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# Birdsong Decreases Protein Levels of FoxP2, a Molecule Required for Human Speech

# Julie E. Miller,<sup>1</sup> Elizabeth Spiteri,<sup>3</sup> Michael C. Condro,<sup>2</sup> Ryan T. Dosumu-Johnson,<sup>1</sup> Daniel H. Geschwind,<sup>3</sup> and Stephanie A. White<sup>1,2</sup>

<sup>1</sup>Department of Physiological Science, <sup>2</sup>Molecular, Cellular, Integrative Physiology Program, <sup>3</sup>Department of Neurology and Semel Institute and Department of Human Genetics David Geffen School of Medicine, University of California, Los Angeles, California

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Miller JE, Spiteri E, Condro MC, Dosumu-Johnson RT, Geschwind DH, White SA. Birdsong decreases protein levels of FoxP2, a molecule required for human speech. J Neurophysiol 100: 2015-2025, 2008. First published August 13, 2008; doi:10.1152/jn.90415.2008. Cognitive and motor deficits associated with language and speech are seen in humans harboring FOXP2 mutations. The neural bases for FOXP2 mutation-related deficits are thought to reside in structural abnormalities distributed across systems important for language and motor learning including the cerebral cortex, basal ganglia, and cerebellum. In these brain regions, our prior research showed that FoxP2 mRNA expression patterns are strikingly similar between developing humans and songbirds. Within the songbird brain, this pattern persists throughout life and includes the striatal subregion, Area X, that is dedicated to song development and maintenance. The persistent mRNA expression suggests a role for FoxP2 that extends beyond the formation of vocal learning circuits to their ongoing use. Because FoxP2 is a transcription factor, a role in shaping circuits likely depends on FoxP2 protein levels which might not always parallel mRNA levels. Indeed our current study shows that FoxP2 protein, like its mRNA, is acutely downregulated in mature Area X when adult males sing with some differences. Total corticosterone levels associated with the different behavioral contexts did not vary, indicating that differences in FoxP2 levels are not likely attributable to stress. Our data, together with recent reports on FoxP2's target genes, suggest that lowered FoxP2 levels may allow for expression of genes important for circuit modification and thus vocal variability.

#### INTRODUCTION

Language and speech deficits accompany a wide variety of cognitive impairments, most prominent examples of which are developmental dysphasia/dyslexia, Specific Language Impairment, and autism spectrum disorders (Fisher 2005; Muhle et al. 2004; Smith 2007). Multi-genetic factors give rise to these disorders, thus presenting a challenge to researchers in understanding their neurological bases and in developing therapies. The gene encoding FOXP2, a member of the forkhead box (FOX) group of transcription factors, has provided a unique molecular entry point into the neural basis of speech since several forms of heterozygous mutations in FoxP2 cause developmental speech and language disorders with prominent features of apraxia (Lai et al. 2001; Watkins et al. 2002). (Of note, by convention, human "FOXP2" is fully capitalized, mouse "Foxp2" is not, and "FoxP2" denotes the molecule in mixed groups of animals. Italics are used when referring to genetic material such as FoxP2 mRNA) (Carlsson and Mahlapuu 2002). In the best-characterized case, members of the KE family have difficulty in the central control of sequential, complex orofacial movements, language skills, and impairments in verbal intelligence (Lai et al. 2001). In keeping with the evolving view that neural substrates for speech and language encompass more than just cortical regions (Lieberman 2007), affected individuals show bilateral abnormalities in subcortical structures, namely the basal ganglia and cerebellum, in addition to cortical abnormalities that include classic language areas like Broca's in the inferior frontal gyrus, all of which are important for human linguistic ability, and motor and reward-based learning. Structural and functional magnetic resonance imaging reveal altered amounts of gray matter in these regions, and their underactivation during tasks of verbal fluency, respectively (Belton et al. 2003; Liegeois et al. 2003; Vargha-Khadem et al. 1998).

Natural constraints on the ability to explore cellular pathways for FOXP2 function in humans create the impetus for developing models in non-human species, particularly other vocal learners (White et al. 2006). Prominent among these are songbirds, which are thought to share mechanisms and pathways for vocal learning with humans (Doupe and Kuhl 1999). Songbirds, like humans but unlike traditional lab animals such as rodents, create new sounds by listening to others and to themselves to learn their vocalizations. Thus, while important advances in understanding Foxp2 function on motor learning, especially those involving the cerebellum, are being made using transgenic mice that lack Foxp2 (Shu et al. 2005) or possess mutant Foxp2 variants (Fujita et al. 2008; Groszer et al. 2008), the impact on learned vocal behaviors may not be observable in this species. A second key strength of the songbird model system is that the neural structures that subserve the learning and production of vocalizations are wellcharacterized, (Fig. 1A), which is less the case for humans (Jarvis et al. 2005). In the zebra finch songbird, Taeniopygia guttata, vocal learning and the underlying neural circuitry is sexually dimorphic (Nottebohm and Arnold 1976). Males, but not females, acquire their song during critical developmental phases by listening to an adult male "tutor," then modify their own vocalizations to match the memorized model. Within the pallium, striatum, and thalamus, the subregions dedicated to song are larger and interconnected in males, a feature that has facilitated their identification and characterization. The anterior forebrain pathway through these regions (Fig. 1A) resembles

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FIG. 1. Schematic of avian song system and experimental design. A, left: primary components of 2 interconnected pathways for birdsong are shown (Jarvis et al. 2005; Reiner et al. 2004b). The posterior vocal motor pathway (solid dark arrows) controls song production (Nottebohm and Arnold 1976; Nottebohm et al. 1982; Wild 1993). The anterior forebrain pathway (AFP, dashed gray arrows), containing Area X, subserves song modification both during juvenile song learning and adult song maintenance (Bottjer et al. 1984; Brainard and Doupe 2000; Kittelberger and Mooney 2004; Leonardo and Konishi 1999; Scharff and Nottebohm 1991; Sturdy et al. 2003; Williams and Mehta 1999). Dark dashed line indicates approximate plan of section. Right: Area X (black) tissue punches were acquired as described in METHODS (figure modified from Poopatanapong et al. 2006). D, dorsal; R, rostral; M, medial. B: timeline for the experimental procedure. Following lights-on (T = 0), nonsinging birds were immediately killed (0-NS) or distracted from singing for 2 h and then killed (2-NS). Singing birds were killed 2 h after the 1st motif sung following lights-on. Songs were continuously recorded for both undirected (2-UD) and directed (2-D) behavioral conditions. In separate birds, blood samples were taken 20 min after lights-on and following 20 min under the nonsinging (NS), undirected (UD) and directed (D) singing protocols.

mammalian cortico-basal ganglia loops (Bottjer and Johnson 1997; Farries 2001; Farries and Perkel 2002; Jarvis et al. 2005; Reiner et al. 2004b).

In contrast to speech, zebra finch song stabilizes at sexual maturity, and each male thereafter sings one song. However, once learned, both speech and birdsong continue to rely on hearing to maintain the quality of the learned vocalizations in adulthood (Brainard and Doupe 2000; Cynx and Von Rad 2001; Nordeen and Nordeen 1992; Williams and Mehta 1999). Adult song can be characterized as either "directed," when a male sings to a conspecific, often a female, or "undirected," when the male practices alone (Dunn and Zann 1996; Hall 1962; Immelmann 1969; Morris 1954). While directed singing is likened to performance, undirected singing is thought to reflect a process of continuous action-based learning that contributes to song maintenance (Jarvis et al. 1998; Nelson and Marler 1994). Although the behavioral output is similar in both contexts, underlying brain activation patterns are distinct (Hessler and Doupe 1999; Jarvis et al. 1998; Teramitsu and White 2006).

Developing human and zebra finch brains exhibit strikingly similar patterns of *FOXP2* mRNA expression in the cortex/ pallium, basal ganglia, thalamus, and cerebellum (Teramitsu et al. 2004). In zebra finches, these developmental patterns persist into adulthood (Haesler et al. 2004; Teramitsu and White 2006) while human adult patterns are not yet known. The persistent expression of *FoxP2* mRNA in adult finches suggests that in addition to *forming* brain regions during embryogenesis, FoxP2 could *regulate* circuits throughout the life of the songbird, during learning and ongoing communication phases. Our prior studies provided support for the latter hypothesis by showing that *FoxP2* mRNA is actively regulated during adult song maintenance within the basal ganglia subregion dedicated to song, known as Area X (Fig. 1*A*), precisely when the bird sings and under certain social interactions (Teramitsu and White 2006). However, the full significance of this observation rests on whether FoxP2 protein follows its mRNA levels as this ultimately determines the effect on FoxP2's downstream transcriptional targets (Spiteri et al. 2007; Vernes et al. 2007). While such relationships may hold in cell culture, the in vivo situation in the brains of behaving animals is more complex.

We thus undertook the development of a new FoxP2 antibody and validated its ability to specifically detect FoxP2 expression in vivo and in vitro in the mature zebra finch brain. We then used it to characterize levels of protein expression within song nucleus Area X in response to different behavioral conditions and to compare these with previously obtained mRNA levels. Further, we investigated whether these conditions, experimentally implemented within the laboratory setting, impact total corticosterone (CORT) levels. We reasoned that CORT, as an indicator of stress in these animals, could present an uncontrolled influence on the relationship between levels of FoxP2, or other molecules, and singing. We find that FoxP2 protein is actively downregulated within Area X in singing birds, compared with nonsingers, and that CORT levels are similar across behavioral conditions. Thus the downregulation is likely due to singing rather than to stress. Recent experimental manipulations resulting in constitutively low levels of FoxP2 expression in Area X cause imprecise song development (Haesler et al. 2007). Our work extends these observations by showing that Area X FoxP2 protein levels are downregulated naturally when birds sing. These findings, by

analogy, provide insight into processes potentially important for human procedural learning and speech.

#### METHODS

#### Subjects

All animal use was approved by the University of California at Los Angeles Institutional Animal Care and Use Committee. Tissue from 14 birds was used for the antibody validation (Fig. 2, *C–E*). Thirty birds were used for the experiments on FoxP2 protein levels as a function of behavioral condition (Fig. 4). Twenty-one birds were used to test levels of stress associated with the different behavioral conditions (Fig. 6).

#### Behavioral manipulations

Adult male zebra finches (120-315 days of age) were moved from our breeding colony and housed individually in sound attenuation chambers (Acoustic Systems; Austin, TX) under a 14:10 h light/dark cycle. Birds were left undisturbed for 2-3 days prior to the experiments to enable acclimation to the new environment. Experiments were conducted in the morning from the time of light onset ("lightson") to the time of death by overdose with inhalation anesthetic (halothane or isoflurane; Halocarbon Laboratories, River Edge, NJ; Abbot Laboratories, Chicago, IL; Fig. 1B). Sounds were recorded and digitized using National Instruments and PreSonus Firepod hardware, with custom LabView (Livingston et al. 2000) and Sound Analysis Pro 1.04 (Tchernichovski et al. 2000) software, respectively. Two groups of nonsinging birds and two groups of singing birds that met our criteria (see following text; Fig. 1B) were generated. Nonsingers were adult males that were killed either at lights-on (0-NS) or 2 h from lights-on (2-NS). The 0-NS group was used for baseline values in the Western immunoblotting quantification because these animals were



not subject to any experimental manipulation and were killed following an undisturbed night of sleep. For the 2-NS group, if birds appeared to make any attempts to sing, they were distracted by the presence of the investigator. If distraction was ineffective, and the bird sang  $\geq 10$  motifs across the 2 h, the bird was excluded from that day's experiments. Singing birds were either males housed alone singing in a solo context (undirected, 2-UD) or males performing continuously to a succession of novel females presented every 4-7 min over a 2-h period ensuring that the male was performing 100% directed behavior (directed, 2-D: Teramitsu and White 2006). The acoustic structure of zebra finch song consists of a set of sound elements, known as syllables, which are repeated in what is referred to as a motif. Singing birds that sang >90 motifs of undirected or directed song within 2 h from song onset (i.e., start of 1st motif) were considered to have met criteria established in prior studies of FoxP2 mRNA (Teramitsu and White 2006) and were killed.

#### Tissue preparation for protein study

After undergoing the behavioral protocols, birds were overdosed, decapitated, and brains rapidly extracted and frozen in aluminum dishes on liquid nitrogen or dry ice and stored at  $-80^{\circ}$ C until use. Brains were mounted in a coronal orientation on a cryostat (Leica Microsystems, Bannockburn, IL). Sections of 40  $\mu$ m thickness were cut prior to visualization of Area X, then bilateral tissue punches of Area X were obtained at a depth of 1 mm using a 20-gauge Luer adaptor (Becton Dickinson, Sparks, MD) attached to a 1 ml syringe. Our previous observations noted that Area X is located ~1,250  $\mu$ m from the rostral-most point of the brain. In some cases, tissue punches of similar size were also taken from the outlying striatal and nidopallial regions, for comparison (see Fig. 2*C* schematic, plus signs) e.g., to determine any regional specificity of the double bands (for further methods and results, see following text). The anatomical precision of

FIG. 2. Antibody specificity is confirmed for in vitro and in vivo FoxP2 proteins. A: immunoblot of FoxP2 protein produced from in vitro transcription and translation (TnT) of a bacterial plasmid containing the full coding sequence for zebra finch FoxP2. Polyclonal antibodies (In-house, left; Abcam, right.) raised against 1 of 2 nonoverlapping peptides in the C-terminus of the FoxP2 recognize a band of similar molecular mass. B: the FoxP2 antibody detects the FoxP2 TnT product (1st lane). Preadsorption of the antibody with 30× excess FoxP2 immunizing peptide (\*, middle lane) prevents antibody binding to FoxP2 protein, whereas preadsorption with a nonantigenic peptide from Gas11- $\alpha$  peptide (NA last lane) does not. C, top: line drawing of anatomical regions highlights song region Area X, observable in the Nissl section below. +, the specific location of tissue punches in the ventral striatum and nidopallium. Right: FoxP2 protein signal is detectable in immunoblots of Area X bilateral tissue punches taken from each of 8 individual birds (50  $\mu$ g/lane). D: immunoblot shows FoxP2 protein in punches from Area X, striatum (Str), and nidopallium (Nido) of 2 male birds (60  $\mu$ g/lane). Preadsorption of the antibody with  $20 \times$  excess peptide (\*) prevents antibody binding to FoxP2 protein from Area X. Bottom: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the loading control. E: immunoblot shows the presence of double bands in Area X, striatum, and nidopallium (40  $\mu$ g/lane) from a single bird. M, mesopallium; HA, hyperpallium apicale; HD, hyperpallium densocellulare.

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the punch technique was demonstrated by post hoc thionin staining of coronal brain sections (Fig. 2*C*). Tissue punches were homogenized in ice-cold modified RIPA lysis buffer: 1% octylphenoxy poly-ethoxyethanol (NP-40 substitute), 0.5% deoxycholate, 0.1% SDS, 1× phosphate-buffered saline; Gibco, Invitrogen, Carlsbad, CA; pH 7.6 with a cocktail of protease inhibitors (No. P8340, Sigma-Aldrich, St. Louis, MO) using a hand-held homogenizer (Kontes, Fisher Scientific) followed by a 10 s homogenization by an ultrasonic cell disruptor (Misonix, Farmingdale, NY) on ice to ensure complete disruption of the nuclear membranes. An aliquot of each sample was removed to determine protein concentration using the RC DC Protein Assay (Bio-Rad, Hercules, CA). Samples were aliquoted in  $2 \times$  Laemmli loading buffer (Bio-Rad) with 0.1% beta-mercaptoethanol. Samples were stored at  $-80^{\circ}$ C until use.

#### Immunoblotting

Samples were heated to 90-100°C for 3-5 min, and lysates were resolved on 10% isocratic (avian tissue samples) or 4-20% gradient (in vitro transcription/translation protein products) SDS-polyacrylamide gels (Promega, Madison, WI). (Observed differences in the molecular mass of FoxP2 between the in vivo and in vitro conditions are likely due to the type of running buffer used and/or to unknown posttranslational modifications of the protein in vivo.) Prestained (Benchmark ladder) or chemilumescent (MagicMark) protein standards (Invitrogen, Carlsbad, CA) were included on gels as molecular mass markers. The Benchmark protein standard gave signals that more closely bracketed the FoxP2 band, and we proceeded to use this same standard in Fig. 2, B-E, and 4, A-C. However, the vendor changed the mass weight for the same standard protein from 79 to 82 kDa over the course of several different lot numbers, and so our blots reflect this. Samples were subjected to electrophoresis in Tris-Glycine-SDS buffer (TGS, Bio-Rad, or Tris-HEPES-SDS, Pierce, Rockford, IL) then transferred in TGS with 20% methanol for 2 h at 400 mA onto 0.45-µm nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1.5 h at room temperature (RT). Blots were probed with FoxP2 antibodies (1:500-1:1,000) in TBST containing 2.5% nonfat dry milk. Following primary antibody incubation (see following text), blots were washed in TBST  $3 \times 10$  min then probed with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution) and anti-mouse IgG (1:5,000 dilution; Amersham Pharmacia Biotech, Piscataway, NJ). Enhanced chemiluminescence with ImmunoStar HRP detection kit (Bio-Rad) was used to develop immunoblots. In some blots, we detected the presence of two bands ( $\sim$ 69,  $\sim$ 66 kDa: see RESULTS) with the lighter band of lower molecular mass potentially representing another isoform of FoxP2. The resolution of this band appeared to depend on the separation characteristics of the gel, including type of SDS-PAGE gel used (gradient gels enable better separation of bands), and the voltage (lower voltage also enhances band separation).

#### Antibodies

Two polyclonal antibodies directed against distinct polypeptide regions within the C-terminus of FoxP2 were employed for our studies. We originally tested a commercially available primary antibody made in goat against a FoxP2 peptide (Abcam). We found that it resulted in high background on Western blots and yielded multiple bands that were difficult to interpret. This motivated us to develop our own antibody. Of note, the vendor subsequently discontinued the goat primary antibody and replaced it with an antibody made in rabbit against FoxP2; see following text. Throughout the text, we refer to the antibody that we generated as the FoxP2 antibody (Spiteri et al. 2007), which was used for all experiments described in this paper unless otherwise noted. We distinguish it from the commercially available

antibody by citing the vendor for the latter (i.e., Abcam). We selected a 14 amino acid sequence, corresponding to amino acids 643-656 of human FoxP2 (EDLNGSLDHIDSNG, Genbank No. AF337817) and predicted from the FoxP2 coding sequences to be identical between humans and zebra finches (GenBank Accession Nos. AY395709 for zebra finch and AF337817 for human). The selected peptide was conjugated with an extra cysteine on the amino terminus and coupled to MBS-KLH and injected into a female New Zealand white rabbit (Sigma-Aldrich) then affinity purified. The second antibody (used only in the in vitro transcription and translation assays, see following text) was a commercially available, polyclonal rabbit antibody against the peptide "REIEEEPLSEDLE," corresponding to amino acids 703-715 of human FOXP2 (No. 16046 Abcam, Cambridge, MA), a sequence also identical in zebra finches. For preadsorption experiments, the FoxP2 antibody was incubated with either immunizing or nonantigenic peptides in excess quantity relative to the antibody concentration. For the nonantigenic peptides, we used a synthetic peptide from Gas11-a (RNYFQLERDKI; gift from R.H. Crosbie, UCLA), a microtubule-associated protein (Bekker et al. 2007). The Gas11- $\alpha$  and FoxP2 peptide sequences are not similar as no two consecutive amino acids are shared. A monoclonal antibody raised against glyceraldehyde 3-phosphate dehydrogenase (No. MAB374, 1:5,000 GAPDH; Chemicon) was used to control for equal protein loading on immunoblots.

#### In vitro transcription and translation of zebra finch FoxP2

FoxP2 bacterial expression plasmids were constructed via directional cloning of PCR amplified zebra finch FoxP2 cDNA (GenBank No. AY395709) using primers designed with restriction sites for EcoRI and NotI into the vector pcDNA3 with the T7 and mammalian CMV promoters (Invitrogen). The correct FoxP2 cDNA sequence was confirmed by the UCLA Sequencing Core using an ABI 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). In three separate experiments, FoxP2 protein was made from 1  $\mu$ g of plasmid DNA using T7 Quick-Coupled in vitro Transcription/Translation System (TnT; Promega). To confirm TnT protein product, Transcend biotinylated lysyl tRNA reagent (Promega) was incorporated into the synthesis mixture, then samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes, probed with streptavidin-conjugated alkaline phosphatase (Promega, 1:1,000), and visualized with Western Blue stabilized substrate (Promega). Identical blots containing TnT product were probed with anti-FoxP2 primary antibodies followed by detection with chemiluminescence reagents as previously described.

#### Quantification and statistical analyses

Parametric statistics with two-tailed probabilities were used unless otherwise indicated. We note that the use of parametric versus nonparametric measures did not alter any experimental outcome. Immunoblots developed by enhanced chemiluminesence were imaged and analyzed using a cooled CCD camera-based image-acquisition system (Chemi-Doc and Quantity One software package, Bio-Rad) and densitometric analysis using Quantity One. A total of eight Western blots (exemplars are shown in Fig. 4, A and C) were probed with FoxP2 antibody. Each blot contained at least one bird from every behavioral condition. To determine whether FoxP2 levels varied as a function of behavioral condition, the FoxP2 value for each lane was normalized to its corresponding GAPDH value to obtain a ratio. The top band in each lane was quantified because of its robust signal strength although inclusion of the lighter, lower band did not alter the relative brain expression levels between groups. For example, in one blot, the normalized mean FoxP2 protein values per group (with 2-3 birds/group) obtained from quantifying the top bands only were: 0-NS: 1.00, 2-NS: 1.15, 2-D: 0.90, 2-UD: 0.90. Similar relationships between means were obtained when both top and bottom bands were

included: 0-NS: 0.99, 2-NS: 1.24, 2-D: 0.96, 2-UD: 0.86 (P > 0.05, paired Student's t-test). To aid in interblot comparisons, these values were normalized using the value obtained from the 0-NS birds within a given blot because the 0-NS group did not undergo any experimental manipulation prior to sacrifice. The normalized values for individual birds are plotted in Fig. 4B, along with the average values obtained per behavioral condition. Means were compared via one-way ANOVA with post hoc Tukey-Kramer tests using JMP statistical software (Cary, NC).

To determine whether FoxP2 levels were correlated with the amount of song in the singing groups, two measures of singing were each compared with the normalized FoxP2 levels. We observed that the distribution of the number of motifs sung by birds in the UD group values did not conform to normal assumptions, using the goodnessof-fit test (Shapiro-Wilcox, P < 0.05). Thus we proceeded to conduct multivariate analyses using nonparametric tests for ranked order. The Spearman's Rho correlation coefficient is reported for all comparisons between FoxP2 protein and motifs. The amount of time spent singing was calculated by selecting 10 random motifs within the 2 h period for each bird and measuring the motif length. An average motif length was obtained and multiplied by the total number of motifs the bird sang to represent time spent singing (in seconds). The amount of song or time spent singing is reported as means  $\pm$  SE with comparisons between groups using Mann-Whitney U tests (Vassar Stats).

#### *Immunohistochemistry*

Within zebra finch striatum, immunohistochemical studies have shown that FoxP2 protein co-localizes with dopamine- and-cAMPregulated phosphoprotein of molecular weight 32 kDa (DARPP-32) (Reiner et al. 2004a) in a subset of medium spiny neurons (Haesler et al. 2004) including within Area X (Rochefort et al. 2007). To further validate our antibody by this additional methodology, we performed immunohistochemistry on adult brain sections containing Area X using our rabbit anti-FoxP2 antibody as the sole primary antibody or together with the mouse anti-DARPP-32 monoclonal antibody used in the prior studies (Fig. 3). Adult male zebra finches were overdosed with inhalant anesthesia and then perfused with prewarmed 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for brain fixation. Brains were extracted and then cryoprotected in 20% sucrose in PB at 4°C. Coronal sections that contained Area X were cut at 40  $\mu$ m and thaw-mounted onto slides (Superfrost, Fisher Scientific) and then stored at  $-80^{\circ}$ C until used for fluorescent immunohistochemistry. Brain sections were encircled by a hydrophobic barrier using a PAP pen (Ted Pella, Reddington, CA) and washed in Tris-buffered saline (TBS) with 0.3% Triton X-100 (Tx) for  $3 \times 5$  min. Sections were incubated for 10 min in 50 mM ammonium chloride in TBS to reduce autofluorescence followed by  $3 \times 5$  min washes in TBSTx. To block nonspecific binding, tissue was incubated in TBSTx with 10% goat serum (Sigma) for 1 h at room temperature followed by  $3 \times 5$  min TBSTx washes in 1% goat serum. Tissue was incubated overnight at 4°C in a TBSTx/1% goat serum solution of the polyclonal primary antibody to FoxP2 at 1:1,000 and the monoclonal primary antibody (from mouse) to DARPP32 at 1:900 (gift of H. C. Hemmings Jr., Weill Cornell Medical College, New York). Following overnight incubation at 4°C, sections were washed  $5 \times 5$  min each with TBSTx then incubated for 4 h at room temperature in a TBSTx/1% goat serum solution using two fluorescence-tagged secondary antibodies against rabbit or mouse IgG, each with distinct emission spectra (Alexafluor 488 nm to detect FoxP2, Alexafluor 350 nm to detect DARPP32; Molecular Probes, Eugene, OR). Sections were washed  $5 \times 5$  min with TBS only. Sections were mounted with coverslips using ProLong Gold Antifade Reagent (Molecular Probes). Images were captured using an Axio-Imager microscope equipped with fluorescence and with the Axiovision 4.4 software program (Carl Zeiss MicroImaging, Thornwood, NY). Coronal sections were imaged with a  $\times 40$  objective of 1.3



FIG. 3. Fluorescent immunohistochemistry shows FoxP2-specific signals. A: exemplar section reveals signals obtained using our FoxP2 antibody against FoxP2 protein. B: lower-magnification view of a thionin-stained section, adjacent to that shown in A. Arrow points to LMAN. Arrowheads demark the striatal-nidopallial border. Box shows region of enlargement shown in A. C: another section processed in the same experiment as that shown in A but without primary antibody lacks detectable signals. D-F:. immunohistochemical co-localization of FoxP2 and DARPP32 within striatal neurons of an adult male zebra finch. In each panel, arrowheads point to the location of the same six neurons, of many, that are immunoreactive for FoxP2. D: FoxP2-positive neurons appear in green. E: DARPP32 signal (blue) seen in the cell body and dendritic processes of neurons. F: FoxP2 and DARPP32 signals co-localize in a subset of striatal neurons (aqua, arrowheads). Scale bar = 40(A), 160(B) and C), and 20 (D-F)  $\mu$ m.

numerical aperture. For determination of cytoarchitectonic boundaries, adjacent sections were processed for Nissl substance using thionin staining.

#### Assessment of corticosteroid levels

Adult male zebra finches (n = 21) >200 day were used. Corticosterone (CORT) is the main avian stress steroid and, in zebra finches, its levels peak at 20 min following the onset of an acute stress and then return to baseline (Evans et al. 2006). To verify our ability to measure a range of total CORT levels, "low stress" blood samples were obtained from six birds housed in the aviary or in the sound attenuation chambers at 20 min after lights-on without prior experimental intervention. "High stress" samples were taken from these same birds but after they were actively restrained—kept captive by the investigator's hand for ~15 min prior to blood sampling. Samples were taken from the brachial vein and treated with heparin (Sigma, St. Louis, MO). All samples except those for the high stress conditions were taken within 3 min of approach and handling before the sampling procedure itself could contribute to CORT concentration in the blood (Romero and Reed 2005; Wingfield et al. 1982).

To determine stress levels associated with different behavioral conditions, 15 males were separated into three groups of five animals in a manner that minimized the difference in mean ages between groups. Similar to the conditions used for the protein study, birds were kept in an enclosed space near the investigator (nonsingers), in a sound-attenuation chamber (undirected singers), or subjected to directed singing conditions as described in the preceding text (Fig. 6A). Baseline CORT measurements were obtained  $\geq 1$  wk prior, at 20 min following lights-on. Experimental samples were taken from these same birds at the 20-min time point for each behavioral condition. In addition to reporting raw CORT values (Fig. 6B), each bird's experimental CORT levels were normalized using its baseline CORT levels to control for individual variability in CORT levels. The normalized data are represented as fold-change from baseline. Two different investigators conducted these experiments, one to observe the birds during the directed or nonsinging behavioral conditions and the other to collect the blood sample. Samples were kept on ice until they were centrifuged for 15 min at 2.3 relative centrifugal force (rcf). CORT measurements were determined by use of an enzyme-linked immunosorbent assay kit (Assay Designs, Ann Arbor, MI) following the procedures outlined by the manufacturer with these changes: plasma samples were diluted 40 times with assay buffer and treated with a steroid displacement agent, as it has been found that substances in avian plasma interfere with the measurement of CORT (Wada et al. 2007).

#### RESULTS

#### Specific detection of FoxP2 protein in Area X

The predicted protein sequence for zebra finch FoxP2 is  $\sim$ 710 amino acids and 98% identical to mouse and human homologs with 100% identity to the human Fox domain (Haesler et al. 2004; Teramitsu et al. 2004). Both our resultant polyclonal antibody and a commercially available one (Abcam, Cambridge, MA), raised against two nonoverlapping peptides, recognize FoxP2 protein generated by in vitro transcription and translation (TnT; Fig. 2A). Preadsorption of our antibody with the immunizing peptide prevented antibody binding to immobilized FoxP2 TnT protein product, whereas preincubation with a nonantigenic peptide sequence from Gas11- $\alpha$  did not block antibody binding to FoxP2 (Fig. 2B). This peptide competition experiment demonstrates that our FoxP2 polyclonal antibody recognizes zebra finch FoxP2 protein.

In zebra finch tissue, the FoxP2 antibody recognizes a protein of the expected molecular mass weight for FoxP2 similar to predicted zebra finch isoform III (69 kDa) (Haesler et al. 2004) in specific brain areas as seen in bilateral punches

taken from Area X of multiple male birds (Fig. 2C) and from nidopallial and striatal regions outside of Area X (Fig. 2D). These protein data suggest that the FoxP2 antibody is both specific and sensitive as it detects protein in Area X tissue punches taken from individual birds. Antibody specificity in vivo is confirmed by the preadsorption control which prevents antibody binding (Fig. 2D, \*). As these experiments were not aimed at quantifying protein as a function of behavioral condition, the birds were in different behavioral contexts. For example, in Fig. 2D, the Area X punch came from a ONS bird, a condition in which we expect lower levels of FoxP2 than at the 2NS time point. This may have contributed to the relatively low FoxP2 protein levels observed in the Area X lanes versus the striatal and nidopallial regions. In some immunoblots, we observed an additional fainter second band of slightly lower molecular mass by several kDa, similar to predicted zebra finch isoform 4 (see METHODS, Figs. 2E and 4C). We wondered whether the second band was region-specific and thus ran additional blots that included tissue from the nidopallium as well as the striatum outlying Area X. We found that these bands are not restricted to Area X but are found in the other tissue extracts as shown in a representative Western blot (Fig. 2E). Rather as noted in METHODS, observation of the second band appeared to depend on the resolution characteristics of the gel (e.g., the blot in Fig. 2D that shows 1 band was run at twice the voltage as that in Fig. 2E showing 2 bands). Further, inclusion or exclusion of the second band in the quantification did not alter the relationships between behavioral condition and FoxP2 protein levels (see METHODS).

## *FoxP2 protein signal colocalizes with that for DARPP-32 in striatal neurons*

Conventional immunohistochemistry using our primary antibody against FoxP2 revealed stronger signals within the dorsal striatum compared with the nidopallium, (Fig. 3, A and B) consistent with mRNA expression data (Haesler et al. 2004; Teramitsu et al. 2004). No detectable signal was observed when the primary antibody was omitted (Fig. 3C). The colocalization of FoxP2 signals (Fig. 3, D and F) with those for DARPP32 (Fig. 3, E and F) in a subset of neurons replicates previous findings (Rochefort et al. 2007) and provides further support for the specificity of our antibody.

#### Singing downregulates FoxP2 protein in Area X

Representative immunoblots show FoxP2 signals obtained from birds under different behavioral conditions (Fig. 4). The summary graph (Fig. 4B) shows values obtained from the blots of FoxP2 protein levels in Area X of individual birds plotted singly and as group means. A one-way ANOVA indicated overall differences between the groups and validated comparisons between them (F = 4.70, P < 0.01; means  $\pm$  SE: 0-NS = 1.00  $\pm$  0.10, 2-NS = 1.40  $\pm$  0.12, 2-D = 0.95  $\pm$  0.10, 2-UD = 0.86  $\pm$  0.09). These comparisons revealed that undirected singers and directed singers had lower amounts of FoxP2 protein than levels in birds that did not sing for 2 h (post hoc Tukey-Kramer, P < 0.05, 2-NS: n = 6; 2-UD: n = 9; 2-D: n = 8). In some cases, FoxP2 levels in Area X of undirected singers could appear as lower than those in directed singers. An example is provided in Fig. 4C to enable a fuller representation

#### BIRDSONG DECREASES FoxP2 PROTEIN IN AREA X



FIG. 4. Behavioral regulation of Area X FoxP2 protein levels. A: exemplar Western blot shows FoxP2 (top) and GAPDH (bottom; used for normalization) signals obtained from Area X punches. Each lane is from a single bird in the indicated group. Of note, the 4th sample on this blot was not measured and not included in the study because at loading, it was noted that the sample volume was off by more than 25%. B: graph of FoxP2 levels by experimental condition shows group means (bar) ± SE (dark gray lines). Each point represents an individual bird. For the 2-UD group, 2 birds with protein values of 0.51 and 0.52 are represented by 1 point. With values from the 0-NS birds used as baseline, levels in 2-NS group are higher than those in the 2-D and 2-UD groups (asterisk, P < 0.05). C: another example blot shows signals obtained from 2 birds in the 2-D group and 2 in the 2-UD group. NS, nonsingers; UD, undirected singers; D, directed singers.

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of the range of results However, across all samples, no significant difference was observed in protein levels between UD and D singers. Other post hoc comparisons between groups did not attain statistical significance.

#### FoxP2 protein levels and amount of song

Previous studies have shown that mRNA and protein levels of the immediate early gene ZENK (acronym for zif-268, egr-1, NGF1-A, and Krox-24) correlate with the amount of singing (Jarvis and Nottebohm 1997; Whitney and Johnson 2005). Thus we considered whether increased singing would increasingly downregulate FoxP2 and examined levels of protein as a function of the amount of song sung for UD and for D singers (Fig. 5). We observed a trend within the undirected group in which higher numbers of motifs resulted in lower FoxP2 levels (Fig. 5, Spearman's Rho, = -0.20, P = 0.59, n = 9), similar to what had been reported for *FoxP2* mRNA (Teramitsu and White 2006). Like the mRNA data, this trend did not reach significance. One UD bird sang 1,236 motifs (not represented in Fig. 5), well beyond the range of song observed in all other UD birds (range = 98–215; means  $\pm$  SE = 143.9  $\pm$  16.5). Removal of this bird's data, however, did not alter the results (Spearman's Rho = -0.30, P = 0.47, n = 8).

In D singers, no trend is observed between protein and singing behavior (Fig. 5, Spearman's Rho = 0, P = 1.0, n = 8). Of note, the amount of song sung by the two singing groups did not differ: the average number of motifs from the 2-D birds (range = 103-405; means  $\pm$  SE = 223  $\pm$  40.6), was similar to the 2-UD birds regardless of whether the UD singer with the highest number of motifs is included (range = 98-1,236; means  $\pm$  SE = 265  $\pm$  122.2; Mann-Whitney U = 45,  $n_1 = 9$ ,  $n_2 = 8$ , P = 0.41) or excluded (range = 98-215; means  $\pm$  SE = 143.9  $\pm$  16.5; Mann-Whitney U = 45,  $n_1 = 8$ ,  $n_2 = 8$ , P = 0.19). Similar, nonsignificant findings were observed when the estimated total amount of time spent singing (calculated per bird as the mean motif length of 10 motifs x total number of motifs sung in 2 h; see METHODS) was used (data not shown).



FIG. 5. FoxP2 protein plotted as a function of the amount of singing in undirected  $(n = 8, \blacksquare)$  or directed  $(n = 8, \Box)$  singers. ---, simple linear regressions.

## Corticosterone levels do not change based on behavioral context

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A potential confound for interpreting biological data obtained from behavioral manipulations such as those used here is that the housing conditions, including the presence or absence of the investigator or conspecifics may induce stress differentially across groups. To test this, we first verified our ability to detect differences in CORT levels in control male zebra finches between conditions of high versus low stress (see METHODS). As expected, when handled, birds had an average approximately twofold higher level of plasma total corticosterone (CORT) than when undisturbed (1-tailed *t*-test: t =-3.79, P < 0.01, n = 6), and these differences did not vary whether birds were housed individually in sound attenuation chambers or grouped in an aviary (2-way ANOVA without replication, F = 1.10, P = 0.46).

Based on this validation of our measurement protocol, we proceeded to examine CORT levels in our experimental birds (see METHODS). No differences in CORT levels were observed across NS, UD, and D groups either when raw CORT values are compared (Fig. 6B, 2-way ANOVA without replication between groups, F = 1.06, P = 0.44; within-group F = 1.71, P = 0.24; means  $\pm$  SE for NS, D, UD in ng/ml: 20.14  $\pm$  1.81, 25.80  $\pm$  4.59, 17.18  $\pm$  1.75) or when each bird's 20 min value

was normalized by its baseline levels (1-way ANOVA F = 1.23, P = 0.33). The average CORT levels for each of the three groups fell between values from the control low and high stress conditions. Additionally, blood samples were obtained at sacrifice for two of the nonsinging birds used for the FoxP2 experiment, see preceding text. Despite having been distracted from singing by the investigator within the 2-h period, we found that these birds had similarly low CORT levels (10.9 and 7.8 ng/ml).

#### DISCUSSION

Here we present evidence for naturally induced regulation of FoxP2 protein in Area X of adult zebra finches, similar to *FoxP2* mRNA. Singing downregulates FoxP2 protein within Area X, the specific subregion of songbird striatum dedicated to song (Scharff and Nottebohm 1991; Sohrabji et al. 1990). Both directed and undirected singers have lower FoxP2 levels at 2 h after song onset compared with nonsinging birds. These data suggest that FoxP2, previously implicated in the formation of vocal control circuitry and in human developmental-onset disorders, also has an on-line function in the adult brain.

Our prior study showed that FoxP2 mRNA is only downregulated by undirected, but not by directed, singing (Teramitsu and White 2006). The difference in social regulation between FoxP2 mRNA and protein, seen here, has been observed for other transcription factors, notably the immediate early gene ZENK in which mRNA is uncoupled from protein levels in sensory versus motor processes (Whitney and Johnson 2005). One interpretation is that singing results in FoxP2 protein turnover, regardless of social context, but that mRNA levels persist in directed singers leading to faster replenishment of the protein molecule. A time-course study that investigates protein and RNA half-lives during singing in the different social contexts may address this and alternative explanations. We do not know whether the singing-driven downregulation in FoxP2 protein in Area X occurs in other song control regions because we did not measure them. No obvious changes in FoxP2 mRNA levels as a function of behavioral state were previously noted in these regions. However, the difference in the social regulation between mRNA versus protein observed here raises the possibility that differential mRNA versus protein regulation could occur in other regions.

In line with our previous study on *FoxP2* mRNA (Teramitsu and White 2006), we observed a trend toward a negative



FIG. 6. Total corticosterone (CORT) levels in experimental animals. A: schematic of experimental groups. B: graph of raw CORT levels in ng/ml with group means represented by gray bars and points for individual birds (n = 5 per group). Gray lines show the SE.

correlation between the amount of undirected singing and Area X FoxP2 levels, whereas no such trend was observed in directed singers. We report this nonsignificant relationship due to a few considerations. First, in the mRNA study, we set a behavioral criterion of 90 motifs for inclusion of animals into singing groups to maximize the behavioral differences between groups. The 90 motif cut-off was preserved here to enable comparison between mRNA and protein studies. While this approach was successful in allowing us to discriminate differences in both mRNA and protein levels based on singing, it is not optimal for determining whether a wide range of singing levels is associated with a gradient in FoxP2 expression as it omits a substantial portion of the range (0-90 on the x axis). Other studies have overcome this limitation by including birds that sang only a few motifs, and even nonsinging birds, in correlations between amount of song and molecular expression (Jarvis and Nottebohm 1997; Jarvis et al. 1998; Poopatanapong et al. 2006). Here, inclusion of data from nonsingers with the UD data would indeed render a significant negative correlation between the number of UD motifs sung and the level of FoxP2 protein. However, nonsinging birds were housed under conditions that were distinct from the UD singers (in a cage next to the investigator vs. undisturbed inside an acoustic-attenuation chamber). It may not be valid to combine data from these groups. For example, it could be that birds that simply did not sing when housed alone in sound-attenuation chambers would have different FoxP2 levels than the nonsinging birds used here.

Perhaps more sensitive signal detection methods (e.g., the use of qRT-PCR for mRNA or of a more sensitive antibody against FoxP2 protein) coupled with inclusion of a wider range of singing values would unveil a robust negative correlation between amount of UD song and FoxP2 protein levels. It is less likely that such a relationship would emerge with the directed singers because the trend is lacking from the present data set on FoxP2 protein, and an opposite trend was observed when mRNA levels were analyzed (Teramitsu and White 2006). Further, other groups have shown that directed and undirected singing are accompanied by distinct brain activation patterns; undirected singing corresponds to higher and more variable levels of neuronal activity and ZENK expression in Area X and LMAN, that is accompanied by slightly greater song variability (Hessler and Doupe 1999; Jarvis et al. 1998; Kao and Brainard 2006; Kao et al. 2005; Sakata et al. 2008).

A working hypothesis is that FoxP2, like other forkhead family members (Carlsson and Mahlapuu 2002), promotes the structural formation of anatomical regions, in this case including striatal areas that subserve vocal learning. This idea is consistent with FoxP2 protein localization to newly born neurons in zebra finch Area X (Rochefort et al. 2007) with the increase in *FoxP2* mRNA expression levels in canary Area X during seasonal periods of song circuit growth (Haesler et al. 2004) and with the structural abnormalities in the striatum of humans bearing FOXP2 mutations (Belton et al. 2003). In contrast to developmental and seasonal roles in promoting growth of specialized brain structures, the behavioral use of such regions may depend on FoxP2 downregulation. Accordingly, a recent study showed that lentivirus-mediated RNA interference to reduce FoxP2 levels in Area X of young birds caused inaccurate imitation of the tutor song (Haesler et al. 2007). The abnormal songs were characterized by spectral and temporal differences in structure and resulted in more adult song variability compared with control birds. The constitutive knock-down of FoxP2, coupled with the naturally occurring dynamic variation in FoxP2 levels shown here, support the idea that low levels of FoxP2 may direct changes in transcriptional activity that promote vocal motor variability. The identification of FOXP2 gene targets (Spiteri et al. 2007; Vernes et al. 2007; see following text) further support this notion. Vocal motor variability has been hypothesized to allow reinforcement and stabilization of correct vocal motor patterns, occurring on both fast (Tumer and Brainard 2007) and slower (Troyer and Doupe 2000a,b) time scales. Future work that compares song stability when FoxP2 levels are high versus when they are low (e.g., in the 2-NS vs. the 2-UD groups used in this study) might reveal corresponding differences in behavioral variability. Unfortunately, the 2-NS birds were killed before any songs were sung—a criterion for group inclusion that precluded obtaining song records for such analysis.

One concern is that so-called behaviorally driven changes in FoxP2, or other molecules, could actually be due to extraneous stress imposed by the experimental manipulations used to alter behavior, rather than the behavior itself. Previous reports have documented the effect of acute stress on memory tasks in zebra finches (Hodgson et al. 2007). Our findings do not appear to be confounded by the endogenous stress levels associated with the different behavioral conditions as plasma CORT concentrations did not depend on the presence of a human or female bird nor on the surrounding environment. Male birds had low CORT at 20 min following experimental onset despite the fact that 20 min corresponds to the peak in the acute stress response of zebra finches (Evans et al. 2006). In a separate exemplar experiment, CORT levels were also low in two birds sampled for FoxP2 protein at the 2-h time point. We were able to document low and high CORT levels in another subset of birds kept in a low versus high stress condition, validating the effectiveness of our measurements. Together these stress steroid measurements alleviate potential concerns that the changes observed here in FoxP2 protein, and previously in FoxP2 mRNA (Teramitsu and White 2006), are attributable to stress. To our knowledge, this is the first analysis of the effects of common laboratory environments (e.g., sound-attenuation chambers vs. aviary) and social-context (alone vs. in the presence of female birds or the investigator) on zebra finch stress. The surprising lack of stressful impact may be partly due to the acclimation period after birds are moved into the sound-attenuation chamber and/or the familiarity of our birds with laboratory personnel as the investigators also provide daily care of our colony.

Identification of FoxP2 gene targets in songbird brain, including within Area X will help to elucidate molecular pathways important for motor learning. Already targets of FOXP2 have been identified in human neural tissue including in fetal basal ganglia and neuronal-like cell lines (Spiteri et al. 2007; Vernes et al. 2007). These targets are associated with neurite outgrowth, dendritic branching, intracellular signaling, and calcium mobilization, all processes important for remodeling of neuronal connections. Additional likely regulators of these target genes include transcription factors, such as CREB, known for their roles in neuronal plasticity (Bourtchuladze et al. 1994; Vernes et al. 2007). Some of these same targets may be shared with songbird brain, and analysis of how these targets vary in the songbird depending on developmental stage may be particularly informative with regard to formative versus on-line roles of FoxP2 as well as for roles shared with or unique to humans. Our current study, which links FoxP2 protein and learned vocal motor behavior, is a step toward shedding insight on the function of FoxP2 in avian vocal learning, and by analogy to humans, in cognitive and motor processes important for speech and language.

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