Activity of FoxP2-positive neurons correlates with begging behavior in a social tadpole

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Abstract

Motor function is a critical aspect of communication in a wide range of taxa. The transcription factor FoxP2 plays an important role in coordinating the development of motor areas related to vocal communication in humans, mice, and songbirds. However, the role of FoxP2 in regulating motor coordination of non-vocal communication behaviors in other vertebrate taxa is unclear. Here, we test the hypothesis that FoxP2 is associated with begging behavior in tadpoles of the Mimetic poison frog (*Ranitomeya imitator*). In this species, mothers provide unfertilized egg meals to tadpoles that perform a begging display to communicate hunger by vigorously dancing back and forth. We mapped the neural distribution of FoxP2-positive neurons in the tadpole brain, where its wide distribution paralleled that of mammals, birds, and fishes. We next evaluated the activity of FoxP2-positive neurons during tadpole begging and found that FoxP2-positive neurons showed increased activation in the striatum, preoptic area and cerebellum. Overall, this work suggests a generalizable function of FoxP2 in social communication across terrestrial vertebrates.

Introduction

Social communication is a key sensorimotor behavior required for survival and fitness across many taxa. In species where parents care for offspring, conveying nutritional needs is a critical infant behavior, including human infants crying [1] and avian chicks display begging behavior [2]. Infants of other less well studied taxa also display begging behavior to solicit resources from parents, such as in amphibian [3,4] and insect [5,6] larvae. These begging behaviors typically involve coordination of motor displays in response to specific social stimuli, although the neural processes that coordinate sociomotor displays in infants is not well understood. Establishing the neural processes underlying infant communication is critical for developing a mechanistic understanding of how infant begging behavior evolves.

Transcription factor Forkhead Box P2 protein (FoxP2) is associated with the motor processes related to communication in many species. Although FOXP2 was once hailed as the human “language gene” [7], it was later established that FOXP2-associated language abnormalities are instead due to the atypical development of motor-related neural pathways [8]. A mutation of a critical residue in the DNA-binding domain of FOXP2 (the R552H mutation) identified in humans leads to impairments of the fine facial movements necessary for speech [9]. These phenotypes can already be observed in children, where small intragenic deletions within the FOXP2 coding gene is linked to childhood apraxia of speech and oral motor dyspraxia [10]. A conserved role for FoxP2 in communication has been extended to mice [11] and birds [12] (human FOXP2 and non-human FoxP2 homologs are upper and lowercase, respectively). For example, ultrasonic vocalizations emitted by mouse pups are influenced by FoxP2 mutations [13]. Anuran amphibians use both vocal and non-vocal communication to solicit mates, defend territories, and communicate within family units [14,15]. While the whole-body distribution of FoxP2 has been described in the early development of *Xenopus* [16], the neural distribution and function of FoxP2 in amphibian life stages with social communication is unknown. Filling this gap is critical to gaining a more holistic view of the role of FoxP2 in sensorimotor development, function, and evolution.

Here, we explored the function of FoxP2 in tadpoles of the mimetic poison frog (*Ranitomeya imitator*). In this species, tadpoles beg parents for unfertilized egg meals by using a robust motor display characterized by vigorously dancing back and forth [17]. This begging display is energetically costly and performed in the absence of brood-mates, as tadpoles are deposited in isolated nurseries [3]. We first mapped the neural distribution of FoxP2 in the *R. imitator* tadpole brain and then tested the hypothesis that activity of FoxP2-positive neurons is associated with begging behavior. We predicted that FoxP2 would show a conserved brain distribution pattern

similar to other vertebrates, and that FoxP2-positive neurons would have higher activity in motor-related brain regions in begging tadpoles.

Methods

Animals

All *Ranitomeya imitator* tadpoles used were bred from our laboratory colony [18]. Adult *R. imitator* females from actively reproducing breeding pairs were used as stimulus animals. All procedures were approved by the Stanford University Animal Care and Use Committee (Protocol #33097).

Begging behavior

We randomly assigned tadpoles (Gosner stage 30-34, no forelimb development and minimal hindlimb development) into one of two experimental groups: exposed to a reproductive adult female (begging, N=16) or exposed to a novel object (a metal bolt, N=15). All behavior trials were conducted between 09:00 and 12:00 hours. Tadpoles were placed into individual square arenas (5 x 5 x 5 cm) filled with 50 mL of tadpole conditioned water. Each arena was placed on a square LED light pad and recorded from above using GoPro cameras (GoPro HERO7 Black, 1080p, 240 fps). Each tadpole acclimated for 10 min in the arena. Then, the stimulus was introduced to the arena and behavior was recorded for 30 min. Stimuli were then removed from the arena and tadpoles were placed in the dark for 15 minutes. Tadpoles were then anesthetized with topical 20% benzocaine and sacrificed by rapid decapitation.

Videos were scored using BORIS software [19] by an observer uninformed of tadpole identity. Begging was quantified by the number and duration of each begging bout. Begging behavior was characterized by rapid vibration of the body and tail, often at a >45° angle with a straight tail.

Immunohistochemistry

Whole tadpole heads were fixed with 4% paraformaldehyde (PFA) in 1X phosphate buffered saline (PBS) at 4°C overnight, rinsed in 1X PBS, and transferred to a 30% sucrose solution for cryoprotection at 4°C overnight. Tadpole heads were then embedded in mounting media (Tissue-Tek® O.C.T. Compound, Electron Microscopy Sciences, Hatfield, PA, USA) and stored at -80°C until cryosectioning at 15 µm into three series. Sections were thaw-mounted onto

SuperFrost Plus microscope slides (VWR International, Randor, PA, USA) and then stored at -80°C until immunohistochemistry.

We used double-label fluorescence immunohistochemistry to detect FoxP2 and phosphorylated ribosomes (pS6, phospho-S6) as a proxy of neural activity [20] as previously described [21]. Slides were incubated overnight in a mix of both primary antibodies [rabbit anti-pS6 (Invitrogen, cat #44-923G) at 1:500 and goat anti-FoxP2 (Abcam, cat #AB1307) at 1:500 in 2% normal donkey, 0.3% TritonX-100, 1X PBS]. Following several washes, slides were incubated in a mix of fluorescent secondary antibodies (1:200 Alexa 488 donkey anti-goat and 1:200 Alexa 568 donkey anti-rabbit in 2% normal donkey serum, 0.3% TritonX-100, 1X PBS) for two hours. Slides were then rinsed in water and cover slipped using Vectashield Hardset Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and stored at 4°C. FoxP2 was restricted to cell nuclei and, as expected, was found in brain regions consistent with other animals. Control slides omitting primary antibodies did not show immunostaining (Supplementary Figure 1).

Fluorescence microscopy and cell counting

Brain sections were imaged on a Leica compound fluorescent microscope connected to a QImaging Retiga 2000R camera as previously described [21]. Brain regions containing FoxP2 were identified using DAPI-stained nuclei while referencing a poison frog brain atlas [21]. For each brain region containing FoxP2, FIJI software [22] was used to measure the area of each brain region within a single hemisphere. The number of FoxP2-positive cells, pS6-positive cells, and colocalized cells were quantified within that area using the “Cell Counter” function. Due to tissue quality, not all brain regions were counted for every individual.

Data analysis

All statistics and figures were generated in R Studio (version 1.1.442) running R (version 3.5.2). We used the glmmTMB R package [23] to analyze cell count data with generalized linear mixed models. For FoxP2-positive and pS6/FoxP2 colocalized cells, we ran separate models using a negative binomial distribution appropriate for count data. For both models, we tested for significant differences between behavioral groups by including group, brain region, and their interaction as main effects. Tadpole identity was included as a random variable to account for repeated sampling of brain regions within individuals and the log of brain region area was included as an offset. For colocalization data, we included the number of colocalized cells as the independent variable and the number of FoxP2 cells as a weight in the model. We then followed

the model with the Anova.glmmTMB function for reported statistical values. When there was a significant interaction between group and brain region, we ran a post-hoc test with the emmeans R package (version 1.5.3) and used a Tukey-correction for multiple hypothesis testing.

Results

Neural distribution of FoxP2

We first determined which brain regions contained FoxP2-positive cells and observed a broad distribution of FoxP2-positive cells throughout the brain (Figure 1, Supplementary Figure 2). The highest densities of FoxP2-positive cells were found in the subpallial forebrain, optic tectum, thalamus, and cerebellum. Notably, there were many FoxP2 cells in regions linked to sensory processing, such as the olfactory bulb (chemosensory), torus semicircularis (acoustic processing), and optic tectum (vision).

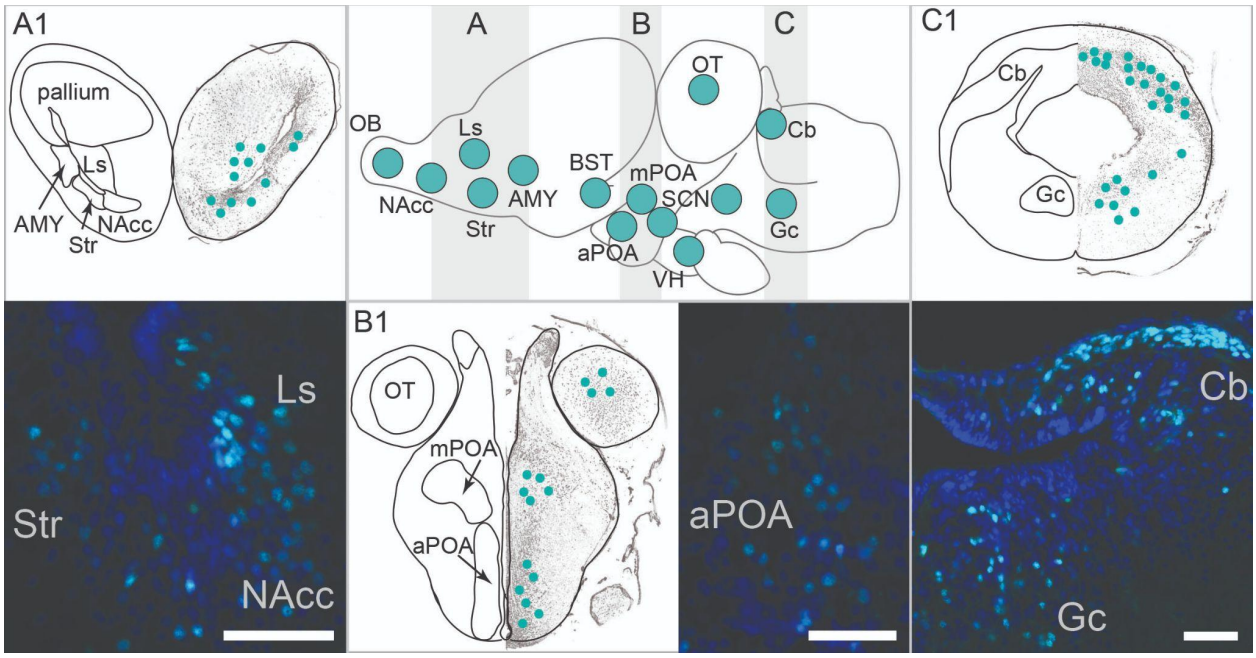

Figure 1. Neural distribution of FoxP2 in amphibians is similar to other vertebrates. FoxP2 is widely distributed throughout the amphibian brain, including the subpallial forebrain (A), midbrain (B), and a few hindbrain regions (C). The center sagittal brain schematic (rostral is to the left) shows brain regions (green) with FoxP2 positive cells. Gray boxes represent areas of interest for more detailed neuroanatomy and micrographs (A1-C1), where green dots represent qualitative presence of FoxP2. Micrographs show FoxP2-positive cells (cyan) and DAPI-stained nuclei (blue); scale bar is 10 μm. The complete neural distribution for FoxP2 can be found in Supplementary Figure 1. Abbreviations: AMY, amygdala; aPOA, anterior preoptic area; BST, bed nucleus of the stria terminalis; Cb, cerebellum; Gc, central gray; Ls, lateral septum; mPOA, magnocellular preoptic area; NAcc, nucleus accumbens; OB, olfactory bulb; OT, optic tectum; SCN, suprachiasmatic nucleus; Str, striatum; VH ventral hypothalamus.

FOXP2-positive neurons are more active during begging

We asked whether FoxP2-positive neuron activity is associated with tadpole begging behavior. We quantified the proportion of FoxP2-positive cells that colocalized with the pS6 marker of neural activity in begging tadpoles compared to controls (Figure 2). Given the wide distribution of FOXP2, we focused our analyses on brain regions associated with dopaminergic signaling, which is required for begging behavior in this species [18]. The activity of FoxP2-positive cells depended on an interaction of behavioral group and brain region (group*region: $F_5 = 300.37$, $p < 0.001$). Begging tadpoles had more active FoxP2-positive cells than controls in the striatum (Str, $z = 2.105$, $p = 0.035$), anterior preoptic area (aPOA, $z = 4.353$, $p < 0.001$) and cerebellum (Cb, $z = 3.696$, $p = 0.0002$), but not the nucleus accumbens ($p=0.146$), lateral septum ($p = 0.195$) or the magnocellular preoptic area ($p = 0.201$). There were no significant differences in the number of FoxP2-positive cells within these brain regions across groups (Supplementary Excel File).

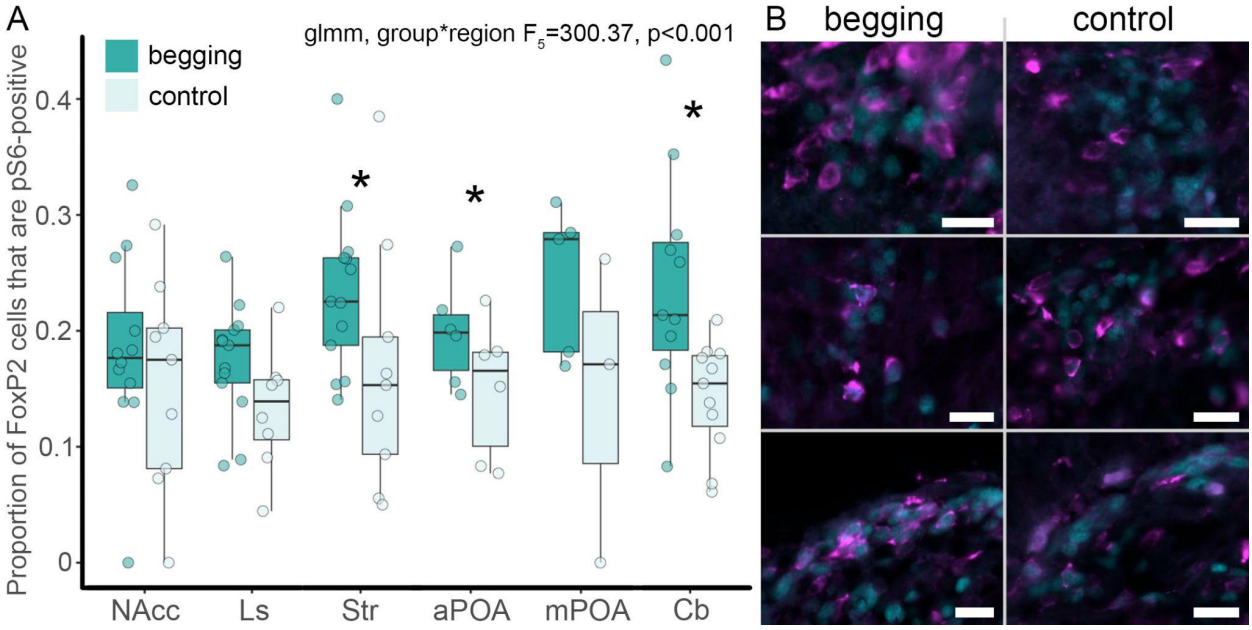

Figure 2. Activity of FoxP2 neurons increases in begging tadpoles. (A) Proportion of active FoxP2-positive cells in begging (dark green) and control (light green) tadpoles are shown in boxplots with individual tadpoles displayed in dots. **(B)** Representative micrographs of FoxP2 (green) and pS6 (pink) colocalization expression of begging (left) or control (right) tadpoles in the striatum, anterior POA, and cerebellum. Abbreviations: aPOA, anterior preoptic area; Cb, cerebellum; Ls, lateral septum; mPOA, magnocellular preoptic area; NAcc, nucleus accumbens; Str, striatum.

Discussion

The ability of offspring to communicate their needs to parents is a behavior critical to survival across taxa [3–5,24]. While there is spectacular variation in the modes of communication by infants, all require complex motor planning and execution. The transcription factor FoxP2 has an important function in the communication in mammals and birds [7,9,10,12,13,25]. Our study expands the role of FoxP2 in communication to amphibians, suggesting a generalizable function of FoxP2 in social-motor coordination across terrestrial vertebrates.

The brain distribution of FoxP2 is conserved across vertebrates

FoxP2 is widespread throughout the amphibian brain, with a distribution pattern consistent with those found in other vertebrates (mammals: [26–28]; birds: [29]; fish: [30–32]). Across these taxa, there is a conserved pattern of expression in brain areas involved in motor output, sensory processing, and sensorimotor integration. In *R. imitator* tadpoles, brain regions that regulate motor output, including the striatum, dopamine-related brain regions, and cerebellum, had many FoxP2-positive neurons. FoxP2 expression is upregulated in striatal regions during vocal learning in zebra finches [12], suggesting that abundance patterns may change with experience, which was not investigated here. We also noted FoxP2-positive cells in many sensory processing regions like the olfactory bulb (chemosensory), optic tectum (visual processing), and torus semicircularis (acoustic processing). In bats, species differences in FoxP2 expression in the olfactory bulb is associated with different feeding habits (frugivorous versus insectivorous) [33], suggesting that FoxP2 may influence olfactory processing. Given this research in bats and considering that *R. imitator* tadpoles rely on smell to distinguish between caregiver stimuli [18], investigating FoxP2's role in sensory integration broadly rather than a narrow focus on vocal communication may be a valuable future research direction. Overall, the distribution of FoxP2 in the amphibian brain suggests a largely conserved pattern across terrestrial vertebrates that function in learning and executing social behaviors important for survival.

Conservation of FoxP2 in motor communication

We found that activity of FoxP2-positive neurons was higher in begging tadpoles compared to controls in several brain regions important for motor output and social behavior, including the striatum, anterior preoptic area, and cerebellum.

The striatum is important for motor skills in many vertebrates [34] and has been linked to communication in several taxa [35]. We found that FoxP2-positive cells in the striatum have

increased activity during tadpole begging, suggesting an important function for this brain region in tadpole motor communication. This is supported by many studies relating to the role of FoxP2 in the striatum in vocalizing birds and mammals. Deficits in songbird vocal communication are observed after FoxP2 knockdown in Area X, a striatal nucleus involved in song learning [12]. At a cellular level, FoxP2 has been implicated in structural plasticity, with FoxP2 modifications influencing spiny dynamics of Area X neurons in zebra finches [36] and dendrite lengths in striatal neurons in mice [37]. In this same study, the variant of FoxP2 expressed in these mice also impacted dopamine concentrations in the striatum and nucleus accumbens. Dopamine signaling is critical to tadpole begging behavior [18], and our results here suggest a potential role for FoxP2 in dopamine-signaling that should be investigated in tadpoles in the future. This general cellular dysregulation can be seen in mice with FoxP2 mutations, where the striatum is generally more active and motor-skill learning is disrupted due to abnormal temporal coordinate of striatal firing [38]. Together, our work links FoxP2 in the striatum to motor communication in amphibians, suggesting a conserved role for striatal FoxP2 in communication across tetrapod vertebrates.

The preoptic area of the hypothalamus plays a well-conserved role regulating social behaviors across vertebrates [39]. This brain region is not commonly integrated into the canonical song circuity of songbirds [40], but it does play a role in courtship-related vocalization in songbirds and mice [41,42]. FoxP2 expression in the preoptic area is observed in various species with vocal communication including midshipman fish [32], bats [27], rodents [26]. We observed increased activity of FoxP2-positive neurons in the anterior preoptic area of begging tadpoles, although whether this pattern is directly relevant to begging requires functional manipulations in a brain region-specific manner. Despite FoxP2 expression in the preoptic area being widely conserved, its specific function in this brain region is not known in any animal, to our knowledge. As the preoptic area extends projections far throughout the brain to modulate behavior and physiology, FoxP2 may facilitate the preoptic area's role in sensorimotor integration, processing, and modulating output, although more research is needed to test this hypothesis.

The cerebellum is a highly conserved brain region across vertebrate evolution that is classically implicated in coordinating voluntary movements, motor learning, and modifying descending commands for precise, adaptive motor control [43]. The cerebellum is also implicated in language [44], as there is higher overall cerebellar activity during language tasks in humans [45]. Mice expressing the humanized FOXP2 with the R552H mutation (that leads to speech-language disorders) have impaired ultrasonic vocalizations and poor dendritic development of FoxP2-positive Purkinje cells in the cerebellum [46]. Expressing the wild type human FOXP2 partially rescues ultrasonic vocalizations, demonstrating that FoxP2 in Purkinje cells of the

cerebellum are important for quality of the vocalization, but not its production. Increased cerebellar FoxP2-positive neural activity during begging in *R. imitator* tadpoles is in line with the importance of these neurons in coordinating communication in neonates. To our knowledge, the role of FoxP2 in the cerebellum during vocal learning in songbirds is unknown, but cerebellar lesions impair song learning [47]. Our study, along with studies in neonatal mice, suggest that investigating the role of cerebellar FoxP2 during communication would resolve whether these patterns are generalizable across taxa.

Summary

We present evidence that FoxP2 has conserved brain expression patterns across vertebrates by filling in a critical taxonomic gap from amphibians and show that communication of nutritional needs by neonates to parents is correlated with activity of FoxP2 in striatal and motor-related brain regions. Overall, this work supports the idea that the FoxP2 transcription factor is part of a molecular toolkit essential for sensory-motor communication via striatal and cerebellar circuits across many animals.

Data Accessibility

All data is included in supplementary materials.

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Author contributions

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Formal Analysis: SCL and LAO

Investigation: SCL and JEM
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Data Curation: SCL
Writing – original draft preparation: SCL and LAO
Writing – review and editing: JEM and JMB
Visualization: SCL and LAO
Supervision: LAO and JMB
Project administration: LAO
Funding acquisition: LAO

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