Molecular Microcircuitry Underlies Functional Specification in a Basal Ganglia Circuit Dedicated to Vocal Learning

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SUMMARY

Similarities between speech and birdsong make songbirds advantageous for investigating the neurogenetics of learned vocal communication—a complex phenotype probably supported by ensembles of interacting genes in cortico-basal ganglia pathways of both species. To date, only FoxP2 has been identified as critical to both speech and birdsong. We performed weighted gene coexpression network analysis on microarray data from singing zebra finches to discover gene ensembles regulated during vocal behavior. We found ~2,000 singing-regulated genes comprising three coexpression groups unique to area X, the basal ganglia subregion dedicated to learned vocalizations. These contained known targets of human FOXP2 and potential avian targets. We validated biological pathways not previously implicated in vocalization. Higher-order gene coexpression patterns, rather than expression levels, molecularly distinguish area X from the ventral striato-pallidum during singing. The previously unknown structure of singing-driven networks enables prioritization of molecular interactors that probably bear on human motor disorders, especially those affecting speech.

INTRODUCTION

Speech and birdsong are examples of the rare ability to learn new vocalizations. Both depend on hearing and are supported by analogous neural pathways through the cortex and basal ganglia (Lieberman, 2006). In humans, such pathways support an array of behaviors, but songbirds like the zebra finch possess well-defined subcircuitry specialized for song learning and production, enabling the design of experiments to uncover vocal-motor-specific function (Figure 1A; Jarvis, 2004). The transcription factor FoxP2, critical for birdsong and the only molecule directly linked to speech and language dysfunction (White, 2010), is expressed similarly in these pathways in both species (Teramitsu et al., 2004). The discovery of FOXP2’s link to vocal-motor dysfunction was a constructive step toward understanding the genetic basis of speech, but learned vocalization is a complex phenotype and probably depends on interactions between many genes. Methodological limitations preclude the study of gene expression in behaving humans, so the neuro-molecular underpinnings of speech remain poorly understood.

Zebra finches, however, are well suited as a model system for neurogenetic investigations of learned vocal-motor behaviors including speech, a notion bolstered by the sequencing and assembly of their genome (Warren et al., 2010).

To elucidate gene ensembles underlying learned vocalizations, we used weighted gene coexpression network analysis (WGCNA; Zhang and Horvath, 2005) to identify and investigate groups of genes coregulated during singing. This biologically inspired method (Supplemental Experimental Procedures, available online) has previously yielded results that could not have been obtained using traditional microarray analyses (Oldham et al., 2008), with gene coexpression groups typically corresponding to functional pathways. Past uses have uncovered novel genes important for human evolution and brain development and have highlighted genes with clinical significance for pathologies such as cancer (Zhao et al., 2010).

Our experimental design was based upon prior studies showing that FoxP2 levels within the song-specialized basal ganglia subregion, striato-pallidal area X, decrease after 2 hr of undirected singing (Miller et al., 2008; Teramitsu and White, 2006; Teramitsu et al., 2010), a form of vocal practice (Jarvis and Nottebohm, 1997; Jarvis et al., 1998), with the magnitude of downregulation correlated to how much the birds sang (Teramitsu et al., 2010). In addition, we observed increased vocal variability after 2 hr of undirected singing (Miller et al., 2010), and another group found abnormally variable acoustic structure in the adult song of birds that underwent knockdown of area X FoxP2 during song development (Haesler et al., 2007). Together, these findings imply that low FoxP2 levels in area X are coincident with increased vocal variability and that genes...
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RESULTS AND DISCUSSION

Prior to network construction, we defined gene significance measures (GS, Supplemental Experimental Procedures) for each probe to relate expression variability to trait variability across all birds (n = 26), e.g., to the act of singing (referred to as GS.singing.X when measured in area X and GS.singing.V when measured in VSP; see Experimental Procedures for explanation of “probe” versus “gene”). In area X, after false discovery rate (FDR) correction, 2,659 probes representing 1,364 known genes were significantly correlated to the act of singing (q < 0.05; GS.singing.X), and 3,709 probes (1,825 known genes) to the number of motifs sung (GS.motifs.X; motifs are neuroethologically relevant sequences of song notes, Hahnloser et al., 2002), with 1,132 genes common to both. In sharp contrast, 0 probes in the VSP had significant GS.singing.V or GS.motifs,V scores (Table S2). We observed small differences in probe expression values in the singing versus nonsinging birds: in area X, only 177 probes (~0.9% of the total) showed > 100% up- or downregulation, 65 probes > 200%, 3 probes > 1000%. In the VSP, only 17 probes showed > 100% up- or downregulation (~0.08%), 6 probes > 200%, and 0 probes > 1000%. We also measured correlations to individual acoustic features such as Wiener entropy (a measure of width and uniformity of the power spectrum (Tchernichovski et al., 2000; GS.entropy) that are typically used to assess song (Figures 2B and S3, Table S2). GS.age was computed for each bird as a negative control. Importantly, GS results did not influence network construction in any way.

During preprocessing, all samples were hierarchically clustered to visualize interarray correlations and remove outliers (Supplemental Experimental Procedures). The area X versus VSP samples segregated into two distinct clusters, as would be expected if tissue source influences gene expression (Figure 2A and S2). There was a significant correlation between the number of motifs sung and Wiener entropy, replicating our prior finding of heightened vocal variability after 2 hr of singing (Figure 2B; Miller et al., 2010).

Essential Network Terminology

To identify ensembles of genes that were tightly coregulated (modules) during singing, we performed WGCNA (Experimental Procedures) of the area X samples and quantitatively related the resulting modules to traits. Coexpression networks were built based exclusively on expression levels, via unsupervised hierarchical clustering on a biologically significant distance...
metric (topological overlap, TO; Experimental Procedures), and relationships between GS and network structure were only examined post hoc. Modules were defined as branches of the dendrogram obtained from clustering and labeled by colors beneath the dendrogram (Figure 3A; probes outside properly defined modules were considered background and colored gray). To study module composition we defined the first principal component of each module as the module eigengene (ME), which can be considered a weighted average of the probe expression profiles that make up the module. Correlating MEs to traits, e.g., number of motifs sung, is an efficient way to relate expression variability within modules to trait variability. The module membership (MM) and intramodular connectivity (kIN) of each probe were defined as the correlation of its expression profile to the ME and the sum of its network connections with other module members, respectively (Experimental Procedures). MM and kIN are closely related; high values for either indicate tight coexpression with most other module genes, signaling increased biological importance.

The Supplemental Experimental Procedures section contains further information on WGCNA methodology, definitions, and advantages.

Multiple Area X Coexpression Modules Strongly Related to Singing

WGCNA yielded 21 proper coexpression modules in area X (Figure 3). Correlations were computed between MEs and traits, and p values were computed for each correlation (Experimental Procedures). After Bonferroni correction (significance threshold \( p = 1.7 \times 10^{-4} \)), the MEs of three modules were significantly related to the act and/or the amount of singing (Figure 3B, Table S3): the blue module (act of singing and number of motifs), the dark green module (act of singing and number of motifs), and the orange module (number of motifs). The positive correlations of the blue module (2,013 probes representing 995 known genes) indicate upregulation of its members during singing and, in general, increased expression with more singing. In contrast, the negative correlations observed for the dark green (1,417 probes representing 824 known genes) and orange (409 probes representing 234 known genes) modules indicate significant downregulation with the act of singing (dark green only) that continued in concert with increased amounts of singing (both). Since Bonferroni correction often results in false negatives (Benjamini and Hochberg, 1995) we also performed a less conservative FDR procedure (Experimental Procedures), yielding two additional significant ME correlations to the number of motifs sung (black and salmon modules) and two to Wiener entropy (blue and orange modules). There were no significant correlations to age.

These five “singing-related” modules contained ~83% of the probes with significant GS.motifs.X and GS.singing.X scores. Compared to the rest of the network, genes in these modules were more strongly coupled to the act and amount of singing, and to Wiener entropy (GS.singing.X, GS.motifs.X, GSentropy.X \( p < 1 \times 10^{-20} \), Kruskal-Wallis ANOVA). The most interconnected probes within the singing-related modules were also the most tightly regulated by singing, as evidenced by the significant correlations of MM to GS.singing.X and GS.motifs.X in these modules (Figures 4A–4C and S3A–S3F), indicating a strong relationship between importance in the network and behavioral relevance. MM-GS relationships such as these were not found in modules unrelated to singing, e.g., the dark red and turquoise modules, indicating that connectivity, and probably the biological functions in those modules, is relatively unspecialized with respect to vocal-motor behavior in area X, at least after 2 hr of singing.

Gene Significance of Area X Song Module Genes Is Not Preserved in VSP

We performed a series of comparisons between area X and the VSP to test the hypothesis that area X singing-related network structure was specific to vocal-motor function and not due to motor function in general. We note that the region of outlying striato-pallidum selected for our analysis, the VSP, is not transcriptionally “muted” during singing; rather, it exhibits immediate early gene (IEG) activation thought to reflect nonvocal movements that co-occur with singing (Feenders et al., 2008). To test whether single probes exhibited similar relationships to singing in both regions, we compared GS scores from area X to those measured in the VSP. As noted above, no probes had significant GS values for the amount or act of singing in the VSP, in contrast to thousands in area X. We compared...
GS.motifs.X and GS.singing.X within each module to GS.motifs.V and GS.singing.V for the same probes in the VSP and found weak correlations overall, especially for genes in the song modules (Figures 4D–4F and S3G–S3L). Thus, genes whose area X expression is tightly coupled to singing have a very different relationship, or none at all, to this behavior in the VSP.

**Area X-Specific Coexpression Patterns Correspond to Singing**

Next, we compared coexpression relationships within each area X module to the coexpression relationships between the same probes in the VSP, assigning each module a preservation score based on statistical comparisons of module composition and structure (Table S3; Langfelder et al., 2011). Area X modules were preserved to varying degrees in the VSP, with the blue, dark green, and orange song modules being the least preserved, and the modules most unrelated to singing (e.g., dark red and turquoise) being the most preserved. The song modules were effectively nonexistent outside of area X, and there was a significant relationship between the strength of ME-singing correlations (Figure 3B) and module preservation ranks (Figures 4G and 4H), revealing a direct link between singing-relatedness and area X-specific network structure in the basal ganglia.

**Area X-Specific Coexpression Patterns Do Not Correspond to Gene Expression Levels**

To test whether the regional differences in singing-related network structure were simply due to differences in gene expression levels, we began by computing correlations between the expression values for each probe in area X and VSP. There was remarkable similarity overall (cor = 0.98, p < 1e-200). Inspection of individual modules revealed a range of strong correlations between area X and VSP expression values (0.94–0.99; Figures 5A–5E). In contrast, we observed a weaker
Figure 4. Module Membership Predicts Relationship to Singing in Area X

(A–C) Area X GS scores for the number of motifs sung are plotted as a function of MM for probes in the blue (left), dark green (center), and orange (right) song modules. Each dot represents one probe. Dashed lines represent the linear regression of GS.motifs.X on MM in each module, with the Pearson correlation coefficient \( r \) and p value (based on Fisher’s \( z \) transformation) shown at top. Arrows indicate approximate locations of the EGR1 (blue module) and FOXP2 (orange module) probes shown in Figure 3A.

(D–F) GS scores arising from the VSP (V) plotted as a function of the values in area X for the number of motifs sung. Each dot represents one probe. Dashed lines represent the linear regression of GS.motifs.V on GS.motifs.X in each module, with the Spearman rank correlation coefficient \( \rho \) and p value shown at top.

(G and H) The magnitude of ME-motifs (left) and ME-entropy (right) relationships in area X (absolute values of correlations represented in Figure 3B heatmap) plotted as a function of the degree of preservation of each module across brain regions. Each circle represents a module, colored accordingly, e.g., the blue, dark green, and orange song modules (upper right) had the strongest ME-correlations and were the least preserved in the VSP. Dashed lines represent the linear regression of ME-motifs and ME-entropy correlations on preservation rank, with Spearman’s \( \rho \) and p value shown at top. The purple and yellow modules overlap in the right panel. See also Figure S3.
overall correlation between area X and VSP network connectivity (cor = 0.61, p < 1e-200), especially within the three song modules (Figures 5F–5J; blue, dark green, orange: mean cor = 0.23; all other modules: mean cor = 0.49).

Activity in certain area X neurons increases during singing (Hessler and Doupe, 1999). One possibility for why the song modules were observed in area X but not VSP is that this increase in neuronal firing leads to increased gene expression levels only in area X. To test this, we computed the normalized median gene expression levels in both brain regions for each bird. In nonsingers, levels were higher in VSP than in area X (Figure 5K). This difference disappeared in singing birds; gene expression levels in area X and VSP became very similar (Figure 5L). These results imply that the area X-specific song modules cannot be accounted for by higher (or lower) area X gene expression levels compared to VSP during singing. Rather, as revealed here by WGCNA, the relevance of transcriptional activity in these regions to singing is determined more by region-specific coexpression relationships, which comprise “molecular microcircuitry” that arises during a specific behavior (singing) within a specific brain region (area X) supporting that behavior. In line with the idea that mere neural activity levels do not account for the song-specialized gene modules, we previously found that activation of the IEG Synaptotagmin 4 (Syt4) is not achieved by overall depolarization of neurons but rather requires the patterned activation underlying singing (Poopatanapong et al., 2006).

**In Silico Validation of Singing-Driven Coexpression Networks**

The new relationships we uncovered between gene coexpression patterns and singing are substantiated by the presence of previously identified area X singing-regulated genes in the song modules (e.g., EGR1, Jarvis and Nottebohm, 1997; FOS, Kimpo and Doupe, 1997; blue module; FOXP2, Teramitsu and White, 2006; dark green/orange modules; by convention, gene symbols are capitalized and italicized and are not meant here to denote the human form, Kaestner et al., 2000). Consistent with prior reports, EGR1 (Jarvis and Nottebohm, 1997) and
Singing-Related Modules Contain Human FOXP2 and are strongly suppressed by singing in our data, and thus selected TRPV1 (3.3e-4, Fisher’s exact test). One of the ion channel genes, (114 probes; Table S2), with six in the orange song module (p < 3e-11), strongly upregulated during song perception. Of these, 42/49 were in our network (kIN.X, p = 4.2e-4), especially in the blue song module, strongly upregulated with singing, including 9/12 probes for FOXP2. Of 302 targets found by a second study in SY5Y cells (Vernes et al., 2007), 119 were in our network (246 probes total; Table S2). Interestingly, these targets showed the opposite regulatory pattern, displaying high MM in modules upregulated with singing (blue: p = 9e-4; black: p = 8.6e-3; Table S2) but low MM in the orange module (p = 9.6e-5; Table S2). The comparison of GS scores from these two groups of genes reiterated their contrary regulation during singing (GS.motifs.X scores were more negative in fetal brain targets, p < 0.04; Table S2). These differences may be attributed to the different tissue types used in each study.

Eleven targets found by both studies were in our network. In line with our prediction, probes representing these 11 targets had strong relationships to singing (29 probes total; absolute values of GS.motifs.X, p = 0.037; GS.singing.X, p = 0.017, Kruskal-Wallis; Table S2), with a trend for greater expression increases in singing versus nonsinging birds (p = 0.064), compared to the rest of the network. Compared to the rest of the module, targets in the dark green song module (GBAS and VLDLR, seven probes total) had high kIN.X and strong negative correlations to GS.motifs.X while showing no difference in expression levels (Figures 6A–6C). This reinforces our finding that the connectivity of genes supersedes expression levels in dictating specification of networks for vocal behavior.

More recently, Vernes et al. (2011) performed a large-scale chromatin immunoprecipitation analysis of all known promoters and expression profiling to identify direct Foxp2 targets in embryonic mouse brain. Of their putative 1,164 targets, 557 were present in our network, with 22 genes among the 300 closest network neighbors of FOXP2 (p < 0.04, Fisher’s exact test). These included NTRK2 and YWHAH, which the authors validated as direct targets. In our network, NTRK2, a blue song module member, was the 3rd-closest neighbor of FOXP2 (probelD = 2758927) and is part of a canonical network involved in posttranslational modification and cellular development, growth, and proliferation that also contains many other close network neighbors of FOXP2 (Figures 6D and 6F; Table S2). It was also found to be regulated during singing in area X by Warren et al. (2010). YWHAH, a gene involved in presynaptic plasticity, was in the blue song module, strongly upregulated during singing, and within the 300 closest network neighbors of FOXP2 (Table S2).
Figure 6. Behavioral Regulation of Gene Expression Coupled with WGCNA Captures Genes Coregulated with FOXP2

(A–C) Barplots show intramodular connectivity (left), GS in area X for number of motifs (middle), and expression level percent change in singing versus nonsinging birds (right), for the dark green module. Left bars in each plot represent values for two direct human FOXP2 targets, GBAS and VLDLR (Spiteri et al., 2007; Vernes et al., 2007); right bars represent the rest of the probes. Error bars = 95% confidence intervals. Kruskal-Wallis p values are shown.
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Two hundred and sixty-four genes were deemed “high confidence” targets by the authors; 95 of these were in our network, including 14, six, and four genes in the blue, dark green, and orange song modules, respectively. Compared to the rest of the network, these 95 genes had relatively high blue MM and low dark green and orange MM (p < 1e-3, Kruskal-Wallis test), a pattern similar to what we observed for FOXP2 targets identified in SY5Y cells (Supplemental Experimental Procedures; Vernes et al., 2007).

Overall, the findings by Vernes et al. (2011) indicate that in embryonic brain, Foxp2 modulates neuronal network formation by directly and indirectly regulating mRNAs involved in the development and plasticity of neuronal connections. This is compatible with our WGCNA results emerging from adult songbird basal ganglia suggesting a role for Foxp2 in singing-related synaptic plasticity via its high interconnectedness with genes linked to MAPKK binding, NMDA receptors, actin/cytoskeleton regulation, and tyrosine phosphatase regulation (see Biological Significance of Singing-Related Modules below).

We also found interesting overlaps between our results and those of two additional studies that identified direct and/or indirect FOXP2 targets. The first study identified genes with differing expression levels in human neural progenitor cells transfected with either the human or the chimpanzee version of FOXP2 (Konopka et al., 2009). Twenty-four such genes were in our network and showed high hIN.X in their respective modules compared to the rest of the network (61 probes total; p = 0.03, Kruskal-Wallis; Table S2). Those in the orange module had especially high hIN.X, compared to the rest of the module (CDC47.L, RUNX1T1: p = 2.7e-3; Table S2). We observed a similar trend for those in the blue module (B3GNT1, HEBP2, NPTX2, TAGLN: p = 0.074) but not in modules unrelated to singing that also contained many of these genes (turquoise, p = 0.9; yellow, dark red, p = 0.76). The second study identified 34 genes whose striatal expression levels (turquoise, p = 0.9; yellow, dark red, p = 0.76) were altered as a result of two human-specific amino acid substitutions introduced into the endogenous Foxp2 locus of mice (Enard et al., 2009). Of these, 13/34 genes were in our network (36 probes), including three in the song modules (ELAVL1: blue, HEXDC and YPEL5: dark green; Table S2), YPEL5 was highly connected in the dark green module and strongly suppressed by singing in our data, and was selected by direct and indirectly regulating mRNAs involved in the development and plasticity of neuronal connections. This is compatible with our WGCNA results emerging from adult songbird basal ganglia suggesting a role for Foxp2 in singing-related synaptic plasticity via its high interconnectedness with genes linked to MAPKK binding, NMDA receptors, actin/cytoskeleton regulation, and tyrosine phosphatase regulation (see Biological Significance of Singing-Related Modules below).

by extension, identified new genes and pathways (see below) that may be critical for speech.

Biological Significance of Singing-Related Modules

We used the functional annotation tools available through the Database for Annotation, Visualization, and Integrated Discovery (DAVID ver. 6.7, Huang et al., 2009) to characterize biological functions represented in the area X modules (Experimental Procedures). Many functional terms were enriched only in one of the singing-related modules, with the majority of these in the blue module; the most significant having to do with actin binding/regulation, MAP kinase activity, or proteasome activity (enrichment threshold = p < 0.1). See Table S4 for all enriched terms in these modules.

To identify the most singing-relevant functions, we defined a measure of term significance (TS) as the absolute value of the product of the mean MM and GS.motifs.X for genes annotated with the term, scaled by 1—the term’s p value. The mean MM, GS.motifs.X, differential connectivity (kIN.diff), and clustering coefficient of genes annotated by terms with the highest TS scores were compared to the rest of the module, allowing us to home in on particularly tight-knit, behaviorally relevant, biological pathways/functions in the singing-related modules (Supplemental Experimental Procedures). For example, 11 genes in the blue module (ARC, C4BP1, CNN3, DLG1, DLG2, DLGAP2, FREQ, HOMER1, IFngr1, NGLN1, and NTRK2) were annotated by the term “GO:0014069—postsynaptic density” (Table S4). Probes representing these genes in the blue module had high MM and GS.motifs.X (27 probes total; mean MM = 0.804, GS.motifs.X = 0.682), and the term “GO:0014069—postsynaptic density” had an enrichment p value of 0.059. Thus TS for this term = 0.804 × 0.682 × (1 – 0.059) = 0.516 (7th highest of 402 enriched blue module terms; Tables S2 and S4). Compared to the rest of the module, probes for the 11 genes annotated with this term had higher average MM (p = 6.2e-7; Kruskal-Wallis test), GS.motifs.X (p = 6.8e-5), kIN.diff (p = 4.7e-6), and clustering coefficient (p = 5.2e-5).

Other top-ranked blue module terms included “GO:0031434—mitogen-activated protein kinase binding” and “IPR019583:PDZ-associated domain of NMDA receptors,” as well as others involving actin, cytoskeleton, and tyrosine phosphatase regulation. Genes associated with these synapse-related functions in the blue module were also some of FOXP2’s closest neighbors, i.e., genes with which it had high TO (Figures 6D–6F, Table S2, Supplemental Experimental Procedures). This may imply a role for Foxp2 in the suppression of synaptic
plasticity, since blue module genes (whose levels increased with singing in these experiments) in high TO with FOXP2 (which decreased with singing) are good candidates for repressed transcriptional targets.

Each of the song modules was enriched for astrocytic markers with developing astrocytes most enriched in the blue module (p = 7.5e-6, Fisher’s exact test) and mature astrocytes in the orange module (p = 4e-3; Cahoy et al., 2008). This observation is consistent with the recent realization that astrocytes are involved in the regulation of neuronal functions, including behavior (Halassa and Haydon, 2010).

We screened the modules for genes associated with Parkinson’s disease (Supplemental Experimental Procedures), since it is a basal ganglia based disorder with a vocal component...
Figure 8. Behavioral Regulation of Hub Genes and Pathways in Area X

(A) Top left: immunoblot of area X protein from four undirected singing (UD) and four nonsinging (NS) birds shows bands for Reelin (~150 kD) and phosphorylated forms of the Dab1 protein (~107 kD, ~61 kD). Top right: Reelin protein is detected in brain extracts from a wild-type mouse (WT), whereas this band is absent in a reeler mutant mouse (~/~), confirming antibody specificity. A band of similar size is observed in zebra finch area X samples from an NS and a UD bird. Bottom panels: box and whisker plots show levels of Reelin protein (left) and of phosphorylated Dab1 isoforms (middle and right) as a function of singing. All three proteins are higher in area X of UD relative to NS birds (Mann-Whitney U two-tailed test, p = 0.03). Middle of each box represents the mean; top and bottom, standard error; whiskers, upper and lower 95% confidence intervals. Data from each bird is shown by individual points. At right, an immunohistochemical section at the level of area X (arrowheads) from a singing bird shows enhanced signals for Dab1 protein within the nucleus relative to outlying VSP. Scale bar = 100 μm. See also Figure S7. M = midline.

(B) Top left: immunoblot of area X protein from three undirected singing (UD) and three nonsinging (NS) birds shows bands at the predicted molecular weight for Ypel5 (~13 kD) that are not apparent in the preadsorption control (*), indicating antibody specificity. Right: quantification of signals from these and additional UD singers revealed a negative correlation between Ypel5 and the amount of singing (Spearman ρ = −0.76; p = 0.03, R² = −0.77). Bottom: photomicrographs of area X from a representative NS (top) and UD (bottom) bird. Immunofluorescent signals for Ypel5 (green) and the neuronal marker NeuN (red) are shown, as well as a no-primary antibody control (Control). All images were obtained at the same exposure. Qualitatively, more cell bodies appear labeled by the anti-Ypel5 antibody in the NS compared to the UD, most noticeable in the merged images where NeuN signals dominate in the UD bird. Scale bar = 200 μm. Insets of boxed areas in the merged images suggest that Ypel5 and NeuN are coexpressed within area X neurons, but in different subcellular regions.
and found enrichment in the black singing-related module (Figure S6). Another module that was moderately singing-related was also enriched for Parkinson’s disease-associated genes, as well as autism susceptibility genes (purple module, \( p = 2.7\times10^{-4}, p = 0.05 \), respectively, Table S2).

**Biological Significance of Other Modules**

The unique presence of the song modules in area X implies that the biological pathways they represent are coregulated in patterns specific to area X during learned vocal-motor behavior. Conversely, functions in modules found in both area X and VSP during singing may typify more general striato-pallidum-wide regulatory networks. To test this, we examined biological functions represented in the dark red, turquoise, and pink modules, the three most preserved in VSP (Figures 4G and 4H, Table S3). The turquoise module was the largest in the network (4,616 probes representing 2,743 known genes; Table S2). It was the only module enriched for many functional terms related to hormone binding, morphogenesis, neurogenesis, and development, implicating it in steroid sensitivity and the ongoing neurogenesis known to occur throughout the adult songbird striatum (Table S4; Nottebohm, 2004; Kim et al., 2004).

The turquoise, dark red, and pink modules were enriched for neuron and oligodendrocyte gene markers (turquoise: genes > 10-fold enriched in oligodendrocytes, \( p = 0.05 \), dark red: genes > 20-fold enriched in neurons, \( p = 0.03 \), Fisher’s exact test; Table S2; Cahoy et al., 2008) and markers of striatal and pallidal neurons (pink: \( p < 0.02 \); Table S2), consistent with the mixed striatal and pallidal nature of what was formerly known as the avian “striatum” (Farries and Perkel, 2002; Reiner et al., 2004). These findings are congruent with the idea that the preserved modules represent functions common across the striato-pallidum.

**Hub Genes and Biological Pathways in Singing-Driven Coexpression Networks**

Given the large number of genes in the song modules, we sought to identify the potentially most important genes for further study. We used two basic approaches (Figure 7): both began by restricting further analysis to the singing-related modules. In one approach, we then focused on song module genes with high GS.motifs.X and MM, i.e., genes highly interconnected within their module (hub genes) and strongly coupled to singing, and screened them for enriched functions and biological features. The other approach is exemplified above in the Biological Significance of Singing-Related Modules section where we functionally annotated the singing-related modules, then prioritized enriched functional terms based on TS scores (Supplemental Experimental Procedures; Table S4), highlighting sets of tightly interconnected singing-related genes that were both important in the module and shared an enriched common feature.

We used these approaches to select pathways in which to test for the presence of constituent proteins in area X. The importance of studying molecules in the context of biological pathways, rather than simply validating mRNA expression, is underscored by our finding that gene coexpression relationships, rather than expression levels per se, determine molecular microcircuitry underlying vocal-motor-specific behavior. As our focus was on the protein level, areas X tissue was isolated from singing and nonsinging birds at 3 (rather than 2) hours following either time from the first motif or lights-on, respectively, to allow for potential translation of mRNA changes (see Supplemental Experimental Procedures for description of tissue processing methods).

WGCNA identified very-low-density lipoprotein receptor, Vldir, a member of the Reelin signaling pathway, as a highly connected member of the dark green song module (mean GS.motifs.X = \(-0.78\), MM = 0.82; Table S2). Vldir was also identified in the literature as a human FOXP2 target (Spiteri et al., 2007; Vernes et al., 2007). In mammals, the Reelin pathway is critical to neuronal migration during development of the neocortex and cerebellum and to regulation of NMDA receptor-mediated synaptic plasticity in the adult hippocampus (Herz and Chen, 2006). Reelin binds to Vldir on migrating neurons and radial glial cells. While this pathway is well established in cortex-containing structures, less is known about the role of these molecules in the basal ganglia of any species. In songbirds, Reelin is expressed in cortical HVC and striato-pallidal area X of adults, but behavioral regulation had not been examined (Balthazart et al., 2008).

In line with behavioral activation of this pathway, expression of Reelin protein was significantly higher in singing versus nonsinging birds (Figure 8A). We also detected Vldir protein expression in area X (Figure S7A). Since in mammals, binding of Reelin to Vldir results in the activation of the cytoplasmic adaptor protein disabled 1 (Dab1) by tyrosine phosphorylation, we tested for singing-driven regulation of Dab1. As expected, we detected a significant increase in phosphorylated forms of Dab1 in area X of singers relative to nonsingers (Figure S8A), Digap2 (aka PSD95, blue module; mean GS.motifs.X = 0.65, MM = 0.82; Table S2) binds Vldir to the NMDA receptor, activating downstream molecules such as the cAMP responsive element modulator (Crem). CREM (blue module; mean GS.motifs.X = 0.83, MM = 0.95) shares high TO with FOXP2 (Figures 6D and 6F; Table S2), implicating FoxP2 in regulation of synaptic plasticity through indirect connections with the Reelin signaling pathway. As noted above, tyrosine phosphorylation and NMDA receptor-related functional terms stood out in the blue module, and DLAGP2 was one of 11 blue module genes annotated by “GO:0014069:postsynaptic density” (Table S4).

A second biological pathway containing yippee-like protein 5 (YpeilS) was selected for further study because of YpeilS’s identification as a putative target of the partially humanized Foxp2 (Enard et al., 2009), its GS.motifs.X score (mean of 3 probes = \(-0.71\)), and MM in the dark green module (mean = 0.86; Table S2). “PIRSF028804: protein yippee-like” and “IPR004910: Yippee-like protein” had the highest TS scores in the dark green module (Table S4). We viewed this as a rigorous test of the predictive power of WGCNA because of the relative lack of information about this molecule in vertebrates (Hosono et al., 2010). In immunohistochemical analyses, we observed signals for YpeilS protein in area X (Figure 8B), as well as for its binding partner, Ran Binding Protein in the Microtubule Organizing Center (Hosono et al., 2010), also in the dark green module (RANBPM aka RANBP9, data not shown). In line with its strong GS.motifs.X score, YpeilS was behaviorally regulated, with lower...
protein levels observed in area X of birds that sang more motifs (Figure 8B). Our results for both Reelin and Ypel5 demonstrate expression of multiple members of their respective signaling pathways in area X, with behavioral regulation of each.

As further validation, we detected protein signals within area X consistent with expression of Transient Receptor Potential Vanilloid Type 1 (Trpv1), a capsaicin receptor. We selected Trpv1 for validation because of its high MM and GS.motifs.X, and its identification as an ion channel positively selected for in the songbird lineage (Figure S7B; Warren et al., 2010). **TRPV1** is in the dark green and salmon singing-related modules (one probe in each; dark green: MM = 0.85, GS.motifs.X = –0.77; salmon: MM = 0.81, GS.motifs.X = –0.51; Table S2) and has been linked to endocannabinoid signaling pathways in the mammalian basal ganglia (Musella et al., 2009; Maccarrone et al., 2008). Cannabinoid exposure during zebra finch development interferes with song learning (Soderstrom and Tian, 2004), potentially through synaptic plasticity mechanisms such as modulation of glutamatergic synapses onto medium spiny neurons in area X (Thompson and Perkel, 2011) and altered area X FoxP2 expression (Soderstrom and Luo, 2010). In keeping with its strong GS.motifs.X score, we observed lower levels of Trpv1 signal in birds that sang more motifs (Figure S7B). These findings provide additional biological and literature-based validation of our WGCNA.

**DISCUSSION**

To our knowledge, this study represents the first identification of basal ganglia gene coexpression networks specialized for vocal behavior, and the first use of WGCNA to link coexpression modules to a naturally occurring, procedurally learned behavior. We found ~2,000 genes within the song-specialized striatal-pallidal area X, but not in VSP, that were significantly coupled to singing, most of which were members of one of five distinct singing-related modules. The three song modules (blue, dark green, orange; Figure 3) were unique to area X, and a given module’s singing-relatedness was highly predictive of its preservation outside of area X, i.e., the more related to singing, the less preserved (Figure 4). The VSP is active during singing, as indicated by IEG expression (Feenders et al., 2008), and we found gene expression levels in VSP and area X to be remarkably similar during singing (Figure 5). Thus, the regional differences we observed in network structure are probably not due to differences in expression levels, and the singing-related modules in area X are probably not a general product of neural activity, but instead reflect area X-specific singing-driven gene regulation patterns.

We predict that WGCNA-type approaches applied to expression data from other song nuclei would likewise reveal song-regulated gene ensembles not found in neighboring tissue, e.g., HVC versus surrounding cortex. The degree to which such hypothetical song modules would conform with the area X coexpression patterns described here, or whether they would represent the same biological pathways, is an open question. Since the different song nuclei apparently support distinct aspects of singing behavior, one might predict that singing-related coexpression patterns would also be distinct, or would at least relate to different song features, e.g., HVC modules might relate to measures of syllable sequencing (Hahnloser et al., 2002).

Prior microarray studies of area X gene regulation were based on singing out differentially expressed genes in singing versus nonsinging birds, then placing them in groups based on the timing of their expression changes. Our approach differed in that we arranged genes into groups based only on their expression patterns, then related them to singing post hoc. This resulted in modules that contained > 1,000 genes previously unknown to be regulated by vocal behavior. The overlap of our findings with those of prior studies is dominated by genes in the blue module, which contained genes with the largest singing-driven increases in expression. This may imply that differential expression approaches are less effective at identifying gene ensembles, especially downregulated ones, with more nuanced regulation patterns. We predict WGCNA-type approaches will be more effective at uncovering biological functions vital to vocal-motor behavior that do not contain genes with massive expression perturbations.

We verified our hypothesis that targets of FOXP2 in human tissue and cell lines would be important members of area X-specific singing-related modules (Figure 6). Future studies could narrow the search for genes that interact with FoxP2 in a vocal-motor context using our results as a guide, beginning by screening for genes with high TO with FOXP2 that also have high singing-related GS and connectivity. We also found enriched functional categories that were unique to the singing-related modules and described a method for prioritizing biological functions and pathways for future investigation, based on testing metrics of network importance and behavioral significance for genes annotated with significantly enriched terms. Combining this method of ranking enriched biological functions by their importance in singing-related coexpression networks with screens for FoxP2 targets, as described above, could prove fruitful for elucidating the molecular underpinnings of learned vocal-motor behavior in songbirds and humans.

We used the WGCNA area X network results and literature sources to identify pathways previously unknown to be regulated by vocal behavior in area X and demonstrated behaviorally driven changes in protein levels in the Reelin signaling pathway and additional molecules (Figures 8 and S7). Finally, enrichment for Parkinson’s disease and autism genes in the song and nonsong modules (Figure S6) supports the use of songbirds not just as a model for speech, but also as a model for exploring pathways in motor disorders with a vocal component.

**EXPERIMENTAL PROCEDURES**

**Behavior**

Animal use was in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California at Los Angeles Chancellor’s Institutional Animal Care and Use Committee. For the microarrays, experiments were conducted in the morning from the time of light onset to death, 2 hr later, according to Miller et al. (2008). During this time, 18 adult male birds sang undirected song of varying amounts. An additional 9 males were designated “nonsingers” (Table S1). If any potential nonsinging bird sang > 10 motifs, it was excluded from the study. Males performing to a female were not included because FOXP2 mRNA levels in such directed singers...
are similar to nonsingers and are not correlated to the amount of song (Teramitsu and White, 2006). For biological validation, 18 nonsingers and 19 undirected singers were collected 3 hr following lights-on or from their first song motif, respectively. Songs were recorded using Shure SM57 microphones, digitized with a PreSonus Firepod (44.1 kHz sampling rate, 24 bit depth), and acquired using Sound Analysis Pro 2.091 (SAP2, Tchernichovski et al., 2000). Acoustic features of song were computed for each bird using the Feature Batch module in SAP2, and the mean values of each feature were obtained to provide one representative number for each bird. Motifs were counted independently by two experimenters via visual inspection of spectrograms in Audacity (version 1.3; http://audacity.sourceforge.net/).

**Antibodies and Assays**

Tissue was processed for immunoblotting or immunohistochemistry following conventional methodologies using primary antibodies to detect the following proteins: Reelin, Vldlr, phosphorylated Dab 1, Dab1, Ypil5, RanBPM, Trpv1, NeuN, and Gapdh. See Supplemental Experimental Procedures for details.

**Microarrays**

Agilent zebra finch oligoarrays (ver. 1) containing 42,921 60-mer cDNA probes were constructed through a collaboration between the Jarvis Laboratory of Duke University, Duke Bioinformatics, and The Genomics group of RIKEN, under the direction of Drs. Erich Jarvis and Jason Howard (http://songbirdtranscriptome.net; Duke University). These arrays represent cDNA libraries obtained from Michigan State University (Dr. Juli Wade), Rockefeller University (Dr. Fernando Nottebohm), the Keck Center of the University of Illinois (Dr. David Clayton), and Duke (Wada et al., 2006; Li et al., 2007; Replogle et al., 2008). Area X and VSP tissue samples were extracted from all birds (n = 27). Each RNA sample was hybridized to a single array, totaling 54 arrays, two per bird. Each slide, containing four arrays, had four hybridized samples: bilateral area X and VSP samples from two different birds. Birds were selected per slide such that low or nonsingers were paired with high singers to minimize possible interslide bias or batch effects (Table S1). During data preprocessing, one area X sample and two VSP samples, all from nonsinging birds, were removed as outliers. See Supplemental Experimental Procedures for details on tissue collection, RNA isolation, array hybridization, and preprocessing.

**Nomenclature: Probes versus Genes**

“Probe” refers to a single probe on the array. GS measurements were computed for each probe. In many cases, multiple probes for a single “gene,” e.g., FOXP2, were present on the array (Figure S5, Table S2). There were 20,104 probes in the network, 16,448 of which were annotated with a gene symbol at the time of analysis (February 2011, see http://songbirdtranscriptome.net for up-to-date annotations). Since many genes were represented by > 1 probe, only 8,015 annotations were unique. Of these 8,015 unique genes, there were 2,496 unique annotations in the five singing-related modules. When we report GS.motifs.X for a gene, that value is the average GS.motifs.X score of all probes for that gene unless otherwise noted. The area X coexpression network was constructed using probes; thus when we report the number of genes in a module we are referring to the number of unique gene annotations found for probes in that module. Due to sources of natural and experimental variability, different probes to the same gene were sometimes assigned to different, though usually similar, modules during network construction, e.g., probes made to different regions of the same gene may bind to alternatively spliced transcript variants with varying levels of efficiency.

**Network Construction**

Many methods exist for analyzing gene expression microarray data. We chose WGCNA because of its biological relevance and other advantages (Supplemental Experimental Procedures). All WGCNA computations were done in the free statistical software R (http://www.r-project.org/) using functions in the WGCNA library (Langfelder and Horvath, 2008), available via R’s package installer. After preprocessing the raw microarray data to remove outliers, normalize, and filter the data from 42,921 to 20,104 probes (Supplemental Experimental Procedures), the correlation matrix was obtained by computing the signed pairwise Pearson correlations between all probes across all birds. The correlation matrix was transformed using a power function \((1 + \text{correlation}) / 2\) to form the adjacency matrix, a matrix of network connection strengths. \(\beta\) was determined empirically using the scale-free topology criterion (signed network: \(\beta = 14\); unsigned: \(\beta = 6\); Zhang and Horvath, 2005). The network is “weighted” because connection strengths can take on any value between 0 and 1, in contrast to “unweighted” networks where connections are binary. Connectivity \((k)\) is defined for each probe as the sum of its connections to all other probes. The intramodular connectivity (\(kN\), Table S2) of each probe is the sum of its connections to other probes in its module. Intramodular connectivity in VSP (\(kN\)) was computed based on the coexpression relationships in VSP of probes grouped by their area X module assignments. See Supplemental Experimental Procedures for details on the scale-free topology criterion and its biological relevance, differential connectivity, signed versus unsigned networks, and FOXP2 neighborhood analysis.

**Module Definition**

WGCNA identifies modules of densely interconnected probes by correlating probes with high topological overlap (TO), a biologically meaningful measure of similarity that is highly effective at filtering spurious or isolated connections (Yip and Horvath, 2007). The TO matrix was computed based on the adjacency matrix (Supplemental Experimental Procedures) and average linkage hierarchical clustering was performed using 1 – TO as the distance metric. Modules were defined using a dynamic tree cutting algorithm to prune the resulting dendrogram (Supplemental Experimental Procedures; Langfelder et al., 2008).

**Relating Network Structure to Singing**

Expression values within each module were summarized by computing module “eigenvalues” (MEs): the first principal component of each module obtained via singular value decomposition. We defined the module membership (MM) of individual probes as their correlations to the MEs, such that every probe had a MM value in each module. To discover any significant relationships between gene expression perturbations within modules and traits, we computed the correlations between MEs and phenotypic measures, including age, acoustic features, number of motifs sung, and whether the bird sang or not (Figure 3B), p values were obtained via the Fisher transformation of each correlation; modules with correlations to singing traits that had p values below the Bonferroni corrected significance threshold \((p < 1.7e-4)\) are referred to as the three “song modules” throughout the text. We also performed the less conservative Benjamini and Hochberg (1995) FDR procedure and found significant correlations to singing for the black and salmon modules. p value corrections were performed using the results from all phenotypic measures listed above, not just those highlighted in Figure 3B.

**Visualization and Functional Annotation**

Lists of unique gene annotations from each module were used for all module enrichment calculations using Fisher’s exact test, functional annotation studies in DAVID and Ingenuity, and when generating VisANT visualizations (Figures 6D–6F and S6, Supplemental Experimental Procedures; Hu et al., 2004).

**ACCESSION NUMBER**

Raw and processed microarray data, and behavioral data for each bird, are available at http://www.ncbi.nlm.nih.gov/geo (accession GSE34819).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.01.005.

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REFERENCES

Balthazart, J., Voigt, C., Boseret, G., and Ball, G.F. (2008). Expression of reelin, its receptors and its intracellular signaling protein, Disabled1 in the canary brain: relationships with the song control system. Neuroscience 153, 944–962.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. Royal Stat. Soc. Series B (Methodological) 57, 289–300.

Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubscher, J.L., Krieg, P.A., Krupenko, S.A., et al. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J. Neurosci. 28, 264–278.

Enard, W., Gehre, S., Hammerschmidt, K., Hölter, S.M., Blass, T., Somel, M., Brückner, M.K., Schreweis, C., Winter, C., Sohr, R., et al. (2009). A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. Cell 137, 961–971.

Farries, M.A., and Perkel, D.J. (2002). A telencephalic nucleus essential for song learning contains neurons with physiological characteristics of both striatum and globus pallidus. J. Neurosci. 22, 3776–3787.

Feenders, G., Liedvogel, M., Rivas, M., Zapka, M., Horita, H., Eara, W., Koud, M., Mortuisen, H., and Jarvis, E.D. (2008). Molecular mapping of movement-associated areas in the avian brain: a motor theory for vocal learning origin. PLoS ONE 3, e1768.

Haesler, S., Rochefort, C., Georgi, B., Licznerski, P., Osten, P., and Scharff, C. (2007). Incomplete and inaccurate vocal imitation after knockdown of Foxp2 in songbird basal ganglia nucleus Area X. PLoS Biol. 5, e321.

Hahnloser, R.H.R., Kozhevnikov, A.A., and Fee, M.S. (2002). An ultra-sparse in songbird basal ganglia nucleus Area X. PLoS Biol.

Jarvis, E.D., and Nottebohm, F. (1997). Motor-driven gene expression. Proc. Natl. Acad. Sci. USA 94, 4097–4102.

Jarvis, E.D., Scharff, C., Grossman, M.R., Ramos, J.A., and Nottebohm, F. (1998). For whom the bird sings: context-dependent gene expression. Neuron 21, 775–788.

Kaestner, K.H., Knochel, W., and Martinez, D.E. (2000). Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev. 14, 142–146.

Kim, Y.H., Perlman, W.R., and Arnold, A.P. (2004). Expression of androgen receptor mRNA in zebra finch song system: developmental regulation by estrogen. J. Comp. Neurol. 469, 535–547.

Kimpo, R.R., and Doupe, A.J. (1997). FOS is induced by singing in distinct neuronal populations in a motor network. Neuron 18, 315–325.

Konopka, G., Bomar, J.M., Winden, K., Coppola, G., Jonsson, Z.O., Gao, F.Y., Peng, S., Preuss, T.M., Wohlschlegel, J.A., and Geschwind, D.H. (2008). Human-specific transcriptional regulation of CNS development genes by Foxp2. Nature 462, 213–217.

Langfelder, P., and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9, 559.

Langfelder, P., Zhang, B., and Horvath, S. (2008). Defining clusters from a hierarchical cluster tree: the Dynamic Tree Cut package for R. Bioinformatics 24, 719–720.

Langfelder, P., Luo, R., Oldham, M.C., and Horvath, S. (2011). Is my network module preserved and reproducible? PLoS Comput. Biol. 7, e1001057.

Li, X., Wang, X.J., Tannenhuaser, J., Podeli, S., Mukherjee, P., Hertel, M., Biane, J., Masuda, S., Nottebohm, F., and Gaasterland, T. (2007). Genomic resources for songbird research and their use in characterizing gene expression during brain development. Proc. Natl. Acad. Sci. USA 104, 6834–6839.

Lieberman, P. (2006). Toward an Evolutionary Biology of Language (Cambridge: Harvard University Press).

Maccarrone, M., Rossi, S., Bari, M., De Chiara, V., Fezza, F., Musella, A., Gasperi, V., Prosperetti, C., Bernardi, G., Finazzi-Agrò, A., et al. (2008). Anandamide inhibits metabolism and physiological actions of 2-arachidonoyl-glycerol in the striatum. Nat. Neurosci. 11, 152–159.

Miller, J.E., Spiteri, E., Condro, M.C., Dosumu-Johnson, R.T., Geschwind, D.H., and White, S.A. (2008). Birdsong decreases protein levels of Foxp2, a molecule required for human speech. J. Neurophysiol. 100, 2015–2025.

Miller, J.E., Hilliard, A.T., and White, S.A. (2010). Song practice promotes acute vocal variability at a key stage of sensorimotor learning. PLoS ONE 5, e8592.

Musella, A., De Chiara, V., Rossi, S., Prosperetti, C., Bernardi, G., Maccarrone, M., and Centonze, D. (2009). TRPV1 channels facilitate glutamate transmission in the striatum. Mol. Cell. Neurosci. 40, 89–97.

Nottebohm, F. (2004). The road we travelled: discovery, choreography, and significance of brain replaceable neurons. Ann. N Y Acad. Sci. 1076, 628–658.

Oldham, M.C., Konopka, G., Iwamoto, K., Langfelder, P., Kato, T., Horvath, S., and Geschwind, D.H. (2008). Functional organization of the transcriptome in human brain. Nat. Neurosci. 11, 1271–1282.

Poopatanapong, A., Teramitsu, I., Byun, J.S., Vician, L.J., Herschman, H.R., and White, S.A. (2006). Singing, but not seizure, induces synaptotagmin IV in zebra finch song circuit nuclei. J. Neurobiol. 66, 1613–1629.

Reiner, A., Perkel, D.J., Bruce, L.L., Butler, A.B., Caillag, A., Kuenzel, W., Medina, L., Paxinos, G., Shimizu, T., Striedter, G., et al; Avian Brain Neurogenomics (SoNG) Initiative: community-based tools and strategies for study of brain gene function and evolution. BMC Genomics 9, 131.

Soderstrom, K., and Luo, B. (2010). Late-postnatal cannabinoid exposure persistently increases Foxp2 expression within zebra finch striatum. Dev. Neurobiol. 70, 195–203.
Soderstrom, K., and Tian, Q. (2004). Distinct periods of cannabinoid sensitivity during zebra finch vocal development. Brain Res. Dev. Brain Res. 153, 225–232.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S.C., Fisher, S.E., Ren, B., and Geschwind, D.H. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am. J. Hum. Genet. 81, 1144–1157.

Tchernichovski, O., Nottebohm, F., Ho, C.E., Pesaran, B., and Mitra, P.P. (2000). A procedure for an automated measurement of song similarity. Anim. Behav. 59, 1167–1176.

Teramitsu, I., and White, S.A. (2006). FoxP2 regulation during undirected singing in adult songbirds. J. Neurosci. 26, 7390–7394.

Teramitsu, I., Kudo, L.C., London, S.E., Geschwind, D.H., and White, S.A. (2004). Parallel FoxP1 and FoxP2 expression in songbird and human brain predicts functional interaction. J. Neurosci. 24, 3152–3163.

Teramitsu, I., Poopatanapong, A., Torrisi, S., and White, S.A. (2010). Striatal FoxP2 is actively regulated during songbird sensorimotor learning. PLoS ONE 5, e8548.

Thompson, J.A., and Perkel, D.J. (2011). Endocannabinoids mediate synaptic plasticity at glutamatergic synapses on spiny neurons within a basal ganglia nucleus necessary for song learning. J. Neurophysiol. 105, 1169–1169.

Vernes, S.C., Spiteri, E., Nicod, J., Groszer, M., Taylor, J.M., Davies, K.E., Geschwind, D.H., and Fisher, S.E. (2007). High-throughput analysis of promoter occupancy reveals direct neural targets of FOXP2, a gene mutated in speech and language disorders. Am. J. Hum. Genet. 81, 1232–1250.

Vernes, S.C., Oliver, P.L., Spiteri, E., Lockstone, H.E., Pulyadi, R., Taylor, J.M., Ho, J., Mombereau, C., Brewer, A., Lowy, E., et al. (2011). Foxp2 regulates gene networks implicated in neurite outgrowth in the developing brain. PLoS Genet. 7, e1002145.

Wada, K., Howard, J.T., McConnel, P., Whitney, O., Lints, T., Rivas, M.V., Horita, H., Patterson, M.A., White, S.A., Scharff, C., et al. (2006). A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. Proc. Natl. Acad. Sci. USA 103, 15212–15217.

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Künstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., et al. (2010). The genome of a songbird. Nature 464, 757–762.

White, S.A. (2010). Genes and vocal learning. Brain Lang. 115, 21–28.

Yip, A.M., and Horvath, S. (2007). Gene network interconnectedness and the generalized topological overlap measure. BMC Bioinformatics 8, 22.

Zhang, B., and Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. 4, e17.

Zhao, W., Langfelder, P., Fuller, T., Dong, J., Li, A., and Hovarth, S. (2010). Weighted gene coexpression network analysis: state of the art. J. Biopharm. Stat. 20, 281–300.
Molecular Microcircuitry Underlies
Functional Specification in a Basal Ganglia
Circuit Dedicated to Vocal Learning

Austin T. Hilliard, Julie E. Miller, Elizabeth R. Fraley, Steve Horvath, and Stephanie A. White

Inventory:
Supplemental information includes 7 figures and legends (S1-S7), 4 tables and legends (S1-S4; actual tables are separate files from this document), Supplemental Experimental Procedures, and Supplemental References. Figure S1 is related to Figure 1, Figure S2 is related to Figure 2, Figure S3 is related to Figure 4, Figure S4 is related to the “In silico validation of singing-driven co-expression networks” section in main text, Figure S5 is related to Figure 6, Figure S6 is related to “Biological significance of singing-related modules” section in main text, Figure S7 is related to Figure 8.
Supplemental Figures

Figure S1: Hierarchical clustering of area X and VSP samples (related to Figure 1).

A: Hierarchical clustering of all samples reveals distinct global gene expression profiles across brain areas: Dendrogram shows average linkage hierarchical clustering of the raw data from all samples. Each leaf represents 1 array, and the y-axis corresponds to sample dissimilarity as measured by 1 – inter array correlation (IAC). Samples are labeled
with bird identifier numbers and appended with either “X” (area X) or “VSP” (ventral striato-pallidum) to denote the brain region from which the sample was taken (also see Table S1). The presence of 2 pronounced large clusters, 1 representing samples from area X (left) and the other representing VSP samples (right), is evidence of distinct global gene expression profiles across brain areas.

**B: Hierarchical clustering of area X samples reveals singing as a profound regulator of gene expression:** Dendrogram shows average linkage hierarchical clustering of processed data from the area X samples using metrics described in A (also see Table S1). For each bird/sample, the number of motifs sung is reported in parentheses. Samples from non-singing birds (circled in red) clustered together on the right with other samples from birds who sang relatively few motifs (mean of right cluster = 68 motifs; Table S1) compared to samples on the left (mean of left cluster = 890 motifs).
Figure S2: Gene expression levels correlate with number of motifs sung in area X but not VSP (related to Figure 2).

**Figure S2.** In area X, bird normalized median gene expression levels across all 20,104 probes used in network construction were positively correlated with the number of motifs sung (left), whereas no correlation was observed in the VSP (right). Individual bird identifier numbers are plotted. Dashed lines represent the linear regression of median expression on number of motifs, with the Pearson correlation and p-value (based on Fisher's z transformation) shown at top. The VSP sample for bird 1 was deemed an outlier during pre-processing and is thus not shown in the right plot.
Figure S3: Relationships between MM and GS in area X, and GS in area X vs. VSP (related to Figure 4).
**A-F: Strength of MM predicts singing relationships of song module genes:** Song MM (blue, dark green, and orange modules) correlations to GS.singing.X and GS.mean.entropy.X. Within each song module, the MM of each probe predicts the strength of its correlation to singing (A-C) or Wiener entropy (D-F). Greater MM correlated to more positive GS values in the blue song module, and more negative GS values in the dark green and orange song modules. Each dot represents 1 probe. Dashed lines represent the linear regression of GS.singing.X (A-C) and GS.mean.entropy.X (D-F) on MM in each module, with the Pearson correlation coefficient \( r \) and p-value (based on Fisher's \( z \) transformation) shown at top of each plot.

**G-L: GS scores in area X of song module genes were preserved to varying degrees in the VSP:** Correlations between GS values in area X and the VSP; GS.singing (G-I), GS.mean.entropy (J-L). For each song module, the weakest correlations were seen for traits with the strongest correlations to the ME (see heatmap in Figure 3B and Table S3 for ME-trait cors). For example, there was less preservation of GS.singing.X (G; cor = 0.097) than GS.mean.entropy.X (J; cor = 0.46) in VSP for blue module genes (blue ME-singing cor = 0.75, blue ME-entropy cor = 0.52). Each dot represents 1 probe. Dashed lines represent the linear regression of GS.singing.V on GS.singing.X (G-I) and GS.mean.entropy.V on GS.mean.entropy.X (J-L) in each module, with the Spearman rank correlation coefficient \( \rho \) (used whenever we compared area X and VSP) and p-value shown at top of each plot.
Figure S4: Previously identified song genes (related to “In silico validation of singing-driven co-expression networks” section in main text).

A-D. Network metric comparisons of song module genes that were previously identified singing-regulated genes: Each panel shows area X measurements for singing-regulated genes from Wada et al. (2006) that were also in 1 of the 5 singing-related modules.
found in our study. GS for motif number (A); GS for singing vs non-singing (B); percent change of expression in singing vs non-singing birds (C); intramodular connectivity (D). On the x-axis are group designations from Wada et al. (2006) that divided genes by their time-course of expression during singing (0.5-I: upregulated expression peaked at 30 min and decreased with continued singing, 0.5-II: upregulated expression peaked at 30 min and remained steady with continued singing, 1-III: upregulated expression peaked at 1 h and decreased with continued singing, 1-IV: upregulated expression peaked at 1 h and remained steady, 3-V: expression rose slowly to peak at 3 h). Genes determined to have peak expression 30 min after singing onset followed by a steady decrease during continued singing showed the largest expression fold-changes between singing vs non-singing birds in our data, whereas genes whose expression was classified as slowly rising to a peak 3 h after singing onset had the highest GS scores. Error bars represent 95% confidence intervals, p-values were computed via a non-parametric ANOVA (Kruskal-Wallis test). Genes from some of Wada et al.’s (2006) groups (e.g. 3-VI) were not in singing-related modules, thus they are not represented in this figure.

E-F: Connectivity, rather than fold expression change predicts GS for previously identified singing-regulated genes: The pattern of group differences in kIN.X (D) more closely mirrored GS differences (A-B) than did percent change (C). Magnitude of expression changes during singing were not correlated to GS.motifs.X for genes from Wada et al. (2006) (E), grouped as shown. Intramodular connectivity (kIN.X) was much more correlated to GS.motifs.X (F), emphasizing the advantage of using network connectivity over expression levels to predict behavioral significance.
Figure S5: Brain-region-specific behavioral regulation of gene expression coupled with WGCNA captures genes co-regulated with FOXP2 (related to Figure 6).

Figure S5: Area X expression levels for all 12 probes for FOXP2 in the network are plotted as a function of the number of motifs sung. Each probe shows significant down-regulation with singing. Bird identifier numbers are shown. Dashed lines represent the linear regression of area X expression on number of motifs for each probe, with the Pearson correlation coefficient $r$ and $p$-value (based on Fisher’s $z$ transformation) shown at top, along with probe ID number. Correlation coefficients shown here are equivalent to GS.motifs.X values for these probes (Table S2).
Figure S6: Parkinson's disease associated genes in the black singing-related module interact with other song module genes (related to “Biological significance of singing-related modules” section in main text).

**Figure S6.** VisANT (Hu et al., 2004) visualization of PD associated genes that were the most interconnected in the black module (large spheres/nodes: \textit{CACNA1D, GRIN2B, GSK3B, TRPM7}), and genes with at least 1 probe in other singing-related modules that they are
known to interact with (smaller nodes). Nodes represent genes; node color symbolizes module assignment in area X. Edges between CACNA1D, GRIN2B, GSK3B, and TRPM7 represent co-expression relationships. Edges between these 4 genes and the smaller nodes represent interactions extracted from the literature by VisANT. The black module was enriched for PD associated genes, a list of which was obtained from the Ingenuity Knowledge Base (p=3e-4, Fisher's exact test; Table S2). Node color represents module assignment (e.g. black: GSK3B, blue: SNCAIP, dark green: NIN, orange: TSC2, salmon: CAMK2A), with the 3 light grey nodes denoting genes known to interact with GSK3B that had representative probes with significant GS.motifs.X scores but were not in a singing-related module.
Figure S7. Presence of Vldlr and behavioral regulation of putative Trpv1 protein in area X (related to Figure 8).

A) Presence of Vldlr protein is confirmed in zebra finch area X. Top: Immunoblot of area X Vldlr protein from 4 NS and 4 UD birds. Bottom right: The anti-Vldlr primary antibody detects protein at the predicted molecular weight ~95kD in zebra finch brain extracts. Preadsorption of the antibody with 20x excess of the immunizing peptide prevents binding (*). Bottom left:
Vldlr protein levels in NS and UD birds (Mann-Whitney U test, p=0.38). Middle of each box represents the mean; top and bottom, standard error; whiskers, upper and lower 95% confidence intervals. Data from each bird is shown by individual points.

B) Immunoblot of putative Trpv1 protein expression in area X of 5 singing (UD) and 5 non-singing (NS) birds reveals bands similar to the predicted molecular weights of two isoforms in zebra finch at 86 and 93kD (http://www.ncbi.nlm.nih.gov/) and reported in immunoblots of rat brain extracts (Tóth et al., 2005). Quantification of Trpv1 signals in UD birds revealed a negative correlation with the amount of singing (Top band – black triangles and dashed line, Spearman Rho= -1, p=1e⁻⁴, R²= -0.87; bottom band – open circles and grey dashed line, Spearman Rho= -0.8, p=0.10, R²= -0.66).
Supplemental Table Legends

Table S1. Tissue processing and trait data.

Each bird in the microarray study was assigned an ID (col. A) and 2 samples from each bird representing area X and VSP (V) were assigned sample numbers including RNA ID, chip barcode, and array (cols. B-F); 27 arrays used. All RNA samples passed a quality control check prior to hybridization (PASS, col. E). Each sample was hybridized to a single microarray so that within a group of 4 arrays per slide, 2 birds were paired together (col. D) – a non-singer/low singer with a higher singer (col. J, K). Taken together, columns B, D, and K show our hybridization scheme aimed at minimizing possible chip biases or batch effects across brain areas. Other columns report bird age and collection dates for tissue and RNA isolation (G-I) and quantitative trait values for acoustic features obtained from song analysis (L-O, abbreviation: FM for frequency modulation). The trait data in this table (col. J-O) were used in the module eigengene – trait correlation computations (Figure 3B, Table S3) and to compute GS values for each probe (Table S2).

Table S2. Area X network data.

This table summarizes the area X network and contains almost all of the results presented in this paper, with the exception of cross-region module preservation statistics and ME – trait correlation information (Table S3), and functional annotation findings (Table S4). Gene symbol and name information for each probe reflect annotations available for the microarray in February 2011. The clone ID# and nucleotide sequence associated with each probe are also provided, which can be used to search for the for the most up-to-date annotation information
for a given probe at http://songbirdtranscriptome.net. The BLAT/BLAST tools provided by the National Center for Biotechnology Information ([http://blast.ncbi.nlm.nih.gov/Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) and on the UCSC Genome Bioinformatics website ([http://genome.ucsc.edu/](http://genome.ucsc.edu/)) are also useful for investigating the alignments of probe nucleotide sequences.

Each of the 20,104 rows contains information for a single probe in the network, with the columns organized as detailed below. As with all Excel files, one can sort the entire table by a given column in order to find genes with, for example, the highest GS scores, connectivity, or module membership. The Excel “Find” function in the Edit menu can be used to look up specific genes, and filtering can be applied to the module color column to view probes in a single module. Due to the large size of the table, we recommend interested readers import it into R ([http://cran.r-project.org/](http://cran.r-project.org/)) as a .csv file or tab-delimited .txt file for more efficient investigation. Simple examples demonstrating relevant searches/filters in R are provided in the code section of our website ([http://www.ibp.ucla.edu/research/white/code.html](http://www.ibp.ucla.edu/research/white/code.html)).

**Columns A-F:** Probe ID# (col. A), clone ID# (col. B), nucleotide sequence of probe (col. C), gene symbol and name (if probe is annotated, else NA; col. D-E), module assignment (col. F).

**Columns G-AH:** GS information for age and 6 song traits, as measured in area X and VSP. Correlations are shown along with their associated FDR-corrected p-values, i.e. q-values. Each trait is represented by a block of 4 columns; the first 2 depict GS and q-values as measured in area X (col. names end with .X), the next 2 depict GS and q-values as measured in the VSP (col. names end with .V). GS measurements with q<0.05 are considered significant.

**Columns AI-AR:** Measurements related to probe intramodular connectivity (area X – kIN.X, VSP – kIN.V, col. AI-AJ) and expression in area X and the VSP. Expression related measures include: the median normalized expression across all samples (col. AK-AL), the percent
change in expression in the singing vs. non-singing birds (col. AM-AN), and the traditional t-statistic with q-value comparing expression in singing vs. non-singing birds (col. AO-AR) in area X and VSP, respectively.

Columns AS-AW: All FOXP2 related: Whether the probe represents a gene found to be a direct/indirect transcriptional target of FOXP2 in neuronal progenitor cells (col. AS; Konopka et al., 2009), a direct target found in fetal human brain (col. AT; Spiteri et al., 2007) or human fetal SH-SY5Y cells (col. AU; Vernes et al., 2007), or found to have significantly changed striatal expression levels in mice bearing a partially humanized form of FOXP2 (col. AV; Enard et al., 2009). Col. AW reports the topological overlap with the FOXP2 probe that had the most significant GS.motifs.X score (probeID=2758927).

Columns AX-BB: Whether probe represents a gene previously found to be regulated during singing in zebra finches, and its grouping according to expression time-course during singing (col. AX-AY; Wada et al., 2006); genes suppressed in the forebrain during auditory perception and likely have undergone positive (pos) selection that were enriched for 6 genes involved in "ion channel activity": CACNA1B, CACNA1G, GRIA2, GRIA3, KCNC2, TRPV1 (col. AZ); or singing-regulated in area X (col. BA; Warren et al., 2010). Column BB are genes associated with Parkinson's disease (Ingenuity Knowledge Base; enriched in black module).

Columns BC-BE: Whether probe represents a gene acting as a marker for neurons (col. BC; >20-fold higher in neurons vs. other cell types, enriched in dark red module), oligodendrocytes (col. BD; >10-fold enriched in oligodendrocytes vs. other cell types, enriched in turquoise module; Cahoy et al., 2008), or the striatum/pallidum in mammals (col. BE; enriched in brown, pink, and orange modules).

Columns BF-CW: Remaining columns contain MM information for every probe in every module followed by the p-value of the correlation. Modules are ordered by the strength of their
ME's correlation to the number of motifs sung, beginning with the dark green, blue, orange, salmon, and black song modules (col. BF-BO). Cols. BP-CW represent the remaining modules.

**Table S3. Area X modules: Preservation in the VSP and relationship to singing.**

Module preservation metrics with accompanying Z-scores and Bonferroni corrected log10 p-values (col. C-AI), and ME – trait correlations (col. AJ-AP; p-values in parentheses). ME – trait correlations that passed Bonferroni correction are colored dark grey, those that passed the less conservative FDR procedure are light grey (Figure 3B). Module preservation statistics reflect the degree to which modules in area X are preserved in the VSP and were obtained using the WGCNA modulePreservation() function. Col. C summarizes the results of the module preservation calculations, ranking the modules by amount of preservation, with larger ranks indicating less preservation. The grey module contains probes that did not fall into a proper module during clustering and tree-cutting, and is considered background. The data in col. AJ-AP are the basis of the heatmap in Figure 3B.

**Table S4. Functional annotation of selected modules.**

DAVID (Huang et al., 2009) functional annotation results for the 5 singing-related modules, a) blue, b) dark green, c) orange, d) black, e) salmon, and the 3 modules most preserved in the VSP, f) turquoise, g) dark red, h) pink (Table S3, Figure 4G-H), are shown. Each row represents 1 enriched term, columns represent the database/category the term is from (col. A), the term itself (col. B), the number and percentage of genes in the module annotated with the term (col. C-D), the enrichment p-value and fold change (col. E-F), corrected p-values (col. G-H), estimated FDR (col. I), the average MM, GS.motifs.X, and differential intramodular
connectivity (kIN.diff) between area X and the VSP of module genes annotated by the term (col. J-L; positive kIN.diff values indicate higher connectivity in area X), the TS score (col. M), a version of the TS score that incorporates kIN.diff (col. N), and gene symbols and Entrez IDs for genes annotated with the term (col. O-P).

**Supplemental Experimental Procedures**

**Animals**

Animal use was in accordance with NIH guidelines for experiments involving vertebrate animals and approved by the University of California at Los Angeles Chancellor’s Institutional Animal Care & Use Committee. Adult male zebra finches between 120-199 days of age for the microarrays and 103-273 days for the biological validation were moved from our breeding colony to individual sound attenuation chambers (Acoustic Systems; Austin, TX) under a 13:11 h light/dark cycle. Birds were acclimated for 2-3 days prior to the experiments. The amount of song sung by singing birds ranged from 134-1,270 motifs for the microarray collection (Table S1) and 448-1885 motifs for the biological validation.

**Tissue Collection**

After undergoing the behavioral protocol, birds were decapitated, and brains rapidly extracted and frozen, then stored at −80°C until use. Tissue punches of area X and VSP were obtained at the same plane of section and histologically validated for anatomical precision (Figure 1B; Miller et al., 2008). Area X and VSP punches were stored separately in RNAlater (Qiagen, Valencia, CA) at -20°C until RNA isolation (see Immunoblotting for protein isolation).
RNA Isolation

Area X and VSP tissue punches were removed from RNAlater and processed separately for each bird (27 birds; 54 samples) using an optimized version of the Qiagen RNeasy Micro Kit protocol for purification of total RNA from animal and human tissues. Modifications were: substitution of QIAzol lysis reagent for β-mercaptoethanol, and additional washes in the RW1 and RPE buffers. Tissue punches were homogenized in QIAzol with a handheld homogenizer (Kontes, Fisher Scientific, Pittsburgh, PA), incubated with chloroform, centrifuged, and the aqueous supernatant retrieved for further processing. Following purification, 1 μl of each eluted sample was analyzed for RNA purity and quantity using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE), and quality using an Agilent Bioanalyzer (Santa Clara, CA). 200 ng of total RNA was converted into labeled cRNA with nucleotides coupled to Cy3 using the Low RNA Input Linear Amplification Kit (Agilent Technologies, Palo Alto, CA) following the manufacturer’s protocol, and labeled cRNA (1.65 ug) from each sample was hybridized to an array.

Microarray Pre-processing

RNA labeling, microarray hybridizations, data collection and background correction procedures were performed by Cogenics Inc. (Beckman-Coulter, Brea, CA). After hybridization, the arrays were washed, scanned, and data extracted from the scanned image using Feature Extraction version 9.5 (Agilent Technologies). Principal component analysis (PCA) performed by Cogenics found the largest source of variability (PC1) to be the nature of the samples, specifically, tissue versus cell culture (2 cell culture samples were included for a separate study). Tissue samples were cleanly sub-divided into 2 by PC2: area X samples
grouped together, while a separate cluster contained all of the VSP samples, except for samples from bird 21 (a non-singer) in which the area X sample grouped with all of the other VSP samples, and vice versa. Samples from bird 21 were thus excluded from further analysis. An additional VSP sample (bird 1, a non-singer) was removed as an outlier, based on hierarchical clustering of the samples, as described below.

All subsequent pre-processing and analysis was done in the freely available statistical software R (http://www.r-project.org). R functions written by ATH for array pre-processing are available at http://www.ibp.ucla.edu/research/white/code.html. First, background corrected data from the remaining 52 arrays were hierarchically clustered on the basis of inter-array correlation (IAC; Oldham et al., 2008). 2 clear clusters emerged, one containing area X samples, the other VSP samples, substantiating the PCA results from Cogenics (Figure S1A).

Next, area X and VSP samples were separated and the 2 datasets were pre-processed separately, but identically, as follows. Control probes were removed and probes with raw fluorescence values >3 standard deviations (SD) away from the mean of measurements across all samples for that probe were removed. Then, samples were hierarchically clustered, again on the basis of IAC, to identify array outliers. For area X, all arrays fell within 2SD of the mean IAC (0.977), and were retained for a total n=26 (18 singing birds and 8 non-singers). Area X samples from non-singing birds fell into 1 of 2 large clusters along with samples from birds who sang relatively little, compared to the higher singers found in the other major cluster (Figure S1B), speaking to the robust effect of singing on large scale gene expression in area X. The clustering of non-singers with birds that sang the least without any distraction suggests that the presence of the investigator under the non-singing condition did not significantly impact gene expression. One VSP sample (1V) was >3SD from the mean VSP IAC (0.974) and was removed, leaving total n=25 (18 singing birds and 7 non-singers). Arrays were
quantile normalized, and a preliminary area X network was built using all 42,921 probes to assess overall connectivity. From this network, the 20,000 probes with the highest connectivity were retained for subsequent WGCNA. Also, 104 probes for genes of interest that were not among the 20,000 most connected were re-inserted into the dataset, thus a total of 20,104 probes were used in the final network construction. These genes of interest included autism associated genes such as CNTNAP2, Parkinson’s disease related genes including PARK2, PARK7, and SNCA, and genes for dopamine receptors, e.g. DRD2 and DRD5.

**Gene Significance**

Pearson correlations between each probe’s expression profile and singing measurements from all birds were computed using the WGCNA library (Langfelder and Horvath, 2008) cor() function, a faster implementation of the standard R version. For each correlation, we computed the Student asymptotic p-value using the WGCNA corPvalueStudent() function, and used the qvalue() function from the R qvalue library to estimate the proportion of false positives at the 0.05 level, generating a q-value for each correlation. Correlations with q < 0.05 were considered significant. In addition to singing vs. non-singing and number of motifs sung, we measured correlations for each probe to individual acoustic features such as Wiener entropy (see Results and Discussion), frequency modulation (GS.mean.FM), pitch (GS.mean.pitch), and pitch goodness (measure of periodicity across the frequency spectrum; GS.mean.pitch.goodness) that are typically used to characterize and assess song. Values for each of these features were obtained for each bird using the feature batch module of Sound Analysis Pro 2.091 (Tchernichovski et al., 2000). This analysis segments all of a given bird’s song recordings into syllables and computes the above features for each syllable. We took
the mean of each feature across all of the birds' syllables in order to obtain 1 representative number for each bird.

**WGCNA – Advantages and Definitions**

Multiple publications provide good overviews of WGCNA methodology (e.g. Zhang and Horvath, 2005; Dong and Horvath, 2007; Zhao et al., 2010; Horvath, 2011). Here, we expand on our rationale for using WGCNA as opposed to other methods available for the study of high-throughput gene expression data. Standard microarray analyses typically begin by identifying individual genes that are differentially expressed between 2 groups, e.g. singing vs non-singing birds. This approach poses statistical challenges and considers genes only in isolation. The selection of WGCNA for analysis of our microarray data is a reflection of its relevance to biological function. Genes interact in hierarchically structured regulatory networks that possess a “small world” or “scale-free” topology. WGCNA uses unsupervised hierarchical clustering based on topological overlap (TO) to exploit the higher-order co-expression relationships present in microarray data, and fit it to this type of structure (Zhang and Horvath, 2005). For a network to be 'scale-free' means that the connectivity distribution of the network follows an inverse power law, i.e. there exist a small number of highly interconnected nodes while the rest have low connectivity. Connectivity \( k \) is defined as:

\[
k_i = \sum_{j=1}^{N} a_{ij}
\]

where \( i \) and \( j \) are probes, \( N \) is the total number of probes, and \( a \) is the adjacency matrix (the transformed correlation matrix).

TO is a biologically meaningful measure of node interconnectedness which compares
patterns of probe connection strengths to quantify similarity in the context of the entire network. In contrast, other similarity measures such as correlation or Euclidean distance only consider each pair of probes in isolation. As a result, TO is highly effective at filtering spurious or isolated connections (Yip and Horvath, 2007), and probes with high TO have an increased chance of being members of the same biological pathways. TO ($\omega$) is defined as:

$$\omega_{ij} = \frac{l_{ij} + a_{ij}}{\min\{k_i, k_j\} + 1 - a_{ij}}$$

where

$$l_{ij} = \sum_{u \neq i, j} a_{iu} a_{uj}$$

and $u$ represents all other probes besides $i$ and $j$. Studies of simulated and real data have confirmed that average linkage hierarchical clustering is better than other types of clustering, e.g. partitioning around medoids (PAM) or k-means, at reproducing hierarchical scale-free networks, especially when the clustering is done on TO, as opposed to other distance metrics (http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/Simulated-01-dataSimulation.pdf).

Finally, all samples are used when building a network in WGCNA and network structure is ultimately related back to phenotypic measurements that can be binary, e.g. singing vs non-singing, or continuous, e.g. number of motifs sung. The ability to study continuous measurements is crucial to maintaining maximum possible ethological relevance. It allows one to study gene co-expression patterns as a function of the gradient of natural phenotypic variability, and avoid artificially dichotomizing data into groups, which often results in
information loss and decreased power (Fedorov et al., 2009). Links between gene co-regulation and continuous behavioral measurements could not have been described using traditional differential expression approaches, which are mostly only appropriate for examination of categorically grouped data. We examined 1 categorical grouping (singing vs non-singing), but found it less informative than motif number, thus we focused on the latter.

As described in the main text, WGCNA can be used to identify hub genes correlated to these network metrics. Together, with cross-referencing of the literature and functional databases, these analyses can predict important biological pathways such as what we show for the Reelin pathway.

WGCNA has advantages in addition to its biological relevance. First, average linkage hierarchical clustering is unsupervised, i.e. the experimenter does not pre-define the number of clusters, such as in k-means clustering. Instead, network structure is defined only by the relationships within the data, and without regard to external phenotypes of interest, e.g. singing. The experimenter cannot bias the results, either with respect to module composition or behavior, since the network is constructed via unsupervised hierarchical clustering without knowledge of birds' singing data. Gene modules are not defined a priori, as in gene set enrichment analysis, and phenotypic information is related to modules post hoc, making significant relationships all the more compelling.

Second, weighted networks retain a significant amount of information that is lost in unweighted networks, wherein a given pair of nodes is only either connected or not, depending on an arbitrary threshold. For example, at a threshold of 0.8, 2 nodes with a connection strength of 0.81 would be considered “connected”, while 2 nodes with a connection strength of 0.79 would be considered “unconnected”. Simulated and empirical
data also show that module identification, connectivity, and relationships between connectivity and external gene significance measures in weighted, as opposed to unweighted, networks are robust to the value chosen for the model fitting (network construction) parameter $\beta$. The value used for $\beta$ is determined empirically by constructing test networks with different $\beta$ values and assessing how closely each approximated a scale-free topology. This is done by linearly regressing $\log_{10}(p(k))$ onto $\log_{10}(k)$, where $p(k)$ is the probability of a given probe having connectivity $k$, and the fit is indicated by $R^2$. Since $R^2$ and $k$ are inversely related, one must choose $\beta$ such that the network approximates scale-freeness as closely as possible while still maintaining enough connectivity to effectively detect gene modules (Zhang and Horvath, 2005).

Third, the multiple hypothesis testing problem inherent to large datasets is effectively sidestepped by WGCNA, since the basic unit of analysis is not single genes, but instead gene modules that can be studied as discrete entities. In our study we went from $n=42,921$ gene probes to $n=21$ gene modules. Rather than statistically testing each individual probe, we only needed to screen individual modules for their relationship to the phenotype. Finally, WGCNA has been shown to produce unique findings, unobtainable via standard techniques, e.g. in Oldham et al., 2006 & 2008, where the authors defined the structure of the human brain transcriptome and highlighted key drivers of evolutionary change.

**WGCNA – Differential connectivity**

Intramodular connectivity in VSP ($kIN.V$, Table S2) was computed based on the co-expression relationships in VSP of probes grouped by their area X module assignments. Thus, the difference in area X and VSP intramodular connectivity ($kIN.diff = kIN.X - kIN.V$) reflects the degree to which a probe is coexpressed with its fellow module members in both regions.
Since $kIN$ is normalized and nonnegative, a $kIN.diff$ score near 1 signifies a probe that is highly connected in its module in area X, but shares little to no co-expression similarity with the same probes in the VSP. A score near -1 signifies the opposite; a probe that is a hub in VSP among the same set of probes with which it shares only weak co-expression similarity in area X. In practice, most $kIN.diff$ scores in our data were not this extreme; 95% fell within the interval $-0.32 < kIN.diff < 0.45$. Most probes (~66%) had higher $kIN.X$ than $kIN.V$.

**WGCNA - Pruning the Dendrogram**

The output of the clustering process can be visualized as a dendrogram (Figures 3A,S1A-B), where the leaves correspond to individual probes, and the height on the y-axis where any 2 leaves are connected represents their distance from one another in the network ($1 – TO$). In order to define discrete modules, the dendrogram must be “pruned”, which amounts to cutting branches at particular heights on the y-axis, i.e. setting the minimum TO a group of probes must share in order to be considered a module. Rather than choosing an arbitrary static height cutoff that might be appropriate for some groups of probes but not others, we pruned the dendrogram using a bottom-up dynamic tree cutting algorithm (Langfelder et al., 2007), which performs an adaptive process of cluster decomposition and combination, allowing the relationships within the data to determine network structure. Studies of simulated data have shown that the dynamic tree cutting algorithm is superior to a static threshold at reproducing hierarchical network structure. See tutorial III on analysis of simulated data on the WGCNA website: [www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/index.html](http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/index.html)

A brief description of the dynamic tree cutting algorithm is as follows, see the relevant reference for details (Langfelder et al., 2007): First, preliminary clusters were identified as
branches that 1) contained a certain minimum number of probes, 2) excluded probes too far from the cluster even if they belonged to the same branch, 3) were distinct from surrounding branches, and 4) had a tightly connected core, defined as the branch tip. Second, previously unassigned probes were tested for sufficient proximity to preliminary clusters, and if the nearest cluster was close enough, the probe was assigned to that cluster. This process was iterated until the number of clusters stabilized.

**WGCNA – Signed vs. Unsigned Networks**

The first step in network construction was to compute the pairwise Pearson correlations between all of the columns in the original expression data matrix to create the correlation matrix $S$, describing for any pair of probes $i$ and $j$, the correlation of their expression profiles across all samples ($S_{ij}$). Many co-expression studies use the absolute value of $S$ as an unsigned co-expression similarity measure, where the network adjacency matrix $A$ is defined as $A_{ij} = |S_{ij}|^\beta$. However, much as weighted networks retain a significant amount of connectivity information that is lost in unweighted networks (see above, WGCNA – Advantages and Definitions), retaining the sign of the correlations in $S$ avoids obscuring biologically relevant information, since unsigned networks cannot distinguish between gene repression and activation (Mason et al., 2009). The adjacency matrix $A$ for a signed network is defined as $A_{ij} = \left( \frac{1 + S_{ij}}{2} \right)^\beta$, to ensure that $0 \geq A_{ij} \geq 1$.

The large (20,104 probes) area X co-expression network discussed throughout the paper, which contained 5 singing-related modules, was constructed as a signed network (Figure 3A). Since probes showing significant downregulation during singing tended to have high TO with other downregulated probes, and vice versa for significantly upregulated probes, the singing-related modules were composed mostly, and in one case completely, of genes either
upregulated (e.g. blue module – 2,010 of 2,013 probes had positive GS.motifs.X scores) or
downregulated (e.g. dark green module – all 1,417 probes had negative GS.motifs.X scores)
during singing. Other (non-singing-related) modules had a more heterogeneous composition,
containing probes both up- and down-regulated during singing, e.g. the dark red module
contained 554 and 612 probes with positive and negative GS.motifs.X scores, respectively
(Table S2). Possible implications are that genes in the dark green module may have been
repressed by genes in the blue song module (which contained probes upregulated during
singing), or that continued singing potentially released the baseline repression of blue module
genes by genes in the dark green module.

Genes in high TO with transcription factors upregulated during singing (such as those
represented in the blue module) are potentially activated by, or activate, the transcription
factors themselves. However, the nature of co-expression relationships of genes known
mostly as transcriptional repressors, especially ones such as FoxP2 that are increasingly
downregulated with continued singing, is not as clear. Genes in high TO with FOXP2, i.e.
FOXP2’s network neighbors, may actually be directly activated by FoxP2, and their
downregulation during singing could reflect the loss of this activation. Alternatively, FoxP2
downregulation may have released repression of blue module genes that in turn repressed,
one uninhibited, FOXP2’s neighbors. This led us to consider using an unsigned network
during further investigation of FOXP2’s network neighbors.

**FOXP2 Network Neighbors**

First, an unsigned version of the area X co-expression network was constructed (β=6), and all
probes were ranked by their TO with the FOXP2 probe with the most significant GS.motifs.X
score (probe ID = 2758927, Table S2). We used an unsigned network here so neighbors of
*FOXP2* could be genes either up- or downregulated during singing, possibly under transcriptional repression or activation by *FOXP2*. By definition, in the signed network all of *FOXP2*'s closest neighbors were genes downregulated during singing, and upregulated genes had very low TO with *FOXP2*. Since the signed network can distinguish between gene repression and activation, we retained module assignments from the signed network when interpreting connectivity in the new unsigned network.

After ranking probes by their TO with *FOXP2*, we filtered out probes unannotated by a gene symbol. For genes represented by multiple probes on the array, we removed all but the probe with the highest TO with *FOXP2*. This left us with a list of probes with unique gene symbols, ranked by TO with *FOXP2*. Then, we examined connections between the top 300 probes/genes (in this case, “probe” and “gene” are now interchangeable) from this list, including *FOXP2*. We used VisANT (Hu et al., 2004; see below) to envisage some of these connections (Figure 6D-E). To construct Figure 6D, we focused on a group of the most highly interconnected probes/genes within *FOXP2*'s 20 closest neighbors, visualizing their connections with *FOXP2* and one another. To make Figure 6E, we screened the list of direct *FOXP2* targets from Spiteri et al. (2007) and Vernes et al. (2007) for those with the highest TO with *FOXP2* in our unsigned network, and again used VisANT to visualize the connections among the 20 closest. For Figure 6F, we converted the list of *FOXP2*'s 300 closest neighbors from gene symbols to Entrez IDs to minimize ambiguity, and uploaded the list to Ingenuity (see below) to screen for any well known canonical networks that might be represented within these genes, using Ingenuity's “Core Analysis” procedure. The network with the highest enrichment score is shown.
**Functional Annotation**

Gene lists for each module were uploaded to DAVID 6.7 ([http://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/); Huang et al., 2009) and converted from official gene symbols to Entrez IDs to minimize ambiguity. The list of all 11,702 unique gene annotations represented by probes on the array was used as the background for all enrichment calculations. Human Entrez IDs were used, as the zebra finch genome remains sparsely annotated, and multiple lines of evidence suggest that the neural systems supporting learned vocalization are highly analogous in humans and zebra finches (Jarvis, 2004). The functional annotation results should be interpreted with caution since they represent comparisons of gene lists obtained from avian systems with mammalian databases. These results are meant to be treated as guides for further investigations. The reliability of gene ontology and functional annotation studies in birds will likely improve with time, making it important to return to our data in the future, including for analyses of probes that are currently unannotated.

**DAVID – Term Significance**

Rather than simply generating lists of enriched terms for each module, we devised a term significance score (TS; Results and Discussion) to identify enriched terms that were particularly relevant to our study, i.e. those that represented sets of genes highly connected within the module and relevant to singing. We defined TS as the absolute value of the product of the average MM (representing network importance) and GS.motifs.X (representing singing-driven regulation) for genes annotated with the term, scaled by 1 – term’s p-value (representing the degree of enrichment). One could also incorporate the average area X-VSP difference in connectivity (kIN.diff, Table S4 – col. N; see above), or any other GS measure, e.g. GS.mean.entropy, for genes annotated with a particular term, depending on one’s
interest. It may also prove useful to incorporate the percentage of module genes annotated with a given term (Table S4 – col. D), or other relevant normalized measurements/metrics. Here, for each of the singing-related modules we ranked enriched terms with p<0.1 by their TS values. For each of the resulting top 10 terms, we compared the average MM, GS.motifs.X, kIN.diff (Table S4 – col. L), and clustering coefficient of genes annotated by the term to the rest of the genes in the module.

Ingenuity
Ingenuity (http://www.ingenuity.com/products/pathways_analysis.html) was used to identify previously known molecular networks represented in the 300 closest network neighbors of FOXP2 in an unsigned network (Figure 6F). The list of FOXP2 neighbors was converted to human Entrez IDs, uploaded, and analyzed using the “Core Analysis” procedure with the annotated human genome as the background, because the zebra finch genome is still sparsely annotated, and the Ingenuity Knowledge Base only includes mammalian data. The canonical pathway with the highest enrichment score was chosen for Figure 6F. We also used Ingenuity to generate a list of Parkinson’s disease associated genes to check for possible module enrichments by searching the Ingenuity Knowledge Base for “Parkinson’s disease” and exporting the resulting list of genes (Figure S6, Table S2).

VisANT
VisANT (http://visant.bu.edu/; Hu et al., 2004) was used to visualize co-expression relationships in our data. Probes for those genes that were represented by multiple probes on the array and in the network were filtered out using connectivity, and in certain cases, TO with FOXP2, keeping only the probe with the highest connectivity and/or FOXP2 TO. After filtering,
gene lists and the relevant connectivity data, i.e. network adjacencies, were loaded in VisANT. For clarity, weak connections (< 0.15 adjacency) were not shown, and after executing VisANT's “spring-embedded relaxing” algorithm for node positioning, nodes were manually placed to maximize intelligibility. Nodes were colored by module assignment, and in Figure 6D-E, edge width was selected to represent connection strength. For Figure S6, after filtering all but 4 of the most connected Parkinson’s disease associated genes in the black module, we queried VisANT to display all other genes with which they are known to interact. Of these, genes in our network with significant GS.motifs.X scores were retained and colored by their module assignment.

**Antibodies**

The following antibodies were used to detect relevant proteins as noted in the main text using immunocytochemistry (IC) and/or immunoblotting (IB): anti-Reelin (mouse monoclonal G10, Millipore, Billerica, MA; 1:100 IB), anti-Vldlr (rabbit polyclonal, Proteintech Group, Chicago, IL; 1:250 IB), anti-Dab1 phosphospecific (to Tyrosine 198, rabbit polyclonal, Invitrogen, Carlsbad, CA; 1:500 IB), anti-Ypel5 (rabbit polyclonal, Proteintech Group; 1:250-1:500 IB, 1:500 IC), anti-Trpv1 (rabbit polyclonal, Novus Biologicals, Littleton, CO; 1:1000 IB), anti-RanBPM (mouse monoclonal, Courtesy of E. Bianchi, Institut Pasteur, Paris, France; 1:5000 IB), anti-NeuN (mouse monoclonal, Millipore, Billerica, MA; 1:500 IC) anti-Gapdh (mouse monoclonal, Millipore; 1:100,000 IB), anti-Dab 1 (rabbit polyclonal, Courtesy of B. Howell, SUNY Upstate Medical University, Syracuse, NY, Howell et al., 1997; 1:1500 IC).

Secondary horseradish peroxidase-conjugated antibodies (GE Healthcare, Piscataway, NJ) for IB were: anti-rabbit IgG (1:2000) and anti-mouse IgG (1:10,000 for Gapdh, 1:2000 dilution for all others); for IC: goat anti-rabbit biotinylated antibody (1:400 for Ypel5, Perkin Elmer,
Waltham, MA; 1:200 for Dab 1) and for immunofluorescence, goat-anti-mouse antibody Alexa Fluor 555 (1:1000, Invitrogen).

**Immunoblotting**

To demonstrate the predictive power of our results, we selected the Reelin, Ypel5, and Trpv1 pathways highlighted by WGCNA based on a filtering approach described in the main text in which to test for constituent protein. Area X tissue from non-singers and singing birds was isolated 3 h following either lights-on for the non-singers or time from the 1st motif for the singers. Tissue punches (Figure 1B) were homogenized in Ripa lysis buffer and protein concentration determined using the Bio-Rad RC DC kit, as in Miller et al. (2008). Brain extract from reeler mice was obtained courtesy of Dr. Patty Phelps (UCLA). Samples were heated to 90–100°C for 3 min, and then run on 10% SDS-PAGE. Prestained dual color standards were used to determine molecular weight. Samples were run in Tris-Glycine-SDS (TGS) buffer (Bio-Rad) at 100V then transferred in TGS with 20% methanol and 1%SDS for 2 h at 400 mA onto 0.45-μm PVDF/nitrocellulose membranes (Bio-Rad). Membranes were blocked with 3% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at RT on a shaker. Blots containing area X samples were probed with selected antibodies as noted above. These antibodies were diluted in TBST containing 3% nonfat dry milk. Following primary antibody incubation, blots were washed in TBST 3x10 min at RT while shaking and probed with horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG (1:2000) and anti-mouse IgG (1:10,000 for Gapdh, 1:2,000 dilution for all others) in 3% milk TBST. Immunoblots were developed using a chemiluminescence HRP detection kit (GE Healthcare, Piscataway, NJ,) and a Typhoon scanner (GE Healthcare). To determine whether protein levels varied as a function of behavior, the value for each lane was background
corrected (except for Trpv1 signals which, based in part on the dual isoforms, precluded clean selection of a background area) then normalized to its corresponding Gapdh value to obtain a ratio using Image Quantity One (Bio-Rad) software. This ratio of protein/Gapdh was plotted for the Reelin, phosphorylated Dab (Figure 8), Vldlr (Figure S7A), and Trpv1 (Figure S7B) data. To enable bird data for Ypel5 to be combined between two blots processed separately (Figure 8B), a second normalization step was performed: the Ypel5/Gapdh ratio per lane was divided by the mean NS value within a blot. Group comparisons between NS and UD birds were performed using nonparametric Mann-Whitney U tests, Spearman’s rho (JMP statistics software, Cary, NC) and graphs plotted in Origin (Northampton, MA).

Immunohistochemistry

To confirm Ypel5 protein expression in zebra finch area X, pairs of male zebra finches were sacrificed 3 h from lights-on following the undirected (n=2) or non-singing (n=2) behavioral protocol described in the Methods. Males were overdosed with inhalant anesthesia and then perfused with prewarmed 0.9% saline followed by ice cold 4% paraformaldehyde in 0.1M phosphate buffer (PB) for brain fixation. Brains were extracted and then cryoprotected in 20% sucrose in PB at 4°C. Coronal sections containing area X were cut at 30µm and thaw-mounted onto slides (Superfrost, Fisher Scientific, Pittsburg, PA) and then stored at -80°C until use. For fluorescent immunohistochemistry, brain sections were encircled by a hydrophobic barrier using a PAP pen (Ted Pella Inc, Reddington, CA) and washed in Tris Buffered Saline (TBS) with 0.3% Triton X-100 (Tx) for 3x5 min. Sections were incubated for 10 min in 50mM ammonium chloride in TBS to reduce autofluorescence followed by 3x5 min washes in TBSTx. Slides were blocked in 5% goat serum in TBSTx for 1 h at RT then washed with TBSTx in 1% goat serum. Sections were incubated in the anti-Ypel5 and anti-NeuN
antibodies (see above) in a solution containing 1% goat serum in TBS with 0.1% Tx, overnight at 4°C. Sections were processed using the Tyramide Signal Amplification (TSA, Perkin-Elmer, Waltham, MA) kit and protocol (kindly provided by Dr. Alice Fleming, UCLA) to increase the protein signals. Tissue sections were washed with TBSTx (0.3%) for 4 x 5 min then incubated with the biotinylated secondary antibody in TBSTx for 1 h at RT, followed by washes. Sections were incubated with Streptavidin-HRP at 1:100 in TBSTx for 30 min at RT, washed for 4 x 5 min then incubated with fluorescein/tyramide (green signal) at 1:50 in amplification diluent for 10 min in the dark. Slides were washed in TBSTx 2 x 5 min followed by TBS alone. To visualize NeuN, sections were incubated with secondary goat anti-mouse antibody, Alexafluor 555 in 1% goat serum in TBS with 0.1% Tx. for 2 h. Sections were then washed 2x with TBSTx 0.3% and then 3x with filtered TBS alone. Slides were mounted using Prolong gold anti-fade mounting media (Invitrogen) and coverslipped. Images were captured using an AxioImager microscope equipped with fluorescence and the Axiovision 4.4 software program (Carl Zeiss MicroImaging, Thornwood, NY). Control slides were processed as described except without the primary Ypel5 and NeuN antibodies.

For Dab 1 immunohistochemistry using the diaminobenzidine (DAB) reaction, free floating 40µm coronal brain sections from an undirected singer were processed at RT as follows: Sections were washed in Phosphate Buffered Saline with 0.1% Tx (PBSTx) and 0.1% Bovine Serum Albumin (BSA) then soaked in 0.3% H₂O₂ with 0.1% azide in PBSTx/0.1% BSA for 30 minutes. Then, sections were rinsed in PBSTx/0.1% BSA and blocked in 10% horse serum with 20% Avidin in PBSTx for 1 h. Tissue was incubated overnight with the primary anti- Dab1 antibody in PBSTx/0.1% BSA with 3% goat serum. This was followed by 3 x 10 min washes in PBSTx/0.1% BSA, then incubation for 1 h with the secondary biotinylated anti-rabbit antibody in PBST/0.1% BSA with 3% goat serum. Sections were incubated in the Vectastain ABC
reaction (Vector Laboratories, Burlingame, CA) for 1 h and reacted with 0.06% DAB substrate for 1 min followed by rinses in PBS. Finally, sections were dehydrated in a series of ethanols (2 x 70%, 90%, 100%) and mounted on slides with permount for viewing with an AxioImager microscope.
Supplemental References

Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubischer, J.L., Krieg, P.A., Krupenko, S.A., et al. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J. Neurosci. 28, 264–278.

Dong, J. and Horvath, S. (2007). Understanding Network Concepts in Modules, BMC Systems Biology. 1:24

Enard, W., Gehre, S., Hammerschmidt, K., Holter, S.M., Blass, T., Somel, M., Bruckner, M.K., Schreiweis, C., Winter, C., Sohr, R., et al. (2009). A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. Cell 137, 961-971.

Fedorov, V., Mannino, F., Zhang, R. (2009). Consequences of dichotomization. Pharmaceutical Statistics 8, 50-61.

Horvath, S. (2011). Weighted Network Analysis: Applications in Genomics and Systems Biology (New York: Springer).

Howell, B.W., Gertler, F.B., Cooper, J.A. (1997). Mouse disabled (mDab1): a Src binding protein implicated in neuronal development. EMBO J. 16(1), 121-132.

Hu, Z., Mellor, J., Wu, J., DeLisi, C. (2004) VisANT: an online visualization and analysis tool for biological interaction data. BMC Bioinformatics, 5, 17.

Huang, D.W., Sherman, B.T., Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc. 4(1):44-57.

Jarvis, E.D. (2004). Learned birdsong and the neurobiology of human language. Ann.NY Acad Sci 1016, 749-777.

Konopka, G., Bomar, J.M., Winden, K., Coppola, G., Jonsson, Z.O., Gao, F.Y., Peng, S.,
Preuss, T.M., Wohlschlegel, J.A., and Geschwind, D.H. (2009). Human-specific transcriptional regulation of CNS development genes by FOXP2. Nature 462, 213-U289.

Langfelder, P., Zhang, B., & Horvath, S. (2007). Defining clusters from a hierarchical cluster tree: the Dynamic Tree Cut package for R. Bioinformatics 24, 719-720.

Langfelder, P., Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics 9, 559.

Mason, M.J., Fan, G., Plath, K., Zhou, Q., Horvath, S. (2009). Signed weighted gene co-expression network analysis of transcriptional regulation in murine embryonic stem cells. BMC Genomics 10, 327.

Miller, J.E., Spiteri, E., Condro, M.C., Dosumu-Johnson, R.T., Geschwind, D.H., and White, S.A. (2008). Birdsong decreases protein levels of FoxP2, a molecule required for human speech. J. Neurophysiol. 100, 2015-2025.

Oldham, M.C., Horvath, S., Geschwind, D.H. (2006). Conservation and evolution of gene coexpression networks in human and chimpanzee brains. Proc. Natl. Acad. Sci. USA. 103, 17973-8.

Oldham, M.C., Konopka, G., Iwamoto, K., Langfelder, P., Kato, T., Horvath, S., and Geschwind, D.H. (2008). Functional organization of the transcriptome in human brain. Nat. Neurosci. 11, 1271-1282.

Spiteri, E., Konopka, G., Coppola, G., Bomar, J., Oldham, M., Ou, J., Vernes, S.C., Fisher, S.E., Ren, B., and Geschwind, D.H. (2007). Identification of the transcriptional targets of FOXP2, a gene linked to speech and language, in developing human brain. Am. J. Hum. Gen. 81, 1144-1157.

Tchernichovski, O., Nottebohm, F., Ho, C.E., Pesaran, B., and Mitra, P.P. (2000). A procedure for an automated measurement of song similarity. Anim. Behav. 59, 1167-1176.
Tóth, A., Boczán, J., Kedei, N., Lizanecz, E., Bagi, Z., Papp, Z., Edes, I., Csiba, L., Blumberg, P.M. (2005). Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. Brain Research. Mol. Br. Res. 135, 162-8.

Vernes, S.C., Spiteri, E., Nicod, J., Groszer, M., Taylor, J.M., Davies, K.E., Geschwind, D.H., and Fisher, S.E. (2007). High-throughput analysis of promoter occupancy reveals direct neural targets of FOXP2, a gene mutated in speech and language disorders. Am. J. Hum. Gen. 81, 1232-1250.

Wada, K., Howard, J.T., McConnell, P., Whitney, O., Lints, T., Rivas, M.V., Horita, H., Patterson, M.A., White, S.A., Scharff, C., et al. (2006). A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. Proc. Natl. Acad. Sci. USA 103, 15212-7.

Warren, W.C., Clayton, D.F., Ellegren, H., Arnold, A.P., Hillier, L.W., Kunstner, A., Searle, S., White, S., Vilella, A.J., Fairley, S., et al. (2010). The genome of a songbird. Nature 464, 757-762.

Yip, A.M., and Horvath, S. (2007). Gene network interconnectedness and the generalized topological overlap measure. BMC Bioinformatics 8, 22.

Zhang, B., and Horvath, S. (2005). A general framework for weighted gene co-expression network analysis. Statistical Applications in Genetics and Molecular Biology 4.

Zhao, W., Langfelder, P., Fuller, T., Dong, J., Li, A. and Horvath, S. (2010). Weighted Gene Coexpression Network Analysis: State of the Art. J. Biopharm. Stats. 20, 281-300.