Supporting Information

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SI Text

Behavioral Procedures

Subjects. All experiments were performed under protocols approved by the University of Illinois Laboratory Animal Care Advisory Committee. Adult male zebra finches (at least 90 days old) were bred and raised in an aviary at the Beckman Institute animal facility. They were housed 3 in a cage (35.6 cm long × 40.6 cm wide × 45.7 cm high) before their use. All birds were kept on a 12-h light/dark cycle. During experiments, a single bird was isolated in a smaller cage (30.5 cm long × 22.9 cm wide × 40.6 cm high) placed inside a sound-attenuation chamber (Tracor Instruments). An incandescent lamp (40 W) was placed in the chamber to maintain the light/dark cycle of the animal quarters. There were 3 treatment conditions in the main microarray and proteomic experiments ($n = 36$ birds): novel, habituated, and silence. The same set of 4 sound-attenuation chambers was used for all treatments, in a sequence of consecutive runs over 6 weeks. Each run (4 birds) involved 2 of the treatment conditions, with 2 birds in each treatment; 1 bird in each treatment was used for the microarray analysis and the other was used for the proteomic analysis. All aspects of the treatments were balanced (by treatment group, chamber used, and position in the sequence over the 6 weeks). For subsequent validation experiments, a fourth group (trained only) was added. All birds stayed in chambers for 3 days and 2 nights. At ≈12:00 p.m. on day 0, birds were put into chambers. On day 1, between 12:00 p.m. and 3:00 p.m., the song stimuli were played back to habituated and trained-only groups through a speaker (model FE103, Radio Shack) located in the chamber. The average sound intensity was adjusted to 70 dB via a sound pressure meter (model 33–2055, Radio Shack). At 12:00 p.m. on day 2, the same acoustic stimuli were played back to both novel and habituated groups for 30 min. The silence group did not get any acoustic stimuli during the 3 days. Half of the birds in each group were decapitated immediately after song offset (30 min after song onset), and the auditory lobules (ALs) were dissected out as described in a previous study (see *Notes on AL Dissection*, below). The other half were decapitated 60 min after song offset (90 min since song onset), and the ALs were also dissected out. All samples were quickly frozen on dry ice and stored at −80 °C until being processed.

Auditory Stimuli. The song stimulus used in this study ("zf101") was recorded some years ago from a bird in a different aviary (1) and has been used as a standard reference stimulus in many studies since (1–6). Stimulus presentation was controlled by computer using the Syrinx program developed by John Burt. We used a single song stimulus design in which a single iteration of 1 song bout (≈2 sec in duration) was repeated once every 10 sec for the duration of the playback period (Fig. 1).

Behavioral Data Analysis. We recorded and analyzed the birds' behavioral responses to song during song playbacks as described before (2, 4). Audio recordings were made with a microphone (model BG 4.1, Shure Brothers) placed inside the chamber; and video recordings were made with a small black-and-white CCD camera (model CVC-120R, Pro-Video by CSI/SPECO) also placed inside the chamber. The duration from song onset to birds' first major movement (groom, hop, fly, call, sing, eat, drink, not including head movement) was defined as "response latency" or "listening duration." Grooming was the most common first movement. A square-root transformation was used to convert the data into normal distribution for Student's t test. In addition to recording responses during song playback, we also recorded the 1 h of spontaneous behavior before the onset of the song. We did not notice any group differences in the amount of spontaneous singing behavior. For all birds, we explicitly counted the amount of singing behavior during the 30 min before euthanasia, as described (7). There were no significant group differences ($P = 0.259$ comparing all 5 treatment groups from both experiments).

Microarray Analysis

Microarray Hybridization. The zebra finch "20K" cDNA microarray and the procedures for its use under the Songbird Neurogenomics Initiative were described in detail previously (8). In brief, the array contains 20,160 addresses, with 19,213 DNA spots representing 18,246 different cDNA probes, which in turn represent 17,582 "nonredundant" targets in the most recent EST assembly and are estimated to represent the products of 11,500–15,000 genes. Arrays were printed at the W. M. Keck Center for Comparative & Functional Genomics, University of Illinois (Urbana, IL). Total RNA was prepared with an RNAqueous-Micro kit (Ambion; average yield = 4 μg/AL pair). A total of 500 ng RNA were amplified using the Low RNA Input Fluorescent Linear Amplification kit (Agilent; average yield = 25 μg). The resulting aRNA was reverse transcribed using an indirect aminoallyl incorporation protocol and labeled with either Cy3 or Cy5 dyes (GE Healthcare). Dye labeling was balanced by group (i.e., half the birds of each group were labeled with Cy3 and the other half with Cy5). A total of 18 arrays were used in the primary experiment ($n = 6$ birds for each of the 3 treatment groups). A 2-channel reference design was used so that each array was hybridized with 1 individual bird sample in 1 fluorescence channel, along with a common reference sample in the other channel. The common reference sample was amplified from the combined total RNA from the whole brains of 30 extra birds (15 males and 15 females) (8). Thus each array represents 1 of 6 biological replicate measurements of 1 experimental condition. In a second follow-up experiment, the same procedure was used for separate hybridizations of 12 additional arrays representing additional birds in the habituated and trained-only groups ($n = 6$ in each group). Slides were hybridized overnight at 42 °C, washed, and scanned using an Axon GenePix 4000B microarray scanner. All slide images were analyzed using GenePix Pro 6.0 software. Analyzed slide images were manually edited and aberrant spots were flagged for exclusion in downstream analysis.

Statistical Analysis. Before analysis, the fluorescence intensities were edited by removing automatic and manual flagged spots that did not surpass minimum quality thresholds. "Background" was defined independently for each spot from its surround and subtracted from each spot value. The data were $\log_2$ transformed and loess normalized within each array using bioconductor R-routines (www.bioconductor.org) (9). Normalized data were imported into SAS (SAS Institute) for a mixed linear model of analysis of variance (SAS proc mixed) with 2 steps (10). The first model normalized data with respect to the global effects of array and dye and their interaction effects. The normalization model was $\log_2(y_{ijkl}) = m + A_i + D_j + (AD)_{ij} + e_{ijkl}$, where m is the sample mean, A_i is the effect of the ith array, D_j is the effect of the jth dye, $(AD)_{ij}$ is the interaction effects of the ith array and

the jth dye, and e_{ijkl} is the stochastic error. The array is treated as a random effect. The residuals were subsequently used as input for the second model, a series of 19,213 spot-specific models, which removed spot-specific biases and calculated least-squares mean estimates of treatment effects for each gene under each treatment. The models at the second step were $r_{ijkl} = m + A_i + D_j + T_{ij} + e_{ijkl}$. T_{ij} is the treatment effect, which is what we are interested in. In this step, the array was also treated as a random effect. Estimated least-squares means were calculated for fixed-effects dye and treatment conditions. The resulting difference between least-squares estimates for 2 different treatments is analogous to a $\log_2$-transformed ratio of gene expression between those 2 treatments. Treatment differences were assessed in 2 ways. ANOVA was used to compare the means of all 3 treatments (in the first experiment) or 2 treatments (second follow-up experiment). Student's t tests were used to compare each song-stimulated group to the silence group to derive the specific regulated gene lists. A false discovery rate (FDR) correction was applied to account for errors because of multiple tests (11). All FDR-adjusted P values that fell below 0.05 were considered statistically significant.

We do not formally report explicit statistical comparisons between the groups in the first experiment (novel, habituation, and silence) and the groups in the second (habituation vs. trained), because these experiments were run at different times on different "batches" of birds and microarray analyses are notoriously sensitive to batch effects and other uncontrolled environmental variables. However, we did execute a mixed-model ANOVA combining treatment groups from both experiments (renormalizing all of the data together in the first stage of the analysis), and the results support the conclusions drawn in the main manuscript. In this combined analysis we found no differences between trained-only and habituated groups at an FDR $P < 0.05$, but several hundred each for the trained–silence and habituated–silence comparisons. Increasing the FDR threshold to 30% yielded just 3 significant trained–habituated spots (2 for ZENK and 1 for NR4A3)—and these are slightly higher in the habituated group versus trained only, consistent with a small residual effect of the "familiar" song presentation to the habituated birds. At this 30% FDR threshold in the combined analysis there are 2,903 significant differences between trained and silence and 2,298 between habituated and silence.

Principal Components Analysis (PCA). PCA was used to group birds according to the similarity of their gene expression profiles (12). The residuals calculated from the global normalization in the previous step were normalized against the reference sample. Thus, on each array, 1 spot has a number that indicates the relative quantity of the RNA in a sample bird comparing to the common reference. The normalized residuals were loaded into TIGR Multiexperiment Viewer (MEV) (13) for PCA analysis.

2.4 Gene Ontology (GO) Analysis. We performed 2 Student's t tests: novel against silence group and habituated against silence group. Totals of 3,140 probes in the habituated group and 616 probes in the novel group were significantly altered (FDR adjusted P value <0.05). From these 2 main gene lists, 4 subgroups were derived according to the directions of change relative to the silence group: novel-up (153 probes), novel-down (463 probes), habituated-up (1,533 probes), and habituated-down (1,607 probes). For this experiment we used the GO annotations imported from the BLAST against Gga Unigene build 33 database at the NCBI, which were described previously (8). Totals of 73.8% of novel-up probes, 43.4% of novel-down probes, 68.8% of habituated-up probes, and 72.2% of habituated-down probes are annotated. The 6 annotated probe lists (2 main lists and 4 subgroups) were loaded onto Onto-Express software for identifying functional categories that are overrepresented in each list (14). The test was based on the hypergeometric distribution (15). All GO terms with an FDR-adjusted P value supporting information (*SI*) Fig. S3. Complete descriptions of all nodes in the tree are given in Table S2. Significant FDR-adjusted P values (<0.05) for all GO terms across all regulation groups are given in Table 2 (main text). Probes that contributed to each significant term are identified (by Gga Unigene ID) in Table S3.

DIGE/MS/MS Analysis

Sample Extraction and Preparation. The NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) was used for protein extraction from dissected frozen AL samples. To each of the stock buffers (CERI and NER) we added added 0.5 μM NaF, 0.5 μM Na3VO4, 5 μM microcystin, 20 μM cyclosporin, and 4 μL/mL protease inhibitor mixture (Sigma-Aldrich). One AL was homogenized by Pellet Pestle (Kontes) in 100 μL CERI solution. Supernatant was collected by centrifuging the homogenates at the highest speed (16,100 $\times$ g), using an Eppendorf centrifuge 5415D for 5 min. The pellet was resuspended in 50 μL NER buffer and the suspension was incubated on ice with occasional vortexing for 40 min. The nuclear fraction was collected as the supernatant after 10 min of centrifugation at the highest speed and combined with the cytosolic fraction. Concentration was determined by BCA protein concentration assay (Pierce). The aliquot samples were precipitated using a ReadyPrep 2-D Cleanup Kit (Bio-Rad) and then dissolved in 20 mM Tris-HCl (pH 8.5), 8 M urea, 4% CHAPS, and 5 mM magnesium acetate.

DIGE Analysis. In this experiment, samples were compared in pairs (each sample from 1 bird), by labeling them with different fluors and electrophoresing them on the same gel along with a reference sample labeled with a third fluor, as follows. A portion (25 μg) of each protein sample was labeled with 200 pmol of either Cy3 or Cy5 (GE Healthcare). A pooled reference sample (12.5 μg of each sample) was labeled with 200 pmol of Cy2. Dye labeling was balanced by group (i.e., half the birds of each group were labeled with Cy3 and the other half with Cy5) to account for dye labeling preference effects. Seventy-five micrograms of Cy2-, Cy3-, and Cy5-labeled proteins were mixed and separated by isoelectric focusing using 24-cm linear IPG DryStrips, pH 3–10 (Amersham Biosciences) in the first dimension. The strips were equilibrated in SDS sample buffer and then were subsequently separated in the second dimension according to molecular weight using SDS–polyacrylamide gels (12%, Ettan DALT Twelve apparatus; Amersham Biosciences). A total of 9 gels were used to compare the 18 samples, producing a total of 27 gel images (representative image in Fig. S2). Gels were scanned at appropriate wavelengths for Cy2, Cy3, and Cy5 fluorescence, using a Typhoon 9400 Variable Mode Imager (GE Healthcare). Individual gel images were merged, analyzed, and quantified using DeCyder Batch Processor and Biological Variation Analysis (BVA) software V6.5 (Amersham Biosciences). An independent 2-stage mixed-model analysis of the data was also performed, following the approach used for the microarrays (which allows for modeling of gel variation and dye effects). Both approaches generated qualitatively similar results. The results of the DeCyder analysis are presented in this report.

Mass Spectrometry. For 51 of the regulated spots, we were able to identify and pick them confidently from a SYPRO Ruby-stained (Invitrogen) preparative gel loaded with 200 μg of unlabeled protein. The gel was run under conditions matched to the 2-dimensional (2D)-DIGE separation (above) and the spots were digested with trypsin (Genotech) and prepped for LC/MS analysis using the Ettan Spot Handling Workstation (GE Healthcare). The resulting peptides were analyzed by LC-MS/

MS, using a QTof API-US or a QTof-II (Waters). The spectra were analyzed using Proteinlynx (Waters) and MASCOT (Matrix Science) database search engines directed to Chordata sequences, using a minimum MASCOT score of 50 as the threshold for significance.

Validation Using in Situ Hybridization

Rationale and Interpretation. Other studies under the Songbird Neurogenomics Initiative have consistently demonstrated a high correlation between microarray measurements obtained under this program and independent measurements of individual probes based on in situ hybridization or quantitative PCR (8, 16–18) (and other manuscripts in preparation). In the present study, our focus was on profiling the overall patterns of transcription on the scale of gene populations (e.g., principal components and Gene Ontology analysis). Hence our interest was in assessing the potential for any large systematic hybridization errors that might affect large numbers of probes and determining whether the birds used in the microarray experiments were representative of these treatment conditions (i.e., testing for errors in biological sampling). To evaluate the potential impact of these 2 types of error in our experiments, we developed 2 sets of in situ hybridization experiments.

In the first validation experiment, we focused on excluding the possibility of major systematic experimental errors. Eighteen probes were randomly chosen from novel-up ($n = 3$), novel-down ($n = 5$), habituated-up ($n = 3$), and habituated-down ($n = 7$) lists. For each probe, 2 treatment conditions were tested by in situ hybridization ($n = 2$ birds per treatment, not the same individuals as used in the original microarray experiment): silence and the song playback condition (novel or habituated) that resulted in a significant difference from silence in the array data. We used the same digoxigenin hybridization protocol as in previous studies (19, 20) and defined "target cells" as pixel groups with area passing a cell size threshold. For each target cell, the "median intensity of all pixels within the cell" times the "area" was defined as target cell intensity. The sum of all target cell intensities within the auditory lobule [fields caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) in the sectioned tissue] was normalized against the size of NCM/CMM and used for statistical analysis. To assess the correlation of microarray data and in situ data, a scatter plot of all 18 probes is shown in Fig. S1*A*, demonstrating a good overall correlation between our microarray and in situ measurements. In other words, both hybridization techniques generally report a similar magnitude and direction of change relative to the silence condition for a sample of probes. From this we can exclude the influence of major systematic errors in our hybridization methods.

In the second validation experiment, to exclude biological sampling error, we performed in situ validations for each of 3 probes, using all 4 treatment groups (silence, novel, habituated, and trained), and with 3 biological replicates in each group. Data are presented in Fig. S1*B* for 1 of the probes (NDUFA12: a NADH dehydrogenase, belonging to mitochondrial complex I). Consistent with the microarray data, this transcript does not respond to novel song but is downregulated in the habituated song group (33% by in situ hybridization versus 42% by microarray). There is also no significant difference between the habituated and the trained-only groups, consistent with the second microarray experiment presented at the end of *Results* (main text). Similar confirmation was obtained for a second probe, RPL15 encoding a ribosome protein (not shown). In the third case (a probe for T-type calcium channel) we did not measure a statistically significant difference across the 4 groups by in situ hybridization with this biological sample size ($n = 3$), although on the microarray ($n = 6$) it was downregulated in the novel group. Additionally, other experiments using in situ hybridization, RT-PCR, and a separate microarray (Agilent oligonucleotides) carried out independently confirmed the pattern of regulation for ZENK and NR4A3 (novel > habituated > silence) in additional samples of birds. Taken together, these results confirm that the general distinctions between treatment groups in the microarray experiment are observed independently in separate collections of animals using different hybridization techniques. From this we can exclude a major error in biological sampling.

In combination, these various results support the broad accuracy of our descriptions of discrete gene expression profiles associated with our different treatment conditions. We acknowledge the caveats that accompany any microarray experiment; i.e., all techniques have false discovery rates, the concordance between any 2 techniques is never perfect, and error rates for any single probe may diverge from the average error rate for a population.

In Situ Hybridization Methods. The whole brain was collected in O.C.T. compound and stored at −80 °C. Later, 10-μm parasagittal slices were prepared in a cryostat and mounted on aminopropyltriethoxysilane-treated slides. After fixation in 3% paraformaldehyde and dehydration, the slides were stored at −80 °C until used for in situ hybridization. The methods for synthesis of digoxigenin-labeled riboprobes and in situ hybridization to assess gene expression are the same as reported previously (19, 20). Probes were made from plasmids purified from stocks prepared at the time of microarray printing (8). Hybridization signals were calculated as the sum of target pixel intensities in the auditory lobule (NCM/CMM), using the Microcomputer Controlled Imaging Device system (MCID) (Imaging Research). An observer blind to treatment conditions set up an intensity threshold for each brain section. All pixels with intensity higher than the threshold were chosen. The groups of pixels with area passing a cell size threshold were defined as target cells. Mean target cell size in the AL was calculated for each section, using the grain counting function of the MCID software. For each target cell, the median intensity of all pixels within the cell times the area of the cell was defined as target cell intensity. The sum of all target cell intensities within the AL was normalized against the area of the AL and used for statistical analysis.

Validation of DIGE/MS/MS Results by Immunoblot

Rationale and Interpretation. Independent validation of large-scale DIGE expression data is problematic. Two-dimensional immunoblots can be used but this method requires comparisons across different electrophoretic gels and blots, introducing sources of technical variation that DIGE is specifically designed to remove. Immunoblot experiments also require validated antibodies to the target proteins. With these limitations and caveats in mind, we attempted 2D immunoblots for 3 of the target proteins identified in Table 3: mitochondria ATPase alpha chain (ATP5A), NADH:ubiquinone oxidoreductase (NDUFS1), and peroxiredoxin III (PRXIII). Commercial antibodies that would potentially cross-react with zebra finch sequences were identified on the basis of available information about the antibody epitopes and predicted zebra finch peptide sequences [goat anti-human ATP5A (sc-49162), goat anti-human NDUFS1 (sc-50131), and mouse anti-human PRXIII (sc-59661), all from Santa Cruz Biotechnology]. The antibody to human PRXIII failed to produce a detectable signal against zebra finch brain extract, but antibodies to ATP5A and NDUFS1 both detected proteins of the expected size; the results for ATP5A are presented in Fig. S4 and the results for NDUFS1 are qualitatively similar. The 2D immunoblots for each antibody revealed a series of proteins with similar molecular weights but different isoelectric points (Fig. S4*B*). Quantification of one-dimensional (1D) immunoblots (where all of the isoforms are collapsed into a single band)

revealed a similar trend for each protein (Fig. S4*C*) to that indicated in the 2D-DIGE analysis (Table 3), but with less statistical power insofar as the DIGE analysis was focused on a single isoform.

Immunoblot Methods. Proteins were extracted as described above and then were separated by either 2D or 1D gel. For 1D analysis, the loading amount was 10 μg; for 2D gel analysis, it was 100 μg. Protein spots were transferred to polyvinylidene difluoride membranes (Invitrogen). For 1D westerns, the membranes were dyed by SYPRO Ruby (Invitrogen) to assess loading before immunoblotting; the images were collected using the ChemiImager System (Alpha Innotech). Next, the membranes were blocked overnight with 5% nonfat dry milk in TBST (20 mM Tris, pH 7.6; 137 mM NaCl; 0.1% Tween-20). The blot was probed with primary antibody (using the conditions suggested by the antibody supplier) for 1 h at room temperature. After being washed 3 times (10 min per wash) with TBST, the membrane was subsequently incubated in HRP-conjugated rabbit anti-goat IgG or HRP-conjugated goat anti-mouse IgG (Cell Signaling Technology) for 1 h at room temperature. Protein spots or bands were visualized using enhanced chemiluminescence (ECL Plus reagent, GE Healthcare) and images were collected via the ChemiImager System. The images were analyzed using AlphaEaseFC software (Alpha Innotech).

Mitochondrial Activity Analysis

Sample Preparation. Extracts were prepared 60 min after test song offset (habituated and novel groups, with trained-only and silence groups collected in parallel) as in the proteomic analysis, from the auditory lobule and also from the anterior forebrain as a nonresponsive control region. The anterior forebrain dissection was made by collecting the tissue anterior to a transverse cut made ≈2.5 mm from the anterior tip of the forebrain. Brain tissue was homogenized and mitochondria were isolated as described in *Current Protocols in Cell Biology* (18.5.11, supplement 19), and the pelleted mitochondria were resuspended to a final volume of 33 μL in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4). Total protein concentration was calculated by measuring absorbance at 280 nm on a SpectraMax M2 microplate reader (Molecular Devices) and used to normalize activity between samples.

Complex I Activity Assay. Adenosine 5′-triphosphate (ATP) levels were measured in 11 μL resuspended mitochondria from each sample pretreated with 1% DMSO versus 11 μL mitochondria pretreated with 100 nM of rotenone, a potent inhibitor of complex I, prepared in 1% DMSO for 2 h using the ATPLite Luminescent Assay Kit (Perkin-Elmer) per manufacturer protocols. Luminescence was then determined using a SpectraMax M2 microplate reader. Complex I activity was then calculated by subtracting the ATP levels (RLU) measured for the rotenone-treated samples from those measured for the corresponding nontreated group. Background levels were also subtracted from each group and final values normalized according to protein concentration.

Complex IV Activity Assay. Complex IV activity was measured in 11 μL resuspended mitochondria from each group, using the Cytochrome *c* Oxidase Assay kit (Sigma) according to manufacturer procedures. Absorbance at 550 nm was measured every 10 s at 25 °C for 1 min via a SpectraMax M2 microplate reader with background values subtracted from each reading. The rate of substrate oxidation (units per milliliter) was then calculated as follows: (ΔA_{550}/min × dilution factor × total reaction volume)/(sample volume × 21.84), with 21.84 representing the difference in extinction coefficients ($\Delta\varepsilon^{mM}$) between reduced and oxidized ferrocytochrome *c* substrate at 550 nm. Values were normalized according to mitochondrial protein concentration and 11 μL of purified cytochrome *c* at a concentration equivalent to the average protein concentration measured for 11 μL resuspended mitochondria were used as a positive control.

Notes on AL Dissection

The term "auditory lobule" was introduced in 2004 (5) as an operational term to describe a piece of tissue obtained following a particular dissection technique. Here we repeat the description of this technique and the rationale for continuing to use this term. Both genomic and electrophysiological responses to song are most evident in the most caudal and medial portions of the nidopallium and mesopallium (under the old nomenclature: NCM and CMHV, see ref. 21). Although this song-responsive subregion has well-defined dorsal, ventral, anterior, posterior, and medial boundaries, it does not have a well-defined lateral border, and it surrounds the primary auditory thalamorecipient area of the telencephalon (field L2a) in which genomic responses to song have not been observed (see Fig. S5) (22). For the biochemical analyses performed here, we needed to be able to dissect this song-responsive region from unstained, unfixed tissue, and the dissection needed to be as rapid and reproducible as possible. In pilot studies, we found that in fresh zebra finch brains bisected down the midline, we could readily observe by eye the ventricular boundaries of the song-responsive area (see ref. 23) and that a single lobe-like unit (wet weight ≈1 mg) could be removed, seemingly intact, by manual dissection, using a fine scalpel to peal away overlying hippocampus and sever connections to more medial tissue at a depth of ≈1–2 mm. Using quantitative RT-PCR, we confirmed that increases in ZENK mRNA of appropriate magnitude could be detected in RNA extracts prepared from this tissue, in birds exposed to song compared to quiet controls (2). Because this dissection is defined operationally and contains partial elements of several formal anatomical regions, we refer to it here by the term AL. The AL dissection is most enriched for the primary ZENK-responsive portion of the NCM, but it also contains some amount of ZENK-responsive caudomedial mesopallium and nonresponsive field L2a. A schematic representation of the AL is shown in Fig. S5.

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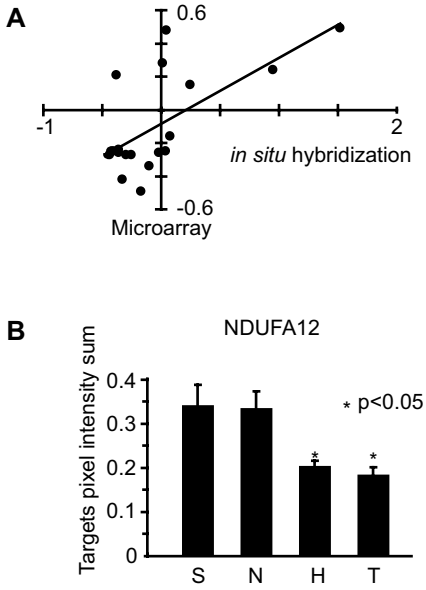

Fig. S1. Correlation of microarray and in situ hybridization results. (*A*) To test the validity of general conclusions based on our microarray data, 18 probes significantly regulated (some up and some down) in the novel or habituated groups were randomly chosen and used for in situ hybridization against brain sections from additional birds collected after the 3 treatments (see *SI Text, Validation Using in Situ Hybridization*). Each dot represents mean signal for 1 probe as measured by in situ hybridization (2 birds) plotted on the *x* axis and by the original microarray experiment on the *y* axis. The data are fitted by simple linear regression with $r = 0.65$ ($P < 0.005$). Thus there is a good overall correlation between these 2 types of measurements in our large dataset. (*B*) The NDUFA12 probe was chosen for detailed in situ analysis. Besides silence (S), novel (N), and habituated (H) groups, a trained-only group ($n = 3$ for each group) was also included as described at the end of *Results* (main text). The transcription of NDUFA12 is significantly downregulated in both the habituated and the trained-only groups, consistent with both the primary and the secondary microarray experiments.

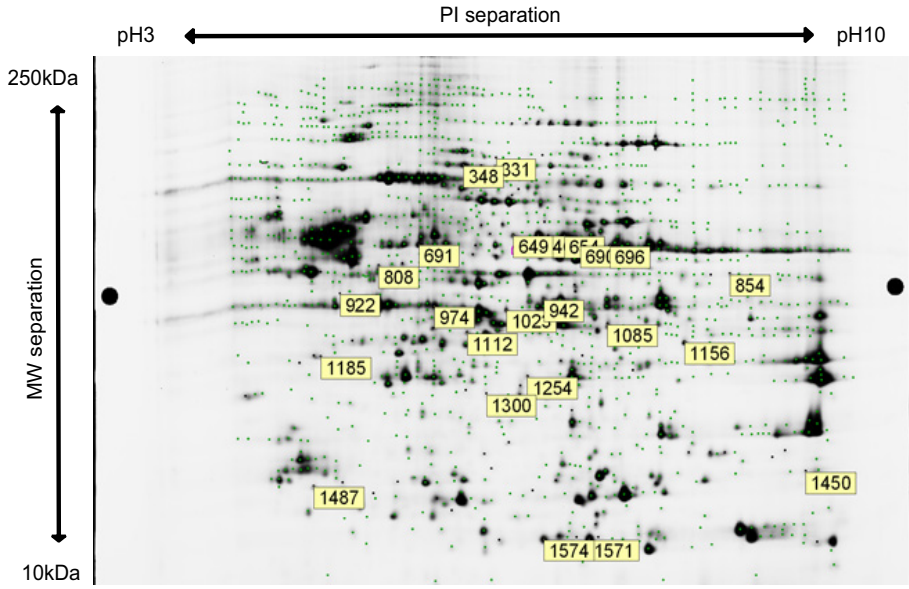

Fig. S2. Representative DIGE gel image from this experiment. Protein spots excised for mass spectral analysis are indicated. The numbers are assigned by BVA software.

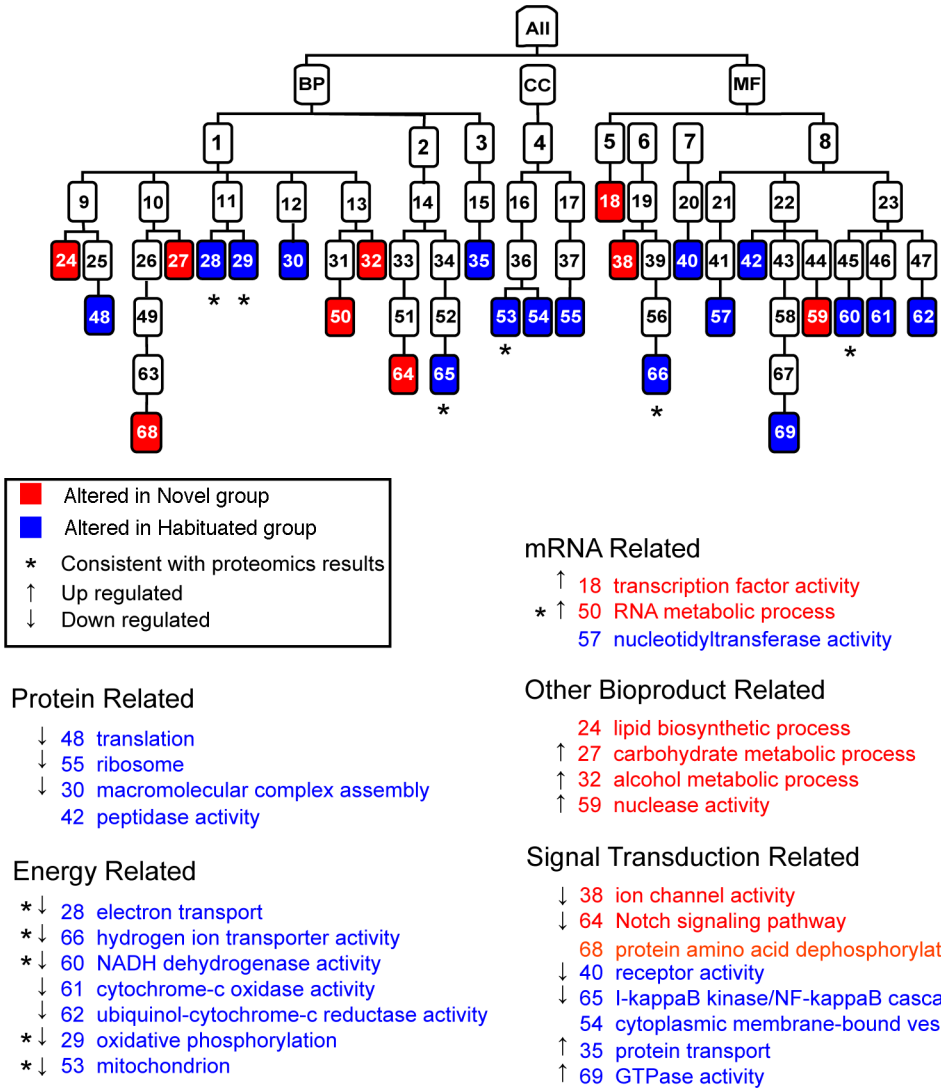

Fig. S3. Directed acyclic graph showing relationships of Gene Ontology (GO) terms overrepresented (FDR $P < 0.05$) in the gene lists for the major regulation patterns defined in Fig. 3*A*. In each box, the number is an index value that is used again in Table 2 (statistical summary), Table S2 (GO hierarchy), and Table S3 (genes that contribute to each term). Red, GO terms overrepresented in the novel list; blue, overrepresentation in the habituated list. Arrows show direction of regulation relative to the silence group; lack of an arrow indicates the term was significant only in the list combining both up- and downregulated genes. Uncolored terms are included to show the position of each significant term in the ontology. BP, biological process; CC, cellular component; MF, molecular function. Asterisks (*) indicate that proteins in the same functional group were also detected and changed in the DIGE/MS analysis (Table 3).

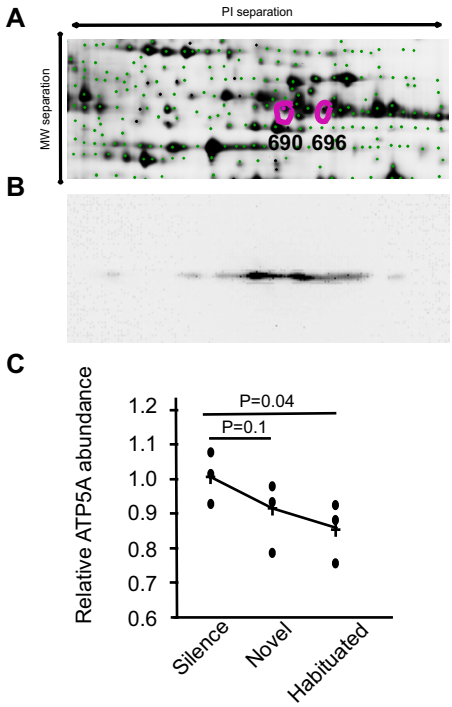

Fig. S4. Immunoblot validates ATP5A regulation pattern detected by DIGE. (*A*) The image is an inset of a representative DIGE gel (same orientation as in Fig. S2) showing positions of 2 spots (circled) identified by MS sequencing as ATP5A; statistical analysis of the DIGE data indicated both spots were downregulated in both song-stimulated groups. Note that both spots migrate with similar molecular weight but different isoelectric points, suggesting alternative posttranslational modifications. (*B*) 2D immunoblot probed with ATP5A antibody revealed a series of proteins with similar molecular weights but different isoelectric points. (*C*) Scatterplot represents 1D immunoblot results. Each spot represents 1 individual bird. All intensities are normalized to the mean intensity of the silence group. The *P* value for each Student's *t* test is labeled. ATP5A protein is downregulated in both novel ($P = 0.1$) and habituated ($P = 0.04$) groups, which is consistent with DIGE results.

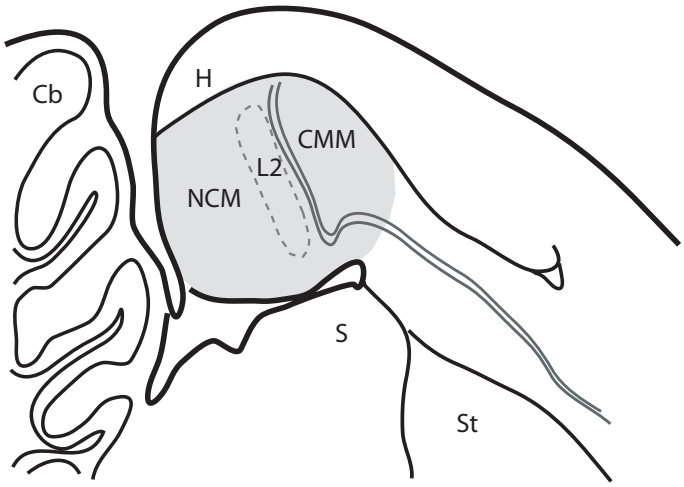

Fig. S5. Schematic representation of brain regions included in the ''auditory lobule'' dissection, viewing a parasagittal section along the midline, anterior to the right, dorsal up. Shading represents the area removed as AL following the dissection procedure described and includes 3 major auditory regions: NCM, caudomedial nidopallium; L2, field L2; and CMM, caudomedial mesopallium. Other labeled landmarks: Cb, cerebellum; H, hippocampus; S, septum; and St, striatum.

Other Supporting Information Files

Table S1
Table S2
Table S3
Table S4
Table S5
Table S6