Vocalization Defects in Conditional Foxp2-KO Mice

Sean Sullivan
sean.sullivan@yale.edu

April 21, 2017

The *FOXP2* gene is known for its important role in speech and language related disorders in humans. In order to better understand these processes, previous work has explored the role of the Foxp2 gene in bird song and other mammalian vocalization. Experimentation on behavioral patterns, physical properties, and vocalizations among Foxp2 genetic variants in mice has yielded robust findings. In particular, abnormalities in the structure and rhythm of adult song associated with courtship have been identified between heterozygous Foxp2 knockout mice and wildtype mice. However, the process yielding this phenomenon is still unclear. This study attempts to pursue these abnormalities to localized areas of the brain by analyzing the vocalization data from mice with conditional Foxp2-KO in the cerebellum and cortex against their wildtype counterparts.

Contents

1 Background

The *FOXP2* gene is known for its important role in speech and language related disorders in humans. First investigated through studies of the KE family, the mechanism the Foxp2 gene controls within speech and language process and development remains unclear. In humans, mutation of one copy of the gene commonly results in Developmental Verbal Dyspraxia (or Childhood Apraxia of Speech), characterized by imprecise control over motor skills in coordinated speech sequences (French 2014). Humans with homozygous mutations affecting Foxp2 have never been recorded (Chabout 2016). Since the Foxp2 transcription factor is highly similar across vertebrate species, the gene has become the target of considerable experimentation. Thus far, disruptions to the gene has been shown to significantly impact systems related to sensorimotor integration and motor learning across species (Groszer 2008). In particular, manipulations in songbirds have helped to elucidate the role of this gene in vocal learning, but findings in non-human mammals have been limited or inconclusive (Chabout 2016). This remains true for mice, which have been the subject of several recently studies. Here we delimit the results of previous experimentation and establish a mouse model for the current experiment.

Targets for Foxp2 experimentation arise in the suggestion of two amino acid substitutions, possibly selected for in human evolution for effects on aspects of speech and language (Enard 2009). The Foxp2-R552H line is a "humanized" model of the R553H substitution in affected members of the KE family, and the Foxp2-S321X line carries a "null" allele model of the human R328X nonsense mutation found in another family (Groszer 2008). Also important for consideration are the vast number of targets of the Foxp2 transcription factor, which activate downstream of Foxp2 expression. SRPX2, a Foxp2 target associated with epilepsy and language disorders in humans, has also been shown to impair development of USVs in mice (Sia 2013). Studies have targeted the behavioral patterns and characteristics of ultrasonic vocaliza-

tions (USVs) between these models and their wildtype counterparts in both pups and adult mice from nonlethal strains.

These models first considered the nature of pups in the Foxp2-R552H "humanized" line. Homozygous expression in mice has been shown to cause severe motor impairment, premature death, and an absence of isolated pup USVs, whereas heterozygous expression leads to some developmental delay (Fujita-Jimbo 2014). Further, homozygous R552H pups show reduced cerebellar growth and low postnatal weight gain, yet, notably, they can still produce complex innate ultrasonic vocalizations (Groszer 2008). Both suffer significant impairment in the frequency of pup USVs in response to isolation. Other than some subtle cerebellar deficits in development, studies have posed that the neural control factors producing vocalizations are normal in heterozygous mice (Shu 2005). More recent studies have explored this further, considering locations in the brain network where Foxp2 expression is affected. These have targeted the cerebellum, identifying affected Purkinje cells and patterns of deficits in modification, not production, of vocalizations (Fujita-Jimbo 2014).

The behavioral and physical characteristics of mice with Foxp2 disruptions further identify possible influences in the brain network related to vocalization. Of particular interest is the expression of Foxp2 in circuits of the cortex, striatum, and cerebellum, which play key roles in motor skill acquisition and sensory-motor learning (Kurt 2012). In one study, heterozygous Foxp2 have been found to decreased exploratory behavior and decreased dopamine concentrations in the brain suggesting that the humanized Foxp2 allele affects basal ganglia (Enard 2009). Another study used a conditioned avoidance paradigm for mice to identify the effects of Foxp2 mutations on learning of auditory-motor associations. In this study, mice were trained on Go/No-go auditory stimuli differing in frequency to measure how well mice were able to learn the association between different frequencies and desirable/undesirable results. Mice heterozygous for either of two different Foxp2 mutations were found to exhibit delays in acquiring new

motor skills, with lower severity in the R553H missense mutation than in the S321X nonsense mutation. Mice in the prior strain learn at a much slower rate than wildtype animals, while mice in the latter strain learn very little at all (Kurt 2012). In addition, R552H heterozygotes display abnormal synaptic plasticity in striatal and cerebellar neural circuits. These findings support the implication that Foxp2 plays a primary role in orofacial motor skills and development, leading to vocalization deficits.

In adult males, USV data has been analyzed for a number of characteristic properties between wildtype and Foxp2-variant genotypes. Reductions in the length and complexity of syllable sequences have been identified in Foxp2-R553H heterozygotes of the C57Bl/6J strain, the same as used for the present study. Although they produce syllables at normal rates, these mice exhibited subtle differences in syllable usage and had an altered sequence structure. Crucially, from measurement on breathing patterns, no respiratory differences have been identified, meaning that these are not derivative of respiratory effects (Chabout 2016). Similar studies conclude that heterozygous Foxp2-KO mice produce significantly fewer long syllables than wildtype mice; this abnormal rhythmic syllable inventory results in distorted song. The findings suggest a deficiency in motor planning and rhythmic motor behavior sequencing, which is supported by the non-vocal motor learning defects observed above (Castellucci 2016). These studies continue the debate on the degree to which these deficits result from impaired vocal learning or innate developmental processes. A further study of adult male song tested heterozygous Foxp2-R552H missense and -S321X nonsense mutations for arousal and emotional content in the different acoustic parameters of adult USVs using stimuli of increasing intensity of arousal. Compared to wild-type animals, heterozygous mutants emitted mainly longer and louder USVs at higher minimum frequencies with a higher occurrence rate of overtones/harmonics and complex frequency jump types. The results correlate USV calling rate with arousal level and sound pressure and call complexity with positive emotion (Gaub 2016).

However, what features of these deficits are coordinated by the different brain regions being targeted remains elusive. In order to separate the influence of Foxp2 disruptions in developmental and online functions in mice, conditional mice in which Foxp2 is inactivated in a spatially and temporally selective manner have been suggested for future testing (Groszer 2008). Previous studies have bred mice with *lox*P sites flanking exons 12-14 of the Foxp2 gene, in which Cre-mediated recombination is able to yield a conditional null allele (French 2007). These allow for more targeted studies of Foxp2 function local to specific tissues in which Foxp2 is expressed. The present study aims to address these questions by analyzing vocalization data from Foxp2$^{\delta 12\text{-}14}$ conditional-KO C57Bl/6J mice active in the cerebellum and the cortex against that of wildtype mice.

2 Methods and Materials

2.1 Animals

A total of 9 C57Bl/6 mice were used as subjects in this study, comprising 2 wild-type mice, 5 [cortex] mice, and 2 [cerebellar] mice. 5 mice were excluded from analysis. Mice were housed in a 12-hour reverse light-dark cycle room and provided food and water *ad libitum*. Mice were genotyped by polymerase chain reaction (PCR) and gel electrophoresis.

2.2 Vocalization Recording

Vocalizations were recorded in a sound-attenuated booth [tech info (Industrial Acoustics, New York, USA)]. An ultrasonic microphone [tech info (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany)] was placed above the recording chamber, in which subject mice were socialized with a random WT female mouse two or three times per week. During socialization sessions, the mice were allowed to interact freely within the range of the microphone. Socialization and recording ranged between around ages P23 and P90 in a rectangular (11.5×7 inches) recording

chamber padded with non-absorbent rubber to reduce noise and facilitate cleaning. Sessions lasted for around 3 minutes. Audio was sampled at 250 kHz, using Avisoft RECORDER software and Avisoft signal conditioner to digitize and record the signal.[1]

2.3 Analysis

Analysis was performed using custom MATLAB (Mathworks, Natick, USA) scripts. Detection parameters were calibrated prior to this investigation using a set of USV recordings evaluated to be typical of wildtype and heterozygous Foxp2 mice (Castellucci 2016). This analysis separated individual syllables and determined acoustic parameters (spectral flatness, dominant frequency, etc.) for each syllable both as a whole and over 1ms-resoluion windows of each syllable. Also calculated were syllable and inter-syllable durations for analysis of syllable inventories and bouts. Further analysis was performed in this experiment to calculate the progression of these acoustic parameters over the course of a single syllable by normalizing the number of time points evaluated for any syllable. Extra figures and code are available in the attached folder.

Rhythmic Properties

The first series of analyses performed considers syllable inventory abnormalities within each genotype of mouse subjects. First, the syllable rate of each animal was plotted over the age of the mouse, and plots were grouped according to genotype to examine syllable rate growth. Next, histograms of the combined syllable set of each mouse were made showing the amount of syllables the mouse created within different duration windows. From here, it was clear whether each genotype exhibits the bimodal distribution of short and long syllables that is expected of wildtype mice but that has been shown to be nonexistent in Foxp2+/- adults (Castellucci 2016). These were analyzed further by producing Ashman's D scores across animals and comparing the averages of these values across genotypes to determine bimodality.

[1] Methods derived from Castellucci 2016.

Acoustic Parameters

The next series of analyses considered the possibility that mouse vocalization differences occur in more specific acoustic parameters of vocalization. First, the average values per mouse of frequency modulation, amplitude modulation, number of pitch jumps, and dominant frequency in a single syllable were found and compared across genotypes and the age of the mouse during the sample. From there, each syllable was split into 20 "time bins" over which acoustic parameter data was linearly interpolated in order to evaluate these properties over the course of a single syllable on average. These were calculated separately for short syllables (dur ¡ 75ms) and long syllables (dur ¿ 75ms). The properties analyzed were peak frequency, amplitude modulation, and spectral flatness.

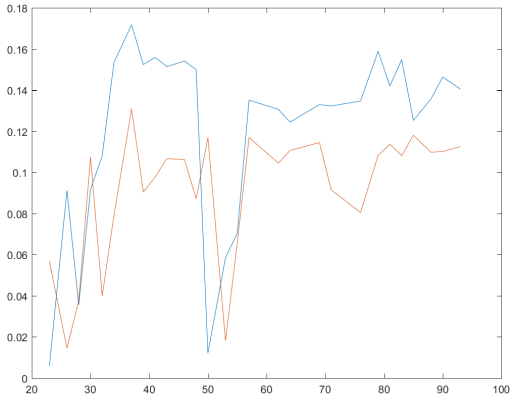
3 Results and Discussion

3.1 Rhythmic Properties

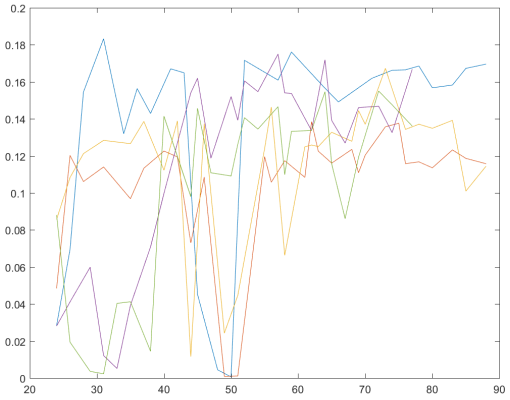
The first series of analyses showed overall that average syllable rates for wildtype mice primarily grows with age as expected. Mice without Foxp2 expression in the cortex or cerebellum seemed to exhibit learning as well, though many mice were much slower to learn than the WT case. The above figure shows an example wildtype bimodal distribution for one of the wildtype subjects. Analysis of Ashman D-scores show no significant difference between each genotype. This supports the hypothesis that the Foxp2-KO in cerebellar and cortex regions do not impact the existence of long syllables.

3.2 Acoustic Parameters

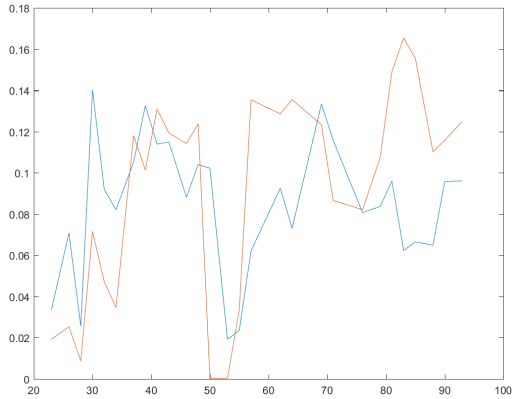
The first pass of analyses revealed no consistent differences between WT-, cortex-, and cerebellum-type mice. Dominant frequencies were always within the same range, and amplitude modulation, frequency modulation, and number of pitch jumps increases steadily throughout aging but



(a)



(b)



(c)

Figure 1: Syllable rate figures for (a) WT, (b) Cortex, and (c) Cerebellum mice for age in days (x axis). Syllable rate (y axis) is calculated in syllables per minute.

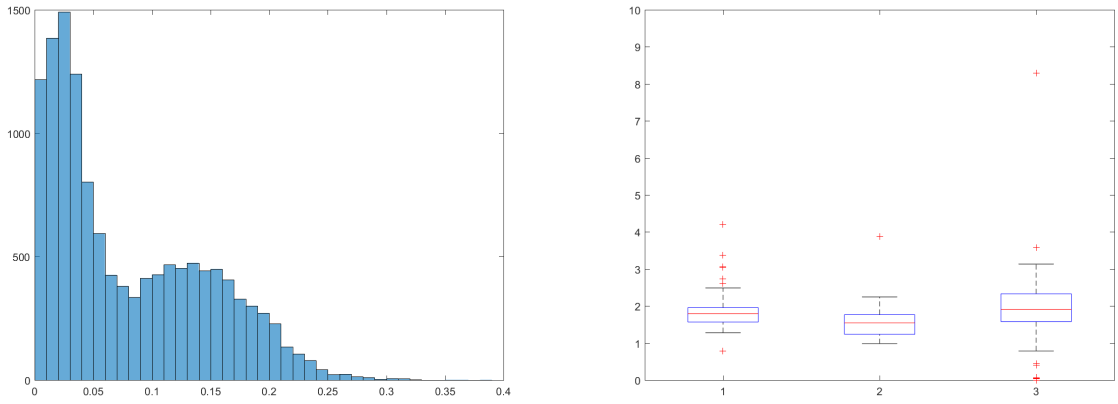


Figure 2: Sample syllable duration histogram for a wild type mouse and corresponding d-score box plot. (1) represents WT mice, (2) represents cortex mice, (3) represents cerebellum mice

at indistinguishable paces. These can be observed in the "plots" folder.

The second pass of analyses determined no consistent differences, although patterns did exist. Spectral flatness increases towards the end of a syllable, peak frequency usually remains flat on average for an animal.

4 Issues and Future Work

This experiment succumbs to a few unfortunate issues. In particular, low sample size of mice for each genotype variant could result in animal-specific differences. For example, it is possible that the Ashman D-scores calculated for each mouse were impacted by low sample sizes. Further, it's possible that further analysis of these samples could reveal more information about the acoustic structure of these animals on a per-syllable basis, since only a limited set of acoustic properties were analyzed here.

As such, it would be worth investing the claims made by this analysis on a larger data set, with more acoustic parameters under analysis. Further, the striatum is among the brain regions which previous studies have localized as a Foxp2 expression site; performing a similar analysis

to these on more brain-localized regions of Foxp2-KO mice could yield greater knowledge about the effects of Foxp2 in the process of motor learning and vocalization.

Acknowledgements

This experiment was made possible by the efforts of McCormick Lab, particularly Gregg Castellucci, who deserve my utmost thanks. Special thanks also go to the Cognitive Science program at Yale Univeristy, specifically Mark Sheskin, for guiding this research process.

5 References

References

1. Castellucci, G., McGinley, M. & McCormick, D. Knockout of Foxp2 disrupts vocal development in mice. *Sci Reports* 6, 23305 (2016).

2. Chabout, J. et al. A Foxp2 Mutation Implicated in Human Speech Deficits Alters Sequencing of Ultrasonic Vocalizations in Adult Male Mice. *Front Behav Neurosci* 10, 197 (2016).

3. Enard, W. et al. A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. *Cell* 137, 96171 (2009).

4. French C., et al. Generation of mice with a conditional Foxp2 null allele. *Genesis* (2007)

5. French, C. & Fisher, S. What can mice tell us about Foxp2 function? *Current Opinion in Neurobiology* 7279 (2014).

6. Fujita-Jimbo, E. & Momoi, T. Specific expression of FOXP2 in cerebellum improves ultrasonic vocalization in heterozygous but not in homozygous Foxp2 (R552H) knock-in pups. *Neuroscience letters* 1626 (2014).

7. Fujita et al. Ultrasonic vocalization impairment of Foxp2 (R552H) knockin mice related to speech-language disorder and abnormality of Purkinje cells. *Proceedings of the National Academy of Sciences* 105, 31173122 (2008).

8. Gaub, S., Fisher, S. E. & Ehret, G. Ultrasonic vocalizations of adult male Foxp2-mutant mice: behavioral contexts of arousal and emotion. *Genes, Brain and Behavior* 15, 24359 (2016).

9. Gaub, S., Groszer, M., Fisher, S. E. & Ehret, G. The structure of innate vocalizations in Foxp2-deficient mouse pups. *Genes, Brain and Behavior* 9, 390401 (2010).

10. Groszer, M. et al. Impaired Synaptic Plasticity and Motor Learning in Mice with a Point Mutation Implicated in Human Speech Deficits. *Current Biology* 18,354362 (2008).

11. Hammerschmidt et al. A humanized version of Foxp2 does not affect ultrasonic vocalization in adult mice. *Genes, Brain and Behavior* 14, 583590 (2015).

12. Kurt, S., Fisher, S. E. & Ehret, G. Foxp2 mutations impair auditory-motor association learning. *PLoS ONE* 7, e33130 (2012).

13. Reimers-Kipping, Hevers, Pbo & Enard. Humanized Foxp2 specifically affects cortico-basal ganglia circuits. *Neuroscience* 7584 (2010).

14. Sia, Clem & Huganir. The human language-associated gene SRPX2 regulates synapse formation and vocalization in mice. *Science* (New York, N.Y.) 98791 (2013).

15. Shu, W. et al. Altered ultrasonic vocalization in mice with a disruption in the Foxp2 gene. *Proc. Natl. Acad. Sci*. U.S.A. 102, 96438 (2005).

16. Usui, N. et al. Sumoylation of FOXP2 Regulates Motor Function and Vocal Communication Through Purkinje Cell Development. *Biol. Psychiatry* (2016).