Striatal FoxP2 Is Actively Regulated during Songbird Sensorimotor Learning

Ikuko Teramitsu1,2a, Amy Poopatanapong3,4b, Salvatore Torrisi2, Stephanie A. White1,2,3*

1 Interdepartmental Program in Molecular, Cellular and Integrative Physiology, University of California Los Angeles, Los Angeles, California, United States of America, 2 Interdepartmental Program in Neuroscience, University of California Los Angeles, Los Angeles, California, United States of America, 3 Department of Physiological Science, University of California Los Angeles, Los Angeles, California, United States of America

Abstract

Background: Mutations in the FOXP2 transcription factor lead to language disorders with developmental onset. Accompanying structural abnormalities in cortico-striatal circuitry indicate that at least a portion of the behavioral phenotype is due to organizational deficits. We previously found parallel FoxP2 expression patterns in human and songbird cortico/pallio-striatal circuits important for learned vocalizations, suggesting that FoxP2's function in birdsong may generalize to speech.

Methodology/Principal Findings: We used zebra finches to address the question of whether FoxP2 is additionally important in the post-organizational function of these circuits. In both humans and songbirds, vocal learning depends on auditory guidance to achieve and maintain optimal vocal output. We tested whether deafening prior to or during the sensorimotor phase of song learning disrupted FoxP2 expression in song circuitry. As expected, the songs of deafened juveniles were abnormal, however basal FoxP2 levels were unaffected. In contrast, when hearing or deaf juveniles sang for two hours in the morning, FoxP2 was acutely down-regulated in the striatal song nucleus, area X. The extent of down-regulation was similar between hearing and deaf birds. Interestingly, levels of FoxP2 and singing were correlated only in hearing birds.

Conclusions/Significance: Hearing appears to link FoxP2 levels to the amount of vocal practice. As juvenile birds spent more time practicing than did adults, their FoxP2 levels are likely to be low more often. Behaviorally-driven reductions in the mRNA encoding this transcription factor could ultimately affect downstream molecules that function in vocal exploration, especially during sensorimotor learning.

Introduction

Forkhead box (FOX) genes encode a family of transcription factors that play regulatory roles during development [1,2]. FOXP2, a member of this family, is the first gene to be directly linked to human language [3–5]. Humans with FOXP2 mutations exhibit deficits in the coordination of sequential orofacial movements, resulting in impaired speech (developmental verbal dyspraxia) [6]. This core deficit is accompanied by additional impairments in receptive linguistic skills and abnormal activation of cortico-basal ganglion regions used in verbal communication [7]. Together, these observations implicate FOXP2 in the organization of neural structures necessary for speech and language.

Birdsong shares key features with speech: it is learned during development, actively maintained in adulthood, requires hearing and relies on pallio-striatal circuits [8,9]. The neuroanatomical structures that subserve song learning and production, known as song nuclei, are well-characterized [10–13]. Songbirds thus provide an important model for the study of neural mechanisms underlying vocal learning. FoxP2 is expressed in the striatum of human embryos and of 1 day post hatch (1d) zebra finches [14]. FoxP2 levels appear to increase in the song nucleus, area X, of developing zebra finches at 35 and 50d [15], followed by an increase in area X volume and the number of new neurons expressing FoxP2 protein at 50 and 75d [16]. Area X is the region of the songbird basal ganglia dedicated to song [17], and contains neuronal phenotypes, including medium spiny neurons, similar to those in mammalian basal ganglia [18,19]. These observations, coupled with the anatomical abnormalities of humans bearing FOXP2 mutations [20], support a role for FoxP2 in the development of neural structures that subserve vocal learning.

In addition to this organizational role, FoxP2 may have post-organizational function(s) in learned vocalizations as its mRNA
and protein are rapidly down-regulated specifically in area X when adult zebra finches practice their songs outside the context of courtship (i.e. sing undirected songs) [21,22]. This idea is supported by the known role of the anterior forebrain pathway, which includes area X, in enabling song modification during development [23,24] and throughout life [25]. Here, we used zebra finches (Taeniopygia guttata) to investigate the role of FoxP2 in song learning. In this species, young males memorize the song of adult male tutors, and then practice their songs during a phase known as ‘sensorimotor learning’ which spans ~30–100 d [26,27].

The learned songs are actively maintained in relatively stable form throughout adulthood [28,29] when the sizes of song nuclei are also relatively stable [30]. Thus, the FoxP2 down-regulation observed in area X of adult zebra finches cannot be due to developing new songs or to significant restructuring of song circuitry. Instead, acute down-regulation may reflect an on-line function for FoxP2 during singing. This function could be to help stabilize mature song. If so, then down-regulation might be lessened or absent in juveniles. Alternatively, acute FoxP2 down-regulation in adults might enable subtle adjustments involved in song maintenance. In this case, reduction of FoxP2 might be similar or greater in juveniles, as they make greater modifications to their songs during learning. In either case, the on-line regulation could be associated with motor control and/or auditory feedback of song.

To probe these possibilities, we first examined basal levels of FoxP2 in area X of non-singing hearing or deafened birds during sensorimotor learning. Our findings suggest that basal FoxP2 levels are associated with structural growth of area X and are not affected by deafening. We then tested for acute down-regulation of FoxP2 in area X of 75 d birds as a function of singing for two hours in the morning. We found that when juveniles sang, FoxP2 levels in area X declined, similar to what we previously reported for adults. Therefore, this regulation is more likely related to song adjustment than to song stability. Interestingly, singing decreased FoxP2 in both hearing and deafened birds, however, levels were only correlated with the amount of singing in hearing birds. Here, we report this evidence for both motor and auditory regulation of FoxP2.

**Materials and Methods**

**Animals and Tissues**

All animal husbandry and experimental procedures were in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California, Los Angeles Institutional Animal Care and Use Committee. Birds were fed seed and calcium-enriched (Calciboost, The Birdcare Company, Los Angeles, CA) to investigate the role of FoxP2 in sensorimotor learning. Our findings suggest that basal FoxP2 levels are associated with structural growth of area X and are not affected by deafening. We then tested for acute down-regulation of FoxP2 in area X of 75 d birds as a function of singing for two hours in the morning. We found that when juveniles sang, FoxP2 levels in area X declined, similar to what we previously reported for adults. Therefore, this regulation is more likely related to song adjustment than to song stability. Interestingly, singing decreased FoxP2 in both hearing and deafened birds, however, levels were only correlated with the amount of singing in hearing birds. Here, we report this evidence for both motor and auditory regulation of FoxP2.

**Sham Surgeries**

Three additional birds underwent sham operations to control for any potential effects of the surgical procedure itself. Sham operations consisted of the same anesthetic protocol and skin incision, but without damage to the tympanic membrane or cochlear extraction. Data from 3 birds that were sham-operated at 25d were compared to that from 3 birds that did not receive sham treatment (untreated). These sham operated or untreated birds were examined at 50d, and their non-singing ‘basal’ FoxP2 levels in area X were compared using in situ hybridization analyses with two distinct probes for FoxP2 as described in Teramitsu & White (2006) and below. Using photomicrographs, the pixel intensity of the hybridization signal in area X relative to that in the outlying striatum of the same hemi-coronal section was calculated as a ratio. Multiple sections per bird were analyzed and a per bird average was computed and used for statistical comparison (see below for more details). As expected, no differences in area X FoxP2 levels were observed between sham operated and untreated birds, indicating that the surgery itself had no effect (mean±SEM levels in area X relative to levels in the outlying striatum, Sham vs. Untreated – 3’ probe: 1.05±0.06 vs. 1.09±0.03, p = 0.38; mid-probe: 1.05±0.04 vs. 1.11±0.04, p = 0.19). Therefore, the results from sham operated and untreated birds are pooled below, and these birds are henceforth referred to as 50NS-H group.

**Basal FoxP2 Levels in Non-Singing Birds**

For analysis of FoxP2 levels in hearing or deafened juvenile birds that did not sing on the day of the experiment, a total of 23
juvenile male zebra finches were used. Birds were monitored in the morning during the 20 minutes from light onset to decapitation to ensure that no singing occurred. To examine the effect of development on FoxP2 expression, 50, 65 and 75d birds were used. As mentioned above, an additional three hearing 75d birds were used in order to facilitate comparison between our measurements and those obtained by another study (Haesler et al., 2004; see Discussion) [15]. This latter group was killed in the morning immediately at light onset rather than within 20 minutes after. Further, brains were sectioned in the sagittal, rather than the coronal, plane. Finally, the region of the outlying striatum that was measured was matched to the region used by Haesler et al. (2004).

To examine the effect of auditory deprivation on FoxP2 expression, a cohort of the birds were deafened at either 25 or 35d (Fig. 1A). Birds that were deafened at 25d were examined at 50d and compared to age-matched hearing birds. Birds that were deafened at 35d were examined at 65 or 75d, and compared with age-matched hearing birds (Fig. 1A).

**FoxP2 Levels in Singing Birds**

To test the effect of singing on FoxP2 expression during sensorimotor learning in the presence or absence of auditory...
feedback, juvenile males were deafened at 35d. Experiments were conducted on these deafened birds and on age-matched hearing birds at 75d, in the morning between 8 AM and noon to minimize circadian variability. Following light onset, the singly housed deafened or hearing males were allowed to sing for 2 hr and then killed for measurement of FoxP2 levels. Digital sound recordings were made using the Song Acquisition Program described in Livingston et al. (2000) [33]. The acoustic structure of birdsong is typically described as being composed of bouts, phrases, motifs, syllables, and notes [34]. Notes are the smallest unit, combining together to form syllables. Syllables are separated from one another by silent intervals. Two or more syllables may group together to form a phrase. A motif is a sequence of notes and/or syllables that are repeated in a stereotyped order. One or more motifs or phrases followed by a second or more of silence comprises a bout of song [35]. In this study, the number of motifs sung by each bird in the hearing group was counted. Because the songs of deafened birds lacked identifiable motif structures, the amount of time spent singing was also measured for both deafened and hearing groups. Silent periods longer than a second were regarded as bout intervals and were not included in the song measurement. For hearing birds, Sound Analysis Pro 1.04 software [36] was used to determine the degree of acoustic variability between syllables. Ten motifs per bird were analyzed for the within-syllable variability via 45 pair-wise comparisons of the acoustic features using the local similarity measure [37]. Resultant scores per syllable were then averaged for each bird.

In Situ Hybridization Analyses

To measure FoxP2 levels in area X of juvenile males, in situ hybridizations were performed following the methods of Teramitsu et al. (2004) [14]. As reported in that study, there are two major splice variants for the coding sequence of zebra finch FoxP2. In addition to the full-length form, a truncated variant lacks the forkhead DNA-binding domain but codes for an additional ten amino acids not present in the full-length form (GenBank DQ285023), similar to the so-called +10 form found in humans [38]. Hence, different hybridizing sequences were chosen to create two probes, one to the middle region of the coding sequence, referred to as the ‘mid-probe’, and the other to the 3’ end of the coding sequence, referred to as the ‘3’-probe’. The former can detect both of these FoxP2 variants whereas the latter will only hybridize to the full-length variant. Of note, because the probes had slightly different specific activities and lengths [14], brain sections that were hybridized with the 3’-probe were exposed to separate films from those hybridized to the mid-probe. This avoided saturation of signals by the stronger probe. Consequently, comparisons of signal intensity between probes are not warranted.

FoxP2 expression in area X of multiple coronal or sagittal brain sections was quantified from digitized images of film autoradiograms using Adobe Photoshop 7.0 (Adobe Systems Inc. San Jose, CA) as previously detailed [14]. Briefly, the background was subtracted from each image, and then the ‘histogram’ tool was used to measure the optical density (OD) values in area X or surrounding medial striatum (striatum mediale; StM). Respective areas for measurements were selected in an unbiased manner by using adjacent Nissl-stained sections precisely overlaid on the film images. For coronal sections, OD measurements were obtained from ~13 hemi-sections per bird (i.e. both right and left hemispheres, if suitable for quantification, were analyzed) spanning the rostro-caudal extent of area X. A similar number of sagittal sections were used spanning the medio-lateral extent of area X. These measurements were averaged to provide a single value per region per bird. OD values from area X were normalized to those from adjacent StM. Thus, ratios of 1.0 indicate that expression levels in the two regions are comparable. Following this analysis, Feenders and colleagues found that gene expression levels in outlying striatum can vary as a function of behavior (e.g. hopping and flying [39]). Thus, in cases where we observed singing-driven regulation of FoxP2 in area X using outlying striatum as a control tissue for normalization, we additionally measured FoxP2 levels in nidopallial regions of the same section (outside of LMAN) and used these for normalization.

Duration of Singing As a Function of Age and Breeding State

To determine how much time juvenile birds spent singing relative to adults, 75d or >120d males from our aviary and >120d pair-bonded males from breeding cages were placed individually in sound attenuation chambers for 5 consecutive days and their songs were recorded during this time. The songs of all subjects were sufficiently mature such that it was possible to identify each bird’s motif, i.e. the kernel of acoustic structure defined by repeated sequences of syllables. For all ages, the number of motifs that each bird sang throughout the entire day on days 2–5 was manually counted using Audacity (v1.3). The circadian pattern of singing was noted by binning into 5 2.5-hour segments, beginning at lights-on (07:30 AM) and ending at lights-off (20:00 PM).

Statistics

Non-parametric methods were used because the data did not conform to parametric assumptions. The effect of auditory deprivation on FoxP2 expression was analyzed using the Kruskal-Wallis test for more than two groups, and the test statistic (H) with degrees of freedom (df) are reported in the relevant figure legends, or in the text in those cases where there is no figure. Mann-Whitney tests were used for comparison of two groups. Two-tailed significance was set at p<0.05, as no a priori hypothesis about the direction of any change in FoxP2 levels between deaf and hearing animals was made. Means±SEMs are reported. Spearman rank tests were used to assess the relationship between amount of singing and FoxP2 expression levels examined in area X.

Results

Deafening at the Onset of Sensorimotor Learning Disrupts Song Development, but Does Not Affect Basal FoxP2 Levels

To confirm that song development was disrupted by deafening [32,40], 75d hearing and deafened groups were recorded. The songs of the hearing males were well-structured with each motif composed of 4–7 readily identified syllables. In contrast, the songs of deafened males were highly disrupted, consisting of a series of amorphous syllables (Fig. 1B). No motif structures were reliably identified in any of the deafened birds. Although chronic auditory deprivation during sensorimotor learning produced abnormal songs, it did not alter basal FoxP2 expression levels. In non-singing birds, FoxP2 levels in area X were similar between hearing and deafened groups at 30, 65 and 75d (p>0.05 at each age, with either probe; Fig. 2).

Interestingly, levels of the full length FoxP2 mRNA slightly increased over development (hearing and deafened birds pooled – 3’-probe: 50d, 1.08±0.01 vs. 65d, 1.11±0.01 vs. 75d, 1.16±0.02, H = 9.8, df = 2, p<0.01). In slight contrast, FoxP2 levels detected by the mid-probe, designed to recognize both long and truncated forms of the molecule, exhibited a transient decrease at 65d
followed by an increase at 75d (H = 6.8, df = 2, p < 0.05). Given that the full length form shows a gradual, consistent rise across these ages, we interpret these data from the mid-probe as a dip in expression of the truncated form at 65d. The developmental changes in FoxP2 expression levels shown here contrast slightly with a trend reported by Haesler et al. (2004) [15]. In that study, ratios of FoxP2 levels within area X increased relative to outlying striatum from 15 to 50d but then appeared to return to 15d ratios at 75d. We were unable to replicate the return of basal area X FoxP2 levels to at, or below, those of outlying striatum at 75d despite testing an additional three birds using methods more similar to that study (see Methods).

### FoxP2 Is Acutely Down-Regulated in Area X When 75d Juveniles Sing Undirected Songs

To investigate whether FoxP2 in juvenile birds exhibits behavioral regulation similar to adults [21], we allowed 75d hearing birds to sing for 2 hours and examined FoxP2 expression levels in area X. We found that FoxP2 in juveniles is also acutely down-regulated by singing (3’-probe, p < 0.02; Fig. 3). Similar results were obtained with the mid-probe (data not shown). Because motor-driven gene expression can occur outside of area X for non-singing behaviors [39], we additionally measured FoxP2 expression levels in a nidopallial region (outside of LMAN) on the same section and used these values for normalization. Akin to the prior analysis, FoxP2 levels in area X were down-regulated by singing when nidopallial areas were used for normalization (3’-probe, p < 0.02).

The extent of FoxP2 down-regulation was correlated with both the amount of time spent singing (Spearman Rho p < 0.02; Fig. 4 left) and with the number of motifs sung (p < 0.02). As expected, the songs of these juvenile birds were less stable than those of the adult males we previously studied (juveniles vs. adults: mean accuracy of syllables (75d range = 76–82%, average = 79% ± 0.9 vs. adult range = 82–90%, average = 86% ± 0.9). However, the extent of FoxP2 down-regulation in juveniles was qualitatively similar to that seen for adults [21]. (Statistical comparison is not justified since the two studies were conducted separately).

### Acute FoxP2 Down-Regulation Occurs Despite Auditory Deprivation

To determine whether or not FoxP2 down-regulation by singing in juveniles depends on auditory feedback, 75d deafened birds were allowed to sing for 2 hours and examined FoxP2 expression levels in area X. Similar to hearing birds, singing also decreased FoxP2 in deafened birds relative to basal levels (3’-probe, p < 0.005; Fig. 3). Similar results were obtained with the mid-probe or when utilizing a nidopallial region instead of outlying striatum for normalization (data not shown). No difference in the extent of down-regulation was observed between the two singing groups (3’-probe: p = 0.38; mid-probe: p = 0.52), revealing that the regulation is driven by the act of singing itself. Interestingly, unlike the hearing group in which FoxP2 levels were correlated with the amount of singing (see above), no correlation was found for the deafened group (Spearman Rho p = 0.60; Fig. 4 right).

Deafened birds sang more than their hearing counterparts (75S-H vs. 75S-D in secs, range: 219–1153 vs. 271–2240, mean ± SEM: 565 ± 129 vs. 1173 ± 209; U = 4.6, p < 0.03). Thus, one concern was that the lack of correlation in the deafened group might be due to maximal down-regulation of FoxP2 (i.e. a ‘floor’ effect) in birds who sang a lot. To gauge the likelihood of this interpretation, we considered whether removing data for the three deafened birds who sang the most (2,240, 2,013 and 1,566 secs) would reveal a correlation. The amount of singing from the remaining subset of deafened birds (range: 271–1477, mean ± SEM: 845 ± 168) was even more similar to that of hearing birds. However, this manipulation failed to reveal any correlation in the deafened birds (Spearman Rho = −0.29; p = 0.54, n = 7). It is important to note that a subject number (n) of 7 was sufficient to observe the correlation in the hearing group. Indeed, during these experi-
ments, we initially collected an n of 6 in both groups and observed a significant correlation in the hearing group, but none in the deafened, as described in preliminary report (hearing vs. deaf: p = 0.045 vs. p = 0.55; Teramitsu & White, Society for Neuroscience Abstracts, 2006). To increase our confidence in these findings, we proceeded to test one additional hearing and three additional deafened birds, and obtained similar results albeit with a more significant p value in the hearing group. Since an n of 6 in the hearing group was sufficient to reveal the correlation, the lack of correlation in the subset of deafened birds (n = 6) or the full cohort (n = 10) cannot be merely attributed to a lack of power.

Juveniles Spend More Time Singing Than Do Adult Birds

Given that song practice lowers FoxP2 levels in both juveniles (here, and adults [21]), we wondered whether the duration of practice differed at different ages. If so, then birds engaged in more singing at one age would presumably experience low FoxP2 levels more frequently than at the other age. To address this, we examined the daily singing patterns of males in three behavioral conditions: 75d juveniles undergoing sensorimotor learning and taken from the group aviary cage; adults (>120 days) taken from the group aviary cage, and pair-bonded adults (>120 days) taken from dedicated breeding cages. Birds were placed individually in sound attenuation chambers for 5 days while their songs were continuously recorded. Compared to both groups of older birds, 75d males tended to start singing sooner and sang more throughout the course of the experiment (Fig. 5 left panel; p < 0.005). The greatest amount of singing occurred following light onset each day (Fig. 5, right panel).

Discussion

Our results demonstrate that basal levels of FoxP2 in area X of juveniles are slightly higher than those in the surrounding striatum, and only decrease acutely when birds sing. In this study, basal levels of full-length FoxP2 remained relatively stable, exhibiting only a modest rise between 50 and 75d, consistent with the constant density of FoxP2 immunoreactive cells observed between 25-100d [16]. Our results (Fig. 2) contrast in one way with a study in which area X FoxP2 levels were reported to rise only up to 50d, but were not statistically tested [15]. We were unable to replicate this change in pattern beyond 50d despite testing additional 75d birds using methods designed to mimic the other study and employing the same subject number (see Methods). Of note, the area X FoxP2 levels observed here in 75d non-singing birds were similar to those that we previously reported in non-singing adults (i.e. slightly higher than the surrounding striatum [21]), making it unlikely that the discrepancy is due to slight differences in the progress of song development between colonies. Differences in the

Figures

**Figure 3. Singing down-regulates FoxP2 in both hearing and deaf juveniles.** A) Representative sections show FoxP2 signals detected with the 3′-probe in hearing and deaf 75d birds. Signals within area X appear slightly stronger than in the surrounding striatum in the non-singer (NS), whereas they appear lower in area X of the singer (S). B) Quantification of the pixel intensity within area X is normalized to that of the outlying striatum. In both hearing (n = 7) and deaf (n = 10) birds, area X FoxP2 levels are higher in the non-singing group (gray boxes) relative to the singing group (white boxes). Mean ± SEM for hearing birds: 75NS-H vs. 75S-H: 1.18 ± 0.02 vs. 0.99 ± 0.04, Mann-Whitney U = 5.7, p < 0.02. Mean ± SEM for deaf birds: 75NS-D vs. 75S-D: 1.15 ± 0.03 vs. 0.94 ± 0.02, Mann-Whitney U = 8, p = 0.005. 'Box and whiskers' plots show the median (line), average (triangle), 25th and 75th percentiles (box) and 5th and 95th percent confidence intervals (whiskers) for each group. Individual values are plotted to the left.

doi:10.1371/journal.pone.0008548.g003

**Figure 4. Hearing links amount of singing with FoxP2 levels.** The amount of time that 75d birds spent singing (x axis) and area X FoxP2 levels measured using the mid-probe (y axis) are correlated in hearing (left; Spearman Rho = −0.86, R² = 0.69; p < 0.02), but not in deaf (right; Spearman Rho = −0.19, R² = 0.04; p = 0.60), juveniles.

doi:10.1371/journal.pone.0008548.g004
Figure 5. 75d birds practice more than adults. Data from 75d males (n = 5) is shown in shades of purple, adult aviary males (n = 4) in gold and adult pair-bonded males (n = 5) in maroon. Left) The amount of song sung while in sound attenuation chambers is shown. Individual data are plotted where squares represent days 2–5 (day one was not counted to allow for acclimation) and color intensity shows percent of total motifs sung each day. Compared with older birds, 75d males tended to sing on the first recording day and sang more overall (Mean ± SEM in secs: 75d = 12,958 ± 1,731, adult aviary males = 4,494 ± 1,042, adult pair-bonded males = 2,034 ± 894; Kruskal-Wallis H = 10.7, DF = 2, p < 0.005). Right) The daily pattern of singing is shown. For each group, the average number of motifs (z axis) is plotted in 2.5 hour time-blocks (x axis) across the 4 days. Each day is represented by one ribbon on the y axis and the 4 days are clustered by group.

doi:10.1371/journal.pone.0008548.g005

probes used to detect FoxP2 in the two studies may contribute to the different findings. The persistent expression of FoxP2 observed here during late sensorimotor learning, the high levels of expression during human [14] and songbird embryogenesis [15], the FoxP2 immunoreactivity observed within newly generated neurons in area X [16], and the structural brain deficits in humans bearing FOXP2 mutations [41] are all consistent with a role for this molecule in the formation of certain brain regions, including the striatum.

Deafening of young birds either shortly before or at the onset of sensorimotor learning did not affect the basal expression pattern of FoxP2 in area X at any of the three ages tested (Fig. 2) despite the expected disruption of song development [32,40]. This suggests that basal (i.e. non-singing) FoxP2 levels in area X are not regulated by auditory input during song development. In contrast to this relatively stable expression, when juvenile birds sang, FoxP2 was acutely down-regulated in area X relative to the surrounding striatum (Fig. 3), similar to what we previously reported for adult birds [21]. Down-regulation occurred in both hearing and deaf birds, indicative of ‘motor-driven’ [42] gene regulation. However, the extent of down-regulation depended on the amount of singing only among hearing birds (Fig. 4), suggesting multiple layers of FoxP2 regulation. To our knowledge, this is the first indication for an effect of audition on FoxP2 such that hearing links levels of the molecule to levels of vocal motor practice. Interestingly, transgenic mice engineered to harbor the KE family mutation in Foxp2 exhibit altered auditory brainstem responses [43]. As noted by the authors of that study, these findings suggest that humans with FOXP2 mutations should be tested for auditory function. Of note, the singing-to-FoxP2 correlation observed here in hearing juveniles was previously observed as a trend in adults for both mRNA [21] and protein [22], but has now emerged as a significant relationship in younger birds.

The precise temporal regulation of FoxP2 that occurs only during singing, and the regional restriction of this regulation to song control nucleus area X strongly suggests that FoxP2 has a post-organizational role in learned vocalizations. Previously, we considered whether the singing driven down-regulation of FoxP2 observed in adults [21] was related to the stereotyped nature of these songs or, alternatively, to their ongoing subtle variability. The latter possibility now seems more likely because down-regulation also occurred here when juveniles sang their more variable songs. Although the magnitude of the down-regulation appeared similar in both adults and juveniles, we found that 75d juvenile birds in our colony engaged in song practice more readily and frequently than did adults (Fig. 5). It follows that FoxP2 levels are also more frequently low during late sensorimotor learning, when song is still changing, than in adulthood, when song is more stable. We note, however, that FoxP2 levels were only measured at a single time point, two hours after song onset in the morning and were compared to levels after two hours of non-singing in control birds. Another difference between adults and juveniles is the stronger link between the amount of song sung and how readily FoxP2 levels decrease at younger ages. This is evidenced by the increased strength of the correlation between these measures in 75d birds (Fig. 4) relative to adults [21,22]. Overall, birds may tacitly ‘self-regulate’ their own FoxP2 levels, depending on how often they engage in vocal motor practice, a relationship that may extend to other learned motor skills and other transcription factors.

FoxP2 could function as a ‘plasticity gate’, either up or down, during both sensorimotor learning and adulthood. In this model, high FoxP2 levels correspond to periods of structural growth and song stability whereas low levels open the gate for vocal variability; the more often that FoxP2 is low, the greater opportunity for variability. Here, we refer to variability that occurs two hours after the onset of undirected singing – a more protracted timescale than the minute-to-minute changes driven by social context, e.g. [44,45]. According to the plasticity gate hypothesis, at some point following song onset, beyond two hours, FoxP2 levels should begin to rise again in order to stabilize motor patterns, a scenario that we are currently testing. This general idea is supported by the observation that after a day of song practice, juveniles exhibit...
more variable songs the next morning [46]. Song practice during the day likely decreased area X FoxP2, at least transiently, in these juveniles, although the full circadian rhythm of FoxP2 levels relative to singing is not yet determined. Moreover, it may be that nighttime ‘song rehearsal’ [46] also decreases FoxP2 levels. Such variability in the representation of vocal motor exploration might be critical for improved imitation, as juveniles exhibiting the greatest morning variability end up producing the best copies of their tutors’ songs. Morning increases in vocal variability decline with maturation, disappearing in adulthood [46]. Concurrently, song improves over the several week period of sensorimotor learning [26] while in adults there is a much more gradual increase in song stability across years [29]. FoxP2 regulation could contribute to these slower changes across the lifetime of the animal, a finding supported by the constant replacement of FoxP2 immunoreactive neurons in zebra finch area X [16]. A direct prediction of this model is that songs of juveniles who sing for two hours, and thus have low FoxP2 levels, should be more variable than the songs of those same individuals when they have not sung and thus have higher FoxP2 levels, a result we have recently confirmed (see Miller et al., companion paper).

The naturally-driven down-regulation by singing observed here complements results from experimentally-induced constitutive down-regulation of FoxP2 during sensorimotor learning [47]. In the latter study, chronic down-regulation of FoxP2 using RNA interference resulted in more variable songs of 90d experimental birds relative to age-matched controls. Without the normal behaviorally-driven fluctuation in FoxP2, the songs of experimental birds were less accurate copies of the tutor. One caveat to this interpretation is that only ~20% of area X was affected, so presumably FoxP2 levels were normally regulated in the remaining portion. FoxP2 is a transcription factor, thus the mechanism by which it exerts its function(s) is through control of downstream genes. Analysis of FOXP2 gene targets in human neural tissues reveal that a subset of these play roles in activity-based sculpting of neural connections, including during learning [48,49]. Together, these findings suggest that while high levels of FoxP2 are important for normal development of neural structures, low levels may enable the fine-tuning of these structures during vocal motor exploration [37].

Acknowledgments

We thank Justine Chen, Michael Condro and Donald Noble for assistance with song and image analyses, Dr. Masakazu Konishi for teaching us the deafening technique and Dr. Richard Mooney for song acquisition software.

Author Contributions

Conceived and designed the experiments: IT SAW. Performed the experiments: IT AP ST. Analyzed the data: IT AP ST SAW. Wrote the paper: IT SAW.

References

1. Carlsson P, Mahlapuu M (2002) Forkhead transcription factors: Key players in development and metabolism. Dev Biol 250: 1–23.
2. Lehmann OJ, Swedan JC, Carlsson P, Jordan T, Bhattacharya SS (2003) Fox’s in development and disease. Trends Genet 19: 339–344.
3. Hurst JA, Baratier M, Auger E, Graham F, Novell S (1998) An extended family with a dominantly inherited speech disorder. Dev Med Child Neurol 40: 352–355.
4. Lai CSL, Fisher SE, Hurst JA, Varga-Khadem F, Monaco AP (2001). A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 413: 519–523.
5. Macdermut KD, Bonora E, Sykes N, Coupe AM, Lai CS, et al. (2005) Identification of FOXP2 truncation as a novel cause of developmental speech and language deficits. Am J Hum Genet 76: 1074–1080.
6. Alcock KJ, Pasingham RE, Watkins KE, Varga-Khadem F (2000) Oral dyspraxia in inherited speech and language impairment and acquired dysphasia. Brain Lang 75: 17–33.
7. Liegeois F, Baldeweg T, Connelly A, Gadian DG, Mishkin M, et al. (2003) Language and cholesterol abnormalities associated with FOX2 gene mutation. Nat Neurosci 6: 1230–1237.
8. Doupe AJ, Kuhl PK (1999) Birdsong and human speech: Common themes and mechanisms. Ann Rev Neurosci 22: 567–631.
9. Jarvis ED, Gunnink O, Bruce L, Collard A, Karten H, et al. (2005) Avian brains and a new understanding of vertebrate brain evolution. Nat Rev Neurosci 6: 151–159.
10. Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. Science 194: 211–213.
11. Wild JM (1993) The avian nucleus retroambigualis: a nucleus for breathing, singing and calling. Brain Res 606: 319–324.
12. Otsuka S, Saito N (1987) Synaptic connections of a forebrain nucleus involved with vocal learning in zebra finches. Br Res Bull 18: 35–44.
13. Botjer RJ, Passingham RE, Watkins KE, Varga-Khadem F (1998) Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. J Comp Neurol 395: 312–326.
14. Teramitsu I, Kudo LC, London SE, Geschwind DH, White SA (2000) Parallel pathways through the song system basal ganglia. J Comp Neurol 423: 2501–2517.
15. Haesler S, Wada K, Nshdejan A, Morrisey EE, Lints T, et al. (2004) FoxP2-expressing neurons to area X varies during song development. Dev Neurobiol 67: 809–817.
16. Doupe AJ, Konishi M (1991) Song-selective auditory circuits in the vocal control system of the zebra finch. Proc Natl Acad Sci USA 88: 11339–11343.
17. Perkel DJ, Farries MA, Luo M, Ding L (2002) Electrophysiological analysis of a songbird basal ganglia circuit essential for vocal plasticity. Brain Res Bull 57: 529–532.
37. Olveczky BP, Andalman AS, Fee MS (2005) Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. PLoS Biol 3: e153.
38. Bruce HA, Margolis RL (2002) FOXP2: Novel exons, splice variants, and CAG repeat length stability. Hum Gen 111: 136–144.
39. Feenders G, Liedvogel M, Rivas M, Zapka M, Horita H, et al. (2008) Molecular mapping of movement-associated areas in the avian brain: a motor theory for vocal learning origin. PLoS One 3: e1768.
40. Price PH (1979) Developmental determinants of structure in zebra finch song. J Comp Physiol Psychol 93: 260–277.
41. Lai CS, Gerrelli D, Monaco AP, Fisher SE, Copp AJ (2003) FOXP2 expression during brain development coincides with adult sites of pathology in a severe speech and language disorder. Brain 126: 2452–2462.
42. Jarvis ED, Nottebohm F (1997) Motor-driven gene expression. Proc Nat Acad Sci USA 94: 4097–4102.
43. Kurt S, Groszer M, Fisher SE, Ehret G (2009) Modified sound-evoked brainstem potentials in Foxp2 mutant mice. Brain Res 1209: 30–6.
44. Kao MH, Brainard MS (2006) Lesions of an avian basal ganglia circuit prevent context-dependent changes to song variability. J Neurophysiol 96: 1441–1455.
45. Sakata JT, Brainard MS (2006) Real-time contributions of auditory feedback to avian vocal motor control. J Neurosci 26: 9619–9620.
46. Derognecourt S, Minza PP, Fisher O, Pytte C, Tchernichovski O (2005) How sleep affects the developmental learning of bird song. Nature 433: 710–716.
47. Haesler S, Rochefort C, Georgi B, Licznerski P, Osten P, et al. (2007) Incomplete and inaccurate vocal imitation after knockdown of Foxp2 in songbird basal ganglia nucleus Area X. PLoS Biol 5: e321.
48. Spiteri E, Konopka G, Coppola G, Bomar J, Oldham M, et al. (2007) Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am J Hum Genet 81: 1144–1157.
49. Vernes SC, Spiteri E, Nicol J, Groszer M, Taylor JM, et al. (2007) High-throughput analysis of promoter occupancy reveals direct neural targets of FOXP2, a gene mutated in speech and language disorders. Am J Hum Genet 81: 1232–1250.