Genetic Components of Vocal Learning

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ABSTRACT: Vocal learning is a rare trait. Humans depend on vocal learning to acquire spoken language, but most species that communicate acoustically have an innate repertoire of sounds that they use for information exchange. Among the few non-human species that also rely on vocal learning, songbirds have provided by far the most information for understanding this process. This article concentrates on the genetic components of vocal learning in humans and birds. We summarize the existing evidence for a genetic predisposition towards acquiring the species-specific human and avian vocal repertoires. We describe the approaches used for finding genes involved in shaping the neural circuitry required for vocal learning or in mediating the learning process itself. Special attention is given to a particular gene, *FOXP2*, which has been implicated in a human speech and language disorder. We have studied *FoxP2* in avian vocal learners and non-learners and review evidence that links both the molecule and its close homologue *FoxP1* to the development of brain regions implicated in vocal learning and to their function. *FoxP2* has a characteristic expression pattern in a brain structure uniquely associated with learned vocal communication, Area X in songbirds, or its analogue in parrots and hummingbirds. In both avian song learners and non-learners *FoxP2* expression predominates in sensory and sensory-motor circuits. These latter regions also express *FoxP2* in mammals and reptiles. We conclude that *FoxP2* is important for the building and function of brain pathways including, but not limited to, those essential for learned vocal communication.

KEYWORDS: zebra finch; seasonal; hummingbird; parrot; budgerigar; transcription factor; basal ganglia

LANGUAGE: THE BALANCE BETWEEN NATURE AND NURTURE

Human language is unique in its capacity to express infinite meaning through combining a finite number of words or signs. Also characteristic for human language is the use of vocal signals to refer to things or concepts, a feature that, despite intense scrutiny, has been found only rarely in other animals.[1] What humans do share with a select group of other animals (songbirds,[2,3] hummingbirds,[4] parrots,[5] bats,[6] whales,[7] seals,[8] and dolphins[9,10]) is the need to learn their vocal repertoire by imi-

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tation. While many other species also communicate with vocal signals, they apparently do not need to learn them.

Yet the fact that language is learned does not imply the absence of a genetic bias towards this learning. Indeed, already Darwin suspected that language acquisition is an "instinct," and since then Chomsky and colleagues have collected convincing evidence for this view.[11] One of the central arguments is that despite the plethora of different languages in the world, all of them follow an intrinsic hierarchical logic termed "universal grammar" by Chomsky.[1] This suggests the existence of a common neural "hardware" that constrains how language is built. The same "predisposed" hardware is also assumed to account for the astonishing speed, ease, and autonomy with which children master the theoretically formidable task of learning thousands of words and understanding the rules of grammar that govern how they can be combined into meaningful sentences.

LANGUAGE GENES?

What then might be the genes involved in building language-ready brains. Should we expect to find one set for "constructing" the language circuitry and others involved in mediating the actual learning? The evidence, while admittedly fragmentary, points definitely towards some generalist and perhaps some specialist genes.

Before speculating about genetic mechanisms, we review evidence for the existence of "language-specialized" neural structures. That such structures exist in humans is indisputable. Damage to the regions around the left Sylvian fissure usually leads to problems with perception and/or production of language, recall of particular classes of words, and understanding or using grammar. Often, only particular domains of language are affected, such as difficulty with recalling objects,[11,12] or fluent grammatical speech devoid of clear meaning.[13] These observations have led to the assumption that different components of language are processed in discrete brain areas (Broca, production; Wernicke, perception; etc.), but this turns out to be a rough approximation at best. Rather, language is supported by distributed neural networks connecting populations of neurons in cortical and subcortical regions throughout the brain, including basal ganglia and cerebellar pathways.[14,15] Thus, the reason that dysfunction of a particular area tends to be associated with impairment of a particular language domain could reflect that this area is itself involved in processing of that particular language domain and/or that it presents a bottleneck for information flow passing through it, from and to other areas of the brain. While this makes it less likely that there are specific genes dedicated solely to construction and function of "language regions," the fact is that some genes might also serve as bottlenecks. Without them, language does not function properly. Although their discovery might not bring us immediately closer to understanding the neural "essence" of language, it might provide us with insights into the molecular machinery involved.

The search for language genes has focused on inherited language impairments, where deficits in language are dissociable from other mental functions.[16] This approach has not been easy, for two reasons.[17,18] First, only a handful of such conditions are known. Among those are verbal apraxia (also called verbal dyspraxia), i.e., the difficulty coordinating mouth and speech movements, developmental speech delay, and stuttering. Second, honing in on mutations causally related to disease is

methodologically far easier for conditions in which a single gene causes the dysfunction than when a number of genes are involved.[19] Essentially, the association of a particular mutation with a particular phenotype is achieved by correlating the trait (i.e., language impairment) with a known DNA marker sequence. In affected individuals, the marker will segregate differently than in unaffected individuals, thus allowing geneticists to zero in on the chromosomal region that is characteristic for the affected individuals. Unfortunately, most hereditary diseases are suspected to be caused by dysfunction of more than one gene, and the elucidation of multigenic diseases has been notoriously difficult.[20]

So far, the search for genes associated with language impairments has yielded a number of linkage associations with chromosomal regions containing large numbers of genes. For instance, specific language impairment (SLI), which has an inherited component that is most evident from twin and adoption studies, is associated with regions on chromosome 7, 13, 16, and 19.[21–23] In the case of linkage to chromosome 7, analysis of the genomic DNA of three generations in the KE family, about half of whose members have impaired speech and language skills,[21] indicated that the affected gene was located among 70 genes on the long arm of chromosome 7. Discovery of the exact location of the mutation, which is inherited in a dominant manner, was facilitated by the identification and investigation of an unrelated individual that suffered from a remarkably similar language disorder and had a balanced translocation between chromosomes 7 and 5. This means that chromosomes 5 and 7 had both "broken" at a certain point along their lengths and that a piece of each had swapped places with the other. One of the breakpoints interrupted the gene *FOXP2*, which is normally located on chromosome 7. (For FoxP2 nomenclature we follow the convention proposed by the Nomenclature Committee for the Forkhead family of genes, i.e., FOXP2 in *Homo*, Foxp2 in *Mus*, and FoxP2 in all other species, proteins in roman type, genes and RNA in italics.[24]) Reexamination of chromosome 7 in the KE family revealed a point mutation in a stretch of the *FOXP2* DNA, which is crucial for the function of the protein. This mutation occurred in all affected family members but in none of the healthy individuals that were investigated. Thus the unlikely scenario described earlier, that a mutation within a single gene leads to dysfunction in a complex behavior, has arisen with the discovery that *FOXP2* is the monogenetic locus for a severe speech and language disorder.[25]

THE *FOXP2* GENE IN HUMANS

Human Behavioral Phenotype

The complex behavioral phenotype of the KE family has been extensively studied since 1990.[26,27] Individuals with the *FOXP2* mutation have difficulty in correctly articulating speech, which has been argued to be a consequence of impaired execution of sequenced movements of the orofacial musculature in general. In fact, affected members of the KE family do perform worse in executing commands like "bite your lip" than unaffected individuals, but perform normally for individual simple oral movements and limb movements, such as the use of a key or brushing one's hair.[28]

In addition, affected family members perform significantly worse than their unaffected relatives on a battery of tests that assess receptive and grammatical lan-

guage. The deficit includes the inability to correctly inflect words (i.e., change tense or number), to match sentences describing subtle relationships between objects with the corresponding pictures, and to distinguish between words and non-words. The low scores on these kind of tests are not paralleled by test scores assessing non-verbal IQ. Even though as a group, the affected individuals score slightly but significantly lower on a non-verbal IQ test than non-affected individuals, there is considerable overlap between the groups.[29–31] Because there is much less overlap in scores for the language-related tasks, it is unlikely that the deficit in language skills is simply a reflection of overall slightly impaired cognitive function.

These findings suggest that the primary deficit in the affected KE family members might reflect a disruption of the sensorimotor mechanisms mediating the selection, control, and sequencing of fine learned movements involving the mouth and face. While it seems improbable that all of the linguistic deficits are symptoms of the articulation problems, it is formally possible that they are a developmental consequence. This interpretation is also compatible with the motor theory of speech perception,[15] which posits that decoding of speech involves part of the motor-production neural machinery. Recent human studies support this idea.[32,33]

Human Structural and Functional Abnormalities

To begin to determine the neural sites that are impacted by a mutation in *FOXP2*, imaging studies were used to examine the gross anatomical morphology of the KE family brains. Brain images from unaffected family members served as the reference point for discerning changes in the affected family members' brains. Across studies, the most consistent finding was a bilateral reduction in the grey matter density of a region of the basal ganglia called the caudate nucleus.[30,34–36] The basal ganglia are composed of striatal regions (caudate and putamen) and pallidal regions (globus pallidus pars externa and pars interna) and are critical for motor planning, sequencing, and cognitive function. Thus, the reduced caudate area observed in the affected family members is generally consistent with their impaired ability to perform motor tasks involving sequential movements, but isn't specifically indicative of orofacial impairments per se.

In addition to those in basal ganglia, cortical abnormalities were observed. In regions that are critical for speech perception (the posterior superior temporal gyrus), speech production (the dorsal inferior frontal and the precentral gyrus), or semantic processing (the angular gyrus) the amount of grey matter differed between affected and unaffected family members. Affected members also had less grey matter in the ventral cerebellum.[34] While structural deficits appear to be bilateral, functional studies revealed more lateralized disturbances. Positron emission tomography activation was lower in the left sensorimotor and supplementary face and mouth region of cortex of affected family members than normal controls during the performance of word repetition tasks.[30] The same subjects showed overactivation of the left caudate nucleus and the left premotor cortex, extending into Broca's area. These latter two areas are needed to generate words fluently.

Two studies used magnetic resonance to image the brains of unaffected versus affected family members during tasks of covert and overt speech.[36,37] In both studies, the left inferior frontal gyrus and the left putamen were consistently less active in affected members. In summary, the *FOXP2* mutation leads to both structural and

functional neural deficits in a corticostriatal network that participates in speech and language. These anatomical findings fit well with the behavioral abnormalities described above. One idea is that the abnormal motor structures, which are bilateral, could represent a "core deficit" that inhibits speech production, which, according to the motor theory of speech perception,[15] could secondarily influence language and cognitive development evidenced by the functional abnormalities on the left side of the brain. As an alternative to the notion of a cascade of deficits over time, the gene could act simultaneously to influence motor, linguistic, and cognitive networks. In either case, the striking difficulty in executing orofacial movements on command, coupled with structural and functional abnormalities of the basal ganglia, suggest a major impairment within the corticostriatal circuitry controlling the sequencing of voluntary, fine, orofacial movements used in speech.

Evolution of FOXP2

Since humans are vocal learners and non-human primates are not, the discovery of the *FOXP2* mutation being causally linked to a language deficit raised the question of whether *FOXP2* might have undergone positive selection in the human lineage. Three studies indicate that this is indeed the case,[38–40] raising the provocative hypothesis that changes in FOXP2 amino acid composition were pivotal for the evolution of learned vocal communication in hominids.

The FOX Gene Family

Can information about the characteristics of FOXP2 protein provide clues about its role in the above described morphological, neural, and behavioral deficits? FOXP2 is a member of the Forkhead (FOX) family of proteins,[25] one of at least 40 that exist in humans. They act as transcriptional regulators, capable of either repressing (decreasing) or activating (increasing) the production of mRNA of a specific suite of molecules. This is achieved by structurally specialized regions, the DNA-binding domains of the protein that contact the promoters of target genes. In all FOX proteins, this domain comprises 80 to 100 highly conserved amino acids. The name "forkhead winged-helix (FOX) domain" stems from the fork-like head structure on *Drosophila* embryos in which the first FOX protein mutant was discovered, and the winged helix refers to the shape of the tertiary structure of this protein domain.[41] It is this FOX DNA binding domain that harbors the point mutation, which causes the language deficit in the KE family; at amino acid position 553 a histidine replaces an arginine.[25]

Within the FOX family there are subfamilies, clusters of proteins that show higher homology to one another than to other such clusters. Those are distinguished by letters, FOXA through (currently) FOXQ. The FOXP subfamily has four members (FOXP1-FOXP4), which, in addition to the FOX domain possess a DNA-binding dependent N-terminal transcriptional repression domain encompassing both a zinc finger and a leucine zipper motif (FIG. 1).[42–44] FOX proteins are involved in a wide variety of biological processes, and mutations in their genes lead to diverse developmental disorders.[26,41] Foxp1 and Foxp2 were initially investigated for their role in lung development. Based on their developmental expression pattern, they appear to coregulate proximal versus distal epithelial lung cell phenotypes.[42] Foxp1 is the

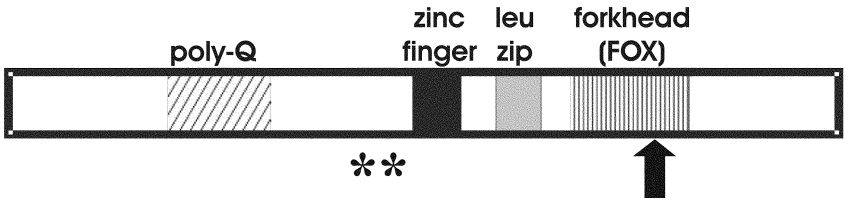

FIGURE 1. Schematic of FoxP2 primary structure. The forkhead/winged helix DNA-binding domain (*vertical stripe*) is common to all forkhead/winged helix (FOX) proteins. The FoxP subfamily is further characterized by a unique forkhead/DNA-binding domain and an N-terminal repression domain that contains a zinc finger and leucine zipper (*black and grey boxes*, respectively). FoxP2 additionally has a polyglutamine tract (*diagonal hatch*).[42,44] *Asterisks* indicate residues 303 and 325 which, among primates, are unique to humans.[25] *Arrow* points to arginine 553, which when mutated to histidine is linked to a rare speech and language abnormality in humans.[38]

closest Forkhead family member to Foxp2, shares a similar N-terminal domain whereby it represses transcription of genes that are also affected by Foxp2, and can dimerize with other Foxp subfamily members.[42,44,45] These findings raise the possibility that FOXP1 could interact with FOXP2 in other tissues, including the brain in regions where both are expressed.

Indeed, while other members of the Forkhead family of transcription factors are thought to function as monomers,[41] recent *in vitro* work demonstrates that Foxp subfamily members require either homo- or hetero-dimerization with each other or other regulatory proteins in order to bind DNA and affect transcription.[44] These functions are mediated by regions within the N-termini of the proteins, called *subdomains 1* and *2*. Within *subdomain 1*, the leucine zipper motif is essential for binding and repression. *Subdomain 2* interacts with a co-repressor protein known as C-terminal binding protein 1 to repress transcription. Foxp1 and Foxp2 possess both subdomains, which may be functionally redundant, while Foxp4 has only the first. These multiple opportunities for interaction may provide a dynamic range of transcriptional control, dependent upon the levels and types of Foxp proteins within a given cell.

SONG LEARNING IN BIRDS AS A MODEL FOR HUMAN SPEECH LEARNING

In view of the relative intractability of studying the cellular and molecular mechanisms of human language learning, birds that learn their songs are an obvious choice as models of vocal learning. The ability to modify innate vocalizations in order to correctly imitate the sounds that constitute the vocal repertoire is essential for both human speech and learned birdsong. In both humans and avian vocal learners, this "learning to play the instrument" proceeds through characteristic stages and relies on the interaction of auditory and motor centers. If the learning does not occur within a "critical period," usually before puberty, imitation is incomplete, as for instance evidenced by people's accents in languages acquired as adults. Similarities between human and songbird vocal learning also exist with respect to social influ-

ences on the behavior.[46,47] Apparently, vocal learning evolved three times independently in the avian lineage, namely in songbirds, parrots, and hummingbirds (see also Jarvis, this volume).[4] Each of these groups of birds needs to learn at least one aspect of their communication sounds by imitating the adult vocalizations of other members of their species. The fact that not all birds are vocal learners provides ready-made control subjects for such studies.

Like human language learning, song learning has the quality of an "instinct"[48,49] and is the product of the interaction of genetic and epigenetic factors. Many of the initial song learning studies were in fact addressing the balance between genetic versus social and cultural influences in different species of songbirds. Evidence for the importance of the genetic background on vocal development comes from a wide range of experimental approaches trying to dissociate the genetic variables from the epigenetic. This may involve rearing different genetic populations of the same species in identical conditions, or mixing genetic backgrounds while keeping auditory input constant (hybrid breeding), or by keeping genetic background constant while changing auditory input. Among the manipulations that alter auditory input are deafening, rearing in white noise, isolation, or presenting different tutors (crossfostering) or tutor tapes. Using these approaches, genetic influences have been found on acoustic characteristics of song, repertoire size, preference for tutor song types, and speed of song development.[50] Probably because vocal learning is such a rare trait and one that is so central to human existence, most studies of birdsong have focused on the mechanism of vocal imitation itself. Thus, much progress has been made in elucidation of the neural circuits involved, their role in behavior, and increasingly their function at the cellular and molecular level.

CANDIDATE MOLECULES: PRIOR STUDIES IN BIRDS

How does one go about identifying genetic mechanisms that might be important for the rare trait of vocal learning? Since the initial observation of specialized nuclei within the telencephalon of song learners,[51] two general strategies have been employed to isolate and characterize functionally significant molecules, i.e., any molecule (gene, protein) that contributes to the formation or function of the vocal learning pathway. The first strategy focuses solely on songbird anatomy and hypothesizes (perhaps naively) that important molecules are those that are more abundant within song nuclei than in surrounding tissue. For a given molecule, abundance (i.e., level of expression) within song areas can be compared to (1) the most adjacent region of the brain that does not subserve song learning; (2) regions of female brain at similar anatomical positions to the song nuclei of males, in species in which only males learn song; and (3) similar coordinates in non-songbirds that do not learn song (e.g., suboscines). Once a molecule is selected, a demonstration that altered molecular expression specifically affects song development and/or production provides the most convincing evidence that the candidate molecule is indeed important for vocal learning.

The second strategy is based on the critical role played by information storage in the song system. Educated guesses at candidate molecules for vocal learning are based on studies of learning and memory in other species, typically rodents. Reagents are developed to identify the avian forms of these molecules (oligonucleotide

probes for detection of gene expression, antibodies for immunohistochemical detection of protein, electrophysiological measures to detect synaptic function). Expression of these putative "memory" molecules within the song circuit is thereby tested, and, as with the first approach, correlations with brain regions, sex, and/or species, as well as aspects of song development are made. Causality is then inferred by the functional effects of manipulations of molecular expression on song behavior. The songbird system thus provides a powerful model allowing analysis of the role of molecules within a functional circuit and facilitating comparisons across brain area, sex, and species.

Molecules Studied Based on Heightened Levels of Expression within Song Nuclei

A classic example of the first approach was the discovery of the unique expression of sex steroid receptors within the telencephalon of songbirds in comparison to non-oscines, in and around song circuit nuclei (see also articles by Harding; Gahr; Brenowitz; and Ball and colleagues, this volume).[52] This unique pattern fits with the general "steroid hypothesis" that sex steroids cause the sexual differentiation of the brain and, specifically, the differentiation of the song circuit in songbirds.[53] Sex steroids strongly regulate the size and function of song control nuclei. However, detailed examination has clarified the contribution of hormonal versus genetic mechanisms to sexual differentiation and has refuted the strictest interpretation of the steroid hypothesis in both songbirds and mammals (see article by Wade and Arnold in this volume). These recent findings illustrate how crossfertilization between observations made in birds and mammals can reveal mechanisms common to both. Further, they raise the interesting question of which gene(s) act(s) in a hormone-independent manner to achieve full sexual differentiation of the song circuit.

The first systematic exploration of relatively abundant molecules in songbird telencephalon[54] did not find any gene expressed selectively in song nuclei. This indicated that song circuit–specific genes, if existent at all, are rare.[55] Subsequently, candidate molecules have been identified by virtue of being more concentrated in song nuclei and some are currently being probed for their potential role in song. For example, insulin-like-growth factor (IGF)-II is strongly expressed in the telencephalic song nucleus HVC, but only in those neurons that project to Area X. Accumulation of the protein in the HVC neurons that project to Area X implies a paracrine mode of action. In canaries, seasonal changes in IGF-II expression covary with changes in adult neurogenesis.[56] Another molecule that is expressed in a highly restricted fashion is an as-yet-unidentified antigen, detected using a monoclonal antibody raised against homogenates of microdissected tissue from song nucleus RA.[57] This antigen is expressed almost exclusively within the song circuit nuclei of the family of estrildine finches, including the zebra finch, and can be induced in female zebra finches upon treatment with masculinizing hormones. Based on its remarkable expression pattern, this single antigen promises to be a "molecular signature" of a functional neural circuit, although its role therein remains to be elucidated.

Differential and subtractive hybridization approaches using songbird brain have also been fruitful in identifying molecules that, while not limited to song nuclei, are expressed at relatively high levels in a subset of them, often during critical stages of song learning. One of these, synelfin, is a homolog to the mammalian protein known

as α-synuclein. This protein is thought to play a role in Parkinson's and Alzheimer's diseases in humans and, in songbirds, is regulated in song nuclei during song learning.[58] Together, these mammalian and songbird studies implicate the protein in memory functions.

Another candidate molecule is retinoic acid, classically known for its role in embryogenesis, now recognized as a necessary protein within the HVC of juvenile songbirds for normal song development.[59,60] Retinoic acid is a ligand for receptor molecules that are potent transcription factors. Targets of retinoid regulation include growth factors and their receptors. Thus, these results suggest that processes of neuron growth, survival, and differentiation continue post-embryogenesis to affect neural plasticity within the developing song circuit (see Mello, this volume).

Molecules Investigated Based on Roles in Mammalian Learning and Memory

A second approach for identifying candidate genes for vocal learning has been to test molecules implicated in synaptic plasticity in rodent learning and memory for their role in songbird song learning. One such molecule is the N-methyl D-aspartate (NMDA) subtype of glutamate receptor, which has been fruitfully characterized in both rodent learning and songbird song circuitry (see Nordeen and Nordeen, this volume). Additional candidate molecules identified in mammals and examined in songbird brain include the endocannabinoids. In rodents, these molecules facilitate the induction of long-term potentiation (LTP) (see Nordeen and Nordeen, this volume) in the hippocampus. Perhaps more relevant to song circuitry, in the rodent striatum they are critical to another form of synaptic plasticity, long-term depression. In zebra finches, endocannabinoids are expressed in the song system where their activation appears to influence sensory-motor learning and perceptual/mnemonic processes without concomitant changes is measures of auditory input.[61–64] Another class of molecules are the immediate early genes *c-fos*[65] and *ZENK* (acronym for zif286, egr-1, ngf1-a, krox-24)[55] (see Clayton, this volume). While these molecules are not limited to song circuitry, their abundance and distinct activation patterns have been extremely useful to probe neural activation pattern involved in song behavior,[66] to map functional vocal learning circuitry across avian evolution,[67,68] and to gain insights into auditory processing of song and calls in females and males[69] (see Theunissen and colleagues, this volume). This list is not meant to be exhaustive, but rather illustrative of the principle of testing candidate learning and memory molecules across model systems. Increasingly, avian and mammalian models offer complementary insights as evidenced by experiments addressing the relationship of critical periods and the maturation of NMDA receptor–mediated synaptic currents.[70,71] Other areas where avian models have stimulated mammalian research are adult neurogenesis,[72–74] and the role of the basal ganglia for vocal learning and production (see Farries; and Perkel, this volume).[75]

FoxP2 AND *FoxP1* IN BIRDS

Although the function of FOXP2 in language and speech remains open, progress has been made in demonstrating its localization in rodent and human embryos. As pointed out above, structural and functional brain anomalies of affected individuals

carrying *FOXP2* mutations consistently implicate the basal ganglia as one of the key affected brain regions.[34,35] The striatum, a component of the basal ganglia, is also the site of high *FOXP2* expression in developing human and rodent brain.[76–79] Since vocal learning in songbirds depends in part on the specialized pathway through the basal ganglia, including striatal vocal nucleus Area X,[80–82] we were motivated to ask the following questions: (1) Does zebra finch *FoxP2* (*zfFoxP2*) bear molecular similarities to human *FOXP2* (*hFOXP2*)? (2) Is *FoxP2* differentially expressed in the brains of avian vocal learners and non-learners? Birds that have only innate vocalizations lack specialized telencephalic "song circuitry" but vocalize via a set of sub-telencephalic nuclei common to both vocal learners and non-learners.[83] (3) How do *FoxP2* and *FoxP1* expression in birds compare to that in mammals, including humans? To address these questions we cloned the *FoxP2* and *FoxP1* genes of a commonly studied vocal learner, the zebra finch, and evaluated expression patterns in brains of eight species of avian "vocal learners," two species of avian "vocal non-learners," a crocodilian, the closest living non-avian relative,[84] and humans.

Cloning of Zebra Finch FoxP2 and FoxP1

We identified the mRNA containing the entire open reading frame encoding *zfFoxP2* as well as some untranslated sequences on either side of it.[79,85] As with mammalian *FoxP2* transcripts, there are multiple isoforms in the zebra finch (FIG. 2). Four isoforms exist that differ based on the presence or absence of two DNA segments, called *splice1* (71 bp) and *splice2* (60 bp), each different at the 5′ end of the gene. *Splice1* introduces a stop codon at position 261 (relative to the first start codon) resulting in predicted protein isoforms III and IV that miss the first 92 amino acids (AA), also reported for human FOXP2.[86] *Splice2* introduces 20 additional AA in-frame into the predicted protein isoforms I and III, not reported in human or mouse. In adult zebra finch brain and lung, four mRNA transcripts are evident, of approximately 9.0 kb, 6.5 kb, 3.5 kb, and 2.5 kb, respectively, some of which correspond in size to the transcripts found in mouse and human.[25,42] The large size of the transcripts relative to the size of the predicted coding region suggests that they contain large amounts of regulatory sequence, perhaps to precisely regulate *zfFoxP2* translation, mRNA location, and/or mRNA stability.

In zebra finch brain, one or both of the long isoforms (I and II) predominate. The zfFoxP2 protein (Isoform I) shares 98.2% identity with human and 98.7% identity with mouse Foxp2, respectively. This emphasizes the remarkable degree of conservation of the *FoxP2* gene[38,39] as ~320 million years ago is the latest time at which modern birds and mammals had a common ancestor.[87] At five AA positions that are identical in mice and human, zfFoxP2 differs from all FoxP2 sequences currently known. At three additional positions, the mouse and zebra finch sequence are identical but the human sequence diverges. Of these three AAs, one also exists in carnivores,[39] one is common to primates, and one is unique to humans. In an analysis of *FOXP2* molecular evolution, the latter has been suggested to result from positive selection during recent primate evolution indicating that hFOXP2 might have been pivotal for the development of human language.[38] Although zfFoxP2 lacks this human-specific AA change, one cannot exclude the possibility that other sequence differences exist between avian vocal learners and non-learners that result from positive se-

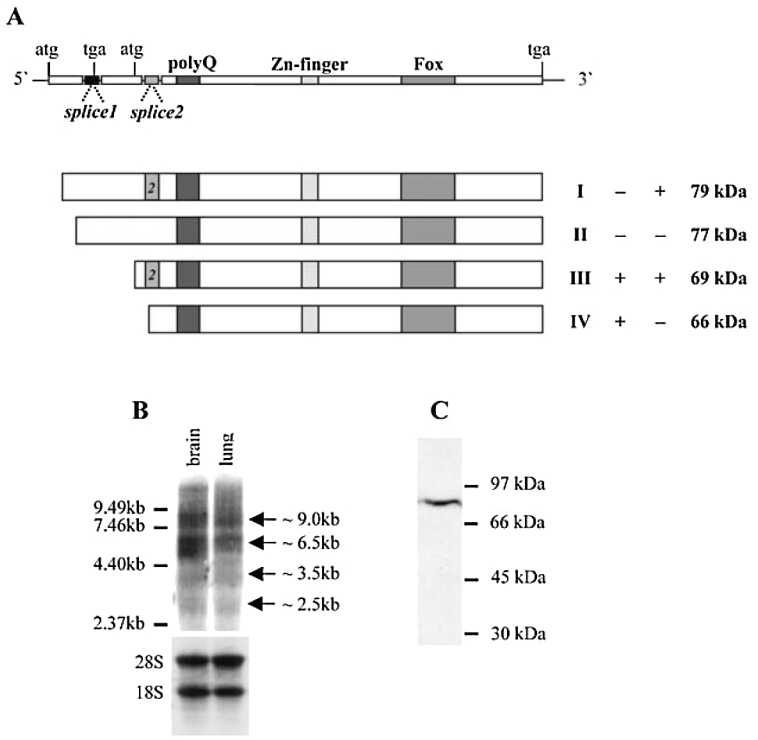

FIGURE 2. Identification of the zebra finch *FoxP2* mRNA (*zfFoxP2*). (**A**) Schematic representation of *zfFoxP2* mRNA structure and its four predicted protein isoforms (I–IV). Positions of start (atg) and stop (tga) codons, the polyglutamine tract (polyQ), zinc-finger (Zn-finger), and forkhead box (Fox) DNA binding domains are shown. Two mRNA segments (*splice1* and *splice2*) are subject to alternative splicing. Presence (+) or absence (−) of splice1 and splice2 leads to variation in length of open reading frames (ORF). *Splice1*contains a stop codon that shifts the frame so that the ORF begins at the second atg, *splice2* inserts 60 base pairs (bp) in-frame into the coding region. The four predicted protein isoforms are shown. For the calculation of their theoretical molecular weight we used Peptide Mass (http://www.expasy.org/tools/peptide-mass.html). (**B**) Northern blot analysis of 20 µg total RNA from adult zebra finch brain and lung was carried out with a ^{32}P-labeled DNA fragment spanning bp 114–959 (relative to first start codon). Ethidium bromide staining of 18S and 28S ribosomal bands demonstrates equal RNA loading. The different *zfFoxP2* transcripts are indicated with *arrows*. (**C**) Western blot analysis of 50 µg brain nuclear protein extract from a 40-day-old male zebra finch reveals a zfFoxP2 protein corresponding in size to either isoform I or II, recognized by a polyclonal antibody raised against amino acids 613–715 of mouse Foxp2.[85]

lection during avian evolution, as proposed for primates. The fact that zebra finches, in contrast to mouse, have a 6.5 kb transcript that corresponds in size to the human transcript also raises the possibility that selection acted on the regulatory sequence.

Expression Pattern of zfFoxP2

Within zebra finch brain, *FoxP2* shows differential expression over development in the song nucleus Area X—a part of the special basal ganglia-like forebrain network required for vocal learning that non-learners do not possess. *FoxP2* expression in Area X stands out, slightly but consistently, from its expression in the surrounding striatum only during the time when young zebra finches learn to imitate song.[85] Comparison of *FoxP2* expression pattern in Area X of adult canaries, zebra finches, bengalese finches, strawberry finches, song sparrows, and in the equivalent regions of Area X in parrots (MMSt) and hummingbirds (VAS) revealed interesting differences. In some species, we found expression in Area X to be higher than in the surrounding striatum, in others it was similar in both regions or lower in Area X compared to the surrounding striatum.[85] Investigating the variables that could account for these differences in *FoxP2* expression in adult Area X, we could rule out singing activity (see Mello, this volume) and song stereotypy. In contrast, a seasonal comparison in canaries, a species with seasonal variation in song plasticity, showed elevated *FoxP2* expression in Area X during the months of the year when song became plastic and less expression during months when song was highly stereotyped (FIG. 3). The differences in *FoxP2* expression within Area X of the other species were also roughly correlated with the likely state of song plasticity at the time of sacrifice.

Both juvenile and adult Area X expression patterns are compatible with a role for FoxP2 in learned vocalization, particularly during development, but also in adulthood even though the function of Area X is more enigmatic in adult songbirds than in juveniles. Experimentally induced lesions of Area X in adult zebra finches that have finished learning their song hardly affect normal song production.[80–82] Yet Area X in adult zebra finches has song-specific motor activity, which is modulated by social context.[66,88] This apparent paradox is reminiscent of the situation in the human basal ganglia, where the absence of striatal regions (e.g., due to stroke) may have less severe functional consequences than disruption of its function (e.g., in Parkinson's and Huntington's diseases). It is thought that Area X in adulthood monitors adult song production and is involved with correction of errors.[89,90] (see articles by Brainard; and Konishi, this volume) Since adult song is less error-prone than developing song, particularly in zebra finches, lesions of Area X might have less apparent effects. Consistent with this hypothesis is the observation that songbirds with different amounts of adult song plasticity apparently rely on Area X for their adult song production to different extents.[81,82,91–93] The differential *FoxP2* expression among avian species might be related to this.

FoxP2 is also expressed in non-vocal striatal regions outside of Area X/VAS/MMSt of all eleven bird species examined, regardless of whether or not they learn their vocalizations. Both vocal learners and vocal non-learners had similar developmental onset of *FoxP2* expression in comparable brain regions and equivalent expression pattern in adults. The strongest signal was consistently observed in the basal ganglia, the dorsal thalamus, the inferior olive, and the Purkinje cells of the

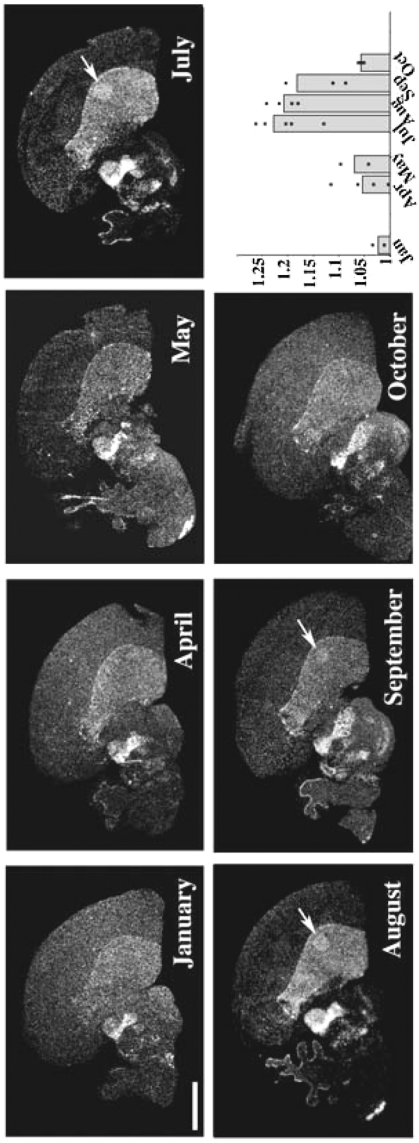

FIGURE 3. *FoxP2* expression in Area X of adult canaries varies seasonally. Area X expressed noticeably more *FoxP2* than the surrounding striatum only during the months of July, August, and September, resulting in higher ratios of Area X to striatum expression. Bar graph shows mean ratios for each month; superimposed points represent values for individual birds.[85]

cerebellum. Less intense but consistent expression was observed in various nuclei related to these regions. In all brain regions that expressed *FoxP2* (as observed by *in situ* hybridization) a Foxp2-specific antibody (used for immunohistochemistry) also recognized strongly labeled nuclei, as is expected for a transcription factor.[85]

Consistent with the reports from developing human and mouse brain,[42,77,78] we saw *FoxP2* expression in the embryonic zebra finch brain as early as stage 23.[85,94,95] The highest expression was in the striatum and dorsal thalamus. In older animals, the dorsal thalamic zone (DTZ),[96] located dorsomedially in the avian diencephalon exhibits distinct subregional labeling.[79] The DTZ is homologous to the mammalian intralaminar, midline and mediodorsal thalamic nuclear complex (IMMC).[96] It consists of multiple nuclei whose boundaries likely underlie the pattern of *FoxP2* expression. For example, nucleus dorsolateralis anterior thalami, pars medialis (DLM), part of the song circuit, expresses *FoxP2* mRNA, while the nucleus dorsolateralis anterior thalami, pars lateralis (DLL) does not. In the vicinity of, but histologically distinct from, the DTZ is the ventrointermediate area (VIA), a region described in pigeons as comparable to the motor part of the mammalian ventral tier.[97] In the zebra finch, *FoxP2* signals are visible in this region just medial to nucleus rotundus.

Striatal and dorsal thalamic expression patterns persist throughout development and adulthood. Expression levels in the striatum decrease slightly with age, but are always higher than in pallial regions, i.e., those dorsal to the striatum, that are low throughout development and in adulthood. The prominent expression in the striatum and caudal dorsal thalamus is common to all species investigated, regardless of sex and of song learning ability. And it is also seen in a crocodile, the closest non-avian relative of birds.[85]

Cellular Identity of zfFoxP2-Expressing Cells

In adult zebra finch striatum, FoxP2 immunoreactivity is characteristically seen in medium or small cells that are uniformly distributed throughout, except for one peculiarity.[85] Small FoxP2-positive cells form distinct, evenly spaced clusters in the lateral striatum (LSt), that abut the pallial-subpallial lamina (PSL, previously called LMD) which separates the pallium from subpallium. In pigeon striatum, similarly arranged patches contain dense choline acetyltransferase (ChAT)–immunoreactive fibers.[98] In zebra finch, these FoxP2-immunoreactive cell clusters are also innervated by ChAT. All FoxP2-immunoreactive brain cells are neurons, some of which also express the polysialylated neural cell adhesion molecule (PSA-NCAM), a marker for cellular plasticity and migration.[34]

To identify the types of striatal neurons expressing FoxP2, we used markers for the three classes of striatal interneurons[99] in conjunction with FoxP2 immunohistochemistry. We used ChAT to detect the large, aspiny cholinergic interneurons, nitric oxide synthase (nNOS) to detect the medium-sized aspiny interneurons that also contain somatostatin, and neuropeptide Y and the calcium binding protein parvalbumin to detect another population of medium-sized aspiny interneurons that also contain GABA and the neurotensin-related hexapeptide LANT6.[99] Neither ChAT, nor nNOS, nor parvalbumin are expressed in the same neurons as FoxP2, suggesting that the striatal neurons that express zfFoxP2 are projection neurons rather than interneurons. The striatal projection neurons in birds, as in mammals, are the site of convergent nigral dopaminergic and cortical (i.e., pallial in birds) glutamatergic input.[99] The adenosine-

3′,5′–monophosphate (cAMP)-regulated phosphoprotein of M_R 32,000 (DARPP 32) is thought to serve as a critical integrator of these two inputs onto the striatal projection neurons.[100] Concordant with our expectation that zfFoxP2 is expressed in striatal projection neurons, we found two indicators of dopaminergic innervation: FoxP2-immunoreactive striatal neurons co-expressed DARPP32, which is indicative of the presence of dopamine D1 receptor, and immunoreactivity for tyrosine hydroxylase (TH), the synthetic enzyme for biogenic amines, was detected around perikarya of neurons with FoxP2 immunoreactive nuclei.

ZfFoxP2 Expression in Subtelencephalic Brain Regions

We also found prominent *zfFoxP2* expression in many subtelencephalic structures. Among these structures there were regions that project to the basal ganglia, such as the substantia nigra/ventral tegmental area and the DTZ. In addition, *zfFoxP2* is expressed in many regions that are involved in relaying and integrating ascending sensory information, including auditory regions (e.g., midbrain nucleus MLd and thalamic nucleus ovoidalis), visual regions (e.g., afferent upper layers of midbrain optic tectum, and thalamic nucleus rotundus), multimodal regions (e.g., layers 10 and 11 of optic tectum,) and somatosensory regions (e.g., sensory trigeminal). In addition, prominent *FoxP2* expression was observed in the Purkinje cells of the cerebellum and the inferior olive, which gives rise to all the climbing fibers innervating the Purkinje cells.[79,85] All species tested, regardless of sex and song learning ability, expressed *zfFoxP2* in these regions. In contrast, *zfFoxP2* expression was not found in midbrain and brainstem motor control areas, such as the vocal nucleus DM, the hypoglossal vocal and tongue nucleus, nXII, nor in most other cranial motor nuclei.[85]

ZfFoxP1 Expression

We also investigated *FoxP1* because studies in mouse lung and *in vitro* demonstrate that Foxp1 (1) is the closest forkhead family member to Foxp2; (2) shares similar N-terminal domains whereby it represses transcription of genes that are also affected by Foxp2; and (3) can dimerize with other Foxp subfamily members.[42,44,45] These features suggest that FoxP1 could interact with FoxP2 within the brain in regions where both are expressed. As with mammalian sequences, zfFoxP2 and zfFoxP1 AA sequences are highly similar and differ mainly by the fact that the longest *zfFoxP1* transcript that we isolated misses the region encoding the polyglutamine stretch and 100 AA on the N-terminus.[85] For human FOXP1, an isoform that lacks the first 100 AA is reported,[101] suggesting that we found a short zfFoxP1 isoform.

Within the zebra finch brain, *FoxP1* exhibited a striking sexual dimorphism, nearly concordant with the sexual dimorphism of the song circuit (FIG. 4).[78,84] Unlike *FoxP2*, *FoxP1* was expressed in the striatal vocal nucleus Area X of all songbirds tested. Also unlike *FoxP2*, within the pallium, *FoxP1* was consistently and prominently expressed in the mesopallium in all species.[85] Interestingly, for the three main songbird pallial vocal nuclei, lMAN, HVC, and RA, *FoxP1* expression differed notably from the expression of the subdivisions in which these nuclei are embedded. HVC and RA strongly expressed *FoxP1,* whereas the surrounding terri-

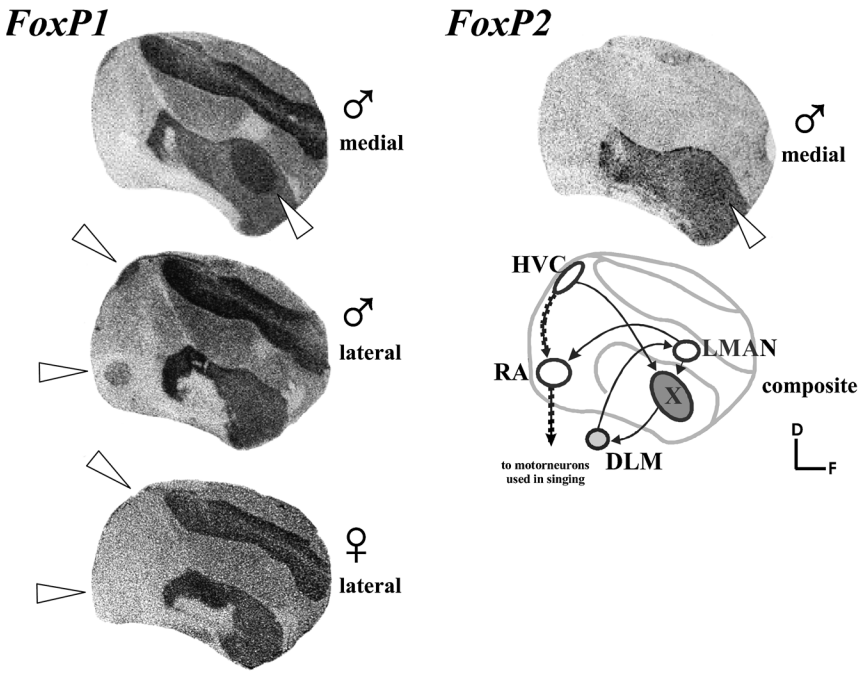

FIGURE 4. Comparison of *FoxP1* and *FoxP2* expression in adult zebra finch telencephalon. *In situ* hybridization of ^{32}P-labeled zebra finch *FoxP1* (*right*) versus FoxP2 (*left*) riboprobes with sagittal sections of adult zebra finch brain reveal areas of overlap, as well as distinct regions of expression. (*Top*) Neural expression of *FoxP1* includes pallial (*top dark diagonal band* of signal) and striatal (*lower band*) regions. *Arrowhead* indicates Area X within the songbird striatum in a medial section from adult male brain. (*Middle*) In a more lateral section, two *arrowheads* point to HVC and RA. (*Bottom*) Section is taken from an adult female at approximately the same plane. *Arrowheads* here are for comparison to above section and indicate the lack of increased signal in regions of sexually dimorphic FoxP1 expression. (*Right*) Top section shows striatal *FoxP2* expression in the same male as on the left. *Arrowhead* points to Area X, which is faintly discernible in this section, consistent with Nissl staining (data not shown). Beneath, composite schematic shows part of the song control circuitry for reference, including the vocal control pathway (*stippled arrows*) and the anterior forebrain pathway (*smooth arrows*).[79]

tories did not. The reverse was true for lMAN, which did not express *FoxP1*, while the region around it did. This was consistent across songbird species. The parrot pallial analogue of HVC, the central nucleus of the nidopallium, had noticeably higher levels than the surrounding nidopallium. *FoxP1* was expressed at high levels in the striatum and in the dorsal thalamus of zebra finches and other birds. A telencephalic expression pattern remarkably similar to the avian brain was found in crocodile, which included high expression in striatal-like and mesopallium-like regions.[85] This suggests that the general *FoxP1* expression pattern in birds was inherited from their common reptilian ancestor.

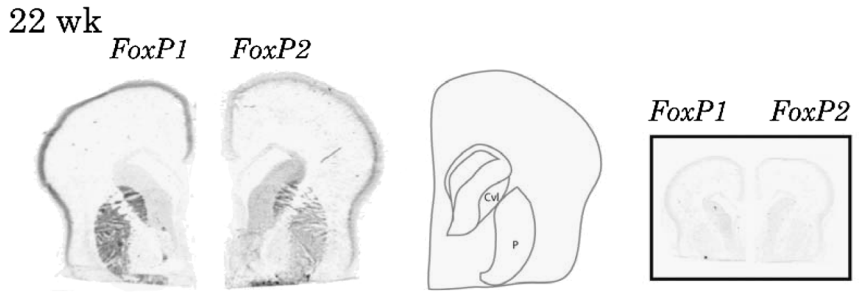

FIGURE 5. *FOXP1* and *FOXP2* expression in human embryonic brain. Coronal sections from 22-week embryo (*left*) show that expression of the two genes overlaps in regions of the striatum, as indicated by the schematic (*middle*), including in the ventrolateral caudate (Cvl) and the putamen (P). In cortex, *FOXP1* is expressed slightly more superficially than *FOXP2*. *Boxed inset* shows sense controls. (Images courtesy of Lili C. Kudo and Daniel H. Geschwind.[79])

FOXP Expression in Humans

The human language phenotype that arises from a mutation in *FOXP2* coupled with the overlapping expression of *FoxP2* with *FoxP1* in the striatum and thalamus of the zebra finch hints at a combinatorial role for these genes in the development of vocal control circuitry. This hypothesis would be supported by a similar overlap in the developing human brain. Thus, *in situ* hybridization analysis was performed on human embryonic brains between 19 and 22 weeks gestation,[79] when subcortical neurogenesis and migration is largely complete and cortical neurogenesis is ongoing.

In cortex, a complementary pattern of *FOXP* gene expression occurs in human embryos, with *FOXP1* localized to more superficial layers than *FOXP2*. Within the striatum, *FOXP1* and *FOXP2* are expressed in highly similar patterns, in the head and tail of nucleus caudatus and putamen where the intensity of *FOXP* label is reminiscent of the enhanced *FoxP* signals within Area X of the songbird striatum (FIG. 5). Interestingly, *FOXP2* shows restricted expression within the globus pallidus (GP) of the basal ganglia.[79] High levels of *FOXP2* expression occur in the GP pars interna, which provides the principal source of output from the basal ganglia to nucleus centrum medianum thalami (CM) and the major motor relay nuclei of the thalamus. As in the zebra finch, human *FOXP1* and *FOXP2* expression overlaps in the thalamus, with *FOXP2* revealing more extensive expression, specifically in CM and nucleus medialis dorsalis thalami, both regions with homologues in the avian DTZ,[96] and in the ventrobasal complex comprised of nucleus ventralis posterior lateralis/medialis thalami. More moderate signals arise from nuclei anterior thalami, dorsal and ventral, and nucleus parafascicularis thalami (Pf). Similar to VIA in the zebra finch,[97] the ventral tier of the human thalamus exhibited strong *FOXP2* expression, including nuclei ventralis anterior, lateralis, and nucleus ventralis posterior lateralis, pars oralis. These thalamic nuclei have strong motor and premotor cortex connectivity.[102] Both genes also demonstrated significant expression in nucleus subthalamicus bilaterally. Additionally, *FOXP2* is strongly expressed in nucleus ruber. The human

brain regions of *FOXP* expression are key relays in essential motor control circuitry involved in motor planning and execution. This pattern of expression in specific subcortical structures for both *FOXP1* and *FOXP2* is entirely consistent with the putative role of these genes in pathways of sensorimotor integration that subserve vocalization and other complex learned motor movements. Note, however that in no case was lateralization of *FoxP* gene expression observed. Given the observations of lateralization in both humans and finch vocal systems, this finding may indicate (1) that expression was measured prior to the time of lateralization in humans; (2) that asymmetric expression was missed due to its occurrence in tissue outside of our samples; (3) that asymmetric differences in *FoxP* expression exist but were undetected by our current methods (e.g., there could be post-translational differences, the cumulative level of all neurally expressed FoxP proteins could be lateralized, quantitation by emulsion autoradiography may be required); and (4) that mechanisms downstream of FOXP account for lateralization; among other possibilities.

CONCLUSIONS

The striking conservation of the *FoxP2* gene sequence and overall brain expression pattern in reptilian and mammalian brains and in the brains of both song-learning and non-song-learning birds indicates that *FoxP2* has a more general role than to specifically enable vocal learning. FoxP2 could be an ancient transcription factor primarily involved in setting up and maintaining subtelencephalic and striatal sensory and sensory-motor circuits, creating a permissive environment upon which vocal learning can evolve if other circumstances/factors come into play. Given the prominent role of many other forkhead transcription factors in early development, this is a likely scenario.[41] Support of this notion also stems from the fact that regions where *FoxP2* is first expressed in the avian embryo are sources of inductive signals that organize adjacent neuroepithelium and neuronal migration during early development. The differences in cortical/pallial *FoxP2* expression between mammals and birds are harder to interpret since direct homologies between avian and mammalian pallial areas remain unresolved.[99]

The common expression pattern of *FoxP2* in birds and humans might provide valuable clues about what constitutes a "permissive environment" for vocal communication and evolution of vocal learning. Learning to imitate acoustic signals requires integration of sensory information with the desired motor output. The basal ganglia as well as the cerebellum in all vertebrates integrate afferent sensory information with descending motor commands and thus participate in the precise control of temporally sequenced muscle movements.[103] Both innate and learned avian and human vocalizations depend on such control,[104] as do many other complex learned behaviors. Anatomical evidence suggests that the specialized regions for vocal learning in birds were elaborated from already modularly connected forebrain regions translating ascending auditory, somatosensory, and visual information into motor commands. Consistent with this, an AFP-like circuit apparently also exists in vocal nonlearners.[105] In humans, the basal ganglia and the cerebellum have attracted far less attention than the cortical speech and language areas, but there is increasing awareness that the basal ganglia and cerebellum are not only essential for the execution but might also be required for the acquisition of human vocal behavior.[75,106]

It has been suggested that the speech and language pathology in humans with *FOXP2* mutations consists of an orofacial dyspraxia core deficit.[26] This could be primarily due to a lack of central control over the peripheral muscles associated with the speech apparatus. However, our data suggest that in birds *FoxP2* is expressed in afferent sensory pathways, and in the striatal projection neurons, which are the site of convergence for both pallial and subpallial projections. Takahashi and colleagues[78] also argue that in rats FoxP2-positive striatal neurons are projection neurons. Expression in these sensory and sensorimotor integration areas makes sense, if *FoxP2* expression indeed highlights a "permissive environment" for vocal learning. Further, many sites of *FoxP2* expression, such as the inferior olive-Purkinje cell pathway, the optic tectum, and the striatum, are known substrates for experience-dependent plasticity.[103,107,108] This highlights the need for more studies investigating the role of ascending visual, auditory, and somatosensory information in complex learned motor skills such as birdsong and human speech.

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