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Developmental shifts in gene expression in the auditory forebrain during the sensitive period for song learning

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Abstract

A male zebra finch begins to learn to sing by memorizing a tutor's song during a sensitive period in juvenile development. Tutor song memorization requires molecular signaling within the auditory forebrain. Using microarray and in situ hybridizations, we tested whether the auditory forebrain at an age just prior to tutoring expresses a different set of genes compared to later in life after song learning has ceased. Microarray analysis revealed differences in expression of thousands of genes in the male auditory forebrain at posthatch day 20 (P20) compared to adulthood. Further, song playbacks had essentially no impact on gene expression in P20 auditory forebrain, but altered expression of hundreds of genes in adults. Most genes that were song-responsive in adults were expressed at constitutively high levels at P20. Using in situ hybridization with a representative sample of 44 probes, we confirmed these effects and found that birds at P20 and P45 were similar in their gene expression patterns. Additionally, 8 of the probes showed male-female differences in expression. We conclude that the developing auditory forebrain is in a very different molecular state from the adult, despite its relatively mature gross morphology and electrophysiological responsiveness to song stimuli. Developmental gene expression changes may contribute to fine-tuning of cellular and molecular properties necessary for song learning.

Keywords

song learning; auditory forebrain; sensory; sensitive period; microarray

INTRODUCTION

Songbirds rely on songs and other learned vocalizations to communicate with each other. Perceptual processing of song appears to be centered in a forebrain area called the “auditory lobule” (AL; Mello et al., 1992; Mello and Clayton, 1994; Cheng and Clayton, 2004; Gentner, 2004; Mello et al., 2004; Theunissen and Shaevitz, 2006; Phan et al., 2006; Gobes and Bolhuis, 2007; George et al., 2008; Woolley and Doupe, 2008; Dong and Clayton, 2008). The AL is composed of three highly interconnected subregions that are functionally analogous to mammalian primary and secondary auditory cortical areas: field L, the caudomedial nidopallium (NCM), and the caudomedial mesopallium (CMM) (Vates et al., 1996; Theunissen and Shaevitz, 2006). Playback of songs can trigger a robust gene response within NCM and CMM, causing transient changes in expression of *ZENK* (*zif268*, *egr-1*, *ngfi-a*, *krox24*; (Mello et al., 1992; Mello and Clayton, 1994), *c-jun* (Nastiuk et al., 1994), *c-*

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fos (Bailey et al., 2002), arc (Velho et al., 2005) and many other genes (Dong et al., submitted). In adults, this genomic response in AL has been linked to the salience and contextual novelty of the playback experience (Mello et al., 1995; Kruse et al., 2004; Terpstra et al., 2004; Vignal et al., 2005; Woolley and Doupe, 2008; Dong and Clayton, 2008).

Songbirds learn to sing by copying a tutor, and this process also requires molecular signaling in the AL (London and Clayton, 2008). In the zebra finch (*Taeniopygia guttata*), tutor song copying is restricted to a sensitive period that extends from approximately posthatch day 30 (P30) to P65 (Bohner, 1990; Slater, 1991; Roper and Zann, 2006). Interestingly, ZENK gene expression in AL undergoes a major shift across this period. At P20, ZENK mRNA is present at a constitutively high level independent of song exposure (Jin and Clayton, Stripling et al., 2001).

At P30, basal ZENK levels (in absence of song stimulation) are so high that song playbacks trigger no further increase despite inducing a significant neurophysiological spike response in AL (Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2003). By P45, basal expression of ZENK has declined to adult-like levels, and an adult-like increase following song stimulation becomes evident (Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2005).

These observations suggest that changes in gene expression in AL might contribute to the developmental trajectory of the song tutoring process. To characterize gene expression patterns in the songbird brain, a broad collaborative approach has been developed under the Songbird Neurogenomics Initiative (Replogle et al., 2008). As part of this initiative, here we used the “SoNG 20K microarray” to establish the profile of gene expression in the AL of male zebra finches just prior to initiation of tutor song memorization (P20). We assessed both the basal state and the state reached 30 minutes after exposure to a novel song, and we compared these profiles to those of adults under the same stimulus conditions. Our results indicate major changes occur in gene expression and regulation in AL between P20 and adulthood. To probe the significance of these changes further, we performed a statistical analysis of functional annotations associated with the regulated gene populations, and additional in situ hybridizations on a subset of RNAs to assess their relative expression at P20, P45 and adulthood. Our findings suggest a complex process of developmental and perceptual maturation in AL.

METHODS

All procedures involving animals were approved by the University of Illinois, Urbana-Champaign Institutional Animal Care and Use Committee.

Subjects

Experiments with juvenile (P20) male zebra finches were conducted over 5 months, following the spontaneous rate of production in the breeding aviary. There is some variability in the morphological development of zebra finches; all birds used in the experiment had grown a full complement of flight feathers and were capable of fledging the nest. The sex of all P20 males was confirmed by visual inspection of the gonads. Individual birds were placed into sound attenuating chambers on P19 and kept there overnight (17 hr) to normalize their immediate experience, so that they were P20 on the day of the experiment. Each chamber was equipped with a speaker for sound playback, and a video camera and microphone connected to an external VCR. P20 zebra finches are still reliant on adults to help feed them and keep them warm. Therefore, for the overall health of the juvenile birds, an adult female was also placed in the cage for the overnight isolation. The adult female was removed immediately upon lights-on the next morning, to balance the

design of the song playback experience with that of the adult males who heard song playback alone (below). To minimize acute effects of the female's removal on the gene expression profile of the juvenile males, we waited 4 hr after removal of the female before proceeding with the playback. The individual P20 males were then exposed to 30 min of pre-recorded conspecific song (SONG) or to silence (SIL; n=6 for each group).

Adult (>P100) males were also placed into sound attenuating chambers overnight and were exposed to either the SONG or SIL condition (n=6 for each group). The adults remained alone for the duration of the sound isolation. These birds were collected as part of another, larger study on adult AL gene expression and were collected approximately 6 months before the P20 tissues. They were processed exactly as the P20 birds were with a microarray methodology and analysis strategy designed to integrate all of the samples (details below).

The song playback for all 24 birds was identical. One unfamiliar zebra finch song bout was looped to play every 10 sec for a total of 180 song bouts in 30 min, a protocol that causes maximal *ZENK* mRNA induction and other robust changes in gene expression in adult AL (Mello et al., 1992; Dong et al., submitted). The period of song playback (or 30 min prior to sacrifice for the SIL condition) for all 24 birds was videotaped to assess the general behavioral state of the P20 birds and the singing quantity of the adult birds. Immediately at the end of the song playback, SONG (and SIL) birds were sacrificed, and the entire AL was bilaterally dissected as in other related studies (Cheng and Clayton, 2004; Dong and Clayton, 2008; Dong et al., submitted). Thus each sample in the microarray analysis comprised the left and right AL from a single bird. Tissue was flash frozen and stored at -80°C until processed for microarray hybridization.

RNA isolation and microarray hybridization

Following the standard procedures of the SoNG Initiative, tissues were provided to K. Replogle who performed all subsequent steps, exactly as with the tissue samples from the other experiments in the community collaboration; the experiment here was identified as Experiment #22 in Replogle et al., 2008. Briefly, total RNA was extracted (RNAqueous Micro, Ambion, Austin, TX), amplified (Low RNA Input Fluorescent Linear Amplification, Agilent, Santa Clara, CA), and Cy3/Cy5 dye-coupled (GE Life Sciences, Piscataway, NJ) as previously described (Replogle et al., 2008). To enable across-batch normalizations and analysis of the P20 and adult tissue, each array was hybridized with one of the experimental samples and a universal reference sample. The universal reference sample was a pooled composite of zebra finch brain mRNA that was hybridized on all of the SoNG microarrays (Replogle et al., 2008). The Cy3/Cy5 dye coupling was balanced between experimental and universal reference samples to control for potential dye incorporation and hybridization biases.

The labeled samples were hybridized to the SoNG 20K microarray, the composition of which is described in detail in Replogle et al., 2008. Briefly, the array contains spotted cDNAs representing 17,214 different RNAs that are non-redundant in that each one contains at least some unique sequence relative to all others on the array. Mapping of these against a larger set of cDNA sequences suggests that they represent the products of 11,500–15,000 genes. Redundancies represent either non-overlapping stretches of the same transcript or alternative splice forms. Additionally, the array contains 368 known control sequences including *ZENK* and β -actin.

The arrays were hybridized overnight at 42°C , washed, scanned (Axon GenePix 4000B, Molecular Devices, Sunnyvale, CA), and visualized with GenePix Pro 6.0 (Molecular Devices) as described for all SoNG arrays (Replogle et al., 2008). Analyzed scan data were

entered into an internet-based database that contains results from all SoNG microarrays (Replogle et al., 2008).

Microarray statistical analysis

Microarray spots that did not meet quality standards were manually removed from each array. Within array biases were normalized with loess regression (Bioconductor, R; Smyth and Speed, 2003). Data quality after within-array normalization was visually inspected to verify that no overt biases remained in the data. We used a boxplot (Bioconductor, R, www.bioconductor.org) to map the M values ($\log_2(\text{Cy5}) - \log_2(\text{Cy3})$) for both the universal reference and AL sample on each array to confirm that the median, distribution, and outlier hybridization intensities for all samples were similar. After within-array normalization, data were analyzed with a two-step mixed linear model of analysis of variance (ANOVA; Wolfinger et al., 2001) in SAS (SAS, Cary, NC). The first-step ANOVA normalized effects of dye, array, and the dye*array interaction to standardize the mean intensity value between all arrays. The resulting residual values (final normalized intensity values for each gene) were used in the second-step mixed linear model ANOVA with dye, age, and song exposure condition as fixed factors, and array (which here represents one AL) as the random factor. The estimated least-squared means were used to identify microarray spots that showed a significant overall change in hybridization intensities.

We performed a total of five statistical comparisons to identify genes that specifically changed with age, song, or their interaction: 1) adult SONG vs. adult SIL, 2) P20 SONG vs. P20 SIL, 3) the interaction between P20 and adult SONG and SIL, 4) P20 SIL vs. adult SIL, and 5) P20 SONG vs. adult SONG. We also cross-referenced the latter two lists and identified genes that were present on both, and therefore independently showed a significant effect of age in both the SONG and SIL conditions. We termed these “core developmental genes.” The results of each of these comparisons were then separated into two subgroups: genes that had increased expression levels in one of the groups (“-up”) and genes that had decreased expression levels in that group (“-down”).

To reduce the potential for type I errors which can be problematic in microarray analysis (Benjamini and Yekutieli, 2005), we used a stringent criterion for significance in these comparisons: the False Discovery Rate (FDR)-adjusted p-value. The FDR p-value accounts for errors that can accumulate from multiple testing. All genes reported as showing significant changes met an FDR $p < 0.05$ criterion; specific FDR thresholds are reported for each comparison.

In addition to specific gene-by-gene statistical analysis, Principal Component Analysis (PCA) was performed using all genes on the array, for all 24 birds (TIGR Multiexperiment Viewer, www.tm4.org). This analysis can illustrate how similar overall the samples were to each other and suggest factors that best describe how the experimental groups segregate.

Functional gene analysis

Statistical analyses identified large numbers of genes that were differentially expressed in our study (Results). To determine whether co-regulated gene sets were statistically associated with particular biological functions, we performed an analysis of Gene Ontology (GO). GO categories are organized in a hierarchical structure to describe the biological functions and subcellular locations of genes identified with standardized gene names such as NCBI UniGene numbers. Each gene on the custom zebra finch brain SoNG microarray was annotated with a unique “SB” identification number but it was not yet annotated with zebra finch UniGene numbers, pending the completion of the zebra finch genome (www.songbirdgenome.org). All sequences on the SoNG array were therefore compared to

the only bird genome currently available, the chicken, and annotated with chicken UniGene numbers. Not all SB sequences, however, were sufficiently similar to the chicken UniGene sequences to acquire gene annotation (Replogle et al., 2008). Therefore, functional gene analysis was performed with the subset of significant microarray genes that could be reliably annotated with chicken “Gga” UniGene names (Replogle et al., 2008).

We used Onto-Express (Khatri et al., 2004; Khatri et al., 2005), a web-based program that utilizes several sources of gene and annotation information to profile functional categories that are overrepresented in gene lists. Onto-Express allows direct comparison of significant gene sets to the complete microarray gene population. This analysis statistically identifies which GO categories are contained within the significant gene lists more often than expected based on the distribution of GO categories on the whole microarray. GO categories with a p-value < 0.05 after hypergeometric analysis were considered significantly over-represented within a given gene list.

We then used Cytoscape (Shannon et al., 2003; Cline et al., 2007) to display the GO results. All GO categories found on the microarray were represented by nodes (circles), the size of which reflected the hierarchical position of the GO category. Nodes were automatically functionally connected with edges based on the GO hierarchy. Significant GO categories within each gene list were then mapped onto this network to visually represent the microarray results.

In situ hybridization

We performed in situ hybridizations to independently confirm the general patterns of gene expression observed in the microarray analysis, and to extend the analysis by adding additional groups of birds. We used a total of 44 genes, representing all five of the major microarray statistical comparisons. 11 probes were chosen from the list of genes that changed across age, and 20 genes were selected for the song response gene lists (including 9 chosen based on p-value instead of FDR p-value from the P20 SONG vs. SIL comparison). We also included 13 genes from the age*song condition interaction comparison. With a single exception (SB03002A1A11.f1, described in Results), the individual genes analyzed with in situ hybridization were not selected based on any specific criterion (for example, magnitude of fold change or gene annotation) thus providing a non-biased investigation into each statistical comparison. In addition to the P20 and adult males in both the SONG and SIL conditions as in the microarray experiment, we also included P45 birds in the SONG and SIL condition, and females at all 3 ages hearing SONG or SIL (n=3 for all groups) in our in situ hybridization analysis. Each of the 44 probes was hybridized to brain sections from all 12 groups of birds.

After song or silent exposure as described for the birds used in the microarray experiment (P20 birds were isolated overnight with an adult female, P45 and adult birds were isolated alone), whole brains were extracted, flash frozen, sectioned to 12µm, and stored at -80°C until use.

Digoxigenin-labeled riboprobes were transcribed using the cDNA clones spotted on the microarray as templates. Hybridization for all genes was performed as previously described (Jin and Clayton, 1997). A full set of P20, P45, adult; SONG and SIL; and male and female AL-containing brain sections were processed simultaneously for quantitative comparisons of hybridization patterns. Images of brain sections were digitally captured on an AxioImager A1 (Carl Zeiss Microimaging, Thornwood, NJ) with a CCD camera (Microfire; Optronics, Goleta, CA). The number and intensity of labeled cells was quantified within the entire AL and the adjacent non-auditory hippocampus (HP) for control purposes (Mello et al., 1992) using ImageProPlus 4.5.1 (MediaCybernetics; Bethesda, MD). All measures were corrected

for the size of the measured area. Hybridization and analysis was performed blind to gene identities.

To remove potential differences in AL staining intensity across batches or from non-specific background staining, each AL value was normalized to the HP value on the same section. Each gene was hybridized to 3 brain sections spanning the medial-lateral extent of AL in each bird. Therefore, after normalizing the AL measures to HP measures on each section, an average hybridization value for the three sections was derived to obtain an overall AL in situ hybridization value for each bird. Normalized AL values from the in situ hybridization were analyzed with one-way ANOVA (SPSS; $\alpha = 0.05$) to test for main effects of age, song exposure condition, and sex. Changes in AL gene expression levels could be due to changes in mRNA levels per cell or in the number of cells that contain the mRNA; we therefore considered both and reported main effects based on either measure. Representative in situ hybridization images shown were modified for contrast to highlight levels of gene expression; modifications were performed equally for all four images per gene.

To determine the general correspondence between the in situ hybridization and microarray expression levels for the 44 genes, we calculated the magnitude of hybridization change between the groups appropriate for the statistical comparison, based on the in situ and microarray hybridization intensity measures. For example, to analyze a developmental gene, we subtracted the adult SONG intensity value from the P20 SONG intensity value for both the microarray and in situ hybridization results. The differences from the in situ hybridization data were then fit to the corresponding microarray values in a linear model in SPSS (SPSS, Chicago, IL).

RESULTS

Quality of P20 and adult microarray hybridizations and analysis

Gene expression profiling was conducted as part of the Songbird Neurogenomics Initiative using the 20K microarray and a set of coordinated procedures that have been independently validated in related studies (Replogle et al., 2008; Lovell et al., 2008). To evaluate the overall quality of the microarray hybridizations within and across the specific arrays in this experiment before beginning between-array normalization and statistical analysis, we plotted the M values of each sample (Fig. 1). Overall, the M value medians and distributions are not systematically different, demonstrating that no overt bias occurred in hybridization of either dye, any sample, or across arrays. Thus, the quality of microarray hybridization was high and consistent across P20 and adult samples.

The overall ANOVA showed that a total of 11060 genes (58%) of the 18848 total genes spotted on the array (including some duplicates) significantly changed across all four experimental groups at a FDR $p < 0.05$.

Robust developmental changes in gene expression

Development was the biggest factor in distinguishing the experimental groups: at a highly stringent FDR $p < 0.001$, 1292 genes showed changed expression across age in the SIL condition and 1789 genes were different across age in the SONG condition. The major effect of development can also be seen in the PCA analysis (Fig. 2); the P20 and adult samples are cleanly divided along PC2.

To identify genes that showed differences in gene expression levels between P20 and adult AL regardless of whether the birds had just heard song or not, we cross-referenced the P20 SONG vs. adult SONG and P20 SIL vs. adult SIL gene lists. This comparison found 925 genes that existed on both lists and therefore showed altered expression levels across age in

both song exposure conditions (Supplementary Table 1). This subset of genes was considered the “core developmental genes” because they showed distinct expression levels in P20 compared to adult AL independently in both song conditions. 488 of the core developmental genes showed greater signal intensities in P20 compared to adult (“P20-up”), and 437 showed greater signal intensities in the adult compared to P20 AL (“ADULT-up”).

To assess the potential functional implications of these developmental changes in gene expression, we performed a statistical analysis of GO terms associated with the genes in these two lists. Distinct functional categories were significantly ($p < 0.05$) overrepresented in the each. Functional categories overrepresented in the P20-up subset include DNA structure and organization, cell cycle, control of cytoskeleton and neurite growth (Fig. 3 and Supplementary Table 1). The ADULT-up group is characterized by genes involved in apoptosis, cellular differentiation, protein transport, those necessary for voltage-gated ion channel activity, and translation. Genes integral to several types of membranes - organelle, nuclear, and vesicular - were also overrepresented in the adult AL (Fig. 3 and Supplementary Table 1).

Robust effects of song on gene expression in adult AL

A total of 296 genes showed a significant difference between SONG and SIL conditions in adult male AL at FDR $p < 0.01$. Of these genes, 244 showed lower signals in the SONG compared to the SIL condition (“SONG-down”) and 52 showed higher signals in the SONG compared to the SIL condition (“SONG-up”). These changes appear to be due specifically to the experience of hearing the song stimulus as there was no significant difference in the amount the birds sang between the SONG and SIL groups ($p = 0.495$).

GO analysis demonstrated unique gene categories that were significantly (p -value < 0.05) overrepresented in the SONG-up and SONG-down gene lists (Fig. 4 and Supplementary Table 2). These categories include ion transport, cell mobility, polysaccharide metabolism, and Notch signaling in the SONG-down group. In the SONG-up gene list, functional categories describing alcohol and RNA metabolism, translational initiation and tRNA activation, as well as tricarboxylic acid-related energy production were overrepresented.

Minimal effects of song on gene expression in P20 AL

In contrast to the robust difference between SONG and SIL gene expression profiles in the adult, there were very few significant differences in P20 birds after hearing song - one gene showed a significant change at FDR $p < 0.05$ and even at an FDR $p < 0.1$, only four genes showed a significant difference between SONG and SIL conditions. Just one of these four genes was functionally annotated, an enzyme called inosine triphosphate pyrophosphatase (ITPase). The only gene that showed a significant change at FDR $p < 0.05$ between P20 SONG and SIL, SB03002A1A11.f1, was not a song response gene in the adult AL and is not currently annotated. PCA analysis of the individual P20 SONG, P20 SIL, adult SONG and adult SIL AL expression profiles illustrated that across PC1, presumed to be the song exposure condition based on the variation between adult SONG and SIL samples, the P20 birds were much more similar to each other (they clustered more tightly together in close proximity along the PC1 axis) than the adult birds (Fig. 2).

The lack of significant gene expression changes after hearing song in P20 birds could be because, like *ZENK*, genes that are “SONG-up” in adults were expressed at constitutively high levels in P20 AL (Jin and Clayton, 1997; Stripling et al., 2001). Similarly, genes that become “SONG-down” in the adult may be expressed constitutively at low levels in the juvenile birds. To test these hypotheses, we analyzed the intensity values for all four experimental groups (adult SONG, adult SIL, P20 SONG, P20 SIL), but only for the genes

classified as SONG-up or SONG-down in adults as described above. One-way ANOVA suggested that both SONG-up and SONG-down genes are expressed at altered levels in the P20 AL compared to the adult AL (p-value <0.001, Bonferroni-corrected post-hoc p <0.016), independent of song exposure condition in the P20 birds. To visualize this effect, we normalized the intensity value of each song response gene from the P20 SONG, P20 SIL, and adult SONG groups to the intensity value of that gene in the adult SIL group. The mean normalized intensity values for the three groups were then graphed to highlight the significant differences in adult SONG and P20 expression patterns amongst the adult song-responsive genes (Fig. 5).

To determine if higher hybridization intensities were a global feature of the P20 microarray data, we also calculated the average hybridization intensity in each of the four groups, just for those genes that did *not* respond to song in the adult AL. In contrast to the adult song response genes, the average hybridization intensity for the adult non-responsive genes was the same in both P20 groups as in the adult groups (p = 0.996).

Investigation of the SONG-up and SONG-down genes in the P20 groups also suggested that there is more variability in the P20 AL than the adult AL expression levels. The average standard deviations of the hybridization intensity values across the entire array are 1.51 and 1.52 for the P20 SONG and SIL birds, respectively. This is not noticeably different from the average standard deviation for the adult groups: 1.57 for both adult SONG and adult SIL. When the average standard deviations of intensity values for the four groups are computed only for the genes that were significantly different in the adult SONG vs. adult SIL comparison, however, the P20 groups did appear to have more variability than the adults: 1.69 and 1.70 (P20 SONG and SIL, respectively) versus 1.27 and 1.08 (adult SONG and SIL groups, respectively), though this difference was not significant (p = 0.406).

In situ hybridization confirms and extends microarray expression patterns

We used in situ hybridization to perform an independent assessment of the factors that influence gene expression in AL. Our goals were to corroborate the broad conclusions of the microarray analysis in a new set of birds using an independent technique, and to extend the analysis to evaluate gene expression at a later point in the song learning period (P45) and in females. For these purposes, we used a total of 44 probes that represented the main comparisons in the original microarray experimental design. These genes included 11 from the age comparisons and 9 from the adult song responsive gene list. We also included the single gene that met the statistical test for a significant song response (FDR p < 0.05) at P20, along with 10 other “P20 possibly-responsive” candidates that failed that test but were significant at an individual p-value < 0.05 (not corrected for multiple testing). Finally, we included 13 genes from a small set of 42 genes that met the formal statistical test (FDR p < 0.05) for an interaction between age and song condition in the microarray.

Riboprobes for each of the 44 genes were hybridized to sections from 36 different birds representing 12 experimental groups: males and females, in SONG and SIL conditions, at P20, P45 and adulthood. A comprehensive table of data for both the microarray and in situ hybridizations is given in Supplementary Table 3.

To assess the overall correspondence of the microarray and in situ hybridization observations, we first asked whether the two methods reported a similar magnitude and direction of change for each of the genes in the P20 vs. adult and SONG vs. SIL comparisons. The microarray and in situ hybridization intensity measurements were in good general agreement as shown by a significant positive correlation between these two methods (R = 0.540, p < 0.001; Fig. 6). We then considered on a gene by gene basis whether the in situ hybridization corroborated the main statistical effect observed in the microarray data,

considering both a significant main effect of cell number or hybridization density. For the 11 representatives of the genes that changed by age in the microarray analysis, 9 also showed a main effect of age in the in situ hybridization analysis ($p < 0.05$, Table 1). These age effects included genes that showed higher expression levels in adult AL and those that showed higher expression levels in P20 AL (Fig. 7). Six of the 9 adult song-responsive genes showed a main effect of song by in situ analysis. Thus, the two major effects evident in the microarray analysis (age, and song stimulation in adults) were also generally replicated in a separate set of birds by in situ hybridization. Similarly, the probes chosen on the basis of weak statistical trends in the microarray data also failed to show a robust effect by in situ hybridization: only one of the 11 “P20 possibly-responsive” genes showed a significant effect of song by in situ hybridization (examples of genes that showed a response to song in the adult, but not P20, AL are shown in Figure 8). Also, only 1 of the 13 genes selected for age*song condition interaction on the microarray showed a significant age*song condition interaction by in situ hybridization.

Across all 44 probes, the in situ hybridization analysis detected a main effect of age for 29. As this analysis also included sections from birds at P45, we conducted post hoc tests for each of these 29 genes and found only one to be significantly different between P20 and P45 (SB02003A1D09.f2, labeled with * in Table 1). Similarly, 10 of the 44 genes showed a main effect of song in the analysis as a whole, but in only one case was there a significant effect of song at P45 ($p=0.039$, SB02019A1D12.f1, labeled with † in Table 1). 8 of the 44 genes showed a significant main effect of sex ($p < 0.05$; Table 1).

DISCUSSION

Song learning in the zebra finch is complex process that requires the integration of sensory and motor processes across multiple brain areas during a restricted developmental period. The neural mechanisms that underlie song learning and its developmental limitations are not known. Here, we focused on the AL, a brain region centrally involved in tutor memory formation during development and song perceptual learning in adults. We combined microarray and in situ hybridizations to ascertain whether AL gene expression is different in juveniles compared to adults, and whether hearing song causes different changes in AL gene expression in juveniles compared to adults. Our approach was inclusive as we deliberately used the entire AL (including field L, NCM and CMM) in order to capture as much molecular variation as possible, following other precedents for biochemical analysis of this anatomical unit (Cheng and Clayton, 2004; Huesmann and Clayton, 2006; Dong and Clayton, 2008; Dong et al., submitted).

We were interested to identify, first, changes that occurred across development independent of whether the birds heard song or not. We found a large number of these “core developmental” transcripts: roughly 10% of the probes on our array measured such changes at a very high significance threshold ($FDR < 0.1\%$). Analysis of the associated functional annotations for this population suggests broad thematic shifts across development. For example, the P20-up set was enriched for genes associated with mitosis. Conversely, the ADULT-up set was enriched for genes associated with apoptosis and cell differentiation. The ADULT-up annotations were also enriched for functions related to ion signaling, membrane changes, and transcriptional control. These may represent the types of functions that are most critical to the efficient operation of the mature system.

These major developmental shifts in gene expression are in sharp contrast to the largely mature gross morphology and electrophysiological function of AL at P20 (e.g., Fig. 7 and Fig 8; and Jin and Clayton, 1997; Stripling et al., 2001). Hence it would seem that developmental gene regulation is associated with functional “fine tuning” of a system that is

already largely assembled by P20. Similar types of gene expression changes may also occur in other parts of the brain responsible for song development; cellular and molecular alterations have been described in all of the major telencephalic song areas even after P20 (e.g. (Konishi and Akutagawa, 1985; Clayton, 1997; Nixdorf-Bergweiler, 2001; Doupe et al., 2004; Bottjer, 2004; Nordeen and Nordeen, 2004). Might such large-scale changes be typical of brain development during adolescence more generally? Large shifts in brain gene expression have been observed during the social maturation of honeybees (Whitfield et al., 2003) and during embryonic development of rodents (Matsuki et al., 2005), but to our knowledge thorough analyses of gene expression in the adolescent mammalian brain have yet to be reported.

Our second goal was to assess the acute effects of song stimulation on gene expression at P20 compared to adults. Song stimulation causes a robust gene response in the adult AL, as shown originally in studies using the *ZENK* gene (Mello et al., 1992) and extended now in a parallel microarray-based experiment under the SoNG Initiative (Dong et al., submitted). Corroborating the analysis of that study, we also detected a bi-directional genomic response to song in adults, with some RNAs rapidly increasing but even more decreasing immediately after song onset. In juveniles, however, we find that both directional components of this genomic response to song are attenuated. Genes that are song-inducible in adults (SONG-up) are constitutively expressed at high levels at P20 (as though always in the induced state), whereas genes that are song-suppressible in adults (SONG-down) are also at constitutively high levels at P20 (as though the ability to down-regulate in response to song has not yet developed). Our data do not suggest that elevation of gene expression is a general property of the juvenile AL; rather, it appears to be a distinguishing feature specifically of the subset of genes that will become song-responsive (either up or down) in the adult. Our microarray data do not, however, allow us to determine if these gene expression patterns are a general property of all cells throughout AL or if there are select subpopulations of cells that contribute to this overall pattern.

On the basis of *ZENK* measurements in juveniles, Jin and Clayton (1997) proposed that there may be a shift from “constitutive plasticity” in the juvenile to “regulated plasticity” in the adult auditory forebrain. If this interpretation is correct, our data here suggest that maturation of the auditory forebrain may involve the emergence of a complex regulatory network that uses both gene activation and gene inhibition in equal measures to manage a dynamic response to ongoing experience. It will be interesting to consider whether the details of this genomic network are different in species that continue to learn song throughout life, as opposed to the zebra finch in which song learning is developmentally restricted.

Complementing our broad microarray-based analysis, we also performed *in situ* hybridizations with 44 gene probes in 12 groups of animals. Most importantly, our *in situ* hybridizations included P45 birds, providing significant insight into the profile of gene expression in AL during the core of the song learning period. Formally we considered two alternative hypotheses. On the one hand, it was possible that P20 and P45 would be similar to each other and different from the adult because, unlike adults, both P20 and P45 birds are receptive to hearing song for the purpose of song copying. On the other hand, it was possible that the P45 AL would be more like the adult AL because, unlike the P20 birds, P45 males are actively singing (albeit immatures). Our results clearly support the first hypothesis, as we found only one core developmental gene to be different in expression between P20 and P45. This suggests that, at least for the sample of genes studied here, twenty-five days of aging and initiation of vocalizations is not sufficient to dramatically alter AL gene expression. Similarly, we found only one gene that showed a song response within the P45 birds. This gene has been annotated as NR4A3. NR4A3 is an immediate early gene that is

also part of the adult song response (Dong et al., submitted). Combined with previous studies that describe an immediate early gene response at P45 (Jin and Clayton, 1997; Bailey and Wade, 2005), these results suggest that emergence of immediate early gene responses may be among the earliest steps in genomic maturation of the AL. Further studies using even more developmental timepoints and genes will be necessary to test this idea.

Song is largely a social behavior. All of the birds used in this experiment, however, were alone when they experienced either song playback or acoustic isolation to eliminate any uncontrolled confounds that may be introduced by the behavior of another bird. We did need to house the P20 males overnight with an adult female to ensure their well-being. Previous studies of young males placed with females showed that this social interaction alone does not detectably affect the *ZENK* response to song (Jin and Clayton, 1997; Stripling et al., 1997), although in adults, hearing a song in some social contexts can alter the magnitude of the response (Vignal et al., 2005; Woolley and Doupe, 2008). We also considered that the removal of the companion female the following morning (on the day of the experiment) likely stressed the P20 males. However, at least in adults, other stressors such as footshock actually increase the *ZENK* response to song (Jarvis et al., 1995) and conditions of restraint and isolation broaden the responsiveness to non-song stimuli (Park and Clayton, 2002). To minimize potential effects of acute stress on the gene expression profile in the P20 birds, we delayed song playback for four hours, beyond the timeframe for demonstrated effects on the *ZENK* response (Mello et al., 1992). To assess their general behavioral state, P20 birds were monitored for the 30 minutes prior to sacrifice. All birds performed behaviors such as perching and grooming, and in most cases vocalized some calls. This suggests that the P20 birds were in a generally alert state, though it remains possible that they were experiencing stress from isolation, and that some individual variation could have contributed to AL gene expression patterns. We note, however, that by in situ hybridization the P20 and P45 birds were very similar to each other and different from the adults in their relative levels of gene expression. Since the P45 birds were alone throughout the isolation period just as the adults were, the juvenile profile of gene expression is likely not due simply to the immediate effects of overnight social experience, the acute effects of removing a female bird four hours previously, or the new state of isolation in the P20 birds. It will be interesting in the future to explore the global effects of social experience on gene expression in the developing zebra finch brain, expanding on previous single-gene studies (Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2003; Bailey and Wade, 2005; Tomaszewski et al., 2006).

The analysis of gene expression has been transformed in recent years by the development of high-throughput technologies for simultaneous measurement of many genes at once. Initially, microarray hybridizations were often approached merely as an efficient way to screen for individual genes that could be studied separately and in depth once they were identified. However, our results here and the results of a number of other studies reveal massive shifts in brain gene expression associated with behavioral development (Whitfield et al., 2003; Wada et al., 2006; Robinson et al., 2008; Cummings et al., 2008; Miyashita et al., 2008). This fits with the lessons of behavioral genetics commonly showing many genes of small individual effect in complex interacting networks (e.g. Anholt et al., 2003; Kendler and Greenspan, 2006). Hence the challenge now is less one of finding “the gene of interest,” than of understanding the function and origin of particular configurations of gene expression. Our results here begin to define two molecular configurations in the developing zebra finch auditory forebrain, one correlated with a potentially receptive developmental learning state and the other with the dynamic but stable response of the mature adult. Further dissection and analysis of these states should be possible by combining high-throughput molecular phenotyping with the behavioral, developmental and physiological manipulations that make the zebra finch such a powerful model system for probing how genes, environment and development interact to shape the behaving brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Boxplot of all 24 microarrays showing high quality of hybridization. Plot shows M values (log₂(Cy5)-log₂(Cy3)) for each sample across all arrays (48 total boxes for the 24 arrays). The similar M value characteristics of each sample illustrate the consistency of hybridization within and across arrays. Boxes shaded gray are represent the experimental samples, white boxes are the universal reference sample. First 24 boxes are arrays hybridized with P20 AL samples, second 24 are arrays hybridized with adult AL samples.

Figure 2.

PCA analysis of the 24 individual birds using all genes on the microarray. Relative distance between birds reflects overall similarity in gene profiles. Principle Components 1 and 2 (PC1 and PC2), suggest that age and song exposure condition, at least in adults, largely separate the samples.

Figure 3. Biological processes network from Cytoscape showing significantly overrepresented categories in the core developmental genes. Entire network represents all GO categories on the microarray. Blue nodes are those categories overrepresented in P20 AL (P20-up) gene list, yellow nodes are those categories overrepresented in adult AL (adult-UP) gene list. For clarity, a subset of category labels are included to illustrate significantly overrepresented nodes.

Figure 4. Biological processes network from Cytoscape for the adult song-silent comparison. Entire network represents all GO categories on the microarray. Blue nodes are those categories overrepresented in SONG-up gene list, yellow nodes are those categories overrepresented in SONG-down gene list. For clarity, a subset of category labels are included to illustrate significantly overrepresented nodes.

Figure 5.

Bar graph showing intensity levels of adult SONG, P20 SONG, and P20 SIL after normalization to the adult SIL group, for adult song responsive genes. Graph illustrates that the adult song response includes both SONG-up and SONG-down genes, and that in the P20 AL, all song response genes are expressed at constitutively high levels. Single asterisks denote significantly higher expression levels in both P20 SONG and SIL conditions compared to the adult SONG condition for the SONG-down genes; double asterisks denote the significantly higher expression levels in P20 SONG and SIL conditions compared to the adult SONG condition for the SONG-up genes. Error bars represent s.e.m.

Figure 6.

Linear fit analysis of in situ hybridization data to microarray data based on 44 genes analyzed with both techniques. Points represent the magnitude of gene expression change between groups (e.g. P20 SONG vs. adult SONG) calculated from intensity measures after microarray and in situ hybridizations. Results show significant ($p < 0.001$) overall correlation between these two techniques.

Figure 7.

In situ hybridization for two developmental genes. A–D) hybridization in adult AL, E–H) hybridization in P20 AL. A, B, E, F) hybridization for SB03013A1G06.f1 showing higher expression in P20 than adult AL. C, D, G, H) hybridization for SB02003A1D09.f2 showing lower expression in P20 than adult AL. Dorsal is up and caudal is left. Scale bar = 1mm.

Figure 8.

In situ hybridization for two adult song response genes showing regulation in adult but not P20 AL. A–D) hybridization in adult AL, E–H) hybridization in P20 AL. A, B, E, F) hybridization for SB03019A1F04.f1, a SONG-down gene, showing the predicted decrease in hybridization in adult SONG (A) compared to adult SIL (B), and high expression in both P20 SONG and SIL (E, F). C, D, G, H) hybridization for SB02019A1D12.f1 (NR4A3), a SONG-up gene, showing the predicted increase in hybridization in adult SIL (C) compared to adult SONG (D), and high expression in both P20 SONG and SIL (G, H). Dorsal is up and caudal is left. Scale bar = 1mm.

Table 1

Summary of in situ hybridization results for 44 genes characterized for effects of age, song exposure, and sex. Genes are indicated with their individual ESTIMA database gene identifiers, and are organized based on what microarray statistical group they were selected from. Check marks indicate genes for which there was a significant main effect ($p < 0.05$) of age, song, or sex by in situ hybridization; dashes are those genes for which there was no significant main effect. Check mark plus asterisk indicates the one gene that in post-hoc test showed a difference between P20 and P45 expression levels. Check mark plus cross indicates the one gene that showed a significant song response in P45 birds.

ESTIMA identifier	age	song	sex
<i>development</i>			
SB03049A2G10.f1	✓	–	–
SB03011B2G05.f1	✓	–	–
SB010003000C11	–	–	✓
SB02031B1E08.f1	✓	–	–
SB02028B1B03.f1	–	–	–
SB02003A1D09.f2	✓*	–	✓
SB03013A1G06.f1	✓	–	–
SB03014A1B02.f1	✓	–	–
SB02013B2G03.f1	✓	–	–
SB02018B1D11.f1	✓	–	–
SB03047A1F11.f1	✓	–	–
<i>adult song vs. adult silent</i>			
SB02016B1B10.f1	✓	✓	–
SB02035B2E10.f1	✓	–	–
SB03001A1D09.f1	✓	✓	✓
SB02018A1F12.f1	✓	✓	–
SB03019A1F04.f1	✓	✓	–
SB03001B2H03.f1	✓	–	–
SB03033A2C05.f1	–	✓	–
SB03026B1F05.f1	✓	–	–
SB02019A1D12.f1	✓	✓ [†]	–
<i>P20 song vs. P20 silent</i>			
SB03042B2D08.f1	✓	–	–
SB03028A1A09.f1	–	–	✓
SB02039A2E12.f1	✓	–	–
SB02045A2F12.f1	✓	–	–
SB03013B1E10.f1	✓	–	–
SB03038B2A04.f1	✓	✓	✓
SB03033A1D10.f1	✓	–	–
SB03026B1B08.f1	–	–	–
SB03002A1A11.f1	✓	–	–
SB03009B1A05.f1	–	–	–
SB02019B2E04.f1	✓	–	–

ESTIMA identifier	age	song	sex
<i>interaction</i>			
SB02033B1G04.f1	–	–	–
SB02025B1A09.f1	√	–	–
SB03039B1B11.f1	–	–	–
SB010006000A04	√	√	√
SB03047B2G09.f1	√	–	–
SB02034A1B02.f1	–	–	–
SB02021A1C06.f1	–	–	–
SB02016B1B10.f1	√	–	–
SB02020A2A08.f1	√	–	–
SB02027B1A05.f1	√	–	–
SB02012A1E04.f1	–	–	√
SB02028A1C05.f1	√	–	√
SB03022B1B08.f1	–	–	–