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Genes and vocal learning

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ABSTRACT

Could a mutation in a single gene be the evolutionary lynchpin supporting the development of human language? A rare mutation in the molecule known as FOXP2 discovered in a human family seemed to suggest so, and its sequence phylogeny reinforced a Chomskian view that language emerged wholesale in humans. Spurred by this discovery, research in primates, rodents and birds suggests that FoxP2 and other language-related genes are interactors in the neuromolecular networks that underlie subsystems of language, such symbolic understanding, vocal learning and theory of mind. The whole picture will only come together through comparative and integrative study into how the human language singularity evolved.

1. Introduction

In 1988, Noam Chomsky pondered, 'Perhaps at some time hundreds of thousands of years ago, some small change took place, some mutation took place in the cells of prehuman organisms. And for reasons of physics which are not yet understood, that led to the representation in the mind/brain of the mechanisms of discrete infinity, the basic concept of language and also of the number system' (Chomsky, 1988). Today, the idea that such a change was restricted to a single molecule and occurred solely in the hominid lineage, referred to as the Grammar Gene' theory, is deemed an extreme position (Bishop, 2009). Rather, the brain system underlying language is likely made up of subsystems, forms of which exist in other taxonomic groups. Language could have arisen in humans as a consequence of the unique intersection of these subsystems. A subsystem identified in non-humans could represent the homologous component in language. Alternatively, if the subsystem emerged at a point that does not feed into the hominid lineage, it could represent convergent or parallel (homoplasous) evolution whereby similar selection pressures drive parallel instances of similar biological solutions (as we, and others (e.g. Jarvis, 2004), have argued for birdsong and speech). Whether homologous or homoplasous, the good news is that this viewpoint opens the door for studying subsystems of language at the biological level using a comparative approach.

This chapter examines one language subsystem, namely the capacity for vocal learning, and the genes expressed in the central nervous system that are hypothesized to contribute to this ability. I focus on human speech and birdsong and define the vocal learning subsystem of language as the experience-dependent modification of one's vocal motor output with the goal of mimicking other members of one's species (conspecifics) or of creating new sounds. Deafness in humans and experiments in animals teach us that vocal learners must hear and attend to the vocalizations of conspecifics (with some exceptions: Feher et al., 2009; Kroodsma et al., 1997; Leitner et al., 2002) and hear their own vocal output in order to produce effective vocal communication signals (for review see Doupe and Kuhl (1999)). Comparison of these sounds to evaluate the match sets the stage for those neural changes that enable adaptive modifications of vocal output. In humans, vocal learning drives the development of speech.

Of course not all animals have been rigorously tested for the vocal learning ability. Tests of vocal learning often rely on deprivation of acoustic inputs during development and evaluation of how closely subsequent vocal output approximates normal song. One measure of how well song develops under these circumstances is whether it serves as an effective communication signal in conspecific interactions. Tests include: deafening early in development which prevents both hearing of others and of self; rearing in the absence of conspecific vocalizations which only prevents the former; and transient distortion of auditory feedback of the animal's own vocal output, affecting only the latter. Non-invasive methods include determining whether changes in vocal output during normal development are more substantial than those expected due to physical maturation of the vocal apparatus (such as the larnyx; Fitch, 1997) or are uncharacteristic of the species-specific behavior. By a majority of these tests, passerine birds of the oscine suborder, known as songbirds, are vocal learners. In addition to humans and songbirds, the short list of animals demonstrated to possess this ability is confined to: parrots and hummingbirds which are in

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separate avian orders (raising the hypothesis that the trait emerged independently three times in the avian lineage); certain marine mammals including harbor seals, dolphins and cetaceous whales; elephants; and certain bat species. As outlined in the introduction to this volume (see contribution by Brenowitz, Perkel & Osterhout, this issue) songbird species such as canaries (*Serinus canaria*), white crowned sparrows (*Zonotrichia leucophrys*), Bengalese finches (*Lonchura domestica*, also known as society finches) and zebra finches (*Taeniopygia guttata*) have relatively short generation times and are amenable to laboratory life, making them extremely practical species in which to conduct controlled studies of the biological basis for vocal learning.

In accordance with the general requirements for vocal learning outlined above, songbirds listen to the songs of their own species as well as their own vocal output (song perception), in order to adaptively modify control of the syrinx, or song organ, and the respiratory muscles used in singing. Experimental deprivation of these auditory inputs generally causes abnormal song in adulthood, but if temporarily applied and then removed, can extend the critical periods for song learning. This phenomenon is analogous to the extension of critical periods for neural organization in the visual system after rearing in darkness (for review see Hooks and Chen (2007)). For example, young zebra finches deprived of tutoring during normal sensory acquisition exhibit an extended critical period such that they can now learn song from a tutor provided after 65 days, the normal close of that critical period (Morrison & Nottebohm, 1993). Similarly, when loud masking noise is used to temporarily deprive finches of auditory feedback from their own vocalizations, sensorimotor learning is extended. Once the noise is turned off, the birds can adaptively modify their songs at ages when normally reared birds do not (Funabiki & Konishi, 2003). In zebra finches, dramatic modifications to song end with sexual maturity at ~100 days when song becomes stable, or crystallized. However, this behavioral stability is maintained by dynamic neural activity and depends on ongoing auditory feedback, as does human speech (Cynx & Von Rad, 2001; Nordeen & Nordeen, 1992; Woolley & Rubel, 1997; for review see Brainard and Doupe (2000)).

Song perception, its constituent neural systems, the genes underlying the formation and function of these systems, and peripheral vocal control are inescapably intertwined with vocal learning. For more information on these topics the interested reader is referred to contributions by Gentner and Goller, in this volume. Other subsystems of language may be better studied in other taxonomic groups. While birdsong can convey individual and species identity and can advertise mating or territorial ownership, it is not 'compositional', i.e. no single song syllable combines with others to build meaning the way that words do. The additional capacity for symbolic content is a necessary step in moving beyond the musicality of birdsong to semantically compositional language. The semantic subcomponent of language may be better studied in non-human primates (see Seyfarth and Cheney contribution, this issue), or, among birds, in parrots (see Pepperberg contribution, this issue). Yet it is worth noting that, in addition to vocal learning, certain songbird species do possess additional subsystems potentially linked to cognitive capacities required for language such as tool use, hierarchical reasoning, and context free syntax. (See Clayton and Emery (2005) for a review of corvid cognition, and Gentner, this volume, for syntax discrimination in starlings.)

2. Strategies used to identify genes for vocal learning

Now that we have defined vocal learning and introduced key songbird species in which to study it, how do we go about identifying genes that function in the song circuit and might generally underlie this rare trait? Since the initial observation of specialized nuclei within the telencephalon of song learners (Nottebohm & Arnold, 1976; see contributions of Margoliash, Schmidt and Kirn (this volume)), two general strategies have been used to isolate and characterize genes that contribute to the formation or function of vocal learning pathways. The first focuses on song circuit neuroanatomy and hypothesizes that important molecules are those that are differentially expressed *within* song control nuclei relative to surrounding tissue. The second approach is to make an educated guess as to candidate molecules for vocal learning. Initially, selection of candidate molecules was based on studies of learning and memory in other taxa or on critical periods in visual system development in rodents (e.g. *n*-methyl D-aspartate (NMDA) receptors). More recently attention has been paid to the handful of genes linked to human language disorders, such as Fragile X syndrome and mutations in FoxP2.

For both approaches, expression of an identified molecule within song control areas can be compared across the developmental phases of song learning. As described above, the timing of these phases can be experimentally manipulated. Thus, the songbird model allows for a unique test of any observed temporal correlation between molecular expression and vocal learning. If expression of a given molecule differs within the song circuit compared to outlying regions during normal sensorimotor learning, sensorimotor learning can be experimentally delayed (Funabiki & Konishi, 2003) to test whether the expression pattern is merely correlated with chronological age, or instead is more directly associated with the learning process. A major challenge in using songbirds to functionally verify the role of genes in vocal learning is that the avian egg is not easily amenable to genetic intervention (Sang, 1994). However, the use of viruses to introduce transgenes into the egg, or into song control regions of the developing brain is increasing, a topic I shall return to later on. Below, I review the genes that have emerged from these general approaches and how further investigation in songbirds contributes to understanding of the way in which they operate in vocal learning.

2.1. Enrichment in song control nuclei

The first systematic exploration of relatively abundant molecules in songbird telencephalon was conducted by Clayton, Nottebohm and colleagues (Clayton, 1997; Clayton et al., 1988). No gene exhibited an expression pattern that was entirely restricted to song nuclei. However, this study and similar approaches in which mRNA expression levels are compared inside versus outside song control regions (e.g. subtractive hybridization, and differential display; see below) have revealed molecules that are enriched in song nuclei. Of these, the first to be experimentally pursued was α-synuclein, a molecule that was independently identified in studies of human neurodegenerative diseases (for review see Clayton and George (1999)). Point mutations, duplications and triplications in the α-synuclein gene cause a rare dominant form of familial Parkinson's Disease (Biskup et al., 2008). Although the precise cellular function for α-synuclein has yet to be determined, evidence suggests that it acts at presynaptic nerve terminals. Its link to Parkinson's Disease coupled with its regulation in the song nucleus LMAN during early stages of birdsong learning suggest that α-synuclein function is critical to neural circuits that underlie the execution of learned motor skills.

Other examples of molecules that are concentrated in various song nuclei include: the biosynthetic enzyme for retinoic acid (Denisenko-Nehrbass et al., 2000) which is enriched in X-projecting HVC neurons; insulin-like growth factor II (IGF-II; Holzenberger et al., 1997), also enriched in these same neurons; and an as-yet unidentified antigen whose expression is largely limited to song

control nuclei (Akutagawa & Konishi, 2001). Both retinoic acid and IGF-II are ligands for growth factor receptors, suggesting they could function in the ongoing neurogenesis that occurs in HVC (for review see Scharff and White (2004)). Inhibition of retinoic acid synthesis or dietary elevation of retinoic acid in juvenile songbirds both result in more variable songs in adulthood, indicating that any intervention in this signaling pathway disrupts song maturation (Wood et al., 2008).

Recently, a gene predicted to encode a small membrane protein containing a fibronectin type III domain and a transmembrane domain has been isolated using a differential display approach (FnTmII, Agate et al., 2007). Agate and colleagues compared transcripts that were expressed at higher levels in HVC relative to RA as a means of biasing their screen for genes involved specifically in learning and in neuronal replacement. They found strong expression of a molecule that they subsequently named FnTmII in neurons of the anterior forebrain pathway. While the predicted intracellular domain of FnTmII is novel and the sequence varies a great deal between rodents and birds, the extracellular domain is conserved and likely mediates specific protein interactions. Although the function of FnTmII is as yet unknown, its highly variable levels of expression within AFP nuclei coupled with its conserved expression across songbirds and rodents in limbic regions led the authors to suggest that FnTmII is involved in motivational states related to learning.

The above overview provides examples of how knowledge of the exact neuroanatomical location of neurons dedicated to vocal learning, a key feature of the songbird model, can be mined for discovery of genes that participate in this process. This list is not meant to be exhaustive but rather serves to illustrate the wide variety of molecular types potentially involved in song learning. Variety is expected since the screen used to isolate these genes makes no assumption as to their role, only that they be enriched in song control regions. This strategy, by definition, is more likely to reveal novel molecules in comparison to the candidate gene approach described below, since the latter depends on scientific knowledge from other systems. As further research is conducted on novel genes, we can expect these studies to lead us to familiar molecules and similar biological solutions to common selection pressures. Conversely, as the functions of candidate genes are revealed, they are pointing to novel molecules not previously considered as genes for vocal learning.

2.2. Candidate genes identified in non-human models – focus on rodent learning and memory

In the candidate gene approach, molecules implicated in either synaptic plasticity of the rodent hippocampus or in critical periods for visual system organization in mammals provide a major focus of investigation in songbirds. NMDA receptors are involved in both processes, and key studies examining their role in the maturation of the song circuit are reviewed in Nordeen and Nordeen (2004). More recently, this same group has examined phosphorylation of calcium/calmodulin dependent protein kinase (CaMKII Singh et al., 2005). CaMKII phosphorylation is a downstream event involved in NMDA receptor-mediated plasticity such as long-term potentiation (LTP) and in many forms of hippocampal based learning, as well as in synaptic plasticity in the rodent striatum (Pittenger et al., 2006). To date within the song circuit, LTP has been demonstrated only in the pallial nucleus, LMAN (Boettiger & Doupe, 2001) and in the medial striatum within Area X (Ding & Perkel, 2004). Of these, Area X exhibits high levels of CaMKII, so Nordeen and Nordeen focused there.

These authors found that zebra finch pupils that heard two hours of song sung by a familiar tutor exhibited increased levels of phosphorylated CaMKII (pCaMKII) in Area X relative to levels in control birds. Control groups included pupils exposed to tutors that did not sing, or to tutors that sang but who were not visible to the pupil (a condition under which pupils do not copy song; Morrison & Nottebohm, 1993), or to new tutors singing unfamiliar song. Isolates tutored for the first time exhibited a more modest rise in pCamKII than did pupils hearing familiar tutor song. None of these changes were observed in zebra finch females who do not develop song behavior.

Interestingly, the increases in pCamKII occurred in medium spiny neurons that express the dopamine and adenosine-3′5′-monophosphate-regulated phosphoprotein, similar to their mammalian counterparts (DARPP-32; Hein et al., 2007)). Based on dopamine's role in goal directed behaviors (see for review Yin et al. (2008)), the authors raise the possibility that Area X '*participates in encoding and/or attaching reward value to the representation of the tutor song*' and may thus guide vocal motor learning. Intriguingly, during song development, these dopamine-sensitive neurons are recruited to Area X and co-express the human language-related transcription factor FoxP2 (Rochefort et al., 2007), a topic we will revisit toward the end of this chapter.

In addition to the dopaminergic system, endogenous cannabinoids play a role in modulating reward processes in the mammalian brain (Solinas et al., 2008). Accordingly, Soderstrom and colleagues investigated endocannabinoid signaling for its potential role in birdsong learning. They found enrichment of the transcript encoding the cannabinoid receptor, CB1, in song control nuclei HVC and RA. CB1 levels appeared to vary over the course of song learning (Soderstrom & Johnson, 2000). Exposure of young and adult zebra finches to daily doses of cannabinoids disrupted song learning in juveniles but had no effect when administered to adults (Soderstrom & Johnson, 2003). The converging view, now supported by this work in songbirds, is that common systems mediate reward signals in vertebrate learning and memory. These studies provide support for the continued use of songbirds to uncover common mechanisms for reward-based, procedural learning.

2.3. Gene candidates – focus on critical periods

Birdsong, like human language, exhibits developmental critical periods for learning (see introduction to this volume and also Kirn contribution). Consequently, genes known to be involved in mammalian critical periods, such as NMDA receptors mentioned above, may similarly function in critical periods for song learning. In the rodent visual system, several research groups have shown that maturation of GABAergic inhibitory circuits control critical period timing (for review see Hooks and Chen (2007)). Recently, Hessler, Hensch and colleagues have investigated the effects of diazepam, a GABAergic agonist, on zebra finch song development (Yazaki-Sugiyama et al., 2007). Diazepam administration to young males prematurely closed the sensory acquisition phase of learning. Birds were tutored by two adult songbirds, sequentially; first, a heterospecific Bengalese finch male, and second, a zebra finch male. Treated juveniles learned only short excerpts of song from the Bengalese male, and failed to learn any song from the second, conspecific tutor. Associated and potentially underlying this premature closure, calretinin, a marker for inhibitory interneurons in HVC, reached adult-like expression levels prematurely.

In the rodent visual field, brain-derived neurotrophic factor has been demonstrated to accelerate both critical period time-course and the development of a class of GABAergic interneurons that robustly express the calcium-binding protein, parvalbumin. In parallel with the decline of critical period plasticity, extra-cellular matrix molecules condense around these parvalbumin-positive cells and form what are referred to as peri-neuronal nets (PNNs, Hanover et al., 1999). Now, Hensch's group has shown that a homeoprotein known as Otx coordinates the postnatal maturation

of the parvalbumin interneurons in mice. Bidirectional manipulation of Otx levels in the developing visual cortex produced opposing effects on parvalbumin neuron development and critical period timing (Sugiyama et al., 2008).

In line with this work, preliminary studies in songbirds conducted by Nick and colleagues have examined the development of PNNs in zebra finch song nuclei, including HVC. PNNs are detected using an antibody against a major component of these nets: chondroitin sulfate proteoglycans. As in mammals, PNNs are concentrated around parvalbumin-containing interneurons in zebra finch brain. PNN levels in normally reared zebra finches exhibit developmental regulation in song nuclei during song learning; they are low during early stages of sensory acquisition, and then rising across development. The researchers then reared young zebra finches as isolates, without exposure to adult male song. As mentioned above, this manipulation can delay the closure of the critical period for sensory acquisition (Livingston et al., 2000; Morrison & Nottebohm, 1993). Isolation reduced the percentage of parvalbumin-containing interneurons in HVC and their association with PNNs, relative to levels in normally reared birds (Balmer et al., 2009) Combined with the evidence from mammals, the strong correlation between PNN expression and vocal learning state in zebra finches suggests that common mechanisms exist for developmentally-regulated critical periods for plasticity. They further suggest that Otx should be tested in the developing songbird to determine if this homeotic gene product regulates the closure of the sensory acquisition phase of vocal learning.

2.4. Gene candidates – focus on immediate early and 'motor-driven' genes

In addition to examining regional enrichment of genes, Clayton, Mello and colleagues also tested immediate early genes as candidate molecules potentially involved in vocal learning. The term immediate early gene (IEG) refers to those molecules whose mRNAs are among the first to increase following cellular stimulation, e.g. growth factor exposure and, in the brain, neuronal depolarization. In the case of neural activity, IEG transcript levels often peak within 30 min of depolarization. Increased IEG expression is independent of new protein translation as it occurs in the presence of protein synthesis inhibitors. Thus, IEGs such as fos, jun, and arc are often called 'primary response genes'. An advantage of examining IEG expression is that their protein products are often transcription factors and thus provide a regulatory link to their target molecules whose function may be critical to a given neuronal system, including vocal learning. In 1992, Mello, Vicario and colleagues showed that the IEG and transcription factor egr-1 is dramatically up-regulated in auditory processing regions of canaries following exposure to previously recorded songs (Mello et al., 1992).

Reasoning that neurons in the song control circuit exhibit auditory responses for the bird's own song, Jarvis and Nottebohm were surprised not to observe enhanced egr-1 expression in the song circuit of birds exposed to song playbacks (Jarvis & Nottebohm, 1997). (Because their increased mRNA levels can reflect neural activity, IEG signals have been used as proxies for neuroanatomical patterns of firing. That said, it is important to note that temporal firing information, the excitatory or inhibitory phenotype of active neurons, and other key electrophysiological features are not revealed by IEG signals but require further testing. Moreover, the relationship between neural activity and IEG expression may depend upon the pattern of neural activity rather than on the absolute level (c.f. Poopatanapong et al., 2006)). Jarvis and Nottebohm went onto discover that egr-1 is dramatically up-regulated in song circuit neurons only when birds sing. Singing related increases occurred even in deafened birds. This observation was termed 'motor-driven gene expression', and, in addition to opening up investigation of egr-1's downstream gene targets, it pioneered the way to identifying suites of genes whose expression is altered as a function of singing.

Similar behavior-based molecular inquiry has been coupled with microdissection and microarray techniques for the ambitious goal of revealing all genes that are activated in song control regions as a function of singing (Wada et al., 2006). In this hybrid between the 'candidate gene' and 'regional enrichment' approaches, groups of birds in varying behavioral conditions are allowed to sing, thereby inducing motor-driven gene expression. At a specified time following song onset, birds are sacrificed, song control regions microdissected, and mRNAs extracted. A labeling reaction converts these to cDNAs which are then exposed to microarrays that contain excerpts of known zebra finch genes. Hybridization between tissue and array cDNAs produces a signal which is then used to determine those genes whose expression levels vary as a function of the behavioral state. Similar approaches are being used by a variety of groups to characterize genes associated with, for example, song learning (Li et al., 2007b). Microarray approaches are also being used to identify hundreds of genes that are differentially expressed within a song control nucleus relative to the outlying area (e.g. Lovell et al., 2008). Of course, such investigations are limited by the number of genes present on the chip (for the current state-of-the-art, see Replogle et al. (2008)) and by the precision of the tissue dissection. Laser capture of mRNA from single, identified neurons (c.f. Lombardino et al., 2006) provides additional finesse. Further, direct deep sequencing methods can be used to provide an unbiased assessment of all genes involved, rather than only those on the chip (c.f. Toth et al., 2007).

2.5. Genes from human disorders: FoxP2 and beyond

None of the genes mentioned thus far have a direct link to human language. In striking contrast, in 2001, a molecule known as FOXP2 was discovered to be the single locus of a mutation underlying an inherited language disorder (Lai et al., 2001). This discovery was based on a case study of a family known by the initials 'KE'. The severe speech disorder exhibited by some KE family members had been investigated by Hurst and colleagues who noted that the inheritance pattern was consistent with a dominant monogenetic locus (Hurst et al., 1990). Indeed, half the KE family suffers from a rare form of Specific Language Impairment (SLI) in which the most prominent deficits lie in sequencing of orofacial movements, especially those required for speech. This disorder is referred to as developmental verbal dyspraxia. Meanwhile, non-learned orofacial control involved in chewing, swallowing or smiling is unimpaired (for review see Marcus and Fisher (2003); Vargha-Khadem et al. (2005)). In addition to core deficits in orofacial control and spoken language, affected individuals are also impaired on tests of verbal fluency and language comprehension. Whether these additional problems are secondary to growing up with speech deficits or are themselves primary is a topic of ongoing investigation, and insight may come from studies in songbirds (see below).

While closing in on the genetic locus of the KE family disorder, Lai, Fisher and colleagues also focused on other probands with the developmental verbal dyspraxia phenotype. They identified an unrelated boy with similar deficits, referred to as CS. Examination of CS's chromosomes revealed a fortuitously detectable rearrangement between chromosomes 5 and 7. One end of this translocation interrupted a gene on chromosome 7 encoding the transcription factor known as FOXP2. Returning to the KE family, the researchers discovered a single point mutation in the FOXP2 gene sequence that segregated with the disorder. No such mutation occurred in the unaffected family members nor in a large sample of unrelated

normal adults. Together, these pieces of evidence pinpointed FOXP2 as a molecule critical for human speech and language.

What is FOXP2 and how does it function? FOXP2 is part of a family of so-called forkhead winged helix (FOX) transcription factors (Carlsson & Mahlapuu, 2002). The founding member of the Fox family was identified in *Drosophila* as the gene locus responsible for the forked-head of mutant embryonic fruit-flies. Fox proteins are transcriptional regulators that can activate or repress the transcription of other genes via DNA binding domains. They are involved in a wide variety of biological processes including patterning of the embryo, and mutations in their genes lead to diverse developmental disorders, dramatically evidenced by the fork-headed fly (Marcus & Fisher, 2003).

2.6. The KE and other mutant FOXP2 forms

Consistent with a role for FOXP2 in embryonic neural patterning, imaging studies have revealed bilateral abnormalities in the brain structure of affected KE family members when compared with unaffected relatives. Differences are seen in the basal ganglia and cerebellum, in addition to cortical abnormalities including in Broca's area in the inferior frontal gyrus. Altered amounts of grey matter in these regions are accompanied by their under-activation during tasks of verbal fluency, coincident with over-activation of diffuse cortical regions not observed in normal controls (see for review Fisher and Marcus (2006)). These findings suggest that a mutant copy of *FOXP2* during development results in the malformation of brain structures later used in the central control of orofacial musculature important for speech. Since 2001, additional mutations in *FOXP2* have been discovered in human cases of developmental verbal dyspraxia cases, providing further confirmation of the link between FOXP2 and speech (Macdermot et al., 2005). Using *in vitro* cell culture systems, Vernes and colleagues showed that the mutant proteins failed tests of their ability to bind to DNA (Vernes et al., 2006). Similar decreased binding of mutant FOXP2 forms in human cases likely results in altered gene transcription in cells that express the protein. This altered transcription appears to affect brain development but curiously other body organs in which the protein exists, such as the lung, appear spared, a topic we shall return to.

2.7. Animal models for FoxP2: focus on birdsong

In addition to cell lines, advances in our understanding the neural function of FoxP2 have been made mainly in mice and songbirds (reviewed in White et al., 2006). As in humans, FoxP2 in these animals is expressed in the cortex/pallium, striatum, and thalamus, among other brain regions, during development; consistent with a role in forming these neural structures (Ferland et al., 2003; Haesler et al., 2004; Lai et al., 2003; Takahashi et al., 2003; Teramitsu et al., 2004). Further, in zebra finches, expression persists into adulthood when *FoxP2* mRNA and protein are down-regulated in Area X of the striatum when adult birds sing (Miller et al., 2008; Teramitsu & White, 2006). This 'on-line' regulation, precisely in the striatal sub-region dedicated to song, and precisely when birds engage in singing strongly implicates the molecule in the functional use of this structure. Tests of an on-line role, in addition to its developmental one, require altering FoxP2 expression during different stages of vocal learning, including in adulthood, and observing the phenotypic outcome.

Accordingly, Haesler, Rochefort and colleagues developed a lentivirus bearing short interfering hairpin RNA (shRNA) constructs designed to knock-down FoxP2 in zebra finches. Virus was injected bilaterally into Area X of 23 day old male finches to determine whether this would interfere with sensorimotor learning (Haesler et al., 2007). Control birds received injections of virus encoding GFP or an shRNA that did not target any zebra finch gene.

All juveniles underwent normal tutoring, and multiple features of their song learning were assessed. Strikingly, at maturity, birds that had received the FoxP2 knock-down construct exhibited less precise copying of their tutors' songs than did the controls. The decreased similarity included omissions, repetitions, and abnormally variable durations of syllables. No difference in the consistency with which knock-down birds ordered their syllables was detected, although the repetitions and omissions suggest that syllable order may have differed from the tutor song. This work represents the first case of genetic interference in songbirds resulting in documented changes to their song. It may be that altering the expression of most any transcription factor in cells that control song would result in song abnormalities. Yet, the fact that FoxP2 is vital for normal human language development is consistent with the idea that the imprecise copying in FoxP2-knock-down birds reflects its specific contribution to vocal learning.

Production of transgenic mice, as opposed to birds, is technically routine. Thus far, four groups have altered *Foxp2* in mice, to either knock it out entirely (Shu et al., 2005), to insert the KE family mutation (Fujita et al., 2008; Groszer et al., 2008) or to insert the normal human-like form (Enard et al., 2009). In brief, although some groups found changes in the unlearned ultrasonic vocalizations emitted by mouse pups, others did not. Interestingly, Groszer and colleagues found altered non-vocal motor learning in mice bearing the KE-like mutation. Heterozygotes exhibited deficits on the accelerating rotorod and the tilted voluntary running wheel tasks. Further in these mice, neurons in the dorsal striatum failed to exhibit a form of synaptic plasticity implicated in the accelerating rotorod learning paradigm (Dang et al., 2006). Because the focus of this contribution is on birdsong and language, the interested reader is referred to Teramitsu and White (2008) for further review of the mouse phenotypes. Meanwhile, the human developmental verbal dyspraxia phenotype that is accompanied by abnormalities in the striatum and cortex of affected KE family members, together with the imprecise song copies in birds with lowered striatal FoxP2, suggest that the neural basis of this subset of human deficits may best be investigated in songbirds. The additional data from mouse studies also emphasize the importance of the striatum in skill learning, be it mouse locomotor coordination, birdsong, or human speech.

2.8. FoxP2 specifics

Given that FoxP2 is expressed in lung and involved in its differentiation, one might ask why the KE family mutant FOXP2 phenotype is restricted to the brain and, within that, to the language system. Indeed, FoxP2 is expressed in most, if not all, body organs (personal observations; Lu et al., 2002; Shu et al., 2007). Part of the answer to distinct functions for FoxP2 likely lies in the distinct co-regulators that are present in different tissues, and the suite of genes that, together, they regulate. To investigate distinct neural targets of FOXP2, Spiteri and colleagues compared human fetal basal ganglia and inferior frontal cortex – two main areas of dysfunction in people with FOXP2 mutations – and human fetal lung (Spiteri et al., 2007). In a technique known as ChIP–chip, they used a primary antibody raised against a FOXP2 peptide to perform chromatin immunoprecipitation on material from each of these tissues. In this method, the antibody 'pulls-down' the transcription factor while it is bound to the non-coding promoter region of its target genes. Following chemical dissociation, the targets were hybridized to promoter microarrays to enable their identification, resulting in a list of 175 molecules. Eight of these were found to be enriched in the basal ganglia and inferior frontal cortex, but not in lung, supporting the idea that FOXP2 plays brain-specific

functions based on brain-specific gene targets. Fourteen FOXP2 targets exhibit accelerated evolution, leading the authors to suggest that these molecules '*comprise a key cohort potentially related to human cognitive specializations integrated by the BG and IFC, including speech and language*'.

These findings are reported together with those of Vernes and colleagues who also used ChIP–chip to identify FOXP2 gene targets in human neuronal-like SH-SY5Y cells (Vernes et al., 2007). Pathway analyses of their identified molecules highlighted Wnt/Notch signaling, among other molecular networks. *Wnt* genes help to pattern the mammalian forebrain during development, consistent with the idea that FOXP2 targets in this pathway contribute to the structural development of these regions. In addition to developmental growth and patterning, other targets identified by both groups suggest a function in activity-based sculpting of neural connections, including during learning. This is intriguing given our own work in zebra finches showing on-line regulation of FoxP2 in the striatal song control region when birds practice their songs (Miller et al., 2008; Teramitsu & White, 2006). These observations support a potential role for FOXP2 in behavioral plasticity in addition to its developmental functions. Further, FoxP2 mRNA in adult songbirds is socially regulated: its levels decrease in the striatal song control nucleus Area X when male zebra finches practice their songs, but remain stable when they perform their songs to females. This social regulation hints that FoxP2 function goes beyond basic motor control, since the motor output in each case is quite similar (Teramitsu & White, 2006). By analogy, differential regulation of FoxP2 may occur in the human brain when we rehearse learned oromotor sequences, versus when we engage in formal oration. Returning briefly to the ChIP–chip analyses, ~30% of the targets identified by Spiteri et al. were also uncovered by Vernes and colleagues. This significant overlap in the FOXP2 targets independently identified from human tissue or neuronal-like cell lines provides important validation for this work and this approach.

2.9. Other language-related genes

Clearly *FOXP2* is not the gene for language or even grammar. Rather, it represents a fortuitous entry point of discovery into molecular networks that, in the human brain, contribute to the language phenotype and, in other brains, may contribute to language sububsystems (c.f. Li et al., 2007a). Will other genes be discovered with such a direct connection to language? This is very possible given that many features of language are spared in affected KE family members (Bishop, 2009). How, then, should such genes be identified? One approach is to examine cases of human cognitive disorders in which impairment of language, if not the major phenotype, is also affected. Genes involved in these disorders may then be of relevance to language. An example is Fragile X syndrome in which an absence of expression of the single gene known as *Fragile X Mental Retardation-1* results in speech delays which accompany other cognitive deficits (Pieretti et al., 1991). Recently, Winograd and colleagues have cloned the gene for and developed an antibody against the zebra finch homolog of the fragile X mental retardation protein. Using young male zebra finches, they show that levels of this protein are enriched within neurons of the song control nucleus RA relative to the surrounding cortical-like pallium, just prior to the onset of sensorimotor learning (Winograd et al., 2008). The authors suggest that the fragile X mental retardation protein may '*participate in the cellular and synaptic changes that are occurring in sensorimotor learning*'.

In contradistinction to Fragile X, it has been argued that any complex disorder, even highly heritable ones such as SLI, autism and schizophrenia, must involve the interactions of many genes, each with their own small contribution. At the same time, it is increasingly recognized that a disorder such as autism is not monomorphic but is actually comprised of many subtypes; an understanding reflected in the new terminology of 'the autisms' and 'autism spectrum disorders' (ASD, Geschwind & Levitt, 2007). We can expect that in some of these subtypes, the links to underlying genes will be stronger than in others. Indeed, mutations in a handful of genes have now been implicated in ASD including Shank, Neuroligin 3 and Neuroligin 4 (Jamain et al., 2003; Stephan, 2008). Core deficits of ASD involve motor stereotypy and atypical social interaction accompanied by marked language deficits. I end by describing one case of a cognitive disorder with autistic features that resulted in the identification of the underlying molecule, providing further inroads to the genetic bases of language and its subsystems.

In 2006, Strauss and colleagues reported on a group of Old Order Amish children who harbored mutations in the gene known as contactin-associated protein-like 2 (CNTNAP2 Strauss et al., 2006). The children exhibited several deficits including intractable seizures, autism, and, relevant here, language regression. The *CNTNAP2* gene encodes a protein called CASPR2, previously shown to play a role in the paranodal localization of potassium channels to the nodes of Ranvier (Poliak et al., 1999). More recently, CASPR2 has been identified as a member of the neurexin gene family. In the brain, association of presynaptic neurexins with postsynaptic neuroligins is thought to be a key component of synaptogenesis (Dean et al., 2003; O'Connor et al., 1993).

In 2008, three separate groups identified *CNTNAP2* as an autism-susceptibility gene in the more general population (reviewed in Stephan, 2008). In one of these studies, certain *CNTNAP2* variants in autistic children were found to be associated with the age at first word (Alarcon et al., 2008). Most recently, Vernes, Fisher and colleagues discovered that in children with SLI, genetic polymorphisms of *CNTNAP2* correlate with their ability to perform a nonword repetition task (Vernes et al., 2008). Intriguingly, cross-species comparisons among mammals demonstrate that the distribution of *CNTNAP2* in the brain differs between vocal learners and non-vocal learners: it is enhanced in language-related cortico-basal ganglia–thalamic circuitry in fetal human brain, in contrast to a broad, non-punctuated distribution in embryonic mouse and rat brain. This neuroanatomical specialization in humans is consistent with the genetic links to language, above. Together, these data provide strong evidence that CNTNAP2 contributes to the regional and functional cortical patterning of the developing human brain that underlies learned vocal communication, potentially link it to FOXP2 (Abrahams et al., 2007).

As mentioned above, a limit on microarray-based gene discovery exists when some genes are missing from the arrays. A variation of the ChIP–chip method is referred to as ChIP-seq in which, following dissociation, the target DNAs are directly sequenced to reveal their identities. This unbiased approach was used by Vernes and colleagues (Vernes et al., 2008) to identify FOXP2 targets not present on the promoter array. Impressively, these studies identified *CNTNAP2* as a direct target of FOXP2 transcriptional repression. In accordance with this regulatory relationship, the human *CNTNAP2* cortical pattern is opposite that of *FOXP2*, i.e. *FOXP2* levels are high where *CNTNAP2* levels are low, consistent with FOXP2 repression of this transcript (Abrahams et al., 2007).

3. Summary

The FOXP2–CNTNAP2 connection fulfills the prediction that – while not the gene for language – FoxP2 is a fortuitous genetic entry point into the molecular networks that support the language phenotype. Indeed, FOXP2 variants have not been convincingly associated with SLI nor with common developmental disorders in which language is delayed or impaired such as autism. In sharp contrast,

recent work discussed above now demonstrates that one of FOXP2's direct transcriptional targets, namely CNTNAP2, is associated with both SLI and autism. This interaction connects FOXP2 with language disorders with which it was not otherwise implicated.

The FoxP2–CNTNAP2 molecular story illustrates how genes linked to language impairments, or to other disorders in which language deficits are prominent, can be fruitfully investigated in songbirds to determine their impact on the vocal learning subsystem and its underlying neural circuitry. Currently, our group is investigating the developmental expression and function of CNTNAP2 in song circuitry (Panaitof et al., Society for Neuroscience Abstracts, 2009). Of course, work in songbirds will not provide the full answer to the neural mechanisms underlying the complex language phenotype. Ideally, multiple animal models will be used that each capture key features of language. Future work in songbirds will rely on improving technologies for altering gene expression with temporal and anatomical precision during phases of song development and in song control circuitry, in order to test the functional consequences on song learning and maintenance. As this technology becomes routine, it will be important to make specific hypotheses about how song might be altered, and to provide data from outside song control areas to determine whether disruption within song control regions specifically affects only song, or causes general disruption of common neuronal function.

Finally, as we move from identification of immediate early genes and other transcription factors to their targets, comparison of the specific molecular networks activated in different brain regions and in different animal models may be ultimately informative as to the adaptations unique to the human linguistic brain (c.f. Oldham et al., 2006). Further, those gene targets that are related to neuronal communication may then bring us back to animal models in which changes in synaptic strengths of key circuits and microcircuits can be functionally related to the behaviors that they control, including vocal learning.

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