Speech-related gene FoxP2 expression in the central vocal pathways of the African clawed frogs

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Abstract

Forkhead box protein P2 (FoxP2) is a transcription factor known to be critical for the development of speech and language in humans; a mutation in FoxP2 gene results in devastating linguistic impairments. Orthologs of FoxP2 are found across vertebrate species including birds, crocodiles, and fish. FoxP2 has highly conserved amino acid sequence as well as the patterns of expression within the brains across vertebrate species. FoxP2 disruption in mice and songbirds results in loss of pup calls and incomplete imitation of adult songs, respectively, suggesting the involvement of the gene in vocal communication in these species. Here, we asked if FoxP2 is expressed in the central vocal pathways of African clawed frogs, Xenopus laevis. Male and female X. laevis generate sex-specific vocalizations to coordinate reproduction during the breeding season. The vocalizations of *X. laevis* are generated by the central pattern generators (CPG) contained in the brainstem that consists of the dorsal tegmental area of medulla (DTAM, homologue of parabrachial nucleus in mammals) and the laryngeal motor nucleus (n.IX-X, homologue of nucleus ambiguus in mammals). The results showed that FoxP2 immunopositive neurons are found in a variety of brain regions including the telencephalon, optic tectum, cerebellum, and brainstem of *X. laevis*. In particular, we found a dense labeling of FoxP2-positive neurons in the DTAM in the two sexes. Given that FoxP2 is known to be expressed in the parabrachial nucleus of mammals, our results represent a conserved expression pattern of the FoxP2 gene. There were no sex differences in the expression patterns of the immunopositive neurons. Further immunohistochemical analyses showed that the FoxP2-positive neurons in DTAM do not express GABA, cholinacetyltransferase, nor parvalbumin. The discovery of FoxP2 expression in *X. laevis* DTAM suggests the contribution of the gene in the vocal communication in amphibians.

Introduction

Forkhead box protein P2 (FoxP2) is critical for the development of speech and language in humans. Interestingly, its orthologs are known to be important for social communication in songbirds and in mice. As a first step to understand the role of FoxP2 in social communication in other vertebrate species, we asked whether FoxP2 is expressed in the central vocal pathways of the African clawed frogs, *Xenopus laevis*.

Male and female X. laevis produce sexually distinct vocalizations under water to coordinate reproduction. The central vocal pathways of X. laevis contain two pairs of nuclei in the brainstem; the laryngeal motor nucleus (n.IX-X, homologue of nucleus ambiguuus in mammals) and the premotor nucleus of the dorsal tegmental area of medulla (DTAM, homologue of parabrachial nucleus in mammals; Fig 1). Previously, we have shown that these two nuclei function as a central pattern generator to produce sex-specific firing patterns of laryngeal motoneurons that underlie vocalizations.

The amino acid sequence of X. laevis FoxP2 is 92% homologous to the human FOXP2 gene. We first used immunohistochemistry to determine if FoxP2 is expressed by the vocal neurons of X. laevis. We next explored the neurochemistry of the FoxP2-positive neurons. Specifically, we tested if FoxP2-positive neurons in DTAM express GABA, cholinacetyltransferase and parvalbumin, using immunohistochemistry. We next tested what types of receptors are expressed by the FoxP2-positive DTAM neurons by conducting constellation pharmacology.

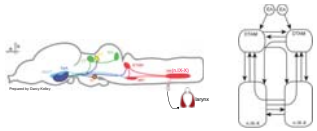

Figure 1. The central vocal pathways of Xenopus laevis. Left, sagittal view. Right, dorsal view. DTAM, dorsal tegmental area of medulla, n.IX-X, laryngeal motor nucleus.

Results

1. DTAM neurons express FoxP2

FoxP2-positive neurons are found in both male and female DTAM, but not in n.IX-X. Other FoxP2-positive neurons are seen in optic tectum, cerebellum, and telencephalon.

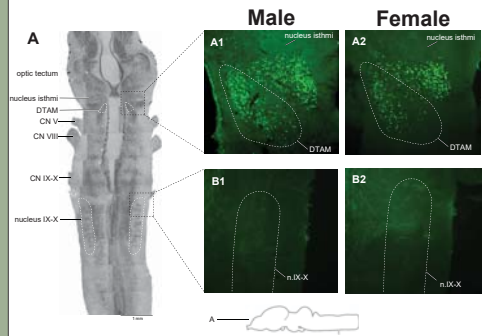

Figure 2. Immunohistochemistry for FoxP2 in the brains of Xenopus laevis. A. Cresyl-violet stained horizontal section of a mid- and hindbrain of Xenopus laevis. A1, A2, FoxP2 positive neurons are found in DTAM (defined by a white dotted line) of both male and female brains. B1, B2, Neurons in n.IX-X were not immuno-positive for FoxP2. The plane of horizontal section is shown in the bottom right cartoon.

2. FoxP2-positive neurons in DTAM are not GABAergic nor cholinergic

Very few neurons in DTAM are immunopositive for GABA or choline acetyltransferase (ChAT), suggesting that FoxP2-positive DTAM neurons use neurotransmitters other than GABA and ACh.

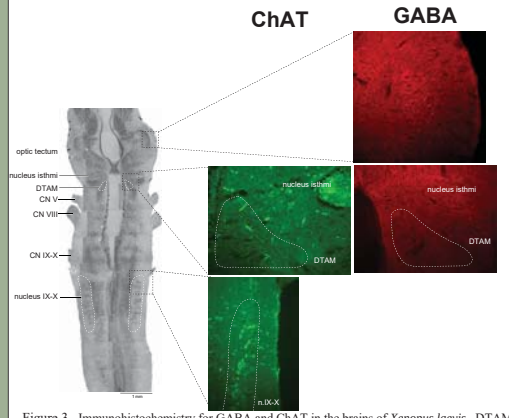

Figure 3. Immunohistochemistry for GABA and ChAT in the brains of *Xenopus laevis*. DTAM neurons were negative for GABA and ChAT. Motoneurons in n.IX-X are positive for ChAT. Some neurons in optic tectum are positive for GABA.

3. Parvalbumin may be expressed by vocal neurons.

RNA-seq gene expression data analyses of the brainstem showed transcripts coding for parvalbumin are expressed at a higher level in brains that generate male-specific calls. In *X. laevis*, treating adult females with testosterone (T) masculinize the vocalizations within 13 weeks. When the abundance of transcripts obtained from the brainstem of males, females, and T-treated females was compared, two gene transcripts coding for parvalbumin were significantly higher in males and T-females than in control females.

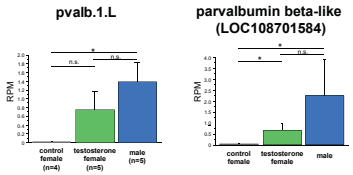

Figure 4. Reads per million mapped reads (RPM) of pvalb.1.L and parvalbumin beta-like gene transcripts obtained from the brainstem of males, females, and testosterone-treated females. Males ane T-females were confirmed to produce male-specific vocalizations prior to RNA extraction. Mann-Whitney U test was used to compare the RPM of the three groups of animals.

4. FoxP2-positive neurons in DTAM do not express parvalbumin.

Although the RNA-seq results above suggested an involvement of parvalbumin in vocal production, the immunohistochemical results showed that parvalbumin is not expressed by neurons in DTAM nor n.IX-X.

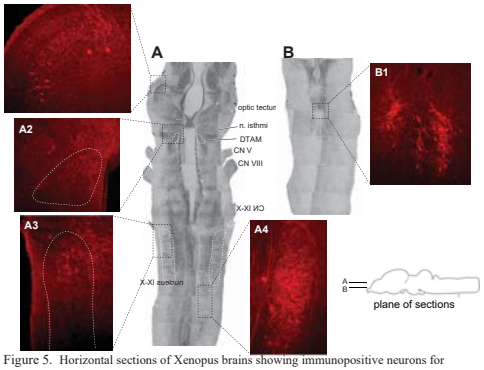

Figure 5. Horizontal sections of Xenopus brains showing immunopositive neurons for parvalbumin. A. Cresyl-violet (CV) stained horizontal section of the mid- and hind-brain of Xenopus laevis. A1, parvalbumin-positive neurons in optic tectum. A2 and A3, there are almost no parvalbumin-positive neurons in DTAM and n.IX-X. A4, parvalbumin-positive neurons are found in caudal reticular formation. B. CV-stained horizontal section of mid- and rostral hindbrain of Xenopus laevis. B1, parvalbumin-positive neurons in ventral tegmentum. The plane of sections are shown in the right bottom cartoon.

5. FoxP2-positive neurons may express NMDA and GABA receptors.

To characterize the types of receptors expressed by FoxP2-positive neurons in DTAM, we carried out constellation pharmacology. Briefly, neurons in DTAM were dissociated, loaded with fura-2, and calcium imaging was carried out while seven types of agonists were applied. FoxP2-positive neurons were identified using immuno-histochemistry *post hoc*. Many of the FoxP2-positive neurons showed responsiveness to NMDA and GABA, suggesting that these neurons express NMDA and GABA receptors.

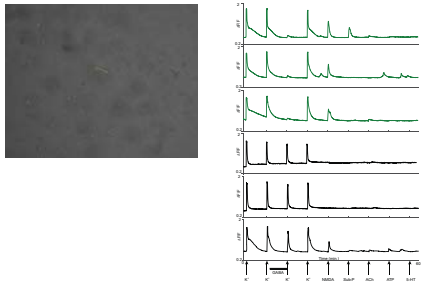

Figure 6. Calcium imaging using neurons dissociated from DTAM region. Left; Dissociated DTAM neurons used for immunohistochemistry. Green neurons are FoxP2 positive neurons. Right; Six calcium imaging traces from six neurons. Top three traces in green are obtained from FoxP2-positive nerons, and bottom three traces in black are obtained from FoxP2-negative neurons. KCl, GABA, NMDA, substance-P, Acetylcholine, ATP, and serotonin (5-HT) were applied to the dissociated cells loaded with fura, and the change in fluorescence was measured to assess the influx of Ca2+ into each neuron. FoxP2-positive neurons tend to respond to GABA and NMDA.

Conclusion: We discovered that premotor nucleus of DTAM - critical for vocal production in X. laevis - contains FoxP2 positive neurons. These FoxP2-positive neurons in DTAM are not GABAergic or cholinergic, and do not express parvalbumin, but may express NMDA and GABA receptors. These pieces of information provide us with a clue into understanding the role of FoxP2-positive neurons in vocal production in *Xenopus laevis*.

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