Elucidating the Neural Circuitry behind Male and Female-directed Song in Songbirds through Immediate Early Genes

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in collaboration with Dr. Marc Schmidt (BIO), Sabina London

Overview

- Male cowbirds sing a song to stimulate a mating posture behavior in females. They also sing to males during the breeding season, which behavioral data suggests is aggressive.
- Though the neural pathway of the song system is well-characterized, little is known about how this system is engaged to produce a superficially similar behavior with different intents.
- We are investigating whether the mechanisms behind the two types of song through looking at key areas within and beyond the song system (Fig 1).

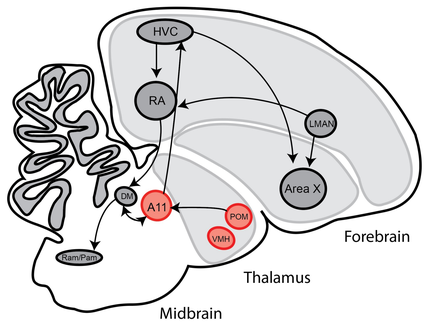


Figure 1. An anatomical diagram of the key areas (grey circles) we wish to look at within song system. Known projections between these areas are mapped out. We also hope to look at A11, POM, and VMH (red), though they are typically not included in the canonical song system.

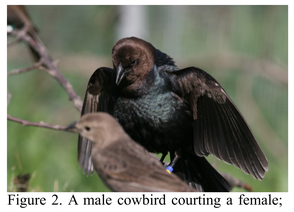


Figure 2. A male cowbird courting a female; Source: Schmidt Lab

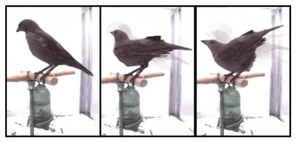
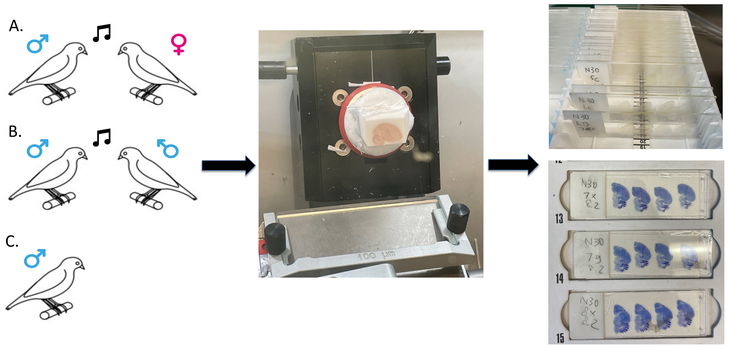


Figure 3. Time lapse of female cowbird going into mating posture behavior upon male song; Source: Perkes et al. (2019)

Methods 1: Slide Preparation

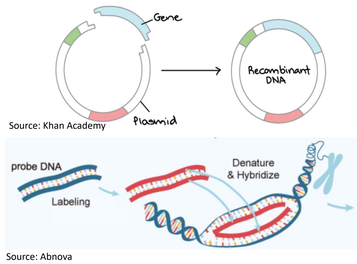


1. There were 3 experimental set ups. Type A was where the male cowbird sang entirely to a female. Type B was where the bird sang entirely to another male. Type C was where the bird sat silently. Singing/silence lasted for 30 minutes, and birds were sacrificed immediately

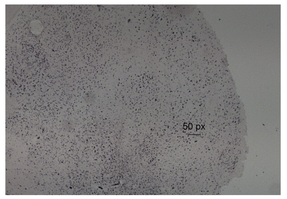
2. Brains were frozen in OCT and cut and mounted on a cryostat at 15µm for in-situ and 30µm for Nissl-staining

3. Slides were separated for in-situ and Nissl-staining. Nissl staining conducted to determine presence of target areas.

Method 2: Probe Synthesis & In-Situ Hybridization



5. Gene of interest was determined and plasmid with a complementary sequence was constructed. However, for PVALB a previously constructed plasmids was used. Then, plasmid was digested to release the insert, purified, and tagged to create the riboprobe. During in-situ hybridization. The probe binds with the target RNA/DNA of interest.



6. Picture shows Arc labeling in a control male. Labelled cells within areas of interest are counted to determine intensity of signal across experimental set

Method 3: Protocol Confirmation

In order to ensure that the in-situ protocol works, we used a known marker of the zebra finch song system, parvalbumin (PVALB) as our initial probe. As expected, the cowbird PVALB signal was consistent with that of the zebra finch (see Zebra Finch Atlas).

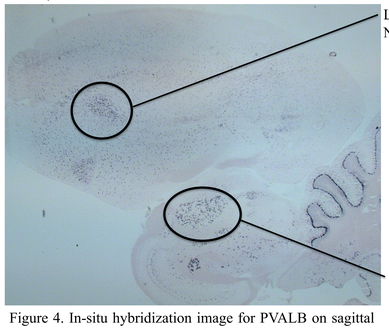


Figure 4. In-situ hybridization image for PVALB on sagittal section showing LMAN and Rt from control male cowbird.

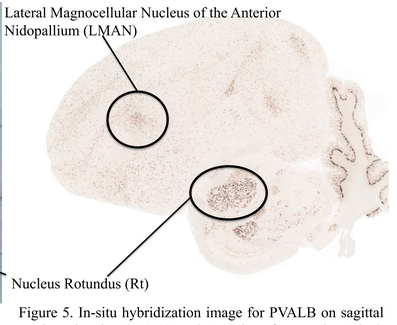


Figure 5. In-situ hybridization image for PVALB on sagittal section showing LMAN and Rt taken from Zebra Finch Expression Brain Atlas.

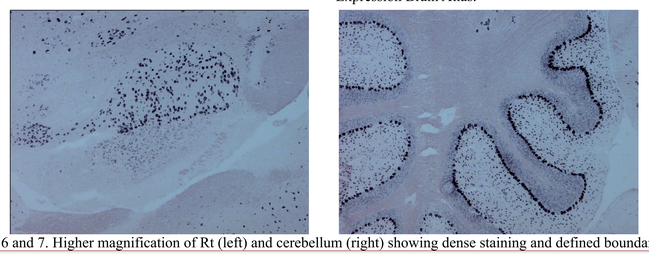


Figure 6 and 7. Higher magnification of Rt (left) and cerebellum (right) showing dense staining and defined boundaries

Moving Forward 1: Timeline

Due to the novelty of the technique to our lab, in my junior year I plan to continue to hone my skills in sectioning on a cryostat and attempt a new in-situ hybridization protocol that has modified for zebra finches.

- **Fall:**

I will create probes from plasmids (courtesy of Mello Lab) for known markers in the zebra finch, including PVALB and GAD, and conduct the in-situ on control birds to ensure that the protocol goes smoothly. Then, I will begin making the probes for the immediate early gene, ZENK, and create a glycerol stock to ensure self-sustainability.

I will also practice my sectioning, this time with more attention towards preventing air bubbles and tears, as well as preservation of the slides to ensure the RNA signal from the IEGs do not degrade.

- **Spring:**

When the breeding season Marc and I will re-conduct the behavioral experiments in the aviary (male-directed, female-directed, and silent controls), and use the new protocol alongside our ZENK, PVALB, and GAD probes to look at expression in the same areas. Furthermore, once we see reliable expression, we will make make ESR1 and VGLUT2 probes to target VMH/POA and A11 respectively.

Moving Forward 2: Probes

Probe	Purpose
ZENK	Overall activity-dependent expression
PVALB	Marker of the song system
GAD	Marker of the song system
ESR1	POA, VMH
VGLUT2	A11

Acknowledgments and Sources

- Plasmids and in-situ hybridization protocol were courtesy of Mello Lab at the University of Oregon, and in particular, Dr. Claudio Mello and Alex Nevue.
- Materials for riboprobe synthesis courtesy of Luo Lab at the Perelman School of Medicine