Differential *FoxP2* and *FoxP1* Expression in a Vocal Learning Nucleus of the Developing Budgerigar

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ABSTRACT: **The forkhead domain FOXP2 and FOXP1 transcription factors are implicated in several cognitive disorders with language deficits, notably autism, and thus play a central role in learned vocal motor behavior in humans. Although a similar role for *FoxP2* and *FoxP1* is proposed for other vertebrate species, including songbirds, the neurodevelopmental expression of these genes are unknown in a species with lifelong vocal learning abilities. Like humans, budgerigars (*Melopsittacus undulatus)* learn new vocalizations throughout their entire lifetime. Like songbirds, budgerigars have distinct brain nuclei for vocal learning, which include the magnocellular nucleus of the medial striatum (MMSt), a basal ganglia region that is considered developmentally and functionally analogous to Area X in songbirds. Here, we used *in situ* hybridization and immunohistochemistry to investigate *FoxP2* and *FoxP1* expression in the MMSt of juvenile and adult budgerigars. We found *FoxP2* mRNA and protein expression levels in the MMSt that were lower than the surrounding striatum throughout development and adulthood. In contrast, *FoxP1* mRNA and protein had an elevated MMSt/striatum expression ratio as birds matured, regardless of their sex. These results show that life-long vocal plasticity in budgerigars is associated with persistent low-level *FoxP2* expression in the budgerigar MMSt, and suggests the possibility that *FoxP1* plays an organizational role in the neurodevelopment of vocal motor circuitry. Thus, developmental regulation of the *FoxP2* and *FoxP1* genes in the basal ganglia appears essential for vocal mimicry in a range of species that possess this relatively rare trait.**
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Keywords: **basal ganglia; budgerigar; *FoxP2*; *FoxP1*; gene expression; vocal learning**

INTRODUCTION

Increasing evidence suggests that the underlying genetic mechanisms for vocal learning are shared between such divergent taxa as humans and several lineages of birds. The neurogenetic basis for vocal learning is not understood completely, but activity of the P2 and P1 forkhead box transcription factors, *FOXP2* and *FOXP1*, in the basal ganglia plays a central role (Scharff and White, 2004; White et al., 2006; Bolhuis et al., 2010; White, 2010). *FOXP2* activity during human embryonic brain development is

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necessary for the organization of cortical and basal ganglia structures involved in sensorimotor integration and fine orofacial motor control. Mutations in this gene in humans produce speech and language pathologies, and neuroanatomical abnormalities, notably in a striatal region of the basal ganglia (Vargha-Khadem et al., 1998; Lai et al., 2001; Watkins et al., 2002; Belton et al., 2003; Lai et al., 2003; MacDermot et al., 2005). Similar to *FOXP2*, expression of *FOXP1* is linked to CNS development and organogenesis (Ferland et al., 2003; Tamura et al., 2003; Jepsen et al., 2008). Moreover, specific mutations and altered *FOXP1* expression levels were found in patients with general cognitive dysfunctions, including intellectual disability and autism spectrum disorders, along with speech related impairments (Hamdan et al., 2010; Horn et al., 2010; Bacon and Rappold, 2012; Chien et al., 2013; Le Fevre et al., 2013; Tsang et al., 2013).

The avian homologs of the *FoxP* transcription factors appear to regulate neural development and plasticity underlying vocal learning abilities in songbirds and possibly other avian vocal learners. *FoxP2* and *FoxP1* show overlapping expression in the basal ganglia of both songbirds and parrots, including a striatal subregion (Area X in songbirds, magnocellular nucleus of the medial striatum or MMSt in budgerigars) that is necessary for vocal learning in both species (Haesler et al., 2004; Teramitsu et al., 2004). In zebra finches (*Taeniopygia guttata*), *FoxP2* expression in Area X peaks late during sensory motor learning, which suggests a positive association with long-term behavioral consolidation (Haesler et al., 2004). Furthermore, during juvenile sensorimotor learning and adulthood in zebra finches, levels of *FoxP2* mRNA in the striatal vocal control region decrease as birds produce a variable "practice" song that is thought to facilitate vocal motor learning (Teramitsu and White, 2006; Teramitsu et al., 2010). The extent of *FoxP2* mRNA and protein downregulation in the striatal vocal control nucleus is related to the amount of singing (Teramitsu and White, 2006; Miller et al., 2008) and associated with coregulation of thousands of genes (Hilliard et al., 2012). Knockdown of *FoxP2* expression in Area X of zebra finches at the onset of sensorimotor learning and continuing into adulthood or during adulthood only resulted in poor learning (Haesler et al., 2007; Murugan et al., 2013), decreased dendritic spine density (Schulz et al., 2010), and abolished dopaminergic (D1R) modulation of vocal variability (Murugan et al., 2013). These investigations in songbirds suggest that *FoxP2* regulates transcription that is associated with structural changes in the basal ganglia that generate vocal variability. FoxP1 in the adult zebra finch brain circuit for vocal control is thought to be involved in the formation of circuits for learned vocal control since its expression closely matches this circuit's well-known sexual dimorphism (Haesler et al., 2004; Teramitsu et al., 2004).

Zebra finch males are close-ended vocal learners in which males learn to sing during an early-life critical period and then lose that ability and cannot learn new vocal patterns in adulthood (Zann, 1996). In contrast, budgerigars (*Melopsittacus undulatus)*, are a small parrot that like humans (Ellis, 1994), are open-ended learners that are capable of using auditory feedback to learn new vocalizations throughout adult life (Brittan-Powell et al., 1997; Heaton and Brauth, 1999; Heaton et al., 1999; Hile and Striedter, 2000; Dahlin et al., 2014). Moreover, humans, songbirds, and parrots are thought to share a homologous basal ganglia substrate for vocal learning [Fig. 1(A,B); Hall et al., 1999; Jarvis and Mello, 2000; Petkov and Jarvis, 2012].

Behavioral phenotype differences between close-ended vocal learners and open-ended vocal learners could arise from neurogenetic differences in their basal ganglia center for vocal control. Here, we examine developmental patterns of *FoxP2* and *FoxP1* in budgerigars to test whether the expression of these genes is developmentally regulated and whether these patterns differ from those found in the zebra finch. We used *in situ* hybridization and immunohistochemistry to detect *FoxP2* and *FoxP1* mRNA and protein expression in the MMSt of juvenile and adult budgerigars of both sexes during 4 distinct developmental periods that coincide with these distinct behavioral stages: (1) at the start of their development of "transitional" immature calls beginning ~20 days posthatch; (2) shortly after fledging ~35 days, around which time these birds produce their first adult-like contact call; (3) ~60 days when these birds typically join their first social group; and (4) during adulthood, a period during which birds continually learn novel group specific calls [Fig. 1(C)].

METHODS

Animals and Acoustic Recording

The budgerigars, used for this study were from our breeding colony at NMSU and maintained on a natural light dark cycle, with ad libitum access to food and water. We used a total of 45 budgerigars, 33 at three developmental timepoints, 11 each at 20, 35, and 60 days old (D). In addition, we used 12 adult male and female budgerigars that were all >D120. Developmental studies show that motor learning begins ~D20, when nonlearned begging calls transition

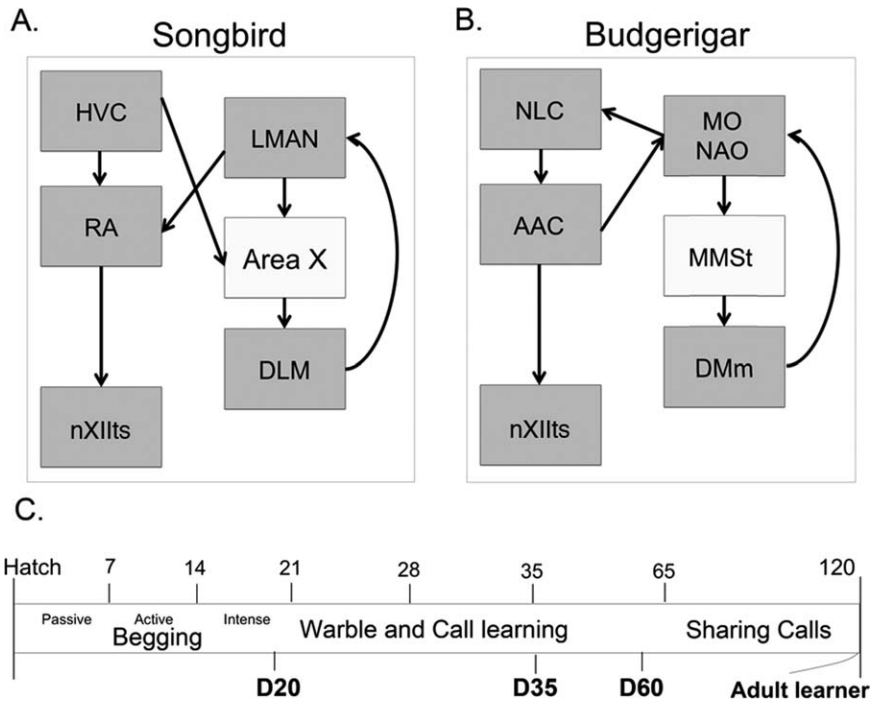

Figure 1 Shown here is a general schematic of interconnected vocal control nuclei in (A) the songbird brain and (B) the budgerigar brain (Nottebohm et al., 1976; Striedter, 1994). Area X and MMSt in the basal ganglia are part of a cortico-basal ganglia-thalamo-cortical loop important for learning acoustic gestures (Petkov and Jarvis, 2012). The songbird and budgerigar CNS via nXIIts projects to the syringeal muscles that produce sound. (C) Vocal development of budgerigars begins after hatch with food begging calls followed by a transitional period around D20 when socially learned vocalizations first appear. Adult like vocalizations begin to emerge around D35, and at around D60 birds begin to join social groups and imitate conspecifics (Brittan-Powell et al., 1997; Hall et al., 1999). Vocal learning in new social groups occurs frequently in adults. The present study used birds that were isolated and recorded at the ages shown in bold, D20, D35, D60, and adults D>120. Abbreviations: Songbird: Area X and HVC are used as proper names: DLM, medial portion of the dorsolateral thalamic nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the archipallium. Budgerigar: AAc, central nucleus of the anterior arcopallium; MO, oval nucleus of the mesopallium; MMSt, magnocellular nucleus of the medial striatum; NAO, oval nucleus of the anterior nidopallium; DMm magnocellular nucleus of the dorsomedial thalamus; nXIIts, tracheosyringeal motor nucleus, a portion of the twelve (hypoglossal) nucleus.

successively into adult-like contact calls for the first time; this is typically completed 2–3 weeks later (Brittan-Powell et al., 1997; Hall et al., 1999). The neural control centers for vocal mimicry also appear as distinct nuclei starting around D20 (Heaton and Brauth, 1999). All of the birds in each age group were used for in situ hybridization, and 5–6 birds from these age groups were used for immunohistochemistry. All budgerigars were euthanized within a 2-day window of reaching the targeted developmental time periods. Of these birds, 18 juveniles were male, 14 juveniles were female, 6 adults were male and 6 adults were female, as determined by sex genotyping using PCR (Pease et al., 2012). Sex genotyping was inconclusive for 1 D60 bird. The birds were individually housed overnight in lab-constructed sound attenuation chambers, the following morning recorded for 2 h after lights-on at 6 a.m., and then immediately euthanized. These birds were acoustically recorded using microphones linked to an 8 channel mixer with digital output to a Windows 7 based PC running Sound Analysis Pro Software (Tchernichovski et al., 2000). The computer digitally captured continuous recordings of all sound events from the chambers The captured files were then visually inspected using spectrogram analysis, filtered for bird vocalization events, and quantified using Raven Pro software (Cornell Ornithology Lab, Ithaca, NY). Within the 2-h period of observation most birds in the D35, D60, and D120 groups did not vocalize and no bird in these groups had more than 2 short vocalization events (warbles or contact calls). Some of the D20 birds did vocalize. Five of these birds produced 1381, 322, 210, 95, and 11

vocalizations each, which all contained a mix of warble and call-like elements. We noted that the call like elements resembled the "transitional" patterned food begging calls found in budgerigars at the earliest stage of sensory-motor learning.

Tissue Preparation

Immediately after being acoustically recorded for at least 2 hours in the morning, the birds were weighed and then euthanized via isoflurane inhalation. The whole brain was extracted within 5 min and flash frozen using liquid nitrogen. The brain was then stored at −80°C and later sectioned at −20°C using a Leica CM1850 cryostat microtome (Leica Microsystems, Buffalo Grove, IL). Sections of 20 μm were then mounted onto positively charged glass microscope slides (Fisher Scientific, Waltham, MA, #12–550-20) in seven replicate series. One series was stained with thionin to enable identification of neuroanatomical structures and to help guide localization of the protein expression patterns for *FoxP1* and *FoxP2* in the MMSt (magnocellular nucleus of the medial striatum) while referencing the budgerigar brain atlas (http://www.brauthlab.umd.edu/atlas.htm). Briefly, this staining procedure involved a series of 1–2 min slide baths in decreasing concentrations of ethanol, 1.5 min in thionin stain, and a water rinse followed by 2 min baths in increasing concentrations of ethanol. Slides were then dipped in xylenes (Sigma-Aldrich, St. Louis, MO, #534056) for 10 min, coverslipped with DPX Mountant (Sigma-Aldrich, St. Louis, MO, #06522) and left to dry overnight. The remaining slides were stored at −80°C until analyzed further using i*n situ* hybridization and immunohistochemistry.

In Situ Hybridization and Analysis

In situ hybridizations were performed using riboprobes as described previously (Teramitsu et al., 2004; Chen et al., 2013). The probes were designed to hybridize to the 3′ region of zebra finch *FoxP1* and *FoxP2*. The *FoxP2* probe corresponded to bp 1870–2127 in budgerigar *FoxP2* coding sequence (GenBank# AY466101.1) and the *FoxP1* probe corresponded to 1731–2035 bp in a predicted budgerigar *FoxP1* coding sequence (NCBI RefSeq XM_005149417.1). The zebra finch *FoxP2* 3' probe and *FoxP1* 3′ probe show 98.8 and 97.4% coding sequence identity to their corresponding budgerigar *FoxP2* and *FoxP1* 3′ regions, respectively. In contrast, the *FoxP2* 3′ probe was only 63.6% identical to budgerigar *FoxP1* sequence and the *FoxP1* 3′ probe was also only 63.1% identical to budgerigar *FoxP2* at the coding sequence level. The pattern of expression we found in budgerigars with the *FoxP1* and *FoxP2* probes was consistent with those reported previously in adult parrots using full-length probes (Haesler et al., 2004). We noted that our zebra finch *FoxP1* 3′ probe sequence did not overlap with a different zebra finch *FoxP1* 3′ probe (Wada et al., 2006) that did not generate a specific hybridization signal in budgerigar brain. Further, specificity of the antisense probes was determined by the absence of a hybridization signal with the corresponding sense probes. To generate probes, the *FoxP* cDNA fragments were amplified by PCR from the pCR 4-TOPO vector (Invitrogen, Carlsbad, CA) using m13F and reverse primers for subsequent *in vitro* translation with T3 (antisense probes) or T7 (sense probes) RNA polymerase. To hybridize these probes, thaw-mounted 20 μm frozen sections were air-dried at RT for 1 h, quickly rinsed in 1X phosphate buffered saline (PBS), and postfixed with 4% paraformaldehyde, pH 7.4. Following acetylation and dehydration, the tissue slides were prehybridized for 1 h in an oven at 55°C while coverslipped in solution containing 50% formamide, 1X Denhardt's, 0.2% SDS, 10 mM EDTA (pH 8.0), 200 mM Tris (pH = 7.8), 1.5 mM NaCl, 250 μg/ml tRNA, and 25 μg/ml poly A. Slides were then hybridized at 55°C overnight in a similar solution that included 10% dextran sulfate and [33P]UTP-labeled RNA probes. Equivalent 8×10^6 counts per minute of riboprobes were loaded on each slide for both *FoxP1* and *FoxP2*. Posthybridization slides were decoverslipped and rinsed at 55°C for 15 min in 4X SSC, washed at RT for 2 h in 2X SSC, treated with RNase A (Sigma) for 30 min, washed twice in 2X SSC for 15 min each at 37°C, and finally washed for 1 h in 0.25X SSC at 60°C before dehydration in graded ethanols, air-drying and exposure to autoradiographic film (BioMax MR film; Eastman Kodak, Rochester, NY). Slides were exposed to autoradiographic film for ∼1 or 2 weeks for *FoxP1* or *FoxP2*, respectively. Developed films were digitized at 600 dpi using a CanoScan 4400P scanner and software (Canon, Ōta, Tokyo, Japan) controlled by a PC running Windows. Film images produced by the 33P decay emissions of the probes were consistent in consecutive tissue sections and similar expression patterns were observed in multiple birds, confirming probe specificity. Adobe Photoshop (Adobe Systems, San Jose, CA) was used to measure mean pixel intensities of the areas of interest after saving the digital image in a tiff format, which allowed for 8 bits per sampled pixel or 256 different shades of gray to be analyzed. These values for two different sections of each brain region in each hemisphere for each animal were imported into JMP software for statistical analysis (SAS Institute, Cary, NC). Mesopallial measurements included both dorsal and ventral regions. One-way ANOVAs and Tukey-Kramer HSD were used to analyze group data.

Immunohistochemistry

Fresh-frozen brain sections containing MMSt and adjoining striatum on microscope slides were used to measure FoxP2 and FoxP1 protein expression. Brain sections were first submerged in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, #P6148) for 5 min and rinsed with 1X PBS three times for 5 min each. To block nonspecific binding, tissue was incubated in PBST (1X PBS with 0.3% Triton X-100) with 5% donkey serum (Jackson Immuno, West Grove, PA, #107175) for 1 h at 4°C. Tissue slides were incubated overnight at 4°C in a PBST/1% donkey serum solution containing the polyclonal goat antibody to FoxP2 (Santa Cruz,

Table 1 Mean Optical Density Values of *FoxP* mRNA Expression in Budgerigar Brains Normalized to Background

	D20 *FoxP1*		D20 *FoxP2*		D35 *FoxP1*		D35 *FoxP2*		D60 *FoxP1*		D60 *FoxP2*		Adult *FoxP1*		Adult *FoxP2*	
Brain Region	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Hyperpallium	57	58	36	37	71	74	52	46	77	80	57	55	78	77	61	68
Mesopallium	119	128	48	47	143	146	71	77	147	146	78	78	152	158	99	103
Nidopallium	49	54	50	42	49	49	42	33	60	53	48	47	59	54	53	60
Basorostral pallial nucleus	18	24	33	39	21	22	24	18	24	19	28	31	22	28	18	27
Medial striatum (MSt)	106	93	92	114	118	122	100	118	107	95	127	110	111	119	91	102
Magnocellular nucleus of the medial striatum (MMSt)	100	87	64	78	130	133	90	101	127	118	104	94	130	139	70	79

Dallas, TX, #sc-21069) at 1:1000, and the polyclonal rabbit antibody to FoxP1 (Abcam, Cambridge, MA, #ab16645) at 1:500. Primary antibody was omitted for negative controls. Target specificity of the primary antibody for FoxP2 had been previously verified in zebra finches (Soderstrom and Luo, 2010), while the primary antibody for FoxP1 was previously verified in rats (Bowers et al., 2013). We note that the staining pattern for FoxP2 closely matched that for FoxP1; overlapping confocal images show coexpression of FoxP2 and FoxP1 (see Fig. 3). Following overnight incubation at 4°C, sections were washed three times for 5 min each with 1X PBS, then incubated for 2 hours at room temperature in PBST/1% donkey serum and 1:200 dilutions of two fluorescence-tagged secondary antibodies (Life Technologies, Carlsbad, CA) against goat or rabbit IgG, each with distinct excitation spectra (AlexaFluor 488 nm to detect FoxP2, Alexa Fluor 594 nm to detect FoxP1). Slides were then washed with 1X PBS 3 times for 5 min each and coverslipped using Vectashield with DAPI (excited by 405 nm; Vector, Burlingame, CA, #H-1200) as a counterstain. Slides were stored overnight at room temperature before confocal imaging.

Confocal Microscopy and Quantification

Fluorescent images of protein expression after immunohistochemistry were captured using a Leica TCS SP5 II Broadband Confocal microscope (Leica, Solms Germany). Cytoarchitectural boundaries were determined using the adjacent thionin stained and FoxP1 and FoxP2 *in situ* hybridized slides. Coronal sections were imaged with at 40X. Optimal beam settings were used for each channel (405 nm for DAPI, 594 nm for FoxP1, 488 nm for FoxP2). For each channel, images of three different tissue sections containing the same brain regions (MMSt and the adjoining striatum) were taken for both brain hemispheres of each animal. These confocal images were converted to an 8-bit gray scale, threshold was manually adjusted, and the image was then made into a binary file. Outliers with a radius of <3 pixels were removed and cell counts were automatically obtained and manually checked using ImageJ software (NIH, Bethesda, MD). The values obtained from cells counts for four brain sections (two from each hemisphere) of each the MMSt and adjoining medial striatum (MSt) were recorded. All FoxP1 and FoxP2 counts were normalized by DAPI to control for varying cell densities. The counts were then averaged for each individual bird. These individual averages were then used to calculate the MMSt/adjoining medial striatum ratio for each animal. The ratios of FoxP1 and FoxP2 expression passed Shapiro-Wilk normality tests, and were analyzed further using a one-way ANOVA with age group as a fixed factor, followed by a *post hoc* pairwise comparison (Tukey-Kramer HSD). JMP software (SAS Institute, Cary, NC) was used for all statistical analyses.

RESULTS

FoxP2 mRNA and Protein Expression

FoxP2 mRNA expression appeared to be consistently elevated in the medial striatum (MSt) compared to the hyperpallium and nidopallium across all age groups [Table 1; Fig. 2(B,C)]. Moreover, mesopallial expression of *FoxP2* mRNA appeared to increase with age. Juvenile D20 animals showed low mesopallial expression levels similar to that in the nidopallium, while adults ($D > 120$) had higher mesopallial expression similar to that found in the striatum at this age [Fig. 2(A–C)]. Further analysis of *FoxP2* mRNA expression in the MSt revealed comparably high levels across all age groups [Fig. 3(A)]. In the MMSt, *FoxP2* mRNA expression varied across development (ANOVA, $F(3,41) = 8.98$, $p < 0.001$). MMSt *FoxP2* mRNA expression was low at D20 and in adults compared to D35 ($p = 0.003$) and D60 ($p < 0.001$) [Fig. 3(A)]. Although *FoxP2* mRNA expression in the MMSt was highest at D35 and D60, the ratio of *FoxP2* mRNA expression in the MMSt expression

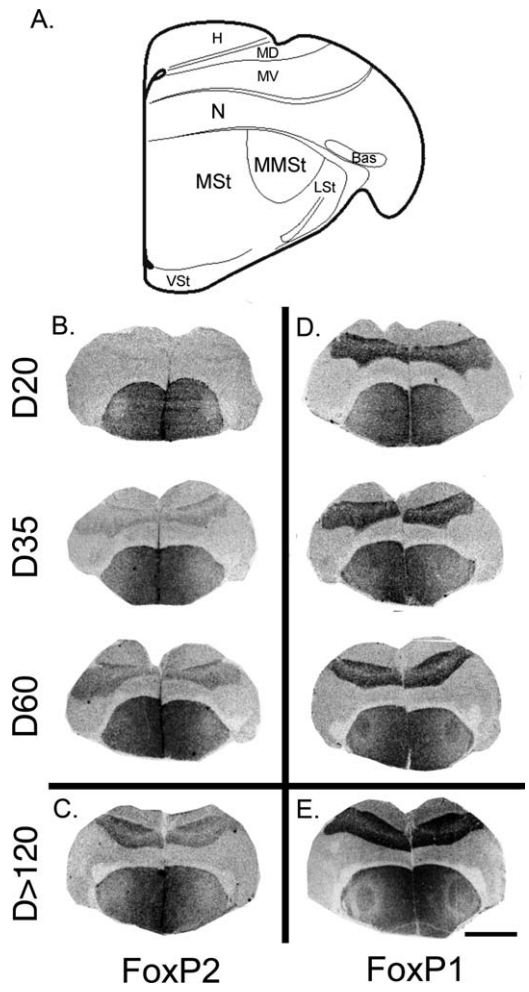

Figure 2 A. Location of the MMSt and adjoining striatum (MSt) in a schematic section from the budgerigar brain atlas at http://www.brauthlab.umd.edu/atlas.htm (for nomenclature see Jarvis et al., 2013). (B) *In situ* hybridized *FoxP2* mRNA in which the MMSt can be found at (B) D20, D35, D60, and (C) in adults, D>120 (all birds are male). Sections of similar male brains show *in situ* hybridized *FoxP1* mRNA in (D) and (E). "*" denotes the adjoining medial striatum where gene expression measurements were obtained. Scale bar in (E) = 4 mm. Abbreviations: H, Hyperpallium; MD, dorsal mesopallium; MV, ventral mesopallium; N, Nidopallium; Bas, Basorostral pallial nucleus; MMSt, Magnocellular nucleus of the medial striatum; MSt, Medial striatum; LSt, Lateral striatum; VSt, Ventral striatum.

relative to MSt was below 1 across all age groups [Fig. 3(C)]. We also found group differences in the *FoxP2* MMSt/MSt expression ratio [ANOVA, $F(3,41) = 15.75$, $p < 0.001$], with a lower MMSt/MSt ratio found at D20 compared to D35 ($p < 0.001$) and D60 ($p < 0.001$). The *FoxP2* MMSt/MSt ratio was also lower in adults compared to D35 ($p = 0.001$). Some D20 birds produced immature vocalizations within the 2-h period before sacrifice ($n = 5$ of 11), and although the *FoxP2* MMSt/MSt ratio at D20 negatively correlated with the amount of vocal production, this relationship only approached significance (Spearman $p = -0.616$, $p = 0.057$). Sex differences in *FoxP2* mRNA in the MMSt/MSt [ANOVA, $F(1) > 0.01$, $p = 0.984$] and its interaction with age ($F(3) = 0.462$, $p = 0.710$) were not significant.

We next evaluated whether the proportion of cells expressing FoxP2 protein also was reduced in the MMSt during development and adulthood [Figs. 4 and 5]. FoxP2 protein expression in the MSt and MMSt across age groups was comparable to that of *FoxP2* mRNA. However, there were differences between age groups in FoxP2 protein expression in the MSt [ANOVA, $F(3,19)$ Ratio = 5.08, $p = 0.009$; Fig. 5(A)]. MSt expression in adults was significantly higher than that of both D20 ($p = 0.008$) and D60 ($p = 0.040$) birds. A one-way ANOVA of protein levels in MMSt with age group as a fixed factor approached significance [$F(3,19) = 2.84$, $p = 0.065$]. *Post hoc* tests showed FoxP2 protein expression in the MMSt at D20 was significantly lower than that from adults ($p = 0.043$). A direct examination of the degree of downregulation in the MMSt using the ratio of FoxP2 protein expression in the MMSt/MSt found no significant differences between the ratios at each age group [ANOVA, $F(3,19) = 0.067$, $p = 0.580$; Fig. 5(C)]. Thus, we find that similar to *FoxP2* mRNA, expression of FoxP2 protein in the MMSt remains lower than that in the surrounding medial striatum throughout periods of learning in both juveniles and adults, albeit with a slight increase in overall protein levels as birds reach adulthood. A main effect for sex on MMSt/MSt FoxP2 protein expression and its interaction with age was not significant [ANOVA, $F(1) = 0.072$, $p = 0.792$, and $F(2) = 0.229$, $p = 0.798$].

FoxP1 mRNA and Protein Expression

We observed increased *FoxP1* mRNA expression in the mesopallium and striatum relative to nidopallial and hyperpallial brain regions across all age groups [Table 1; Fig. 2(D,E)]. Although *FoxP1* mRNA expression in the MSt appeared to increase over development, these differences did not reach statistical significance [ANOVA, $F(3,41) = 1.43$, $p = 0.246$; Fig. 3(B)]. However *FoxP1* mRNA expression in the MMSt was significantly different across the age groups [ANOVA, $F(3,41) = 3.23$, $p = 0.320$; Fig. 3(B)]. D20 birds had significantly less expression than birds at D35 ($p = 0.048$) and D>120 ($p = 0.046$). Moreover, the ratio of *FoxP1* mRNA expression in the MMSt relative to the MSt was significantly lower at

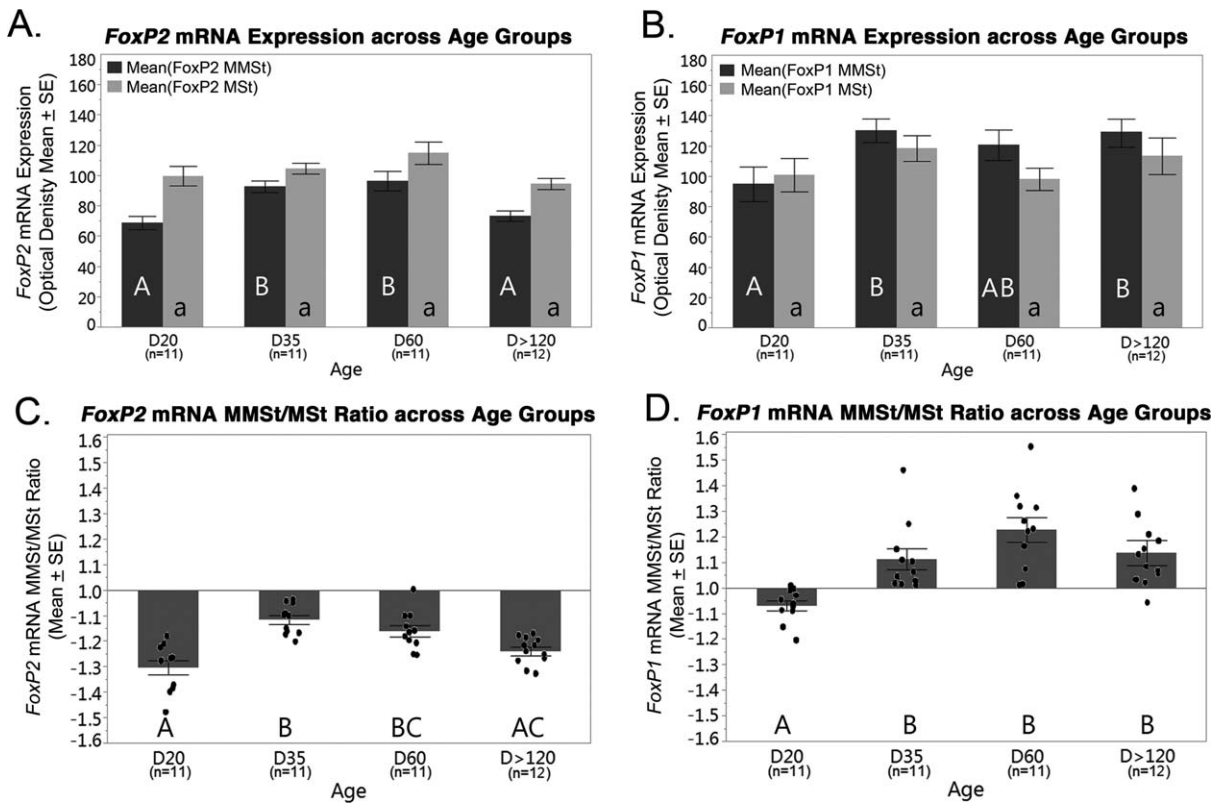

Figure 3 (A) *FoxP2* mRNA expression in the MMSt (dark gray) and MSt (light gray) across age groups. No significant differences were found between groups in the MSt ($p < 0.05$). (B) *FoxP1* mRNA expression in the MMSt and MSt across age groups. No significant differences were found between age groups for both the MSt. *FoxP1* mRNA expression in the MMSt was significantly lower at D20 compared to D35 and D>120. (C, D) *FoxP2* and *FoxP1* mRNA expression ratios (MMSt/MSt) show significant differences between age groups ($p < 0.05$). Points in (C) and (D) represent individual birds. For all graphs, significant mRNA expression differences in the MMSt, MSt, (A, B, respectively) or a ratio thereof (C, D), between the four age groups is denoted with different letters. Bars with different letters are significantly different from each other ($p < 0.05$). The letter case is used to denote significant differences between age groups separately for the MMSt (uppercase) and MSt (lowercase). Error bars = SE.

D20 compared to all other age groups ($p < 0.001$–0.007). *FoxP1* mRNA expression showed no relationship to vocalizing in D20 birds (Spearman $p = 0.058$, $p = 0.873$). A main effect of sex and its interaction with age for MMSt/MSt *FoxP1* mRNA expression was also not significant [ANOVA, $F(1) = 0.004$, $p = 0.286$, and $F(3) = 1.096$, $p = 0.363$].

Comparable differences were found in FoxP1 protein expression between age groups. Significant differences were found in FoxP1 protein expression between age groups in the MSt [ANOVA, $F(3, 19) = 10.19$, $p < 0.001$], where adult birds had higher expression compared to D60 ($p = 0.026$), D35 ($p = 0.024$), and D20 birds ($p < 0.001$). Furthermore, there was an age group difference in FoxP1 protein expression in the MMSt [ANOVA, $F(3,19)$ Ratio = 12.64, $p < 0.001$], and *post hoc* tests revealed that FoxP1 expression in D20 birds was significantly lower than that of D35 ($p = 0.010$), D60 ($p = 0.003$) and adult birds ($p < 0.001$). D35, D60 and adult birds did not differ from one another [Fig. 5(B)]. A ratio of FoxP1 expression in the MMSt compared to the MSt showed the degree of FoxP1 protein expression was also different between the age groups [ANOVA, $F(3,19) = 7.96$, $p = 0.001$; Fig. 5(D)]. The ratios of D20 birds were significantly lower than that of D35 ($p = 0.010$), D60 ($p = 0.001$) and adults birds ($p = 0.038$). Thus, in MMSt, FoxP1 mRNA and protein expression appears to increase after D20. We did not find a significant main effect of sex on MMSt/MSt FoxP1 protein expression [ANOVA, $F(1) = 0.083$, $p = 0.778$], and its interaction with age was also not significant, and [$F(2) = 0.557$, $p = 0.587$].

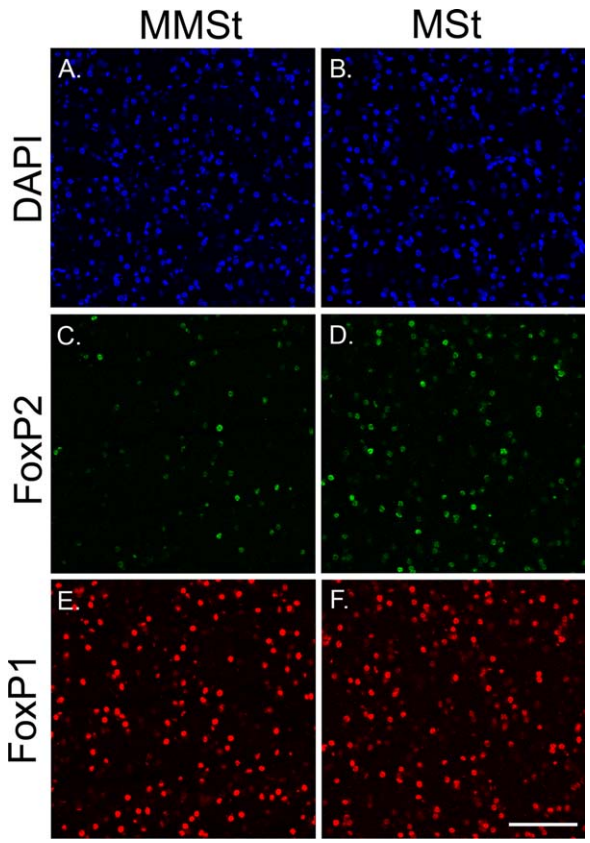

Figure 4 Confocal images taken with a 40X objective to detect FoxP2 and FoxP1 protein within the MSt and MMSt. Example images are shown from a female animal at D35. From top to bottom: (A, B) DAPI stained cells in 405 nm within the MMSt and MSt; (C, D) FoxP2 protein expressing neurons in 488 nm within the MMSt and MSt; (E, F) FoxP1 expressing neurons in 594 nm within the MMSt and MSt; Scale bar in $H = 50$ μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FoxP2 and FoxP1 Coexpression in the MMSt

As expected, most of the cells expressing FoxP1 protein in the MMSt and MSt overlapped with FoxP2 protein expressing cells (Fig. 6). To further explore the possibility of a potential interaction between FoxP2 and FoxP1 in the MMSt we analyzed an expression ratio of FoxP2/FoxP1 protein and found a significant difference across age groups [ANOVA, $F(3, 19) = 6.429$, $p = 0.004$). In D20 bird, the FoxP2/FoxP1 protein expression ratio was significantly higher compared to that ratio in birds at D60 ($p = 0.004$) and D120 ($p = 0.026$). Although the FoxP2/FoxP1 ratio was highest at D20, it remained <1 across the age groups; mean expression was 0.47, 0.39, 0.30, and 0.35 for D20–D$>$120, respectively (Fig. 7).

DISCUSSION

We investigated developmental *FoxP2* and *FoxP1* mRNA and protein expression within a basal ganglia vocal learning nucleus in the budgerigar, a parrot species with open-ended vocal learning. Our results suggest that these genes play a conserved role for vocal learning in evolutionarily diverse species. Moreover, the developmental *FoxP2* expression pattern we observe here in budgerigars differs from that found during zebra finch development, and is consistent with persistent vocal plasticity in budgerigars. The developmental *FoxP1* expression we observed in the MMSt provides support for its role, as previously suggested, in the development of vocal motor neural circuitry. Thus, the divergent developmental expression patterns we find for *FoxP2* and *FoxP1* suggests the possibility that these genes may have distinct contributions to the processes underlying vocal ontogeny in species with vocal learning.

Functional Implications of FoxP2 Expression in Budgerigars

Previous research in adult and juvenile zebra finches found that downregulation of *FoxP2* in Area X is related to the production of undirected songs that lack a particular social target (Teramitsu and White, 2006; Miller et al., 2008); such singing is understood to be a form of vocal practice (Olveczky et al., 2005). This singing-dependent downregulation of *FoxP2* is consistent with a postorganizational role for *FoxP2* in the modulation of neural vocal motor circuits for learning. We could not determine whether learned vocal production in fledgling juveniles or adults downregulates *FoxP2* expression, as these budgerigars rarely vocalized during the 2-hour period of observation prior to euthanization. Moreover, the relationship between vocal practice and FoxP2 protein expression in D20 birds could not be fully explored here due to a lack of statistical power as only 3 of 6 D20 birds vocalized, and only 2 of these 3 D20 birds produced $>$11 vocalizations within the 2 h period before sacrifice. Nonetheless, we did find a trend in D20 birds toward lower *FoxP2* mRNA expression as vocal production increased, consistent with a role for *FoxP2* in modulating plasticity in the budgerigar.

Downregulation of *FoxP2* mRNA and protein expression within the MMSt relative to the MSt was seen in both juvenile and adult budgerigars, and both are capable of learning new vocal patterns. We hypothesize that the persistent low level *FoxP2* expression in the MMSt maintains this region in a state that allows for persistent plasticity; thus permitting

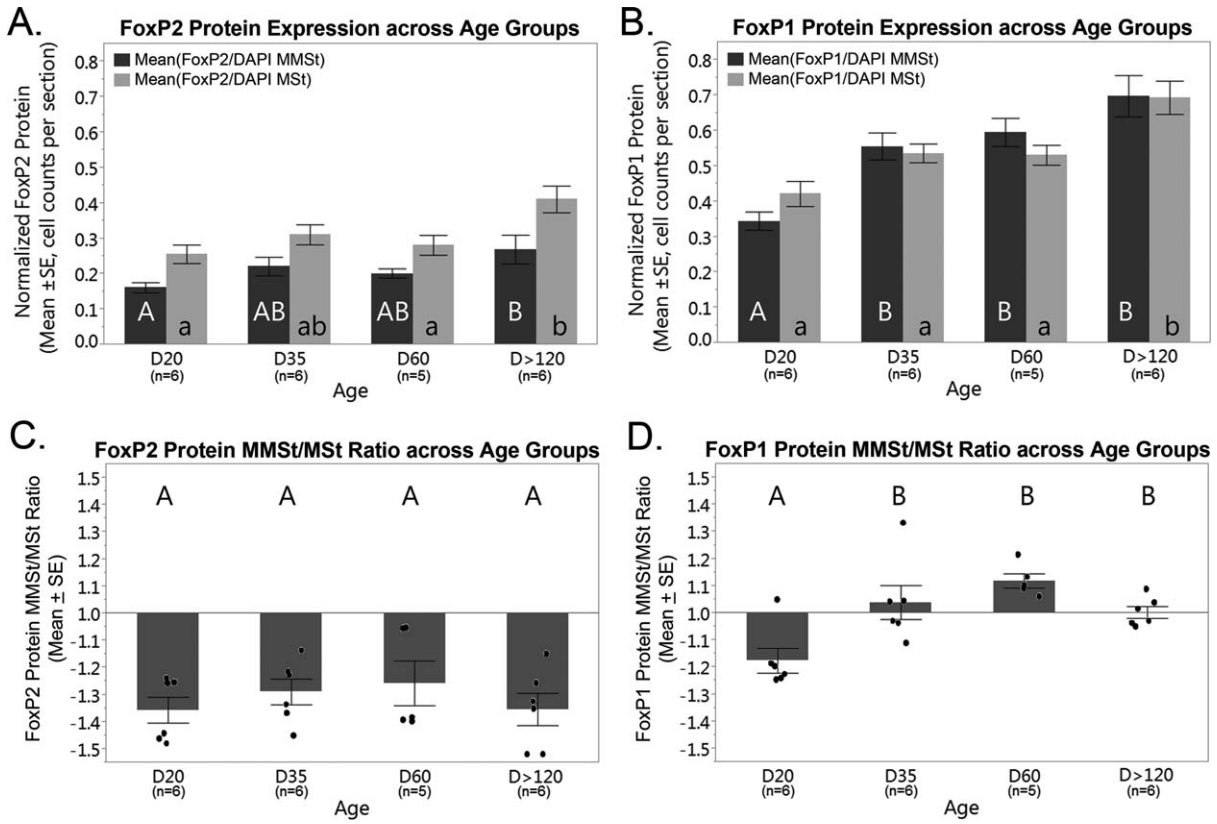

Figure 5 (A) DAPI normalized FoxP2 protein expression across age groups in the MMSt (dark gray) and MSt (light gray). Significant differences between age groups in the MMSt and MSt. are shown with bars not connected by the same letter ($p < 0.05$). (B) DAPI normalized FoxP1 protein expression across age groups in the MMSt (dark gray) and MSt (light gray) show significant differences in D20 and adult birds using bars with different letters ($p < 0.05$). (C) FoxP2 and (D) FoxP1 MMSt/MSt protein expression ratios across age groups. No significant differences in FoxP2 expression ratios were found between groups. Ratios were <1 for all age groups. (D) MMSt/MSt FoxP1 protein expression ratios were significantly lower at D20 ($p < 0.05$). Points in (C) and (D) represent individual birds. Error bars = SE.

mature vocal learning circuits to encode the necessary motor patterns to produce learned vocalizations. If true, then perhaps the extent of vocal learning in budgerigars correlates with the low level of *FoxP2* expression in the MMSt. Although it is beyond the scope of this study, we are pursing the question of whether vocal modification in the budgerigar may be influenced by social contexts such as group membership status (novel or stable), and its potential to influence *FoxP2* expression. We should point out here that we are not proposing that *FoxP2* is not necessary for vocal learning. Rather, our results are consistent with results from zebra finches showing that downregulation of *FoxP2* during undirected singing is associated with greater plasticity in song (Teramitsu and White, 2006), and that *FoxP2* is a transcriptional regulator of a suite of other genes in their Area X (Hilliard et al., 2012). Together, these results suggest the hypothesis that downregulation of *FoxP2* in the budgerigar MMSt is a key regulatory event that allows for the vocal plasticity seen in both juvenile and adult of this species.

Previous studies in budgerigars suggested that *FoxP2* expression would be elevated in the MMSt relative to the adjoining striatum during early vocal learning periods and then decline as birds entered adulthood, as was found in developing zebra finches (Teramitsu et al., 2004; Haesler et al., 2004). Such regulation in zebra finches suggested a role for *FoxP2* in the formation of circuits for learned vocalizations. Developmental expression studies in human brain also support a role for *FoxP2* in the development of motor-related circuits (Lai et al., 2003). One explanation for the developmental *FoxP2* expression differences between zebra finches and budgerigars, suggests an alternative, though not mutually exclusive, role for *FoxP2*. That is, the upregulation of *FoxP2* in D35 and D50 zebra finches may be related to long-term consolidation within the neural circuits

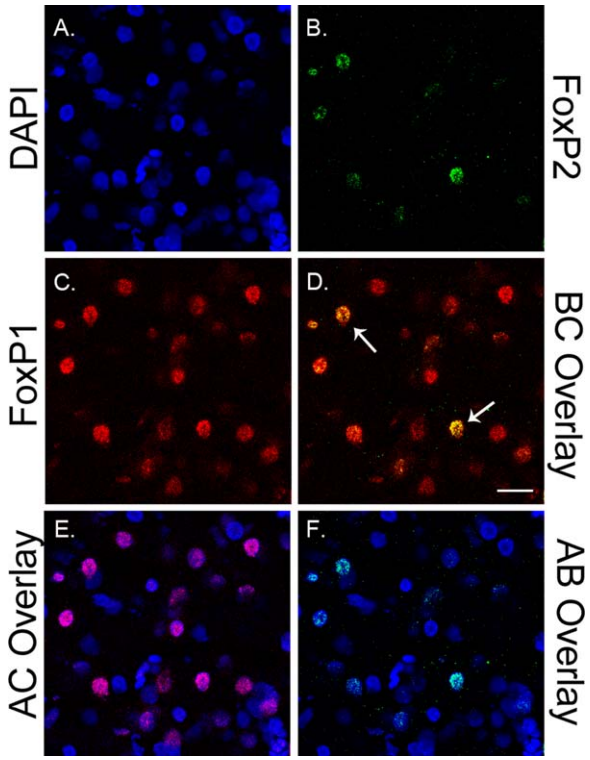

Figure 6 Representative confocal images taken with a 63X objective show overlapping FoxP2 and FoxP1 protein expression within the MMSt of a female animal at D20. A, B and C show DAPI, FoxP2 and FoxP1 labeled cells. D, E, and F show different combinations of overlapping expression between A, B, and C. In (D) arrows indicate example cells coexpressing FoxP2 and FoxP1; scale bar = 20 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

underlying a specific behavioral performance, for example, the crystallization of a stereotyped song. We found that *FoxP2* mRNA expression was increased at D35 and D60 compared to D20 and adults, but this expression did not equal or exceed the surrounding striatum at any developmental timepoint we observed. As budgerigars are open-ended vocal learners they may experience this crystallization to a lesser degree; thus there may be no point in their development during which *FoxP2* expression is upregulated in the MMSt relative to the MSt. This hypothesis is consistent with a finding in mice, showing that *FoxP2* regulates gene expression crucial for modulating synapse formation (Sia et al., 2013).

The Role of FoxP1 Expression and Interactions with FoxP2

The *FoxP1* mRNA and protein expression ratio for the MMSt relative to the MSt was <1 in the D20 birds, but increased significantly in D35, D60, and adult birds, showing that FoxP1 in the MMSt is being upregulated relative to the adjoining striatum as birds matured. This finding is similar to those found in vocal learning songbirds, where *FoxP1* mRNA expression in zebra finches was also upregulated in Area X relative to the MSt in juvenile and adult male birds. However in zebra finches, Area X/outlying striatum *FoxP1* mRNA expression appeared to peak in younger (D35) birds, whereas in budgerigars, this *FoxP1* mRNA and protein ratio was highest at D60. This expression pattern was unlike that of *FoxP2* and suggests that upregulation of *FoxP1* expression in the MMSt (or Area X) plays a role in the development and adult function of basal ganglia circuitry that is required for vocal plasticity. A similar role for *FoxP1* has been described in developing mouse brain (Ferland et al., 2003). Differences in the timing of peak *FoxP1* expression in the motor circuitry could reflect differences in the corresponding rates of maturation in different species.

FoxP1 may interact with *FoxP2* and other genes in the FoxP family to regulate genes involved in the development and maintenance of vocal learning circuits. *FoxP2* and *FoxP1* act in cooperation to regulate development of mouse lung and esophageal tissues (Shu et al., 2007) so perhaps these genes cooperate to establish and modify connections in the brain as well. In songbirds, *FoxP2* and *FoxP1* are coexpressed in the striatum (Chen et al., 2013). Here we found in budgerigars overlapping FoxP2 and FoxP1 protein expression in the same MMSt cells and that the ratio of MMSt FoxP2/FoxP1 protein expression was highest in nestling birds, significantly decreasing as birds aged. The deceasing ratio was due to a prodigious

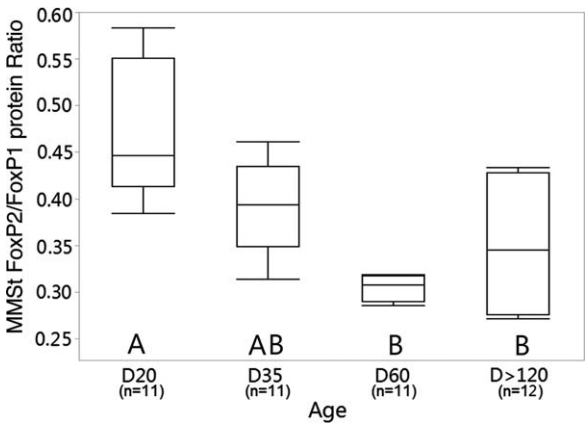

Figure 7 Box plots show a ratio of FoxP2/FoxP1 protein expression in the MMSt. FoxP expression is DAPI normalized. Top and bottom whisker lines, and box line represent maximum, minimum and median values, respectively.

increase in FoxP1 protein expression as birds aged. These results suggest that a primary role for *FoxP1* expression, during early development and not later, could be interacting with *FoxP2* for the cooperative regulation of gene expression.

Lack of Sex Differences but a Mesopallial Increase in FoxP Expression

Unlike in the zebra finch, where *FoxP2* and *FoxP1* mRNA expression is sexually dimorphic, we detected no differences in *FoxP2* and *FoxP1* gene expression between male and female budgerigars. This result is consistent with the vocal learning behavior observed in this species, as both sexes have been shown to learn new vocalizations, even as adults (Farabaugh and Dooling, 1996; Hile and Striedter, 2000; Dahlin et al., 2014). Interestingly our data suggest a role for the FoxP genes outside of the striatum. We found a striking difference in *FoxP2* and *FoxP1* mRNA expression in the mesopallium, where expression gradually increased throughout development into adulthood, in parallel with the acquisition and increase of the vocal repertoire. Although this region of the mesopallium does not contain vocal control nuclei, in general the mesopallium is enlarged in birds with high cognitive abilities, like parrots (Lefebvre et al., 2004; Iwaniuk and Hurd, 2005; Chen et al., 2013). Cognitive complexity in birds may be dependent on mesopallial brain organization and, as our data suggests, its underlying gene activity that includes the *FoxP2* and *FoxP1* genes.

CONCLUSIONS

Vocal learning has evolved independently in various groups of birds and mammals (Petkov and Jarvis, 2012), yet the exact physiological components of this complex behavior are not completely understood. The results from these experiments shed light on some of the neuromolecular mechanisms that allow vocal learning in juvenile and adult animals, and add to the increasing evidence for common neurogenetic mechanisms underlying learned vocal communication. Further investigation of FoxP gene regulation in budgerigars is a promising route for increasing our understanding of the neurogenetic processes underlying vocal learning in both juveniles and adults.

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