Glutamatergic Vocal Motoneurons in *Xenopus laevis*: An Immunohistochemical Analysis of Neurotransmitters in Frog Vocal Motoneurons.

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INTRODUCTION

African clawed frogs, *Xenopus laevis*, have sexually distinct vocalizations based on the sex-specific firing rate (Fig 1). The central vocal pathways of *X. laevis* is an ideal model to explore the neural circuitry underlying behavior because the vocalizations are independent of the respiratory system, allowing us to more clearly see the relationship between neural activity and the behavioral output.

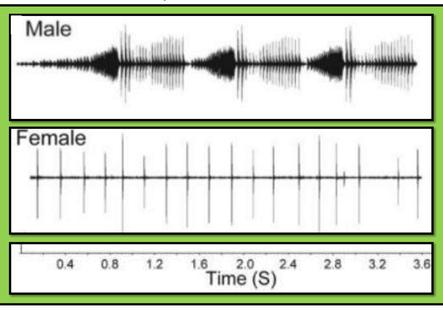

Figure 1: Amplitude envelopes of male (top) verses female (bottom) vocalizations over time.

To be able to gain a deeper understanding of the neural behavior of *Xenopus laevis,* the goal of this project is to determine the neurotransmitters used by the vocal motoneruons. Traditionally, motoneurons are known to release acetylcholine to regulate skeletal muscles. Previously, it was discovered that the Xenopus vocal motoneurons have unique feedback pathways to project back to the premotor neurons in addition to regulating the activity of the laryngeal muscles. Here, we hypothesize that glutamate is used as a neurotransmitter by the vocal motoneurons to drive the feedback pathways. To test this hypothesis, we will label vesicular glutamate transporter (VGluT1) protein selective for glutamatergic neurons using immunohistochemistry.

Figure 2: *Xenopus laevis*

ACKNOWLEDGEMENTS

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METHODS

Indirect Immunohistochemistry is an immunostaining visualization technique using primary antibodies and secondary antibodies conjugated with a fluorescent dye (Alexa Flour 555 or 594). Here we used two types of polyclonal primary antibody (rabbit and guinea pig anti-VGluT1) that are predicted to bind (i.e., >80% sequence homology) to the VGluT1 of *X. laevis.*

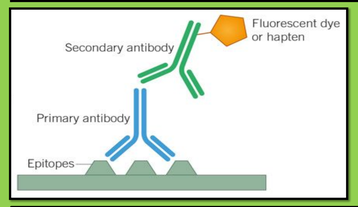


Figure 3: Indirect method of immunohistochemistry on a cellular level. Primary antibody attaches to the epitopes (in our case, VGluT1) first. Then secondary antibody attaches to the primary conjugated with fluorophore.

Tissue Preparation

- **Fixation:** Trans-cardial perfusion is performed in *Xenopus laevis* to fix the brain tissue.
- **Microtome Sectioning:** The brain is sectioned into 40 micron thick sections and mounted onto slides.
- **Antigen Retrieval:** The sections are boiled in citrate buffer to recover epitopes that were masked during fixation.
- **Blocking:** To control for background staining and eliminate false positives, sections are blocked with skim milk acting as a dilute protein solution binding to all sites that may be available for unspecific binding.

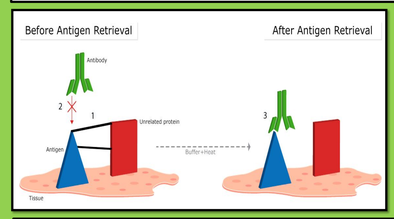


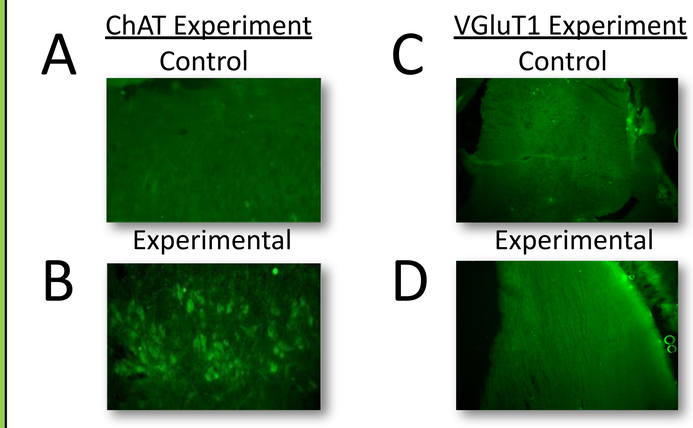
Figure 4: Antigen retrieval. During fixation proteins form crosslinks that can block the antibody from attaching to the antigen. After antigen retrieval the epitope is recovered allowing the antibody to bind to the antigen.

Experiment

- **Primary Antibody:** rabbit anti-VGluT1 (AB77822) 1:100 concentration, guinea pig anti-VGluT1 (AB5905) 1:1000 concentration. Tissue was incubated with primary antibody which binds to the antigen (VGluT1).
- **Secondary Antibody:** donkey anti-rabbit 1:200 concentration, donkey anti-guinea pig 1:1000 concentration. Tissue was incubated with secondary antibody which binds to the primary antibody and adds fluorescence that allows us to visualize the location of the antigen.
- **Control Group:** Processed in the same way as experimental group but without the primary antibody.
- **Experimental Group:** Processed with primary and secondary antibodies.

RESULTS/CONCLUSION

The current focus of this project to label glutamatergic neurons has not yet been successful to date. Previous studies using *in situ* hybridization have shown that VGluT1 mRNA is present in distinct parts of the *Xenopus* brain (Gleason et. al 2003), indicating that we should be able to obtain positive staining using immunohistochemical (IHC) technique. Since the initial attempt to label VGluT1 failed, antigen retrieval step was added, and an additional primary antibody from a different commercial source, and mouse brain sections kindly made available by Dr. Saijoh were added to the study. Despite all these efforts, the labeling is yet to be successful (Fig D). Compared to the choline acetyltransferase (ChAT)-positive neurons labeled in *Xenopus laevis* (Fig B), experimental tissues of VGluT1 showed no obvious labeling of neurons.



Moving forward, we would like to try cryosectioning the tissue. Previously during antigen retrieval, the sections were coming off the slides which may have hindered the effectiveness of the treatment and cryosectoning may help solve this issue. In the future after successfully staining VGluT1 positive neurons, the next step would be to double stain with both ChAT and VGluT1 to identify potential co-transmission in vocal motoneurons.

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Abstract

The nervous system is intricate; it involves multitudes of neurons that can lead to complex behaviors. The African clawed frog, *Xenopus laevis*, has sex-specific vocalizations that are independent of the respiratory system which allows for a clearer look at the relationship between neural activity and behavior. Motoneurons that innervate skeletal muscles release acetylcholine as neurotransmitters. Previously, unusual feedback pathways from the motoneurons to the premotor neurons were discovered in the central vocal pathways of *X. laevis*. Here, we hypothesize that along with acetylcholine, glutamate is also used as a neurotransmitter by the vocal motor neurons to drive feedback neurons in the central vocal pathways of *X. laevis*. To test this hypothesis, it is necessary to: 1) establish a method for labeling vesicular glutamate transporters (VGluT1) expressed selectively by the glutamatergic neurons in the frog brain and 2) double stain for glutamate and acetylcholine. My project deals with the first step. Through immunohistochemistry we stain the tissue and label for VGluT1, a transporter protein that moves glutamate across a membrane, using rabbit anti-VGluT1 as the primary antibody and donkey anti-rabbit conjugated with a fluorophore as the secondary antibody. Initial attempts to stain for glutamate using two different primary antibodies have been unsuccessful despite an extra step we added to enhance our chance of success and require further troubleshooting. Once my project is complete and glutamate has been successfully labelled for, we would then use immunohistochemistry to double stain for acetylcholine and glutamate to look for possible co-transmission.