genes differentially regulated by different singing conditions. I anticipate that I will be able to categorize POM neuronal cell types based on their gene expression markers and develop CellREADR constructs that specifically target POM neural subpopulations.

1.4. Potential Pitfalls and Alternative Strategies

If I encountered technical difficulties with the intersectional viral approaches, I could inject green and red fluorescent beads into two different POM projected regions to retrogradely label the POM cell bodies. If there are overlaps between green and red fluorescent beads in the POM cell bodies, it indicates that the same POM neurons have efferent projections to the two different regions. If there is no overlap between the beads, I can conclude that the same population of POM neurons only projects to one destined region.

Since POM is a relatively small brain region, I will need to dissect the POM from multiple animals to collect enough cells for single-cell RNA sequencing. However, the single-cell dissociation process is time-sensitive. Collecting multiple brains and combining the samples may take a longer time and cause a decreased number of healthy cells in the fresh brain tissues. If this is the situation, I will perform single-nucleus RNA sequencing instead, which is not time-sensitive and works with frozen tissues. However, single-cell RNA sequencing is still preferred because it measures not only nuclear transcripts, which single-nucleus RNA sequencing mainly measures, but also cytoplasmic transcripts. Another concern is that it might be hard to manually dissect the POM without obvious landmarks. An alternative strategy is to inject retrograde fluorescent beads in a POM-projected brain region three weeks before the tissue collection day. With the guide of the fluorescently labeled cells in the POM, I will be able to find and dissect the POM under a microscope. Our collaborator Dr. Dmitry Velmeshev is an expert in single-cell genomics and spatial transcriptomics, and I am confident to perform RNA sequencing and analysis in the finch POM.

Aim 2. Determine the contribution of the POM cell types in motivating different types of singing

2.1. Rationale

Here we seek to test the functional roles of distinct POM cell types and their efferent projections in driving motivational signals of zebra finches for directed and undirected singing. First, to test the necessity and sufficiency of the POM activity for singing, I will chemogenetically and optogenetically suppress or stimulate the neuronal populations of the POM and measure the effects on directed and undirected singing. Second, to differentiate the roles of different POM cell types and their projections in the motivation for singing, I will collaborate with Dr. Josh Huang at Duke to develop and exploit CellREADR to manipulate the activity of specific POM neuronal populations or their axon terminals and examine the effects on singing behaviors. In addition to determining the causal link between POM cell types and motivation for singing by manipulating POM neuronal activity, I will also build the correlative link between the activity of the POM neuronal subpopulations and natural singing behaviors, which reflects their causative functions. I will perform miniscope imaging to visualize single neurons' activity in the POM and relate neuronal ensemble dynamics to different aspects of singing behaviors. Completion of this aim will contribute to our understanding of the mechanisms underlying motivation to sing and the functions of distinct cell types in POM in motivating singing.

2.2. Experimental Design and Methods

Determine whether POM cell types motivate singing: My preliminary results showed that by infusing muscimol, a γ -aminobutyric acid type A (GABA A) receptor agonist, into the POM using microdialysis, both undirected and directed singing of male zebra finches were suppressed (Figure 5), suggesting that POM is necessary for both types of singing. To manipulate the POM neuronal activity in a more temporally and regionally precise manner, I will virally express inhibitory or excitatory Designer Receptors Activated Only by Designer Drugs (iDREADDs or eDREADDs) by injecting AAV9-hSyn-hM4D-Gi-mCherry or AAV9-hSyn-hM4D-Gq-mCherry in the bilateral POM of male zebra finches. Three weeks later, I will administer clozapine N-oxide (CNO) to induce chemogenetic inhibition or activation of the POM neurons or saline as control through intraperitoneal injection. I will quantify the effects on the singing rates and measure non-vocal behaviors, including postural changes and head gestures, using a supervised learning algorithm (DeepLabCut)¹¹. In a second approach, I will virally express Archaerhodopsin (ArchT) or Channelrhodopsin-2 (ChR2) in the POM neurons by injecting AAV2/9-CAG-ArchT-mCherry or AAV2/9-CAG-ChR2-mCherry and implanting optic ferrules in the unilateral POM. Three weeks later, continuous pulses of green light (400-1000ms duration, 532 nm, 8-15mW) will be delivered to the POM to induce optogenetic inhibition or stimulation. I will measure the effects on singing and behaviors as previously described. In addition to pan-neuronal manipulations in the POM, I will apply CellREADR (in collaboration with Dr. Josh Huang at Duke) to target specific neuronal cell types selected from scRNASeq results. By combining CellREADR with chemogenetic or optogenetic approaches, I can differentiate the roles of different POM cell types and their projections in regulating singing behaviors.

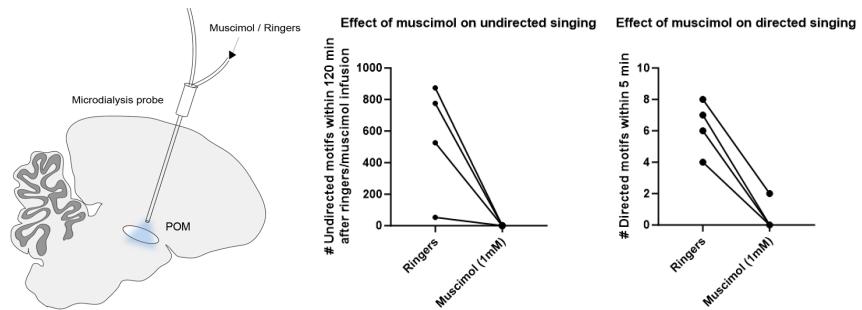


Figure 5. Left: Implantation site of microdialysis probe for muscimol (1mM) or ringers (control solution) infusion. Right: Effects of muscimol on undirected singing and directed singing.

Monitor POM activity and relate its activity to singing: I will virally express GCaMP in the POM neural subpopulations of male zebra finches and image neural activity before, during, and after undirected and directed singing. I will inject AAV2/9-CAG-GCaMP6s in the POM to image the POM neural dynamics at single-cell resolution. Additionally, I will inject CellREADR constructs to express Cre recombinase in specific cell types and inject AAV2/9-CAG-flex-GCaMP8S to image neural subpopulations in the POM. Furthermore, I can identify POM neurons that project to VTA and A11 through an intersectional approach. I will inject scAAV-DJ/9/2-hCMV-Cre in VTA or A11 to retrogradely transport Cre recombinase into the POM and inject AAV2/9-CAG-flex-GCaMP8S in POM to express GCaMP in POM neurons that project to VTA or A11. I will image neural activity with a standard single-color miniscope through a 1-mm diameter GRIN lens implanted at the lateral boundary of POM. I will use constrained nonnegative matrix factorization for microendoscopic data (CNMF-E) to extract regions of interest corresponding to neurons¹². I will align extracted traces and regions of

interest to vocal and non-vocal behaviors before, during, and after undirected and directed singing, which are recorded during imaging, and analyze using MATLAB software.

2.3. Expected Outcomes

I anticipate that chemogenetically inhibiting bilateral POM neural activity through using iDREADDs will suppress both undirected and directed singing and associated behaviors, while chemogenetically activating bilateral POM neural activity through using eDREADDs will augment undirected and directed singing and associated behaviors. I also expect that optogenetic inhibition or stimulation will lead to similar outcomes as the chemogenetic approaches. Additionally, optogenetic methods will provide more insights into the temporal relationship between POM activity and motivation to sing. By combining CellREADR with chemogenetic or optogenetic approaches, I expect that the manipulations of different neural cell types in the POM will differentially affect undirected and directed singing.

By imaging POM activity at single-cell resolution during singing, I anticipate finding different neurons whose activities align with different vocal and non-vocal behaviors and stimuli, including onsets of undirected and directed singing, the appearance of female finches, head orientation, body movements, etc. For example, I may find neurons displaying consistently increasing neural activity that is triggered by the appearance of a female finch and reaches its peak activity at the onset of directed singing. CellREADR techniques and the intersectional approach for labeling specific cell types or projection neurons will further elucidate the identity of imaged neurons and the circuit organization of POM in regulating the motivation to sing.

2.4. Potential Pitfalls and Alternative Strategies

It is possible that the same neuronal subpopulation is responsible for both undirected and directed singing, while their efferent projections towards different brain regions differentially regulate undirected and directed singing. To elucidate this circuit mechanism, I can optogenetically manipulate POM inputs in its projected brain regions by expressing G_{i/o}-coupled opsin parapinopsin (PPO) in the POM neuronal subpopulations. I can inject CellREADR constructs targeting POM cell types and AAV2/9-CAG-PPO-Venus into the POM and deliver laser in VTA or A11 to block them from receiving POM inputs. By quantifying both vocal and non-vocal behaviors as previously described, I can distinguish the circuit organization of the POM in regulating the motivation to sing.

Timetable

Aims	Objectives	Year 1	Year 2	Year 3	Year 4
1	Characterize anatomical connectivity of the finch POM	X	X		
	Characterize POM cell types activated for undirected and directed singing	X	X	X	
2	Determine whether POM cell types motivate singing	X	X	X	
	Monitor POM activity and relate its activity to singing		X	X	X

Future Directions

If a juvenile finch is more motivated to practice singing, would he learn faster and better? The current study will provide us with an opportunity to explore the causal relationship between motivation and learning. Juvenile zebra finches listen and memorize tutor songs between 30 and 60 dph and practice singing between 60 and 90 dph. I could virally express CellREADR constructs with ChR2 or ArchT in the POM neuronal subpopulations of juvenile male zebra finches. Then, I would expose them to live tutors between 30 and 60 dph. Between 60 and 90 dph, I would deliver laser pulses to the POM to optogenetically stimulate POM neural cell bodies to augment or suppress their motivation to sing. I could apply variational autoencoder (VAE) methods¹³ for quantifying vocal variability across syllable sequences to assess how stimulating or suppressing the motivation to sing in juvenile zebra finches affects juvenile learning trajectories. The natural song-learning behaviors of finches and the powerful machine-learning tool will lead to significant advances in understanding the neural mechanisms of motivational signals and learning outcomes.

Bibliographies

1. Jacques Balthazart, Philippe Absil, Agnes Foidart, Marc Houbart, Nobuhiro Harada, and Gregory F. Ball (1996). Distribution of Aromatase-Immunoreactive Cells in the Forebrain of Zebra Finches (*Taeniopygia guttata*): Implications for the Neural Action of Steroids and Nuclear Definition in the Avian Hypothalamus. *Journal of Neurobiology*, 31(2), 129-148.
2. Gregory F. Ball, Catherine J. Auger, Daniel J. Bernard, Thierry D. Charlier, Jennifer J. Sartor, Lauren V. Riters, and Jacques Balthazart (2004). Seasonal Plasticity in the Song Control System: Multiple Brain Sites of Steroid Hormone Action and the Importance of Variation in Song Behavior. *Annals of the New York Academy of Sciences* 1016(1): 586–610.
3. Mor Ben-Tov, Fabiola Duarte, Richard Mooney (2023). A neural hub for holistic courtship displays. *Current Biology*, 33(9): 1640-1653.
4. Katherine L. Anderson, Lionel Colón, Violet Doolittle, Raysa Rosario Martinez, Joseph Uraga, and Osceola Whitney (2023). Context-Dependent Activation of a Social Behavior Brain Network during Learned Vocal Production. *Brain Structure and Function* 228(7).
5. Panzica GC, Viglietti-Panzica C, Balthazart J. The sexually dimorphic medial preoptic nucleus of quail: A key brain area mediating steroid action on male sexual behavior. *Front Neuroendocrinol*. 1996b;17:51–125.
6. Jacques Balthazart, Philippe Absil, Agnes Foidart, Marc Houbart, Nobuhiro Harada, and Gregory F. Ball (1996). Distribution of Aromatase-Immunoreactive Cells in the Forebrain of Zebra Finches (*Taeniopygia guttata*): Implications for the Neural Action of Steroids and Nuclear Definition in the Avian Hypothalamus. *Journal of Neurobiology*, 31(2), 129-148.
7. Tanaka M, Sun F, Li Y, Mooney R (2018). A mesocortical dopamine circuit enables the cultural transmission of vocal behaviour. *Nature*, 563(7729):117-120.
8. Tschida K, Michael V, Takatoh J, Han BX, Zhao S, Sakurai K, Mooney R, Wang F (2019). A Specialized Neural Circuit Gates Social Vocalizations in the Mouse. *Neuron*, 103(3):459-472.e4.
9. Shuyun Xiao, Valerie Michael, Richard Mooney (2023). Nested circuits mediate the decision to vocalize *eLife*, 12:e85547.
10. Masahiro Morishita, Kaito Kobayashi, Moeri Mitsuzuka, Ryo Takagi, Kota Ono, Rami Monma, Yosuke Tsuneoka, Shuhei Horio, Shinji Tsukahara (2023). Two step actions of testicular androgens in the organization of a male-specific neural pathway from the medial preoptic area to the ventral tegmental area for modulating sexually motivated behavior. *Journal of Neuroscience*, JN-RM-0361-23.
11. Mathis, A., Mamidanna, P., Cury, K.M., Abe, T., Murthy, V.N., Mathis, M.W., and Bethge, M (2018). DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nat.Neurosci.* 21,12811289.
12. Pengcheng Zhou, Shanna L Resendez, Jose Rodriguez-Romaguera, Jessica C Jimenez, Shay Q Neufeld, Andrea Giovannucci, Johannes Friedrich, Eftychios A Pnevmatikakis, Garret D Stuber, Rene Hen, Mazen A Kheirbek, Bernardo L Sabatini, Robert E Kass, Liam Paninski (2018) Efficient and accurate extraction of in vivo calcium signals from microendoscopic video data *eLife* 7:e28728.
13. Jack Goffinet, Samuel Brudner, Richard Mooney, John Pearson (2021) Low-dimensional learned feature spaces quantify individual and group differences in vocal repertoires *eLife* 10:e67855.
14. Riters & Alger (2004). Neuroanatomical evidence for indirect connections between the medial preoptic nucleus and the song control system: possible neural substrates for sexually motivated song. *Cell Tissue Res* 316:35-44.
15. Moffitt JR, Bambah-Mukku D, Eichhorn SW, Vaughn E, Shekhar K, Perez JD, Rubinstein ND, Hao J, Regev A, Dulac C, Zhuang X. Molecular, spatial, and functional single-cell profiling of the hypothalamic preoptic region. *Science*. 2018 Nov 16;362(6416):eaau5324.
16. Bayless DW, Davis CO, Yang R, Wei Y, de Andrade Carvalho VM, Knoedler JR, Yang T, Livingston O, Lomvardas A, Martins GJ, Vicente AM, Ding JB, Luo L, Shah NM. A neural circuit for male sexual behavior and reward. *Cell*. 2023 Aug 31;186(18):3862-3881.e28.
17. Wu YE, Pan L, Zuo Y, Li X, Hong W. Detecting Activated Cell Populations Using Single-Cell RNA-Seq. *Neuron*. 2017 Oct 11;96(2):313-329.e6.
18. Qian, Y., Li, J., Zhao, S., Matthews, E. A., Adoff, M., Zhong, W., An, X., Yeo, M., Park, C., Yang, X., Wang, B. S., Southwell, D. G., & Huang, Z. J. (2022). Programmable RNA sensing for cell monitoring and manipulation. *Nature*, 610(7933), 713–721.
19. Fee MS, Goldberg JH. A hypothesis for basal ganglia-dependent reinforcement learning in the songbird. *Neuroscience*. 2011 Dec 15;198:152-70. doi: 10.1016/j.neuroscience.2011.09.069. Epub 2011 Oct 13. Erratum in: *Neuroscience*. 2013 Dec 26;255:301.

20. Sossinka, R., & Böhner, J. (1980). Song types in the Zebra Finch *Poephila guttata castanotis*. *Zeitschrift für Tierpsychologie*, 53(2), 123–132.
21. Zann, R. A. (2002). Vocalisations. In *The zebra finch: A synthesis of field and laboratory studies*. Oxford Univ. Press.