

Profiling of experience-regulated proteins in the songbird auditory forebrain using quantitative proteomics

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Abstract

Auditory and perceptual processing of songs are required for a number of behaviors in songbirds such as vocal learning, territorial defense, mate selection and individual recognition. These neural processes are accompanied by increased expression of a few transcription factors, particularly in the caudomedial nidopallium (NCM), an auditory forebrain area believed to play a key role in auditory learning and song discrimination. However, these molecular changes are presumably part of a larger, yet uncharacterized, protein regulatory network. In order to gain further insight into this network, we performed two-dimensional differential in-gel expression (2D-DIGE) experiments, extensive protein quantification analyses, and tandem mass spectrometry in the NCM of adult songbirds hearing novel songs. A subset of proteins was selected for immunocytochemistry in NCM sections to confirm the 2D-DIGE findings and to provide additional quantitative and anatomical information. Using these methodologies, we found that stimulation of freely behaving birds with conspecific songs did not significantly impact the NCM proteome 5 min after stimulus onset. However, following 1 and 3 h of stimulation, a significant number of proteins were consistently regulated in NCM. These proteins spanned a range of functional categories that included metabolic enzymes, cytoskeletal molecules, and proteins involved in neurotransmitter secretion and calcium binding. Our findings suggest that auditory processing of vocal communication signals in freely behaving songbirds triggers a cascade of protein regulatory events that are dynamically regulated through activity-dependent changes in calcium levels.

Introduction

Processing of auditory information is critical to the social biology of songbirds. Appropriate perception of auditory communication signals (songs) is required for territorial defense, mate selection and individual recognition (Nowicki & Searcy, 2004; Zeigler & Marler, 2004). In addition, songbirds have the rare trait of vocal learning, which is the ability to learn vocalizations through imitation, rather than through instinct (Jarvis, 2004). Vocal learning behavior is a requirement for the acquisition of spoken language in humans and involves the processing and encoding of auditory information associated with a tutor and the individual's own vocalizations (Doupe & Kuhl, 1999; Jarvis, 2004).

Given that auditory processing is required for many behaviors in songbirds, a significant effort in the field has been put forth towards understanding the functional organization of auditory areas, and how these circuits change as a function of auditory experience. One auditory area in the songbird telencephalon, the caudomedial nidopallium (NCM), has received substantial attention in this regard because it is involved in auditory processing of birdsong and is one of the final stations of the ascending auditory pathway (Fig. 1A). Anatomical connectivity and gene expression findings have been used to suggest that NCM is analogous to parts of the mammalian auditory

cortex (Mello *et al.*, 1998, 2004; Mello & Pinaud, 2006). Auditory input to NCM drives vigorous electrophysiological responses (Chew *et al.*, 1995, 1996; Terleph *et al.*, 2006) and the expression of activity-dependent genes, in particular the immediate early genes *zenk*, *c-jun*, *c-fos* and *arc* (Mello *et al.*, 1992, 2004; Nastiuk *et al.*, 1994; Mello & Ribeiro, 1998; Velho *et al.*, 2005; Mello & Pinaud, 2006). Recent studies have also revealed post-translational changes of signal transduction proteins after auditory stimulation upstream of immediate early gene activation, such as phosphorylation of the extracellular-signal regulated kinase (ERK) (Cheng & Clayton, 2004). Electrophysiological and gene expression studies have shown that NCM cells respond more strongly to same-species songs than to different-species songs and artificial stimuli (Mello *et al.*, 1992; Chew *et al.*, 1996). Furthermore, NCM responds most robustly when the species-specific songs are novel; these responses decrease as songs become familiar to the bird (Chew *et al.*, 1995). Finally, recent gene expression, electrophysiological and lesion experiments suggest that NCM is a possible site where auditory memories required for vocal learning or song discrimination are formed and/or stored (Bolhuis *et al.*, 2000; Terpstra *et al.*, 2004; Bolhuis & Gahr, 2006; Phan *et al.*, 2006; Gobes & Bolhuis, 2007). The underlying molecular substrate for these processes in NCM presumably involves large-scale proteomic changes; however, the regulation of only a few immediate early genes as a result of song stimulation is currently known.

In the present study we aimed at understanding the dynamic regulation of proteins that takes place in NCM as a result of two

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variables: auditory experience and time. We approached this question by identifying proteins that are up- or down-regulated in NCM after stimulating adult songbirds with conspecific songs, for different lengths of time. We assayed the levels and post-translational modifications [revealed by isoelectric point (pI) shifts due to charge change] of high-abundance proteins in NCM by two-dimensional differential in-gel expression (2D-DIGE)-based proteomics, coupled to extensive data analyses, protein quantification and identification by mass spectrometry. Confirmation of the differential regulation of selected proteins was performed with immunocytochemistry (ICC) and Western blots. Our results suggest that auditory experience triggers extensive changes in the expression of specific proteins in NCM. These changes may underlie experience-dependent neuronal modifications that provide the basis of auditory discrimination and perhaps long-term changes for the formation of auditory memories required for vocal learning. To the best of our knowledge, the results presented here provide the first quantitative and systematic proteomic screening conducted in the brain of a songbird species.

Materials and methods

Animals and stimulation procedure

We used 39 adult female zebra finches (*Taeniopygia guttata*; $n = 27$ for 2D-DIGE experiments and $n = 12$ for ICC experiments). We chose females because they do not sing in response to hearing songs, which can be a confounding factor in males given that protein regulation can result from self-stimulation. Animals were individually placed overnight in sound attenuation chambers and randomly divided into four experimental groups: Group 1, controls ($n = 9$) without song auditory experience; Group 2, 5 min ($n = 6$) of continuous auditory stimulation, which consisted of a medley of three conspecific songs (each song ~ 1.5 s in duration, played every 30 s); Group 3, 1 h ($n = 6$) of continuous auditory stimulation (same stimulus set as 5-min group); Group 4, 3 h ($n = 6$) of continuous auditory stimulation (same stimulus set as 5-min group) (Fig. 1B). After stimulation, animals were killed and their brains were processed for 2D-DIGE. The same protocol was repeated for the ICC experiments ($n = 3$ per group). Animal protocols were approved by the Duke University IACUC committee and are in accordance with NIH guidelines.

For 2D-DIGE experiments, animals were killed by decapitation, brains were quickly dissected out of the skull, cut in the sagittal plane and mounted on a vibratome stage. A total of 2–3 parasagittal sections (300 μ m thick) starting from the midline were obtained from each hemisphere, placed in a Petri dish with phosphate-buffered saline (PBS) at room temperature (RT), and examined with the naked eye under a bright light against a black laboratory countertop. We then dissected the medio-lateral extent of NCM (0.1 to ~ 0.9 mm from midline). In cases where the first section was obtained more laterally, we excluded the third section to ensure that regions lateral to NCM were not collected. For the first section, NCM typically detached itself from the rest of the brain without manipulation. For subsequent sections, the thalamo-recipient field L2, which is easily identified by its lighter staining due to a higher fiber density, was used as a reference to define the anterior border of NCM (Fig. 1C). The ventricles were used to delineate the dorsal, ventral and caudal borders of NCM. A razorblade was used to isolate NCM from the remainder of the section. NCMs from all sections of an individual animal were pooled and placed into lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS [3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate], 20 mM Tris, pH 8.5, complemented with protease inhibitors). The total time between the animal being killed and NCM dissection never exceeded 10 min.

For ICC experiments, animals were anesthetized and perfused transcardially with 40 mL of PBS (0.1 M, pH 7.4) followed by 60 mL of an ice-cold 4% solution of paraformaldehyde in PBS. Brains were dissected out of the skull and placed overnight in a 30% sucrose solution. Tissue was then dried, placed in embedding medium (Tissue-Tek, Sakura) and fast-frozen in a dry-ice/ethanol bath. Sections of each brain were obtained in the parasagittal plane (30 μ m thick), thaw-mounted on Superfrost-Plus slides (Fisher Scientific), dried at RT overnight and frozen at -80 °C until further processing.

Sample isolation, protein extraction and quantification

Tissues were gently ground using plastic pestles in 1.5-mL Eppendorf vials, vortexed for 5 min at 4 °C in ice-cold water, and sonicated with the tip of the sonicator next to the vial in ice (Fisher model 100, power output 4), twice, for 30 s each. After sonication, the samples were vortexed for 5 min at RT and pelleted by centrifugation at 15 000g for 20 min at 4 °C. The supernatant (protein lysates) was cleaned with the 2D Cleanup kit (GE-Healthcare, Piscataway, NJ, USA) following the manufacturer's instructions. The final pellet was resuspended in focusing buffer (8 M urea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5) and total protein concentration was determined with the 2D Quant kit (GE-Healthcare).

Cy-dye labeling

The internal standard methodology was used to determine protein differential expression (see Fig. 1D) (Alban *et al.*, 2003; Friedman *et al.*, 2004; Osorio *et al.*, 2006): 120 μ g of total protein was taken from one sample of each time point (control, 5 min, 1 h and 3 h). Each of these samples was then labelled with 8 pmol/ μ g of Cy3 for control and Cy5 for 5 min, 1 h and 3 h, using a described protocol of the GE-Healthcare Kit (25800983). Additionally, 60 μ g of each of the four samples were pooled and labelled with 8 pmol/ μ g of Cy2 to create the internal standard (IS) sample. The proteins were labeled for 30 min on ice in the dark. The labeling reaction was stopped with 1 μ L of 10 mM lysine for 10 min on ice in the dark. The samples were then combined in pairs as shown in Fig. 1D (control vs. 5 min, control vs. 1 h, control vs. 3 h), along with the IS. Combining samples minimizes sample-to-sample variations and allows for pair-wise comparisons in a single gel with different labels, minimizing gel-to-gel variations. The resulting volume was diluted with an equal volume of 2 \times sample buffer [8 M urea, 4% CHAPS, 20 mg/mL DTT, 2% v/v IPG (Immobilized pH Gradients) Buffer 3-10 (GE-Healthcare)] and placed on ice for 15 min. Samples were then supplemented with rehydration buffer (8 M urea, 4% CHAPS, 2 mg/mL DTT, 1% v/v IPG Buffer 3-10) to give a final volume of 250 μ L.

2D gel electrophoresis and imaging

Labeled samples (250 μ L) were applied to IPG strips (13 cm, pI ranges 3–10, GE-Healthcare) on the rehydration tray (GE-Healthcare) and focused using an Ettan IPGphor II (GE-Healthcare) as follows: active rehydration at 30 V for 14 h, followed by isoelectric focusing for a total of 28 kV.h (step to 500 V for 1 h, step to 1000 V for 1 h, step to 8000 V to a total of 28 kV.h). After isoelectric focusing, disulfide bonds were reduced by placing the strips for 10 min in 20 mL equilibration buffer (6 M urea, 50 mM Tris, pH 8.8, 30% glycerol, 2% SDS) containing 5 mg/mL DTT. The strips were then incubated for 10 min in fresh equilibration buffer with 45 mg/mL iodoacetamide.

For the second dimension, the IPG strips were placed on 12% homogeneous polyacrylamide gels (4% stacking). Gels were cast using low-fluorescence glass plates (13 cm plates, GE-Healthcare) previously treated with bindsilane (GE-Healthcare). Each SDS-PAGE was run at 9 mA for 16 h in a HOEFER SE-600 system. Individual images of Cy2-, Cy3- and Cy5-labeled proteins of each gel were obtained using a Typhoon 9410 scanner (GE-Healthcare) with excitation/emission wavelengths of 480/530 nm for Cy2, 520/590 nm for Cy3 and 620/680 nm for Cy5. After imaging the gels were stained with colloidal Coomassie (Bio-Rad, Hercules, CA, USA).

DIGE analysis

2D-DIGE gels were analysed with DeCyder 2D 6.5 software (GE-Healthcare) to identify proteins displaying differential expression levels. Comparisons of abundance changes were examined across three of the six replicate experiments, and for pair-wise comparisons of individual Cy3–control and Cy5–time points (Fig. 1D). We chose the three highest quality gels of the six replicates at each time point, because this increased statistical power by eliminating artifacts. The gels that were not considered for further analyses either exhibited low resolution, more background or both. The high-quality gels were manually edited to remove dust particle signals and out of range spots. Two standard deviations from the mean volume ratios (95th percentile confidence) were used as threshold to determine levels of significance for a given set of samples. Statistical analysis and gel-to-gel comparison was performed with the Biological Variation Analysis (BVA) module (GE-Healthcare) (Fig. 1D). Clustering of the experimental groups (control, 5 min, 1 h and 3 h) was analysed by Principal Component Analysis (PCA) (Kim *et al.*, 2007) using a K-means algorithm included in the Extended Data Analysis (EDA) module of the Decyder 6.5 computer program (Fig. 1D).

Protein identification

Protein spots that showed significant changes in levels were processed for protein identification by tandem mass spectrometry at the UNC-Duke Michael Hooker Proteomics Center of the University of North Carolina, Chapel Hill. Briefly, gel plugs were submitted to in-gel digestion with modified trypsin, and MALDI-MS/MS (matrix-assisted laser desorption ionization-mass spectrometry) data were acquired using a 4700 Proteomics Analyser MALDI-TOF/TOF [matrix-assisted laser desorption ionization-time of flight; Applied Biosystems, Inc. (ABI), Framingham, MA, USA]. MS and MS/MS peak spectra were acquired and the 15 most intense peaks with a signal-to-noise ratio greater than 20 were selected automatically for MS/MS analysis. The peptide mass fingerprinting and sequence tag data from the TOF/TOF were evaluated with GPS Explorer scores (ABI). The MS and MS/MS spectra were used by the Mascot search engine to identify proteins from non-redundant databases (NCBI, MSDB) (Parker *et al.*, 2005). The MALDI data and protein annotations were also manually verified against the GenBank translated protein database. We included MS/MS and ion scores, per cent coverage and total number of peptides assigned to each protein in Tables 1 and 2.

Immunocytochemistry (ICC)

To confirm the experience-dependent regulation of proteins detected with the 2D-DIGE approach, we used ICC with commercially

available antibodies against selected proteins of interest. The ICC protocol has been previously described (Pinaud *et al.*, 2002b, 2004; 2006). Briefly, thaw-mounted sections on slides were placed for 30 min in PBS (0.1 M, pH 7.4) for hydration. The slides were then placed in a humid chamber and sequentially incubated in the following solutions: (i) blocking buffer (BB; 0.5% albumin and 0.3% Triton X-100 in 0.1 M PB) for 30 min at RT; (ii) primary antibody in BB overnight at 4 °C; (iii) appropriate species-specific fluorescent secondary antibodies coupled with either Alexa-488 or Alexa-594 (1 : 200 dilution in BB; Vector Laboratories, Burlingame, CA, USA), for 2 h at RT. The steps above were separated by three 10-min washes in PBS. Sections were finally washed for 30 min in PBS and coverslipped with Anti-Fade mounting medium (Invitrogen, Eugene, OR, USA). The primary antibodies were anti-calbindin 2, also called calretinin (raised in goat; 1 : 1000 dilution; Swant, Switzerland); anti-pyruvate kinase (raised in goat; 1 : 1000 dilution; Novus Biologicals, Littleton, CO, USA); anti-14-3-3 (raised in rabbit; 1 : 2000 dilution; GeneTex, San Antonio, TX, USA); anti-synapsin II (raised in mouse; 1 : 1000 dilution; BD Transduction Laboratories, San Jose, CA, USA); anti-egr-1 (raised in rabbit; 1 : 1000 dilution; Santa Cruz, Temecula, CA, USA); anti-CRMP-2 (raised in mouse; 1 : 500–1 : 2000; IBL, Japan). Omission of primary antibodies resulted in complete elimination of immunostained profiles.

Western blots

Specificity of primary antibodies not previously determined in songbird tissue was assessed by Western blot analysis with protein lysates isolated from either whole zebra finch brains or NCM dissections. Samples were isolated, extracted and quantified as described above for the 2D-DIGE experiments. For Western blots, a total of 10 µg of protein of each sample (each experimental group) was brought to a volume of 10 µL by adding Laemli loading sample buffer. Samples were denatured by boiling for 5 min in a water bath. Subsequently, proteins were fractionated in a 10% SDS-PAGE at 100 V for 1 h. Fractionated samples were transferred from gels onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) in a solution of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 40 V/160 mA for 18 h at 4 °C. Membranes were then incubated for 1 h in a BB consisting of 5% dry milk in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween 20), followed by a rinse in distilled water (1 min) and two incubations in TBS-T (10 min each) at room temperature. Subsequently, membranes were incubated, under agitation, in a solution containing the primary antibodies of interest (detailed above) in BB: anti-synapsin II (1 : 5000/90 min incubation); anti-14-3-3 (1 : 1000/overnight incubation); anti-PKM2 (1 : 1000/overnight incubation); anti-calretinin (1 : 1000/90 min incubation). Membranes were then rinsed in TBS-T and incubated with appropriate peroxidase-coupled secondary antibodies in BB, so that enhanced chemiluminescent (ECL) protocols could be used, as follows: for anti-synapsin II signal (AbCam, Cambridge, MA, USA, 1 : 2500 dilution, for 30 min), for anti-14-3-3 signal (GE Healthcare, 1 : 2000 dilution, for 1 h), for anti-PKM2 signal (CalBiochem, San Diego, CA, USA 1 : 2000 dilution, for 1 h), for anti-calretinin signal (AbCam, 1 : 2500 dilution, for 30 min). After incubation on secondary antibody solutions, membranes were again rinsed in TBS-T, and subsequently incubated in a solution of ECL (ECL-plus, GE Healthcare), following the manufacturer's instructions. After ECL detection, blots were scanned on a tabletop scanner and digitized to a computer. Adobe Photoshop software was used to assemble the final Western blot plates.

Cell counts, optical density measurements and statistical analysis

Immunopositive cells were counted as profiles, essentially as detailed previously (Pinaud *et al.*, 2004), in a minimum of five grids of either 100 × 100- μm (for pyruvate kinase and 14-3-3) or 500 × 500- μm (for calbindin 2/calretinin) areas that were sequentially placed across the rostro-caudal and dorso-ventral aspects of NCM; the larger size grid was used for calbindin 2 because few cells express this protein in NCM, and thus the smaller size grid would yield large quantification errors. Qualitatively, each antibody yielded a relatively homogeneous pattern of immunoreactivity across NCM, except for pyruvate kinase, which was expressed at high levels in the rostral NCM and low levels in caudal NCM. In this case, these domains were quantified separately to gain further insight into the activity-dependent regulation of this protein across subdomains of NCM. For synapsin II, because immunoreactive profiles are restricted to the presynaptic terminal, we obtained optical density measurements for 100 × 100- μm grids as previously described (Pinaud *et al.*, 2001, 2002a). One-way analysis of variance (ANOVA) was used to analyse significant treatment effects across groups. Tukey's HSD test was used for *posthoc* comparisons with the criterion level set at $P < 0.05$.

Statistical analyses of the 2D-DIGE data were calculated with DeCyder 2D 6.5 software. First, differential expression was calculated from the three replicates of each time point for which the *t*-test and the ANOVA were determined. Differential protein expression was considered significant if $P < 0.05$ with fold-changes in protein expression falling inside of two standard deviations (95% confidence). PCA was used to determine clustering of the experimental groups and was performed with the K-means algorithm included in the EDA module. This algorithm classifies the data by minimizing the sums of the squares of the distances between the data and the centroid of the corresponding cluster (Lloyd, 1957).

Imaging and figure assembly

Digital images were obtained with a Spot Camera III (Spot Diagnostics) that was coupled to a Leica DMRXA2 microscope. Photomicrograph plates were assembled in Adobe Photoshop.

Results

Protein regulation as a function of auditory experience

We used 2D-DIGE-based proteomics to study how the population of proteins in NCM is dynamically regulated by auditory stimulation. Our animals were divided into four groups (controls, 5 min, 1 h and 3 h; see Methods). These stimulation times were chosen to investigate fast changes, such as post-translational modifications (5 min), the first wave of immediate early gene responses (1 h) and a second wave proposed to consist of late gene responses (3 h) (Chew *et al.*, 1995; Pinaud & Tremere, 2006). We screened for approximately 2500 spots per condition, and determined by manual selection that ~2000 were reliably identified as proteins. With these 2000, extended data analysis via PCA revealed that the protein population profile for each experimental group (5 min, 1 h and 3 h) was significantly different from each other (Fig. 2). Importantly, no significant differences were detected when comparing different gels within each time point, as these clustered together in the PCA. These results indicate that the NCM proteome significantly changes across the time points studied. In addition, this analysis revealed that our replications of the experiments

and the proteome changes across animals were highly consistent (Fig. 2).

Quantitative analysis revealed no statistically significant effects of auditory stimulation on the protein spots of the 5-min condition, as compared with control animals. However, significant changes in the NCM proteome were detected after 1 h and 3 h of auditory experience. In the 1-h group, 16 protein spots were found to be differentially regulated (Fig. 3, left column). Of these, 12 protein spots were significantly up-regulated (range 9–37% above silent control levels) and four were significantly down-regulated as a function of auditory experience (range 37–117% below silent control levels; Table 1). In the 3-h group, 16 protein spots were differentially regulated (Fig. 3, right column). Of these, seven protein spots were significantly up-regulated (range 13–40% above control levels) and nine were significantly down-regulated (range 10–44% below control levels; Table 1).

Identification and functional characteristics of differentially regulated proteins

All differentially expressed spots from both the 1-h and the 3-h conditions were selected for MALDI-TOF/TOF mass spectrometric analysis to obtain protein identity. After MS analysis, 12 (of 16) spots were reliably identified with high confidence (> 95%) scores in the 1-h condition, and nine (of 16) spots were reliably identified in the 3-h group. The inability to identify a spot was related to low amounts of protein in that particular spot, which in turn led to low confidence (< 95%) protein identification by MS. The identities of the spots, their predicted and observed molecular weights, isoelectric points (pIs), accession numbers to hits in the NCBI GenBank database, and the MS and Ion scores are detailed in Table 2. The 12 spots from the 1-h group consisted of nine proteins; the nine spots from the 3-h group consisted of seven proteins. This redundancy of proteins within each group is likely to result from post-translational modifications that cause changes in spot location of a single protein within a gel. For example, two spots that showed differential regulation (620 and 621 in the 1-h group; 619 and 624 in the 3-h group; Fig. 3) were both identified by MS as synapsin II (SYN2). These spots have identical molecular weights but clearly different pIs (Fig. 3; Table 2), providing evidence for a potential change in charge of SYN2. Both forms of SYN2 appear to be regulated by song stimulation. There was also redundancy of proteins across the 1-h and 3-h groups, where SYN2 and pyruvate kinase (PKM2) were present, and differentially regulated, at both time points. This finding suggests that these two proteins are significantly regulated by 1 h, with a persistent change at 3 h after song stimulation. In all, when taking into account intra- and intergroup redundancy, we found 14 proteins regulated in NCM as a function of song auditory experience.

We grouped the 14 proteins according to their function and cellular location (Fig. 4). The proteins exhibited a multitude of functional roles that ranged from metabolic and catabolic processing, transcriptional regulation, molecular chaperone activity, and synaptic activity (Fig. 4A), which are expressed across most cellular domains, with the largest fraction in the cytoplasm (Fig. 4B). The majority of proteins (43%) were enzymes (Fig. 4A; Table 2); these included PKM2, phosphoglycerate kinase (PGK), the collapsin response-mediator protein-2B (CRMP-2B) that has hydrolase activity, glutamate dehydrogenase (GLUD), ATP synthase alpha subunit (ATP5A1), and ATP synthase beta subunit (ATPB). Most of these enzymes function in the mitochondrion (Fig. 4B). A fraction of the proteins (14%) exhibited synaptic functions; these were SYN2, a protein involved in the regulation of the size of readily releasable pool of vesicles (Hilfiker *et al.*, 1999; Pinaud, 2005), and the synaptosomal-

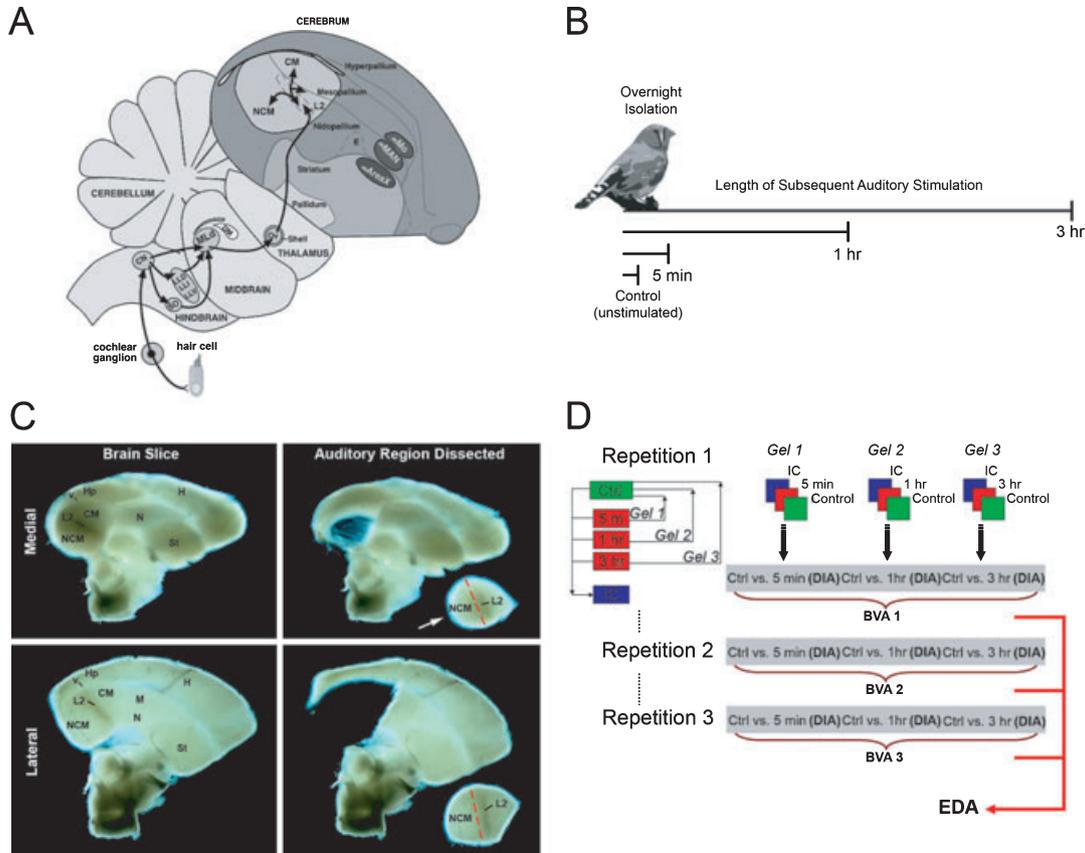


FIG. 1. (A) Schematic representation of a parasagittal section through a zebra finch brain detailing the connectivity of the main stations of the ascending auditory pathway. The focus of the present study, NCM, receives input from the thalamo-recipient layer Field L2, and has reciprocal connectivity with the caudal mesopallium (CM). (B) Experimental approach. All animals were isolated overnight in sound-proof boxes and randomly included into one of four experimental groups. Control animals were killed after isolation without stimulation. The remaining animals were stimulated with playbacks of conspecific songs for either 5 min, 1 h or 3 h. (C) Representative brain sections were obtained with a vibratome for dissection of NCM. The red dashed lines indicate the approximate locations where sections were taken to isolate NCM. Note the darker colour of the thalamo-recipient field L2 is in these preparations, which significantly facilitates the isolation of NCM. (D) Representation of the 2D-DIGE experiments conducted in the present work. Gels were run for individual comparisons across experimental groups (control vs. 5 min; control vs. 1 h and control vs. 3 h). Each experiment was repeated three times with different animals. Intra-gel (DIA), inter-gel (BVA), as well as across-repetition (EDA) comparisons were conducted in a quantitative manner in order to identify statistically significant differences across these variables (see Methods for further details). Anatomical abbreviations not mentioned above or in the text: CN, cochlear nuclei; DM, dorsal medial mesencephalic nucleus; E, entopallium; H, hyperpallium; Hp, hippocampus; LL, lateral lemniscal nuclei; M, mesopallium; MLd, dorsal lateral mesencephalic nucleus; N, nidopallium; Ov, ovoidalis; St, striatum; v, ventricle; SO, superior olive.

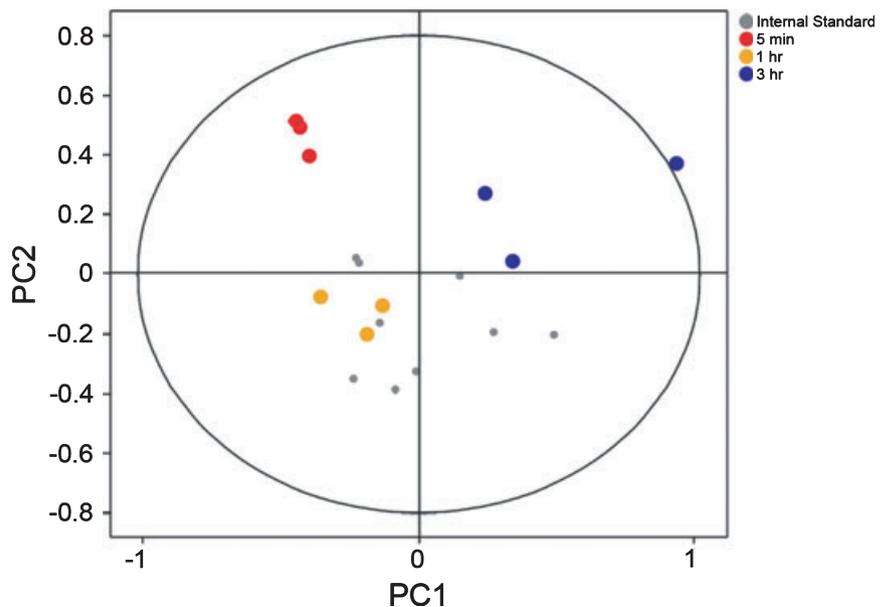


FIG. 2. Principal Component Analysis (PCA) of ~2000 protein spots per condition reveals that the NCM proteome is significantly different across experimental conditions. Independent gels for each of the conditions are not significantly different from each other. The circle represents the 95% confidence interval. Values that cluster in a quadrant are significantly different than values falling within other quadrants. See Methods for description of internal standard (IS).

TABLE 1. Quantitative analysis of differentially regulated spots

Control vs. 1 h		Control vs. 3 h	
Spot number	Average ratio	Spot number	Average ratio
425	1.27	417	-1.35
458	1.09	619	1.14
620	1.20	624	1.13
621	1.16	760	1.19
686	-2.35	783	-1.16
707	1.11	806	-1.31
760	1.19	890	-1.10
782	-1.36	942	1.19
783	-1.31	965	1.16
811	1.20	1101	1.40
812	1.19	1189	1.20
814	1.24	1267	-1.17
822	1.37	1273	-1.44
855	1.21	1310	-1.28
856	1.35	1318	-1.39
1222	-1.37	1322	-1.40

Indicated are spot numbers of differentially regulated proteins ($P < 0.05$) and the average expression difference between control and experimental samples. Positive ratios indicate spots where experimental samples have higher expression levels compared with control samples (up-regulation as a result of auditory experience) and negative ratios indicate spots where the control samples have higher expression levels compared with experimental samples (down-regulation as a result of auditory experience).

associated protein-25 (SNP25), a t-SNARE/Q-SNARE (soluble N-ethylmaleimide-sensitive factor-attachment receptors) involved in calcium-dependent neurotransmitter release (Catterall, 1999; Zamponi, 2003). SNAREs participate in intracellular membrane trafficking mediated by the formation of protein complexes between SNAREs

contained in vesicles and target membranes. A similar fraction (14%) was associated with microtubules; these were the microtubule binding protein tropomyosin 3 gamma (TRM3) and tubulin beta chain (TBB). The remaining proteins included the chaperone heat shock protein 108 (hsp108), the calcium-binding protein calbindin 2 (CALB2; also known as calretinin) and the adaptor protein 14-3-3 gamma (1433G; also known as PKC inhibitor). In addition, one identified protein exhibited similarity with the homeobox transcription factor Nkx-3.1 (Nkx3-1). Even though this protein spot yielded a low protein score, it exhibited an Ion score that allowed for high confidence protein identification.

Confirmation and anatomical profiling of selected differentially expressed proteins

Although the 2D-DIGE approach we used was highly quantitative and is known to yield a low percentage of false-positive detections, we chose to confirm the differential regulation of selected proteins of interest using ICC in brain sections containing NCM, from a new group of stimulated animals. ICC also allowed us to study the anatomical distribution of the cells expressing the proteins of interest, as well as to perform further quantification on the effects of auditory experience. However, although ICC can easily reveal protein expression level changes in tissue sections, it cannot simply reveal post-translational changes to proteins (as the 2D-DIGE approach can), without antibodies directed at specific modifications (e.g. a phosphorylated residue in a given protein of interest). For ICC analysis, we chose the following proteins: (i) SYN2, (ii) PKM2, (iii) 14-3-3, (iv) CALB2 and (v) CRMP-2B. When available, we used the predicted protein sequences from cDNAs of a songbird cDNA database (<http://songbirdtranscriptome.net/>) to purchase commercially available

TABLE 2. Hearing-regulated proteins in NCM at 1 h and 3 h as determined from 2D-DIGE experiments and MS/MS analyses

Spot no.	Protein name	Abbreviation	Accession ID	Species	Predicted MW (kDa)*	Observed MW/pI	Protein score	Ion score	Coverage (%)	No. of peptides
1-h group										
425	Heat shock protein 108	hsp108	Q90WA6	<i>G. gallus</i>	91.1	91.2/4.86	83	42	17	13
620	Synapsin II	SYN2	Q6RSD2	<i>T. guttata</i>	23.8	71.3/7.35	126	106	19	4
621	Synapsin II	SYN2	Q6RSD2	<i>T. guttata</i>	23.8	71.3/7.18	68	40	20	5
707	Collapsin response mediator protein-2B	CRMP-2B	Q71SG1	<i>G. gallus</i>	62.2	62.2/6.05	679	515	53	21
760	Pyruvate kinase	PKM2	P00548	<i>G. gallus</i>	57.8	58.0/7.29	178	121	31	13
812	Glutamate dehydrogenase	GLUD	P10860	<i>R. norvegicus</i>	61.3	61.4/8.05	133	92	25	11
811	Glutamate dehydrogenase	GLUD	P00368	<i>G. gallus</i>	55.6	59.0/7.84	181	119	30	13
814	ATP synthase alpha subunit	ATP5A1	Q8UVX3	<i>G. gallus</i>	60.1	59.0/7.97	300	213	34	15
822	ATP synthase alpha subunit	ATP5A1	Q8UVX3	<i>G. gallus</i>	60.1	58.4/8.47	735	608	39	19
855	Similar to NK-3 transcription factor	Nkx3-1	P97436	<i>M. musculus</i>	26.8	57.0/6.24	39	23	13	3
856	ATP synthase beta subunit	ATPB	Q5ZLC5	<i>G. gallus</i>	56.5	56.7/5.23	914	758	47	19
1222	Tropomyosin 3 gamma	TRM3	Q5ZLJ7	<i>G. gallus</i>	28.7	34.3/4.58	285	151	42	19
3-h group										
619	Synapsin II	SYN2	Q6RSD2	<i>T. guttata</i>	23.8	71.2/7.58	140	85	39	7
624	Synapsin II	SYN2	Q6RSD2	<i>T. guttata</i>	23.8	71.1/7.89	52	32	19	4
760	Pyruvate kinase	PKM2	P00548	<i>G. gallus</i>	57.9	58.0/7.29	233	184	29	12
806	Tubulin beta chain	TBB	P07437	<i>H. sapiens</i>	49.7	59.9/4.5	145	64	26	13
942 [‡]	Phosphoglycerate kinase	PGK	Q76BF6	<i>O. latipes</i>	41.2	50.7/7.5	104	50	28	10
965	Phosphoglycerate kinase	PGK	Q76BF6	<i>O. latipes</i>	41.2	49.4/7.97	323	287	26	8
1273	14-3-3 protein gamma (PKC inhibitor)	1433G	Q5F3W6	<i>O. aries</i>	17.9	31.8/5.23	195	80	58	11
1310	Calretinin	CALB2	Q08331	<i>M. musculus</i>	22	31.3/4.94	125	73	37	8
1318	Synaptosomal-associated protein-25	SNP25	Q5R505	<i>P. pygmaeus</i>	23.1	30.3/4.28	90	78	20	3

Spot nos. are labeled according to the gels of Fig. 3. Accession ID are those in NCBI. *Predicted MW from MASCOT. Observed MW and PI calculated from the 2D-DIGE gels. [‡]Oxidized.

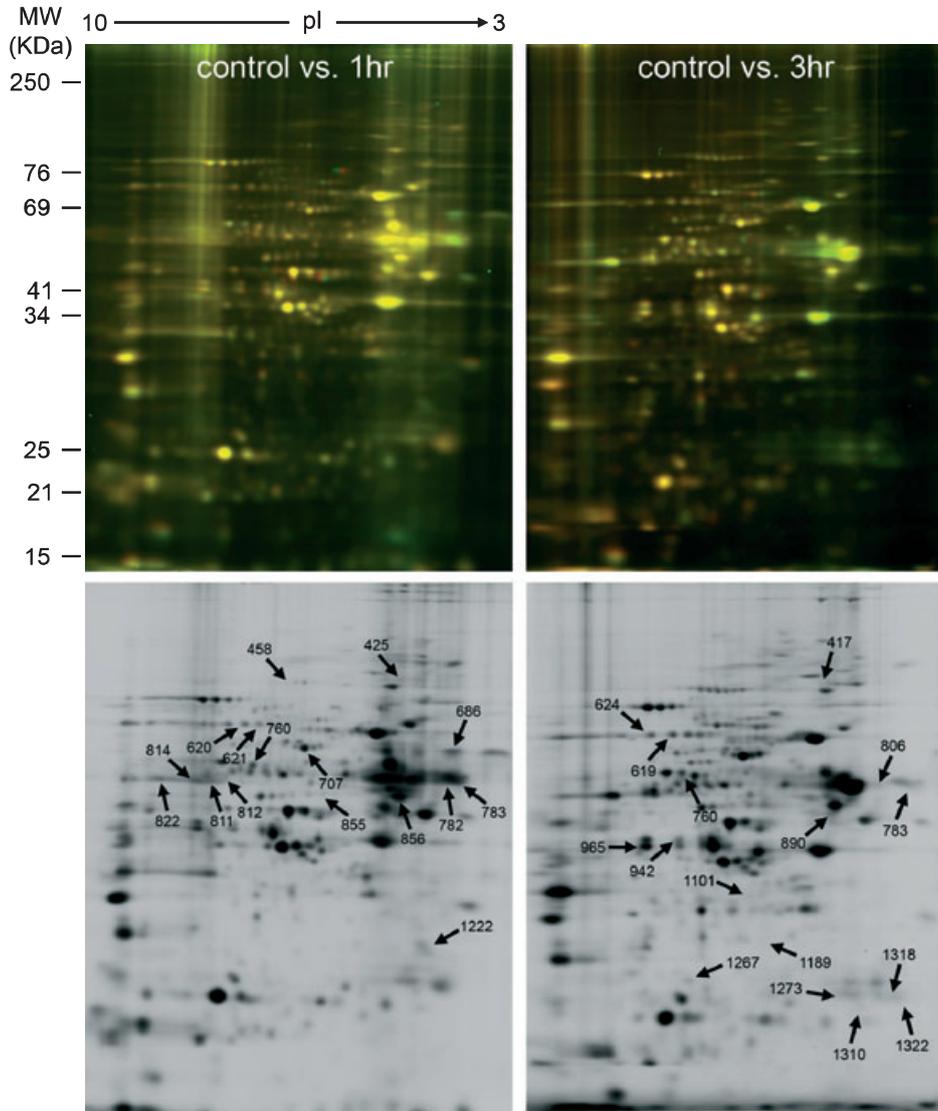


FIG. 3. Top: representative 2D-DIGE gels illustrating fractionated proteins from NCM in comparisons for the 1-h (control vs. 1 h; left) and 3-h (control vs. 3 h; right) groups. Control samples were labeled with the fluorophore Cy3 (green) while experimental samples were labeled with Cy5 (red). Internal control samples were labeled with Cy2 (blue, not shown; see Methods for details). Bottom: Comassie blue-stained gels illustrating differentially regulated spots (arrows) in the 1-h (left) and 3-h (right) conditions, as revealed by quantitative and statistical analyses with DeCyder software. All differentially regulated spots underwent protein fingerprinting by mass spectrometry (see Methods for details).

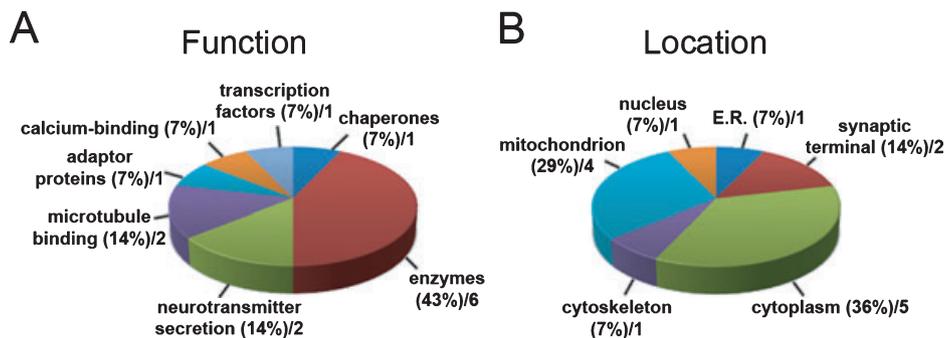


FIG. 4. Pie charts illustrating the cellular location (A) and molecular function (B) of the proteins identified in the present study (from Table 2). Data are expressed as percentage of the total number of differentially expressed proteins (in parentheses). Numbers adjacent to the parentheses indicate the number of proteins identified in that category. Categories were determined based upon known gene ontologies. No protein was grouped into more than one category for A and B. We note, however, that synapsin II is involved in both calcium binding and neurotransmitter secretion; we placed it in the latter category as its main function.

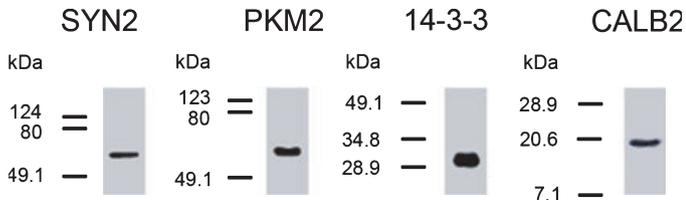


FIG. 5. Western blots illustrating the specificity of antibodies used for ICC experiments. These blots revealed the specific detection of SYN2, PKM2, 14-3-3 and CALB2 proteins, from left to right, in the zebra finch brain. The single bands were within the predicted molecular weight for the proteins in question with each antibody, indicating that these antibodies specifically recognize these four proteins in the zebra finch NCM. Blots were conducted with NCM tissue obtained from a bird that was killed after 1 h of song playbacks.

antibodies that were made against homologous peptides of other species (see Methods). We tested the specificity of the above-mentioned antibodies in zebra finch brain tissue, including in NCM (Fig. 5). All antibodies yielded specific Western blot signals, as evidenced by single band detection with the predicted molecular weights of the proteins of interest (Fig. 5), except for CRMP-2B, which was therefore excluded from further analysis and quantification. We used the above mentioned antibodies to generate reliable ICC signals for further analyses. In addition, we used an antibody against the *zenk* protein (ZENK), which has been previously shown to specifically recognize this protein in zebra finch brain tissue, as a positive control for song-induced protein expression in our animals (Mello & Ribeiro, 1998; Mello *et al.*, 2004; Mello & Pinaud, 2006). Individual results are detailed below.

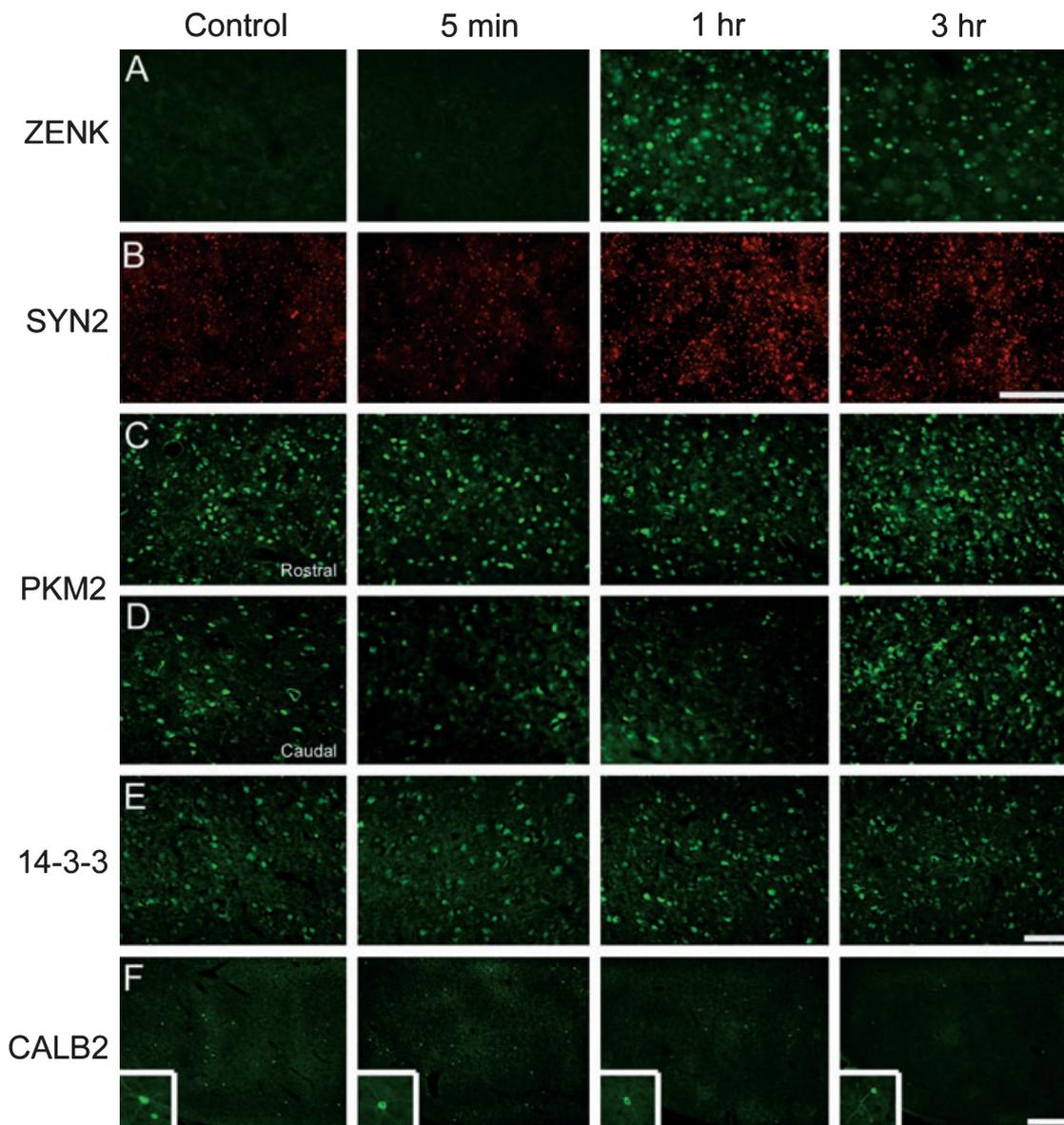


FIG. 6. ICC directed against selected proteins identified in the 2D-DIGE screening. Shown are representative photomicrographs of NCM sections of animals from each experimental group (columns). All photomicrographs depicted here for each individual antibody were reacted as a single immunocytochemical batch. (A) *egr-1*/ZENK protein is expressed in cell nuclei. (B) Synapsin II is expressed in neuropil, as suggested by the punctate staining, which is consistent with presynaptic terminals. (C and D) Pyruvate kinase is expressed in mitochondria; sections from both caudal and rostral parts of NCM are shown. (E) 14-3-3 and (F) Calbindin 2 are both expressed in the cytoplasm. Calbindin 2-positive cells were found sparsely in NCM and, thus, the images shown are at lower magnification; the insets show high-power images of representative cells for each of the conditions. Scale bars: A, C-E, μm ; B, 25 μm ; F, 200 μm .

ZENK

As expected (Mello & Ribeiro, 1998; Mello *et al.*, 2004; Mello & Pinaud, 2006), we found that our positive control protein ZENK was expressed in a negligible number of neurons in the control and 5-min time points, while a robust up-regulation was observed 1 h after stimulus onset (Fig. 6A). In the 3-h time point, also as expected (Mello & Ribeiro, 1998), there was a clear habituation in the ZENK response compared with for 1-h animals; however, these levels were still higher than those found in the control group. These findings reinforce the notion that *zenk* expression is highly sensitive to song auditory stimulation in the NCM of freely behaving animals (Mello *et al.*, 1995; Mello & Ribeiro, 1998; Mello & Pinaud, 2006).

Synapsin II (SYN2)

2D-DIGE experiments revealed that the SYN2 protein was up-regulated in both the 1-h and the 3-h time points relative to control levels. ICC analysis revealed a high density of SYN2-positive neuropil covering the full extent of NCM; qualitatively, a higher density of immunolabeled terminals appeared to be present in both the 1-h and the

3-h groups, as compared with silent control animals (Fig. 6B). This high density pattern of neuropil staining in the absence of apparent staining of axonal processes is consistent with a presynaptic location of SYN2 as described in several vertebrates, such as chicken and mammals (Landis *et al.*, 1988; Valtorta *et al.*, 1988; Hilfiker *et al.*, 1999; Zidanic, 2002). To quantify differences across experimental groups, we obtained optical density measurements using previously described protocols (Pinaud *et al.*, 2001, 2002a). Quantitative analysis on ICC material confirmed these results (Fig. 7A), revealing a significant up-regulation in SYN2 levels by 1 h. Similar to ZENK, a significant increase in SYN2 immunolabeling was detected after 3 h of auditory stimulation relative to controls and these levels were less marked than those found in the 1-h group (Fig. 6B).

Pyruvate kinase (PKM2)

The 2D-DIGE experiments showed that the spot containing PKM2, a metabolic enzyme involved in glycolysis, was significantly increased in both the 1-h and the 3-h time points compared with controls. ICC analysis in control animals revealed a high prevalence of immunolabeled cells in rostral NCM, but few immunostained cells in the

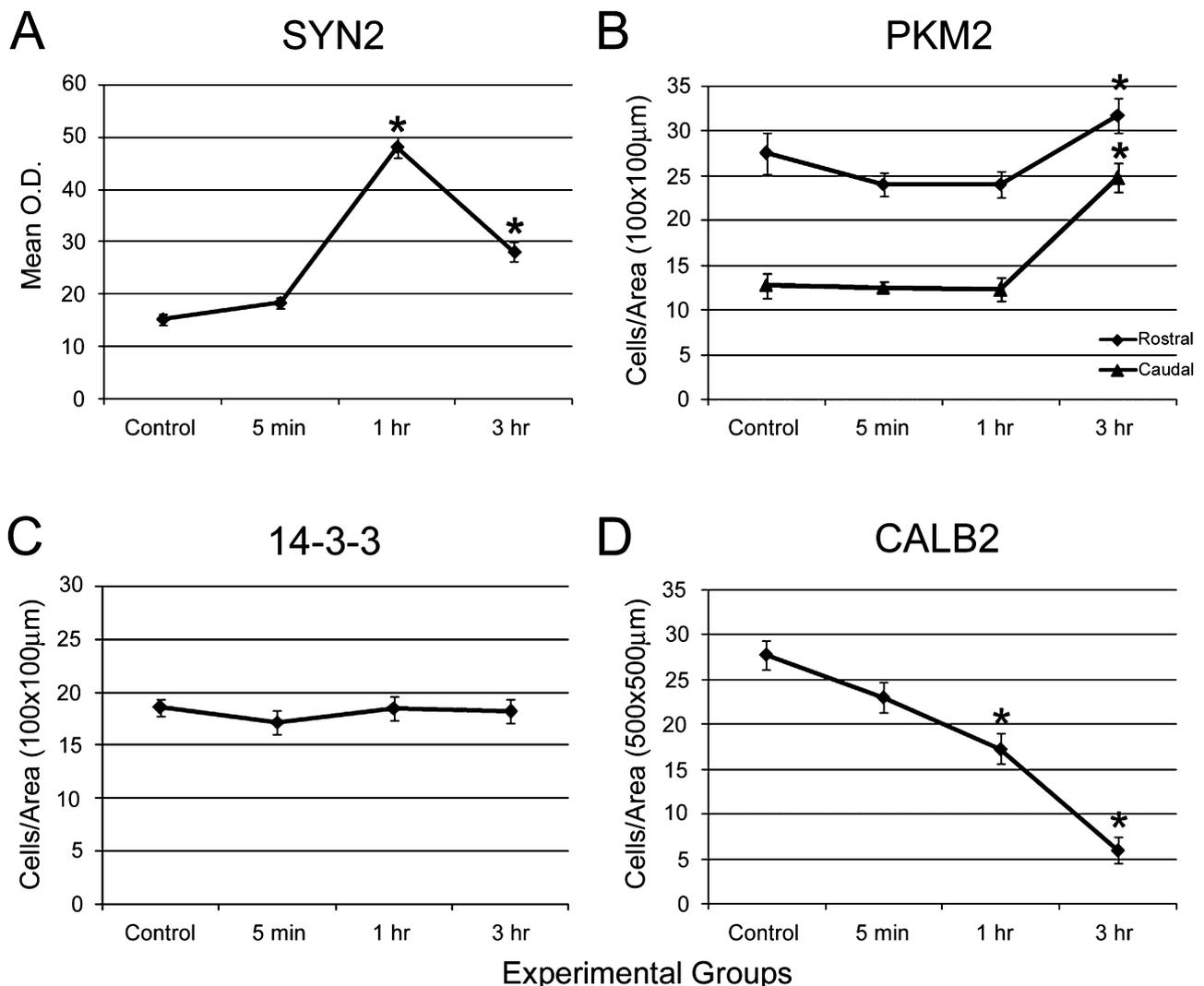


FIG. 7. Quantification of immunocytochemistry data. Due to its typical terminal labeling, SYN2 immunoreactivity was quantified with standard optical density measurements (A). Immunolabeled neurons were counted as profiles for PKM2 (B), 14-3-3 (C) and CALB2 (D). With the exception of 14-3-3, significant regulation was found for all proteins. Asterisks depict significant differences ($P < 0.05$, ANOVA) across groups.

caudal aspect of this area (Fig. 6C and D). Because of this asymmetric expression pattern, we quantified these two domains (rostral vs. caudal) separately. In contrast to the results obtained with 2D-DIGE, we were unable to detect significant differences in the expression levels of PKM2 in the 1-h time point (Fig. 7B). However, a significant increase in the number of PKM2-positive cells, in both rostral and caudal NCM domains, was detected by 3 h. Interestingly, the most robust up-regulation of PKM2 (~250%) was found to take place in the caudal NCM, as compared with the rostral NCM (~130%) (Fig. 7B).

14-3-3 (PKC inhibitor)

The spot for the regulatory protein 14-3-3 was found to be significantly down-regulated in animals belonging to the 3-h group, as revealed by 2D-DIGE. ICC analysis revealed a high density of 14-3-3-positive cells, which appeared to be homogeneously distributed throughout NCM (Fig. 6E). Quantification of the number of 14-3-3-immunolabeled neurons failed to reveal significant differences across experimental groups (Fig. 7C). To determine if the lack of a detectable difference was due to a potential difference in the overall amount of protein per cell (instead of the number of cells expressing this protein), we performed a Western blot analysis for all three time points. Similarly to the ICC results, this approach did not detect a measurable difference in 14-3-3 expression levels across experimental groups, as indicated by densitometric analysis conducted with NIH Image software (data not shown).

Calbindin 2 (CALB2)

The 2D-DIGE experiments showed that the protein spot containing the calcium-binding protein CALB2 was significantly decreased in the 3-h time point. ICC in control animals revealed few immunopositive cells in NCM (Fig. 6F) that were strongly labeled (Fig. 6F, insets). These cells were distributed homogeneously throughout the dorso-ventral, medio-lateral and antero-posterior extents of NCM. Interestingly, CALB2-positive neuronal processes covered a large extent of NCM, forming a low-density mesh. In accordance with data obtained with our 2D-DIGE experiments, CALB2 expression was significantly down-regulated in response to auditory stimulation in the 1-h and 3-h groups (Fig. 7D). This decrease in the number of CALB2-positive cells was almost linear across the time points examined.

Discussion

In the present study, we identified 14 proteins in NCM that are regulated by song stimulation. This effort more than doubled our knowledge on the identity of proteins regulated by auditory experience in songbirds. Below, we discuss our findings in the context of using proteomics for large-scale screening of experience-regulated proteins and potential protein cascades associated with auditory processing of songs.

Methodological considerations for large-scale screening of experience-regulated proteins

We used quantitative 2D-DIGE to investigate hearing-driven protein regulation in the auditory area NCM of the zebra finch. A clear advantage of this approach is that the number of true-positives is high ($\geq 75\%$), as revealed by multiple representations of the same protein spots across time and by our ICC verifications. This high rate is presumably due to the extensive data analysis performed on the protein spots. False-positives are presumably 'diluted' when comparing

multiple groups and replications of the experiment. The differential expression of a single protein (14-3-3) was not confirmed and, thus this was presumed to be a false-positive. However, down-regulation of the 14-3-3 spot was consistently detected, and was quantitatively and qualitatively the largest change of all spots in the 3-h group. It is therefore possible that 14-3-3 is post-translationally modified, without changes in the 14-3-3-expressing cell density or in the steady-state levels of this protein in NCM. This hypothesis remains to be explored in subsequent studies. Overall, the relatively low number of differentially expressed proteins detected in our experiments, combined with low false-positive rates, and the redundancy of a fraction of regulated proteins in multiple spots and across experimental groups, attests to the reliability of this approach for mapping the dynamic regulation of the zebra finch proteome in response to sensory experience. It also indicates that we can reliably consider that most of the proteins identified in our 2D-DIGE experiments are indeed regulated during song-stimulation in awake, freely behaving animals.

Differentially regulated proteins were detected in both the 1-h and the 3-h groups, but not in the 5-min group. We interpret the lack of measurable changes in the NCM proteome in the 5-min condition to suggest that this time point may be too close to the control group to yield significant, measurable, changes in protein expression. By 5 min, mostly post-translational modifications are likely to have taken place in NCM, as seen with the rapid phosphorylation of the ERK protein (Cheng & Clayton, 2004), but these may be few or below the threshold of detection of our method.

Overall, a lower than expected number of proteins was found, in that it has been proposed by many investigators that there may be up to ~100 activity-regulated proteins in the brain (Wada *et al.*, 2006). We believe that multiple factors may account for such results. First, we screened approximately 2000 protein spots per condition, which likely accounts for a small fraction of the NCM proteome. This fraction may relate to the fact that our 2D-DIGE experiments detect mainly highly abundant proteins. High-abundance proteins may overlap and mask differentially regulated spots of low-abundance proteins. Identifying these low-abundance proteins would require removing the high-abundance proteins by immunoprecipitation and then running the gels on the remaining proteins. Second, highly acidic (pH < 3) or highly basic (pH > 10) proteins were not fractionated and were therefore collapsed together in our 2D gels. This limitation is imposed by the range of the pH strips used to fractionate proteins in the first dimension (which ranged from 3 to 10) and can be overcome by shifting the range of pHs to more acid or basic. Third, the quantitative analyses employed in the current study were highly stringent and contributed to minimizing the detection of false-positive proteins; however, these may also have complicated the identification of additional true positives. In this regard, we identified a number of other proteins in the 2D-DIGE gels that showed differential expression in all animals of each time point, however, due to high variance within a time point, these spots failed to reach statistical significance. Another possibility is that the number of regulated proteins in one part of the auditory pathway, such as the NCM, reflects a subset of protein regulatory events detected across the entire pool of auditory telencephalic structures. For instance, in the song control circuit, although 33 singing-regulated genes have been identified to date, smaller fractions of this total are expressed in each individual nuclei of this circuit (Wada *et al.*, 2006). Therefore, it is possible that if additional stations of the auditory pathway were included in the present study, a larger population of hearing-regulated proteins would be identified. Despite the limitations discussed above, this work represents the first unbiased quantitative screening of differentially regulated proteins in the songbird brain.

Hearing-regulated proteins

Previous studies investigated the role of auditory-induced molecular changes in NCM based upon proteins known to change in response to neural activity in the mammalian brain. These have resulted in the identification of four transcription factors (Mello *et al.*, 1992; Nastiuk *et al.*, 1994; Velho *et al.*, 2005) and two signaling molecules (Cheng & Clayton, 2004; Huesmann & Clayton, 2006). [See note at end of Discussion.]

With our unbiased quantitative 2D-DIGE approach, a large fraction of the identified proteins were enzymes, most of which are involved in ATP synthesis. The most conservative interpretation for their consistent appearance is that auditory stimulation triggered basic metabolic processes required for cell function, such as cellular respiration. Alternatively, the regulation of these enzymes may reflect the engagement of long-term alterations in metabolic processing triggered by acute sensory stimulation. Future studies should shed light into these questions.

The best characterized regulated protein change in NCM is the robust and transient song-induced up-regulation of the transcriptional regulator ZENK (Mello *et al.*, 2004; Mello & Pinaud, 2006). The *zenk* gene has been proposed to play a critical role in the coupling of neuronal activity and activity-dependent long-term changes in neuronal function, and possibly the formation of auditory memories required for song learning (Mello *et al.*, 2004; Mello & Pinaud, 2006). Consistent with this notion, *zenk* expression depends on the activation of NMDA glutamate receptors, which are implicated in calcium-dependent enhanced synaptic efficacy and neural plasticity (Cole *et al.*, 1989; Pinaud, 2005). Furthermore, recent evidence suggests that calcium-driven *zenk* expression in NCM is dependent on the activation of the ERK pathway (Cheng & Clayton, 2004), a major biochemical pathway underlying experience-dependent neuronal plasticity (reviewed in Pinaud, 2005). Our quantitative proteomics screening was not able to detect changes in ZENK protein expression or other previously identified song-regulated proteins in NCM. The pIs for ZENK (pH 8.7) and c-jun (pH 8.9) fall in the upper limit of the resolution of our 2D-DIGE gels, where at that range, most basic proteins tend to collapse together, thereby preventing a reliable quantification of their differential expression. 2D-DIGE analysis using a more basic pI range (e.g. 6–11) and more detailed temporal analysis should be a successful approach to fractionate and quantify differences in expression of ZENK, c-jun and other protein changes induced by auditory stimulation. This approach may also shed light on whether ZENK is post-translationally modified as a result of auditory experience, a question that is currently unknown.

Although inspected, we did not detect significant changes in the levels of *c-fos* and *arc*, two additional immediate early genes known to be regulated by song in NCM (Velho *et al.*, 2005), and whose pIs fall within high-resolution ranges of our gels (4.8 and 5.6, respectively). One likely possibility for these findings is that although a significant number of cells express *c-fos* and *arc* mRNA in NCM, as a result of auditory stimulation, the actual change in the overall concentration of these proteins could be below the threshold of detection of our method. An additional possibility is that both proteins are of relatively low abundance as compared with the proteins that are fractionated in the conditions used for the current report. Future studies aimed at investigating low-abundance proteins should shed light on these possibilities. Finally, the time course for ERK phosphorylation in NCM has been reported to be only 2 min and with recovery by 5 min (Cheng & Clayton, 2004). Thus, we could have missed the peak accumulated alteration of this protein in our 5-min group. At the other end of the temporal spectrum, we found that a number of protein changes overlapped at the 1-h and 3-h time

points, indicating that the two predicted waves of protein expression (Chew *et al.*, 1995; Pinaud & Tremere, 2005; Pinaud, 2005) contain some of the same proteins.

Consistent with the notion that activity-dependent gene expression may play a critical role in the structural and functional organization of NCM, we detected a series of proteins whose regulation is impacted by sensory experience in this auditory structure. For instance, the down-regulation of the calcium-binding protein calbindin 2 could play a direct role in the regulation of intracellular calcium levels that are likely to result from auditory stimulation in NCM. That is, down-regulation of this protein could, theoretically, increase the availability of intracellular calcium to trigger downstream events that couple neuronal activation to genomic responses, including the activation of the ERK pathway (Fig. 8). This hypothesis remains to be tested experimentally.

Although we did not detect protein expression level changes for the regulatory protein 14-3-3 in the ICC experiment, the alternative possibility exists that this protein undergoes post-translational modifications as a function of auditory experience, as suggested by the 2-DIGE gels. 14-3-3 has been shown to play significant roles in cell physiology, including apoptosis, cell cycle control and signal transduction (Darling *et al.*, 2005). It plays a role in the heterodimerization of Raf-1 and B-Raf, which are critical for the activation of the ERK pathway (Rushworth *et al.*, 2006). In addition, 14-3-3 activation plays a role in experience-dependent plasticity of neural circuitry (Nelson *et al.*, 2004). If 14-3-3 is post-transcriptionally activated in NCM, it would be an expected secondary modulatory step in the activity-dependent biochemical cascade that leads to ERK and *zenk* activation (Fig. 8).

Given that ZENK is a transcription factor well positioned to mediate experience-dependent alterations in neural circuits (Mello, 2002; Mello *et al.*, 2004; Pinaud, 2004, 2005), a significant effort has been placed at characterizing the target genes that are potentially regulated by this protein. Two of the best described ZENK targets are promoters of the synapsin I and the synapsin II genes, as shown in cells in culture (Thiel *et al.*, 1994; Petersohn *et al.*, 1995). The synapsin proteins contribute to the regulation of the size of the readily releasable pool of neurotransmitter vesicles in the presynaptic terminals and therefore play a major role in activity-dependent, calcium-regulated, neurotransmitter release. As far as we are aware, our finding is the first *in vivo* result showing co-induction of one of the synapsin proteins and ZENK, suggesting that perhaps *in vivo* ZENK also up-regulates synapsin II as a result of auditory experience (Fig. 8; see Velho *et al.*, 2002). This co-induction includes significantly lower induced expression levels of both proteins over time. This decrease in the synapsin II response is parallel to a fast and long-lasting decrease, or 'habituation', in the amplitude of the electrophysiological responses in NCM that occurs after repeated presentations of conspecific songs (Chew *et al.*, 1995, 1996; Stripling *et al.*, 1997). Habituation has been proposed as a possible mechanism for auditory discrimination and learning in NCM. However, the neural basis of this phenomenon remains unclear. Given that synapsin II is involved in the regulation of neurotransmitter release (Hilfiker *et al.*, 1999), its down-regulation following repeated song presentation may account, at least partially, for the long-term habituation of electrophysiological responses that occurs under these conditions. Future studies involving double labeling, temporally refined time course delays and gene manipulations are necessary to establish a causal link between ZENK expression, synapsin II expression and experience-induced habituation of electrophysiological responses in NCM.

Our results may also shed light on the functional organization of NCM. We found that PKM2, a metabolic enzyme involved in

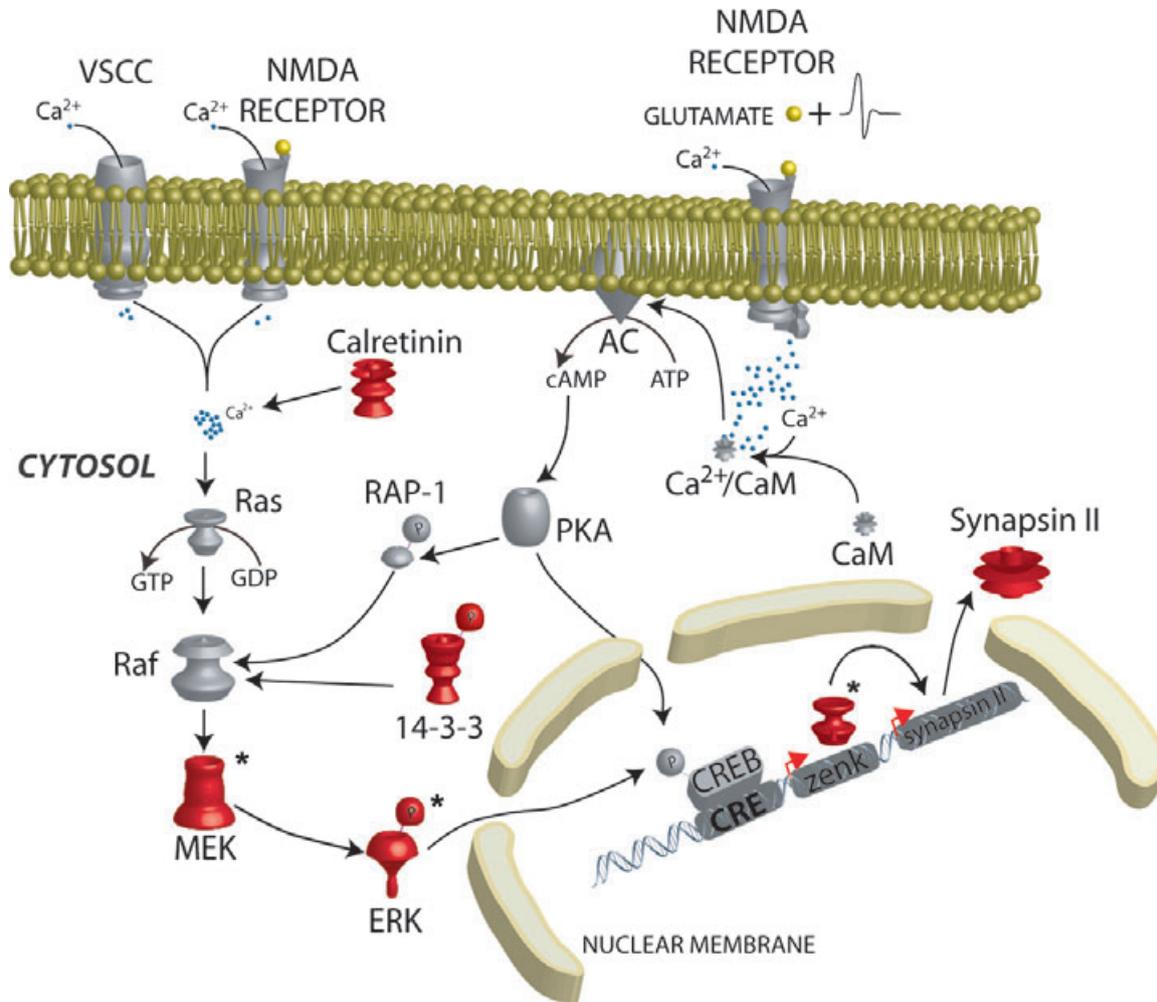


FIG. 8. Schematic representation of known interacting biochemical pathways of proteins detected in this study mapped onto previously known pathways. Proteins identified to be regulated by auditory stimulation in the current and previous studies are colour-coded in red, and identified without and with asterisks, respectively. Most of the interactions of the molecular signaling pathways shown here have been determined in non-songbird species, including the ZENK binding site in the synapsin II promoter. For a detailed discussion of potential mechanisms see Discussion section and Pinaud (2005). Figure modified from Pinaud (2005).

glycolysis, has a distinct pattern of expression in NCM: in rostral NCM of control animals, a significant number of immunopositive neurons was observed, while few PKM2-immunolabeled cells were detected in caudal NCM. Interestingly, a similar, but mirror-image asymmetric distribution exists for the calcium-binding protein calbindin 1 in NCM (Pinaud *et al.*, 2006). A high density of calbindin 1-positive cells was detected in caudal NCM, as compared with the few immunolabeled neurons that were observed in the rostral aspect. This supports the idea that NCM has different subdomains that can be defined based on neuroanatomical, neurochemical and electrophysiological grounds (Chew *et al.*, 1995; Vates *et al.*, 1996; Pinaud *et al.*, 2006). The functional differences between these putative subdomains remain to be determined, but one possibility is that the more caudal division of NCM processes more complex auditory stimuli, such as conspecific songs (Pinaud *et al.*, 2006).

Given that our studies were conducted in females, the possibility exists that there may be fundamental molecular differences in the auditory pathway across sexes. Although future investigations will be required to systematically test this possibility, we consider it unlikely that significant sex differences in the hearing-regulated proteome of the adult NCM will be observed. In fact, to the best of our knowledge,

the expression levels of three proteins have been shown to differ between sexes in the songbird NCM: ZENK, C-fos and calbindin 1. The first two have been shown to be differentially regulated in NCM only during early development (Bailey & Wade, 2003), but not after 45 days of age (Bailey & Wade, 2005). Calbindin 1 is expressed in a sexually dimorphic manner in the adult NCM, but is not regulated by auditory stimulation (Pinaud *et al.*, 2006). Therefore, the proteins studied thus far have been found either to not differ, or differ in a small quantitative manner in restricted developmental periods in the NCM of males vs. females.

In summary, we have used a quantitative approach to derive a larger-scale understanding of how proteins are dynamically regulated in the songbird NCM as a result of auditory experience. Proteins that are known to be regulated by activity and calcium influx, and that have been shown to play fundamental roles in experience-dependent neuronal plasticity in a number of preparations, were consistently found in our screenings. Future studies will be necessary to identify the expression of less abundant proteins and to determine the functional roles of the identified regulated proteins in neuronal maintenance functions, auditory processing and learning, or sensorimotor-learning of songs. Finally, our results further strengthen the use

of proteomic approaches, in particular 2D-DIGE, to quantitatively dissect dynamic and large-scale changes in protein expression from the brains of awake behaving animals.

Note. A recent study (Wada *et al.*, 2006) used cDNA microarrays to identify 33 genes (including the four mentioned transcription factors) that are regulated by singing in another zebra finch brain pathway, the song control system, which is involved in the motor learning and production of learned songs. Of these genes, only one (ATPB) overlapped with the current study, even though we noted that cDNAs of most of the 14 proteins (except SYN2, CRMP-2B and 14-3-3) were on the arrays. Thus, either regulation of the mRNAs for the proteins of this study was missed in the array experiments or the known specializations of the song system (Wada *et al.*, 2004; Wada *et al.*, 2006) may lead to a non-overlapping set of proteins regulated by singing in the song system vs. hearing in the auditory system.

Acknowledgements

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Abbreviations

1433G, 14-3-3 gamma; 2D-DIGE, two-dimensional differential in-gel expression; ATP5A1, ATP synthase alpha subunit; ATPB, ATP synthase beta subunit; BB, blocking buffer; BVA, Biological Variation Analysis; CALB2, calbindin 2; CRMP-2B, collapsin response-mediator protein-2B; EDA, Extended Data Analysis; ERK, extracellular-signal regulated kinase; GLUD, glutamate dehydrogenase; hsp108, heat shock protein 108; ICC, immunocytochemistry; IPG, Immobilized pH Gradients; IS, internal standard; MALDI-MS/MS, matrix-assisted laser desorption ionization-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NCM, caudomedial nidopallium; PCA, Principal Component Analysis; PGK, phosphoglycerate kinase; PVDF, polyvinylidene difluoride; RT, room temperature; SNARE, soluble N-ethylmaleimide-sensitive factor-attachment receptors; SNAP25, synaptosomal-associated protein-25; TBB, tubulin beta chain; TRM3, tropomyosin 3 gamma.

References

- Alban, A., David, S.O., Bjorksten, L., Andersson, C., Sloge, E., Lewis, S. & Currie, I. (2003) A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics*, **3**, 36–44.
- Bailey, D.J. & Wade, J. (2003) Differential expression of the immediate early genes FOS and ZENK following auditory stimulation in the juvenile male and female zebra finch. *Brain Res. Mol. Brain Res.*, **116**, 147–154.
- Bailey, D.J. & Wade, J. (2005) FOS and ZENK responses in 45-day-old zebra finches vary with auditory stimulus and brain region, but not sex. *Behav. Brain Res.*, **162**, 108–115.
- Bolhuis, J.J. & Gahr, M. (2006) Neural mechanisms of birdsong memory. *Nat. Rev. Neurosci.*, **7**, 347–357.
- Bolhuis, J.J., Zijlstra, G.G., den Boer-Visser, A.M. & Van Der Zee, E.A. (2000) Localized neuronal activation in the zebra finch brain is related to the strength of song learning. *Proc. Natl Acad. Sci. USA*, **97**, 2282–2285.
- Catterall, W.A. (1999) Interactions of presynaptic Ca²⁺ channels and snare proteins in neurotransmitter release. *Ann. NY Acad. Sci.*, **868**, 144–159.
- Cheng, H.Y. & Clayton, D.F. (2004) Activation and habituation of extracellular signal-regulated kinase phosphorylation in zebra finch auditory forebrain during song presentation. *J. Neurosci.*, **24**, 7503–7513.
- Chew, S.J., Mello, C., Nottebohm, F., Jarvis, E. & Vicario, D.S. (1995) Decrements in auditory responses to a repeated conspecific song are long-lasting and require two periods of protein synthesis in the songbird forebrain. *Proc. Natl Acad. Sci. USA*, **92**, 3406–3410.

- Chew, S.J., Vicario, D.S. & Nottebohm, F. (1996) A large-capacity memory system that recognizes the calls and songs of individual birds. *Proc. Natl Acad. Sci. USA*, **93**, 1950–1955.
- Cole, A.J., Saffen, D.W., Baraban, J.M. & Worley, P.F. (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature*, **340**, 474–476.
- Darling, D.L., Yingling, J. & Wynshaw-Boris, A. (2005) Role of 14–3–3 proteins in eukaryotic signaling and development. *Curr. Top. Dev. Biol.*, **68**, 281–315.
- Doupe, A.J. & Kuhl, P.K. (1999) Birdsong and human speech: common themes and mechanisms. *Annu. Rev. Neurosci.*, **22**, 567–631.
- Friedman, D.B., Hill, S., Keller, J.W., Merchant, N.B., Levy, S.E., Coffey, R.J. & Caprioli, R.M. (2004) Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. *Proteomics*, **4**, 793–811.
- Gobes, S.M. & Bolhuis, J.J. (2007) Birdsong memory: a neural dissociation between song recognition and production. *Curr. Biol.*, **17**, 789–793.
- Hilfiker, S., Pieribone, V.A., Czernik, A.J., Kao, H.T., Augustine, G.J. & Greengard, P. (1999) Synapsins as regulators of neurotransmitter release. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **354**, 269–279.
- Huesmann, G.R. & Clayton, D.F. (2006) Dynamic role of postsynaptic caspase-3 and BIRC4 in zebra finch song-response habituation. *Neuron*, **52**, 1061–1072.
- Jarvis, E.D. (2004) Learned birdsong and the neurobiology of human language. *Ann. NY Acad. Sci.*, **1016**, 749–777.
- Kim, J.K., Bamba, T., Harada, K., Fukusaki, E. & Kobayashi, A. (2007) Time-course metabolic profiling in Arabidopsis thaliana cell cultures after salt stress treatment. *J. Exp. Bot.*, **58**, 415–424.
- Landis, D.M., Hall, A.K., Weinstein, L.A. & Reese, T.S. (1988) The organization of cytoplasm at the presynaptic active zone of a central nervous system synapse. *Neuron*, **1**, 201–209.
- Lloyd, S. (1957) Least squares quantization in PCM. *IEEE Trans. Inf. Theory*, **28**, 128137.
- Mello, C.V. (2002) Mapping vocal communication pathways in birds with inducible gene expression. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.*, **188**, 943–959.
- Mello, C., Nottebohm, F. & Clayton, D. (1995) Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. *J. Neurosci.*, **15**, 6919–6925.
- Mello, C.V. & Pinaud, R. (2006) Immediate early gene regulation in the auditory system. In: Pinaud, R. & Tremere, L.A. (Eds), *Immediate Early Genes in Sensory Processing, Cognitive Performance and Neurological Disorders*. Springer, New York, pp. 35–56.
- Mello, C.V. & Ribeiro, S. (1998) ZENK protein regulation by song in the brain of songbirds. *J. Comp. Neurol.*, **393**, 426–438.
- Mello, C.V., Vates, G.E., Okuhata, S. & Nottebohm, F. (1998) Descending auditory pathways in the adult male zebra finch (*Taeniopygia guttata*). *J. Comp. Neurol.*, **395**, 137–160.
- Mello, C.V., Velho, T.A. & Pinaud, R. (2004) Song-induced gene expression: a window on song auditory processing and perception. *Ann. NY Acad. Sci.*, **1016**, 263–281.
- Mello, C.V., Vicario, D.S. & Clayton, D.F. (1992) Song presentation induces gene expression in the songbird forebrain. *Proc. Natl Acad. Sci. USA*, **89**, 6818–6822.
- Nastiuk, K.L., Mello, C.V., George, J.M. & Clayton, D.F. (1994) Immediate-early gene responses in the avian song control system: cloning and expression analysis of the canary c-jun cDNA. *Brain Res. Mol. Brain Res.*, **27**, 299–309.
- Nelson, T.J., Backlund, P.S. Jr & Alkon, D.L. (2004) Hippocampal protein-protein interactions in spatial memory. *Hippocampus*, **14**, 46–57.
- Nowicki, S. & Searcy, W.A. (2004) Song function and the evolution of female preferences: why birds sing, why brains matter. *Ann. NY Acad. Sci.*, **1016**, 704–723.
- Osorio, C., Sullivan, P.M., He, D.N., Mace, B.E., Ervin, J.F., Strittmatter, W.J. & Alzate, O. (2006) Mortalin is regulated by APOE in hippocampus of AD patients and by human APOE in TR mice. *Neurobiol. Aging*, **28**, 1853–1862.
- Parker, C.E., Warren, M.R., Loiselle, D.R., Dicheva, N.N., Scarlett, C.O. & Borchers, C.H. (2005) Identification of components of protein complexes. *Meth. Mol. Biol.*, **301**, 117–151.
- Petersohn, D., Schoch, S., Brinkmann, D.R. & Thiel, G. (1995) The human synapsin II gene promoter. Possible role for the transcription factor zif268/egr-1, polyoma enhancer activator 3, and AP2. *J. Biol. Chem.*, **270**, 24361–24369.
- Phan, M.L., Pytte, C.L. & Vicario, D.S. (2006) Early auditory experience generates long-lasting memories that may subserve vocal learning in songbirds. *Proc. Natl Acad. Sci. USA*, **103**, 1088–1093.

- Pinaud, R. (2004) Experience-dependent immediate early gene expression in the adult central nervous system: evidence from enriched-environment studies. *Int. J. Neurosci.*, **114**, 321–333.
- Pinaud, R. (2005) Critical calcium-regulated biochemical and gene expression programs involved in experience-dependent plasticity. In: Pinaud, R., Tremere, L.A. & De Weerd, P. (Eds), *Plasticity in the Visual System: from Genes to Circuits*. Springer, New York, pp. 153–180.
- Pinaud, R., Fortes, A.F., Lovell, P. & Mello, C.V. (2006) Calbindin-positive neurons reveal a sexual dimorphism within the songbird analogue of the mammalian auditory cortex. *J. Neurobiol.*, **66**, 182–195.
- Pinaud, R., Penner, M.R., Robertson, H.A. & Currie, R.W. (2001) Upregulation of the immediate early gene arc in the brains of rats exposed to environmental enrichment: implications for molecular plasticity. *Brain Res. Mol. Brain Res.*, **91**, 50–56.
- Pinaud, R. & Tremere, L.A. (2005) Experience-dependent rewiring of retinal circuitry: involvement of immediate early genes. In: Pinaud, R., Tremere, L.A. & De Weerd, P. (Eds), *Plasticity in the Visual System: from Genes to Circuits*. Springer, New York, pp. 79–95.
- Pinaud, R. & Tremere, L.A. (2006) *Immediate Early Genes in Sensory Processing, Cognitive Performance and Neurological Disorders*. Springer, New York.
- Pinaud, R., Tremere, L.A., Penner, M.R., Hess, F.F., Barnes, S., Robertson, H.A. & Currie, R.W. (2002a) Plasticity-driven gene expression in the rat retina. *Brain Res. Mol. Brain Res.*, **98**, 93–101.
- Pinaud, R., Tremere, L.A., Penner, M.R., Hess, F.F., Robertson, H.A. & Currie, R.W. (2002b) Complexity of sensory environment drives the expression of candidate-plasticity gene, nerve growth factor induced-A. *Neuroscience*, **112**, 573–582.
- Pinaud, R., Velho, T.A., Jeong, J.K., Tremere, L.A., Leao, R.M., von Gersdorff, H. & Mello, C.V. (2004) GABAergic neurons participate in the brain's response to birdsong auditory stimulation. *Eur. J. Neurosci.*, **20**, 1318–1330.
- Rushworth, L.K., Hindley, A.D., O'Neill, E. & Kolch, W. (2006) Regulation and role of Raf-1/B-Raf heterodimerization. *Mol. Cell Biol.*, **26**, 2262–2272.
- Stripling, R., Volman, S.F. & Clayton, D.F. (1997) Response modulation in the zebra finch neostriatum: relationship to nuclear gene regulation. *J. Neurosci.*, **17**, 3883–3893.
- Terleph, T.A., Mello, C.V. & Vicario, D.S. (2006) Auditory topography and temporal response dynamics of canary caudal telencephalon. *J. Neurobiol.*, **66**, 281–292.
- Terpstra, N.J., Bolhuis, J.J. & den Boer-Visser, A.M. (2004) An analysis of the neural representation of birdsong memory. *J. Neurosci.*, **24**, 4971–4977.
- Thiel, G., Schoch, S. & Petersohn, D. (1994) Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. *J. Biol. Chem.*, **269**, 15294–15301.
- Valtorta, F., Villa, A., Jahn, R., De Camilli, P., Greengard, P. & Ceccarelli, B. (1988) Localization of synapsin I at the frog neuromuscular junction. *Neuroscience*, **24**, 593–603.
- Vates, G.E., Broome, B.M., Mello, C.V. & Nottebohm, F. (1996) Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches. *J. Comp. Neurol.*, **366**, 613–642.
- Velho, T.A., Pinaud, R. & Mello, C.V. (2002) Synapsin II, a candidate ZENK target, is regulated by song in the songbird NCM. *Soc. Neurosci. Abstr.*, **28**, 382.5.
- Velho, T.A., Pinaud, R., Rodrigues, P.V. & Mello, C.V. (2005) Co-induction of activity-dependent genes in songbirds. *Eur. J. Neurosci.*, **22**, 1667–1678.
- Wada, K., Howard, J.T., McConnell, P., Whitney, O., Lints, T., Rivas, M.V., Horita, H., Patterson, M.A., White, S.A., Scharff, C., Haesler, S., Zhao, S., Sakaguchi, H., Hagiwara, M., Shiraki, T., Hirozane-Kishikawa, T., Skene, P., Hayashizaki, Y., Carninci, P. & Jarvis, E.D. (2006) A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. *Proc. Natl Acad. Sci. USA*, **103**, 15212–15217.
- Wada, K., Sakaguchi, H., Jarvis, E.D. & Hagiwara, M. (2004) Differential expression of glutamate receptors in avian neural pathways for learned vocalization. *J. Comp. Neurol.*, **476**, 44–64.
- Zamponi, G.W. (2003) Regulation of presynaptic calcium channels by synaptic proteins. *J. Pharmacol. Sci.*, **92**, 79–83.
- Zeigler, H.P. & Marler, P. (2004) *Behavioral Neurobiology of Birdsong*. New York Academy of Sciences, New York.
- Zidanic, M. (2002) Cholinergic innervation of the chick basilar papilla. *J. Comp. Neurol.*, **445**, 159–175.