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ZENK Protein Regulation by Song in the Brain of Songbirds

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ABSTRACT

When songbirds hear the song of another individual of the same species or when they sing, the mRNA levels of the ZENK gene increase rapidly in forebrain areas involved in vocal communication. This gene induction is thought to be related to long-term neuronal change and possibly the formation of song-related memories. We used immunocytochemistry to study the levels and distribution of ZENK protein in the brain of zebra finches and canaries after presentation of song playbacks. Birds that heard the playbacks and did not sing in response showed increased ZENK protein levels in auditory brain areas, including the caudomedial neostriatum and hyperstriatum ventrale, fields L1 and L3, the shelf adjacent to the high vocal center (HVC), the cup adjacent to the nucleus robustus archistriatalis (RA), and the nucleus mesencephalicus lateralis pars dorsalis (MLd). No ZENK expression was seen in song nuclei in these birds. Males that sang in response to the playbacks showed, in addition to auditory areas, increased ZENK protein in several song control nuclei, most prominently in HVC, RA, area X, and the dorsomedial nucleus (DN) of the intercollicular complex. The rise in ZENK protein followed that described previously for ZENK mRNA by a short lag, and the distribution of ZENK-labeled cells was in agreement with previous analysis of mRNA distribution. Thus, ZENK protein regulation can be used to assess activation of brain areas involved in perceptual and motor aspects of song. Possible implications of ZENK induction in these areas are discussed. J. Comp. Neurol. 393:426–438, 1998.

Indexing terms: plasticity; memory; auditory; avian; immediate-early gene

Vocal communication through song is a central aspect of songbird biology (Kroodsma and Byers, 1991), and song memories are essential for several aspects of a bird's social life, including song learning and sexual and territorial behaviors (Immelmann, 1969; Brooks and Falls, 1975; Kroodsma, 1982; Marler and Peters, 1982; Godard, 1991; for a recent review, see Kroodsma and Miller, 1996). Recent insights into possible mechanisms related to the formation of song memories originated from the finding that the immediate-early gene (IEG) ZENK, also known as zif-268 (Christy et al., 1988), egr-1 (Sukhatme et al., 1988), NGFI-A (Milbrandt, 1987), and Krox-24 (Lemaire et al., 1988) as well as the c-jun gene (Nishimura and Vogt, 1988) are rapidly induced in the brain of songbirds when they hear playbacks of conspecific song (Mello et al., 1992; Nastiuk et al., 1994). This induction is most prominent in the caudomedial neostriatum (NCM) and the adjacent caudomedial hyperstriatum ventrale (CMHV; Mello and Clayton, 1994), both areas thought to participate in auditory processing (Chew et al., 1995, 1996; Vates et al., 1996; Stripling et al., 1997). ZENK induction in NCM is highest for conspecific songs, lower for heterospecific songs, and absent for pure tones (Mello et al., 1992), suggesting a tuning of NCM neurons to complex auditory stimuli, in particular, those of behavioral relevance. ZENK induction decreases after repeated presentations of the same song but can be elicited again upon presentation of a different conspecific song stimulus (Mello et al., 1995), supporting the notion that NCM participates in auditory processing and song discrimination. More recently, ZENK mRNA induction has also been observed in several telencephalic song control nuclei as a result of active singing behavior in captive as well as in wild songbirds (Jarvis and Nottebohm, 1997; Jarvis et al., 1997).

The findings described above prompted a series of electrophysiological studies to assess directly the role of ZENK-positive brain areas in birdsong processing. Such experiments have demonstrated that NCM neurons fire in response to song presentations and that their responses habituate upon repeated presentations of the same stimulus

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(Chew et al., 1995; Stripling et al., 1997). This habituation is song-specific and long-lasting, according to the relevance of the stimulus presented (Chew et al., 1995, 1996). Moreover, the maintenance of habituation to song depends on genes expressed at discrete time windows after song presentation, as demonstrated by local injections of RNA and protein synthesis inhibitors (Chew et al., 1995, 1996). Presently, long-term habituation in NCM represents one of the most intriguing candidate mechanisms for the formation of long-term song auditory memories.

ZENK encodes a zinc finger transcriptional regulator (Christy and Nathans, 1989) and is thought to exert its action by controlling expression levels of downstream genes that contain ZENK-binding motifs in their promoters (Gupta et al., 1991). Interestingly, ZENK induction also correlates with induction of hippocampal long-term potentiation, or LTP (Cole et al., 1989; Wisden et al., 1990), a form of activity-related synaptic plasticity that depends for its maintenance on RNA and protein synthesis (Nguyen et al., 1994) and that has been postulated as a substrate for memory formation in rodents (Bliss and Collingridge, 1993). A possibility suggested by this parallel in mammals is that ZENK represents an early component of the gene-regulatory cascade necessary for long-term habituation of NCM neurons to song (Goelet et al., 1986; Morgan and Curran, 1989; Chew et al., 1995, 1996). To elucidate the precise functional significance of ZENK induction, however, one needs to determine precisely when and where the ZENK protein is present, so that it can exert its action on downstream genes.

We have used an immunocytochemical assay to determine the time course of ZENK protein expression and the brain distribution of ZENK protein after presentation of song playbacks to zebra finches and canaries. We demonstrate that the ZENK protein is expressed in areas involved in song perception and production, in accordance with previous descriptions of ZENK mRNA distribution after song presentation. In NCM, ZENK protein levels peak 1-2 hours after stimulus onset, but cell nuclei labeled for ZENK protein can be observed as early as 15 minutes after start of stimulation. Our results provide a detailed map of brain areas involved in vocal communication in songbirds and establish ZENK immunocytochemistry (ICC) as a powerful method for functional brain mapping, at cellular resolution, of a naturally occurring behavior.

MATERIALS AND METHODS

Song presentation

A total of 30 adult male zebra finches (*Taeniopygia guttata*) and ten (four males and six females) adult canaries (*Serinus canaria*) bred in aviaries located at the Rockefeller University Field Research Center (Millbrook, NY) were used. The protocols utilized were approved by the Rockefeller University's animal care facility (LARC) and animal use committee and are in conformity to NIH guidelines. The birds were placed individually in soundproof chambers for 1 day and were then presented with playbacks of conspecific song; unstimulated controls heard only silence. The stimulus tapes contained song from three different males of the same species, as detailed previously (see Mello and Clayton, 1994). The stimulation period was 30 minutes, during which the birds' vocal responses were monitored continuously. The playbacks were presented through speakers installed inside the isolation chambers; during both the isolation period and the stimulation period, the birds were kept inside the isolation chambers, and no novel stimuli other than the playbacks were presented. For analysis of the anatomical distribution of ZENK protein, birds were divided into two groups, according to their response to the song playbacks (as described in Jarvis and Nottebohm, 1997): 1) Most birds did not sing in response to the playbacks and were therefore designated the "hearing-only" group; this group includes all stimulated canaries (of both sexes) that did not sing in response to playbacks under our experimental conditions as well as the zebra finches used for the analysis of ZENK induction kinetics; and 2) a small number of zebra finches (n = 3) that had a high frequency of spontaneous singing responded to the song playbacks with countersinging and was designated the "hearing-and-singing" group. After a survival period that varied, as detailed in Results, the birds were killed. Another group of birds (n = 3) was killed 90 minutes after injection with metrazole, a potent γ-aminobutyric acid (GABA)ergic antagonist that leads to overall brain depolarization and widespread ZENK gene induction (Mello and Clayton, 1995).

Immunocytochemistry

Two rabbit polyclonal anti-ZENK antisera that recognize the same carboxy-terminal peptide in the rodent ZENK protein sequence were used. The first antiserum was generated by Dr. David Hancock at the Imperial Cancer Research Fund (United Kingdom) and was used previously in studies of olfactory learning in rats (Brennan et al., 1992). The second antiserum was a commercial anti-egr-1 antiserum (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) raised against the carboxy-terminus of mouse egr-1. Although both antisera gave essentially the same results, we utilized the first antiserum only for pilot experiments in the initial phases of this project; all of the data presented in this report, including ICC and immunoblot, were obtained with the commercial antiserum. Birds were killed under deep Nembutal anesthesia (Abbott Laboratories, Chicago, IL) and perfused sequentially with phosphate-buffered saline (PBS), pH 7.4, and 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were then dissected out, washed overnight at 4°C in 0.1 M PB under agitation, submerged in 20% sucrose in 0.1 M PB, and frozen in embedding medium (TissueTek; Sakura Finetek, Torrance, CA) in a dry-ice/ethanol bath.

Twenty-micrometer serial parasagittal sections were cut on a cryostat, mounted onto slides precoated with chromalum or 3-aminopropyl triethoxysilane (TESPA; Aldrich, Milwaukee, WI), and air dried overnight. Particular care was taken to align all brains in the same orientation prior to cutting and to keep track of section number from the midline in order to minimize variability in the resulting morphology of brain structures across animals at each particular parasagittal level. Slides were then sequentially incubated as follows: 1) 30 minutes at room temperature in blocking solution (0.5% albumin and 0.3% Triton X-100 in 0.1 M PB); 2) 36–48 hours at 4°C in blocking solution containing anti-ZENK antiserum (0.1 μg/ml; Santa Cruz Biotechnology); 3) 15 minutes at room temperature in avidin blocking solution followed by 15 minutes at room temperature in biotin blocking solution (Vector blocking kit, dilutions as recommended by Vector Laboratories, Burlingame, CA); 4) 2 hours at room temperature in blocking solution containing biotinylated goat anti-rabbit

immunoglobulin (IgG; 1:200 dilution; Vector Laboratories); and 5) 2 hours at room temperature in avidin/biotin complex (ABC) reagent (Vector Laboratories). Each of the steps above was followed by three washes (10 minutes each) in 0.1 M PB. The slides were then developed by incubation in 0.03% diaminobenzidine (DAB), 0.15% nickel-ammonium sulfate, and 0.001% H_2O_2 in PB followed by rinsing in PB, dehydration, and coverslipping with Permount. Controls were run by omitting the primary anti-ZENK antiserum or by using a preabsorbed antiserum in step 2 above. The preabsorption was performed by incubating the anti-ZENK antiserum at the working dilution with a tenfold excess (by weight) of the corresponding preabsorption peptide, essentially as recommended by the manufacturer (Santa Cruz Biotechnology). Adjacent sections were stained with cresyl violet to allow visualization of brain structures. We follow the anatomical nomenclature as detailed in Stokes et al. (1974) and Vates et al. (1996). For brain location and orientation of the structures shown throughout this report, please refer to Figure 3.

Immunoblot

A protocol modified from Harlow and Lane (1988) was used. Birds stimulated with song playbacks or injected with metrazole were killed under deep Nembutal anesthesia and quickly perfused with PBS. The brains were quickly removed, and NCM and other brain regions were dissected out, frozen, and stored at −70°C. The tissue samples were thawed, placed in 1.5-ml plastic tubes, and homogenized with small plastic pestles in five volumes of sample buffer (60 mM Tris, pH 6.8, containing 100 mM dithiothreitol [DTT] and 2% sodium dodecyl sulfate [SDS]). The homogenate was then boiled for 5 minutes, sheared by ten passes through a 26-gauge needle, and centrifuged at ×12,000 g for 20 minutes at 4°C. The pellet was discarded, and protein in the supernatant was quantified by spectrophotometry. Ten micrograms of protein in sample buffer (containing 0.005% bromophenol blue) were then resolved with unidimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted overnight at 4°C onto nitrocellulose. The filters were washed in 0.1 M PB, stored dry, and then incubated sequentially as follows: 1) 30 minutes in blocking solution (3% fresh skim milk in 0.1 M PB); 2) overnight at 4°C in blocking solution containing anti-ZENK antiserum (0.01 µg/ml); 3) 2 hours at room temperature in blocking solution containing biotinylated goat anti-rabbit IgG (1:200 dilution); and 4) 2 hours at room temperature in ABC reagent (Vector Laboratories) in PB. Each of the steps above was followed by several washes in 0.1 M PB, and the blot was developed by the enhanced chemiluminescence (ECL) method (Amersham, Arlington Heights, IL).

Quantification of ZENK kinetics

To describe the kinetics of ZENK protein expression in NCM after onset of the playback, a quantification of the relative increase in the population of ZENK-labeled nuclei per unit area as a function of time was performed. For this purpose, we chose one particular parasagittal plane (150 µm from the midline) and counted ZENK-labeled nuclei within NCM at this same parasagittal plane for all birds in the kinetic analysis. This particular plane was chosen for two primary reasons: 1) At this level, other structures, including field L and CMHV, are absent from the caudomedial telencephalon, and NCM is very clearly defined as an approximately spherical structure bound by the ventricular epithelium on all sides (as depicted by the diagram in Fig. 3A and the maps in Fig. 2B); thus, the issue of the lateral boundary of NCM, which is still a matter of contention, can be avoided. 2) This plane is close to the midline and medial to field L; thus, it is well within the area that presents by far the highest levels of ZENK gene expression within the auditory telencephalon (as in Mello and Clayton, 1994).

To avoid sampling bias, all ZENK-labeled nuclei present within NCM in each section analyzed were counted, and the resulting number was divided by the total NCM area in that particular section to generate a density value; two immediately adjacent sections were counted per bird, and the results were averaged. The actual mapping and counting of ZENK-labeled nuclei were performed with a computer-yoked microscope (Alvarez-Buylla and Vicario, 1988). The mappings were performed blind by using an inclusive criterion for labeling, i.e., both weakly and strongly labeled nuclei (see Fig. 2A) were counted; no attempts were made to quantify the relative amounts of protein per labeled nucleus. The counts obtained for each time point were compared with values obtained for unstimulated controls by using the Mann-Whitney U test and a significance criterion of $P < 0.05$.

To control for a possible effect of song presentation on the total NCM area per section or on the morphology of ZENK-labeled nuclei, which could affect our density estimates, we compared 1) the average area occupied by NCM in our parasagittal sections between unstimulated controls and all song-stimulated birds used in the kinetic analysis; and 2) the average area occupied by randomly picked, ZENK-labeled nuclei in our ICC preparations between unstimulated controls and song-stimulated birds from the 30-minute survival group. The resulting values (presented as means ± S.E.M.) had nearly normal distributions, and the comparisons were performed by using an unpaired t-test and a significance criterion of $P < 0.05$ (see Results).

Figure preparation

Selected brain areas were photographed with TechPan film (Kodak, Rochester, NY), and negatives were digitized with a Nikon scanner (Tokyo, Japan). Image-analysis software (Adobe Photoshop; Adobe Systems, Mountain View, CA) was used to adjust gray level and for formatting and lettering of figures without making any changes to the data.

RESULTS

Specificity of anti-ZENK antisera

The ZENK amino acid sequence is very conserved between songbirds and mammals (Milbrandt, 1987; Sukhatme et al., 1988; Mello, 1993). The antisera used in the present study were raised against the peptide consisting of the last 19 amino acid residues predicted by the rodent and human sequences (Wisden et al., 1990; Brennan et al., 1992). A direct comparison of this peptide with the predicted amino acid sequence of canary ZENK (Fig. 1A) reveals a high degree of conservation (close to 70% identity at the amino acid level) among these different animal groups; note that two out of the six substitutions are conservative. When it was run on a GeneBank homology search, the sequence encoding this peptide revealed similarities only to ZENK homologues cloned in various species.

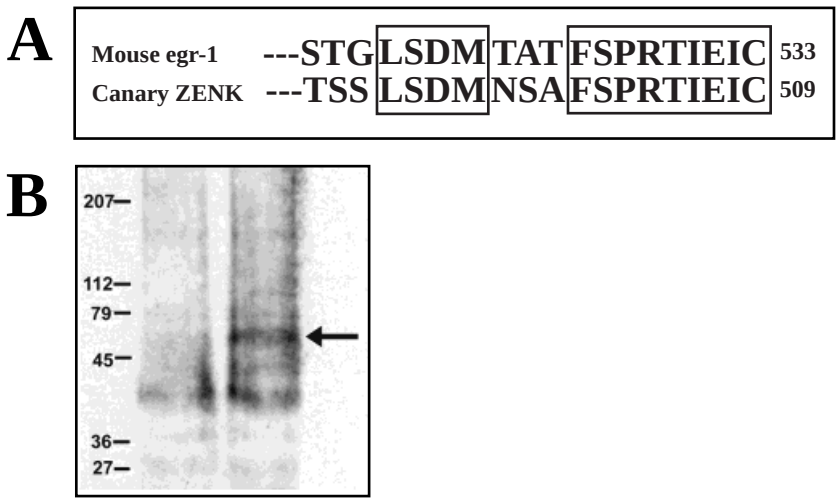

Fig. 1. **A:** Sequence comparison between the predicted carboxy-terminal peptide of the mammalian (top line) and canary (bottom line) ZENK homologues against which the antisera used in this report were raised. Shown are the last 19 amino acids; the regions of identity are enclosed by rectangles, and the numbers on the right indicate the number of the last amino acid residue in each respective sequence. **B:** Immunoblot detection of ZENK protein. Brain extract from a bird killed 2 hours after a metrazole injection (**right lane**) is compared with that of an unstimulated control (**left lane**). An inducible band specifically labeled with the anti-ZENK antiserum is indicated by the arrow. Other noninduced bands on this blot are revealed by incubation with the detection system alone (avidin/biotin complex [ABC] reagent plus enhanced chemiluminescence [ECL]; not shown) in the absence of incubation with the primary antiserum; thus, they are nonspecific.

On immunoblots, a protein of 62.5 kDa apparent weight could be recognized that was induced by brain depolarization (Fig. 1B, arrow). The blot shown in Figure 1B was incubated with the same detection system used for ICC (ABC reagent; Vector Laboratories) before the ECL step. When incubation with the primary antibody was omitted, the band indicated by the arrow was not detected, although the other noninduced bands still appeared under such conditions (not shown). Thus, the other bands are a result of nonspecific binding of the detection system to some tissue components. This effect can be minimized both on blots and with ICC by using Vector's Elite kit and/or performing the avidin/biotin blocking steps.

In the mammalian brain, the ZENK gene is expressed in neuronal cells, and the corresponding protein has a nuclear localization (Worley et al., 1991; Herdegen et al., 1993; Okuno et al., 1995). In songbirds, ZENK mRNA induction by song playbacks or metrazole is also known to occur in neuronal cells (Mello and Clayton, 1994, 1995). The anti-ZENK antisera used here, as expected, recognized a nuclear protein present in relatively few cells in unstimulated birds. The number of these cells increased greatly after song playbacks (Fig. 2A, compare top and middle photomicrographs) or metrazole administration (Fig. 9). In both cases, the resulting staining was restricted to nuclei, and cytoplasmic labeling was absent. When incubation with the primary antiserum was omitted, no nuclear staining was observed, although long incubations in DAB led to increased generalized background staining in all animals, particularly when the avidin/biotin blocking step was not performed. In addition, preabsorption of the antiserum with the specific peptide against which it was raised resulted in complete loss of specific nuclear staining, even in sections from stimulated birds (Fig. 2A, bottom). We conclude that the antisera used for ICC specifically recognize the protein encoded by the avian homologue of ZENK in our songbird brain sections. Interestingly, the intensity of the staining varied from cell to cell: Some nuclei were labeled very strongly, and others were less so (Fig. 2A, middle), indicating differences in ZENK protein content per nucleus. We did not explore this observation any further.

Kinetics of ZENK protein regulation in NCM

We initially focused our analysis on NCM, a region where ZENK induction by song has been well characterized at the mRNA level (Mello and Clayton, 1994; Mello et al., 1995). To study the time course of ZENK protein expression, male zebra finches were presented with song playbacks for 0.5 hour and were killed at 0.5, 1.0, 2.0, 4.0, or 6.0 hours (n = 3 per time point) after stimulus onset. Another four birds served as unstimulated controls. Brain sections through NCM at the 150-μm parasagittal plane (as indicated by the dashed rectangle in Fig. 3A) were processed for ZENK ICC and then mapped and counted for ZENK-labeled nuclei. The results are shown in Figure 2B,C. Relatively few ZENK-positive cells could be seen in unstimulated controls. A large number of ZENK-labeled nuclei above control levels was seen as early as 30 minutes after start of stimulation, but their number peaked between 1 and 2 hours after stimulus onset and declined thereafter. The density of ZENK-labeled nuclei in song-stimulated birds was significantly higher than in unstimulated controls for all time points analyzed ($U = 0$; $P < 0.05$; Mann Whitney U test), except for the 6-hour survival group. In addition to the time points shown on the graph (Fig. 2C), an increase in ZENK-labeled nuclei was apparent as early as 15 minutes after start of the stimulus, but not at 7 hours (not shown; n = 1 for these time points).

In contrast to the density of ZENK-labeled nuclei, no significant differences were detected (Student's t-test; criterion of $P < 0.05$) when the average area occupied by NCM in our preparations was compared between unstimulated controls (1.355 ± 0.045 mm^2; n = 4) and song-stimulated birds (1.378 ± 0.048 mm^2; n = 15). Neither

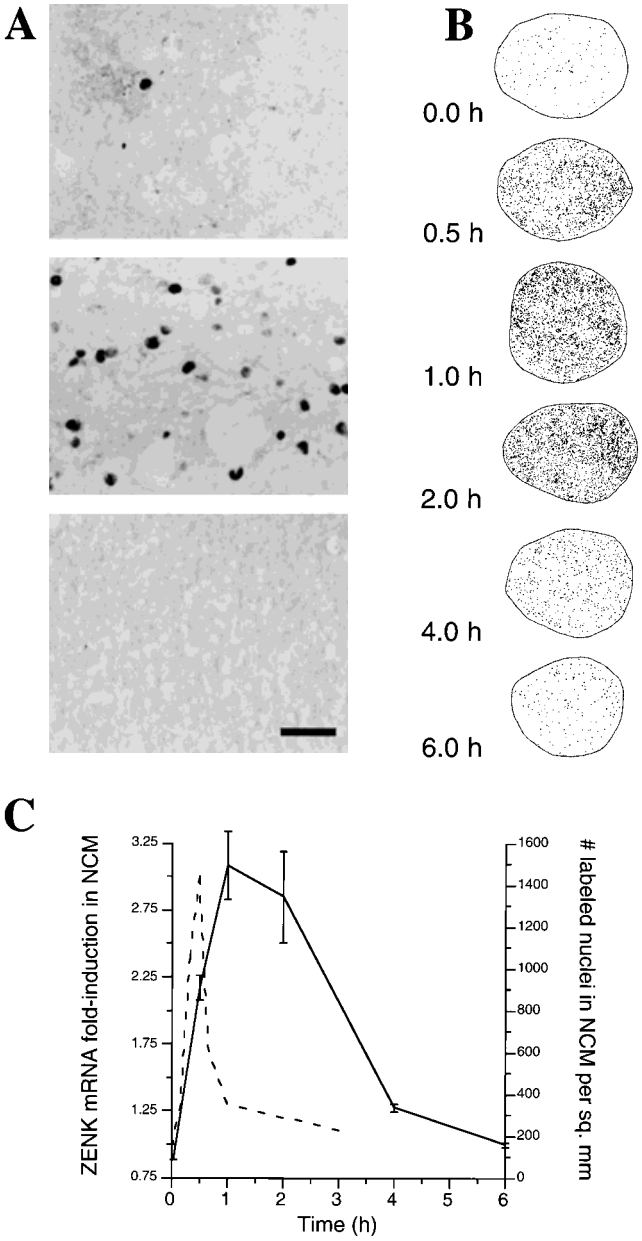

Fig. 2. ZENK expression at the protein level in the caudomedial neostriatum (NCM) after song presentation. **A:** High-power views of sections through NCM from an unstimulated control (**top**) and from a stimulated bird from the "hearing-only" group (**middle**) processed for ZENK immunocytochemistry (ICC). The staining observed is essentially nuclear, with some cell-to-cell variability in intensity; notice the marked increase in the number of ZENK-labeled nuclei after song. Preincubation of the ZENK antiserum with the peptide against which the antiserum was raised results in complete loss of nuclear staining (**bottom**; section adjacent to that shown in the middle). **B:** Kinetics of ZENK protein increase in NCM after song. Maps of ZENK-labeled nuclei at several delays after song stimulation are shown (one representative example per time point). The section level is indicated in Figure 3A. **C:** Quantification of ZENK protein expression in NCM after song presentation. Shown are the average densities (number of nuclei per unit area ± S.E.M.; y axis on the right) of ZENK-labeled nuclei (n = 3 per time point). Unstimulated controls (n = 4) are plotted at time = 0. The values for 0.5, 1.0, 2.0, and 4.0 hours are significantly higher than unstimulated controls (U = 0; $P < 0.05$; Mann-Whitney U test). For comparison, the time course of ZENK mRNA induction by song in NCM (adapted from Mello and Clayton, 1994; Mello et al., 1995) is represented diagrammatically by the dashed line (y axis on the left). Scale bar = 50 µm.

were systematic differences observed in the shape of NCM across the various groups (as illustrated by the maps in Fig. 2B). These findings attest to the consistency of our histological preparations. Similarly, no significant changes were detected (Student's t-test; criterion of $P < 0.05$) in the average area occupied by ZENK-labeled nuclei in a comparison between unstimulated controls (19.43 ± 0.72 µm^2; n = 101 nuclei) and song-stimulated animals from the 30-minute survival group (20.55 ± 0.51 µm^2; n = 192). In addition, no consistent changes after song exposure were observed in the shape of ZENK-labeled nuclei (Fig. 2A) or in their distribution throughout NCM, which remained relatively homogeneous at all times analyzed (Fig. 2B). These findings support the conclusion that exposure to song does not lead to obvious, rapid morphological changes in ZENK-labeled nuclei in NCM, which could have offset our estimate of the increase in their density in this area.

Brain distribution of ZENK protein after song presentation

We analyzed series of parasagittal sections throughout the brain at levels containing various auditory and song control structures in which ZENK mRNA induction by song had been characterized previously (Mello and Clayton, 1994). These structures are represented by the dashed rectangles in the camera lucida drawings in Figure 3 and are shown in greater detail in Figures 4–9. No differences in brain distribution of ZENK-labeled nuclei were observed in comparisons between female and male canaries or between canaries and zebra finches. This was true for birds in both the unstimulated controls and the hearing-only groups. All of the results presented below for unstimulated controls, for the hearing-only group, and for the hearing-and-singing group were obtained in zebra finches (no canaries sang back to the playbacks in our study).

In general, relatively few ZENK-positive cell nuclei were seen in most structures throughout the forebrain of unstimulated controls (n = 4), including all auditory and song control areas analyzed. Cell nuclei labeled for ZENK protein in controls were more numerous in the rostral and rostromedial portions of the hyperstriatum accessorium (HA) and hyperstriatum dorsale (HD) and were somewhat less numerous in the hyperstriatum ventrale (HV) and paleostriatum (P; not shown), an effect that had not been noticed previously for ZENK mRNA. In HA and HD, there was probably a partial overlap with areas of visual representation (Karten et al., 1973). Expression in the areas above was unaffected by song presentation or singing behavior.

For song stimulation, we analyzed birds killed 1.5 hours after stimulus onset (n = 4), which is well within the peak of ZENK protein expression in NCM, as shown in Figure 2. The results were in agreement with previous descriptions of ZENK mRNA induction after song stimulation (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). Besides NCM (Figs. 2, 4B,4D), ZENK-labeled nuclei were also abundant in the caudal hyperstriatum ventrale in stimulated birds from both the hearing-only (Fig. 4D) and the hearing-and-singing (not shown) groups, but not in unstimulated controls (Fig. 4A,C). This effect was observed in both the medial and the lateral subdivisions of the caudal hyperstriatum ventrale, respectively CMHV (Fig. 4D) and CLHV (not shown). For a detailed definition of these areas based on their connectivity, see Vates et al. (1996).

Other regions that contained a marked increase in ZENK protein expression after song stimulation included field L (subdivisions L1 and L3) and the caudodorsal

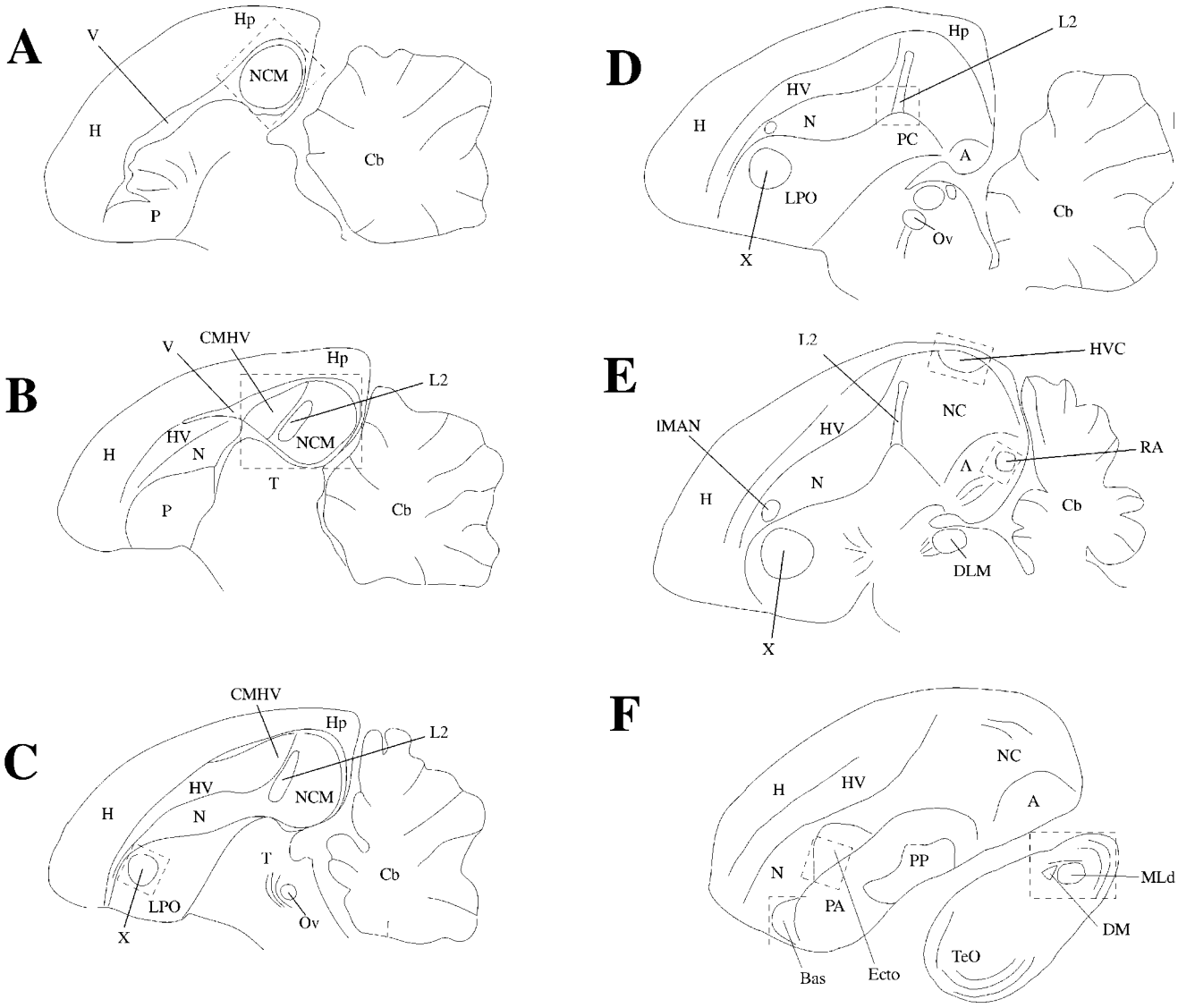

Fig. 3. Camera lucida drawings of serial parasagittal sections of the brain of an adult male zebra finch indicating the brain levels presented in this report. Represented are the planes 0.15 mm (**A**), 0.25 mm (**B**), 1.0 mm (**C**), 1.4 mm (**D**), 2.2 mm (**E**), and 2.8 mm (**F**) lateral to the midline. Orientation: Dorsal is up, and rostral is to the left in A–F. The dashed rectangles enclose various auditory regions, song control nuclei, and primary sensory areas that are shown in detail in Figures 4–10. Some dashed rectangles (in A, E, and F) were rotated relative to this figure to generate the images in Figures 4A,B, 6, 7, and 10C; in Figures 4–10, all other views are shown at the same orientation as that in this figure. A, archistriatum; Bas, nucleus basalis; Cb, cerebellum; CMHV, caudomedial hyperstriatum ventrale; DLM, medial dorsolateral nucleus of the thalamus; DM, dorsomedial nucleus of the intercollicular complex; Ecto, ectostriatum; H, hyperstriatum; Hp, hippocampus; HV, hyperstriatum ventrale; HVC, high vocal center; L2, L2 subfield of field L; LPO, lobus paraolfactorius; lMAN, lateral magnocellular nucleus of the anterior neostriatum; MLd, nucleus mesencephalicus lateralis, pars dorsalis; N, neostriatum; NC, caudal neostriatum; NCM, caudomedial neostriatum; Ov, nucleus ovoidalis; P, paleostriatum; PA, paleostriatum augmentatum; PC, caudal paleostriatum; PP, paleostriatum primitivum; RA, robust nucleus of the archistriatum; T, thalamus; TeO, optic tectum; V, ventricle; X, area X. Scale bar = 1 mm.

paleostriatum (PC; Fig. 5). In the latter region, several ZENK-labeled nuclei were present in unstimulated controls, but their number increased visibly after song presentation. ZENK-positive nuclei were also abundant within a 300–500 μm band immediately ventral to song nucleus high vocal center (HVC) after song stimulation (Fig. 6B,C), but not in unstimulated controls (Fig. 6A). This distribution corresponds to the HVC shelf, as defined by its connectivity, i.e., the region containing terminations of fibers that originate in fields L1 and L3 and CLHV (Kelley and Nottebohm, 1979; for a detailed description of the HVC shelf, see Vates et al., 1996). Fewer nuclei labeled for ZENK were seen in the archistriatum adjacent to song nucleus robust of the archistriatum (RA), particularly its rostroventral portion (for a bird from the hearing-and-singing group, see Fig. 7B), i.e., the RA cup (Kelley and Nottebohm, 1979). This is a relatively small but significant effect, because ZENK-labeled nuclei are practically absent in this area in unstimulated controls (Fig. 7A). ZENK-labeled nuclei were also seen in auditory nucleus mesencephalicus lateralis, pars dorsalis (MLd) in stimulated birds (Fig. 9A), but not in unstimulated controls (not shown). In contrast, ZENK protein was notably absent in fields L2a (Fig. 5B) and L2b and in thalamic nucleus ovoidalis (not

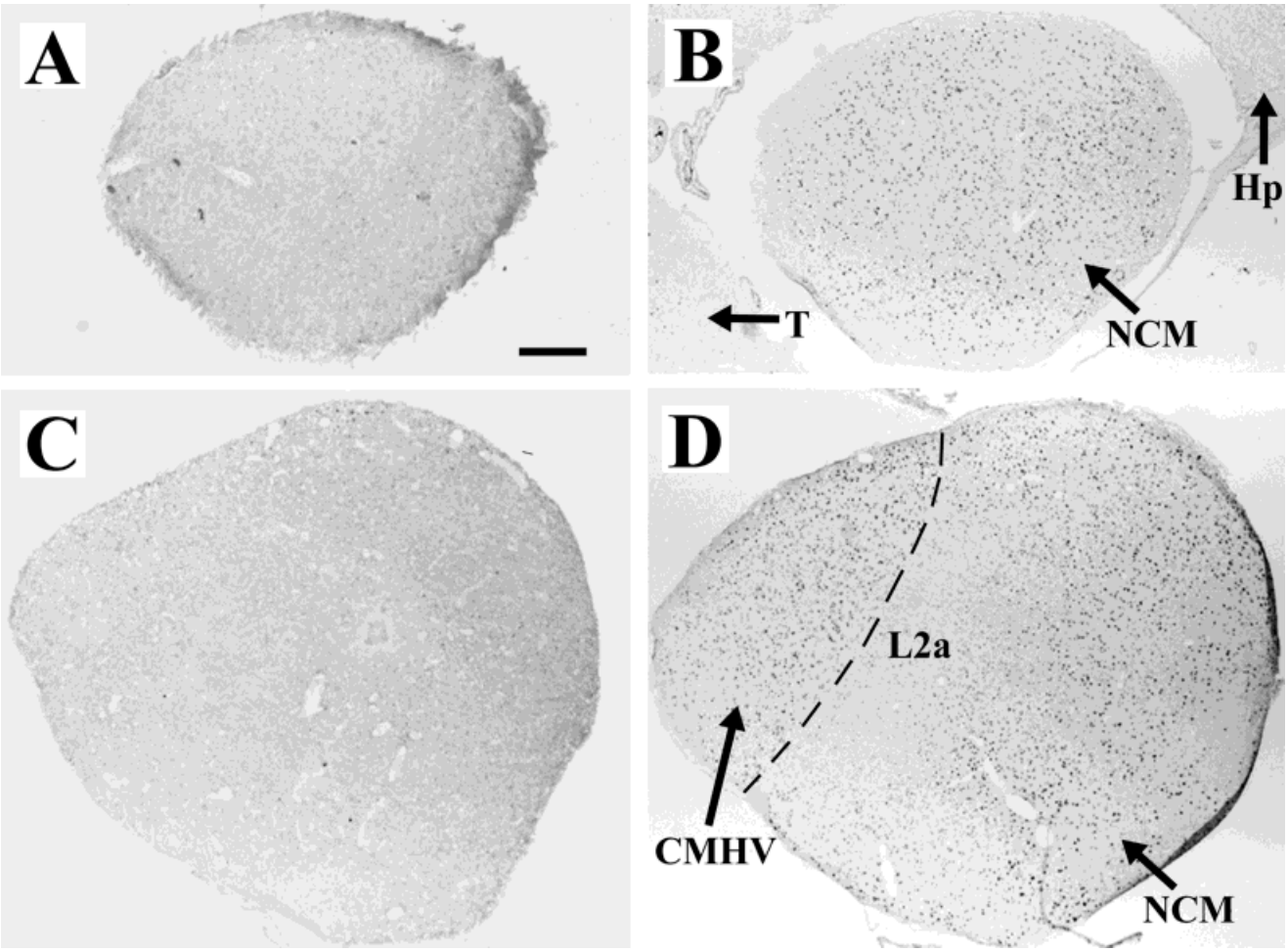

Fig. 4. **A-D:** ZENK expression in the caudomedial telencephalon. The areas shown in photomicrographs correspond to the areas enclosed by the dashed rectangles in Figure 3A (0.15 mm parasagittal plane) and in Figure 3B (0.25 mm parasagittal plane), respectively. Notice the presence of numerous ZENK-labeled nuclei after song stimulation in caudomedial neostriatum (NCM) and caudomedial hyperstriatum ventrale (CMHV) but not in L2a in a bird from the hearing-only group (B,D) in contrast to an unstimulated control (A,C). For orientation, see Figure 3. Hp, hippocampus; L2a, L2a subfield of field L; T, thalamus. Scale bar = 500 µm.

shown) in all birds studied. No apparent increase in ZENK protein expression occurred in other brain areas. The nuclei that precede MLd in the ascending auditory pathway were not studied.

The induction described above for auditory structures occurred in all stimulated birds, irrespective of their behavioral response to the playbacks. In contrast, ZENK protein expression in several nuclei of the song control system (Nottebohm et al., 1976, 1982; Bottjer et al., 1984) depended on the birds' vocal response to stimulation. In the hearing-only group, ZENK-labeled nuclei were conspicuously absent in all song nuclei analyzed, including HVC (Fig. 6B), area X of the paleostriatum (Fig. 8A), and the dorsomedial (DM) nucleus of the intercollicular (ICo) complex (Fig. 9A) as well as RA, the medial and lateral magnocellular nuclei of the anterior neostriatum (mMAN and lMAN, respectively), the nucleus interfascialis (NIf), and the thalamic nucleus uvaeformis (Uva; not shown). In contrast, ZENK-labeled nuclei were abundant in several of these nuclei in birds from the hearing-and-singing group. The most pronounced expression occurred in HVC (Fig. 6C), RA (Fig. 7B), and area X (Fig. 8B).

A high number of ZENK-labeled nuclei in the animals that sang was also observed in DM (Fig. 9B). This nucleus is seen in our ICC preparations of parasagittal brain sections as a small and distinct area in the rostral intercollicular (ICo) complex that stands out from the surrounding tissue due to a higher background staining. We established the identity of this area by comparing it with previously published definitions of DM in zebra finches, based on its connectivity (Vicario, 1991; Wild et al., 1997; for an alternative classification and nomenclature of nuclei within the general avian ICo area, see Puelles et al., 1994). ZENK-labeled cells were also seen occasionally in the ICo outside of DM (Fig. 9B), but their occurrence and distribution was not consistent across individuals or groups. Similarly, some ZENK-labeled nuclei were seen in lMAN and in the medial dorsolateral nucleus of the thalamus (DLM) in the hearing-and-singing group (not shown), but there was considerable variability in these areas, and, in some cases, ZENK-positive cells were absent. The intensity of the staining in lMAN was also rather weak, suggesting a lesser content of ZENK protein per cell. In DLM, several ZENK-labeled nuclei were already present

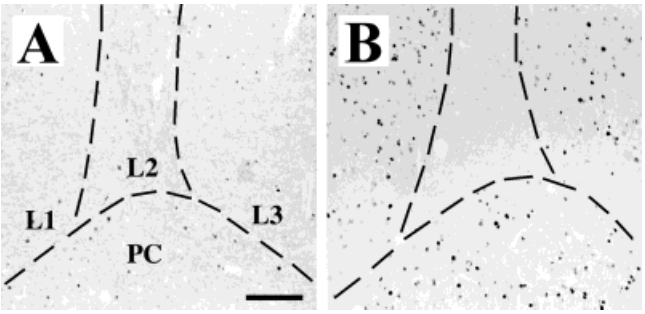

Fig. 5. ZENK expression in field L and paleostriatum. The area shown in photomicrographs **A** and **B** corresponds to the area enclosed by the rectangle in Figure 3D (1.4 mm parasagittal plane). The dashed lines are drawn over the boundaries that separate field L subdivisions and the paleostriatum. Notice the marked increase in ZENK-labeled nuclei in L1, L3, and PC, but not in L2, in a stimulated bird from the hearing-only group (B) compared with an unstimulated control (A). Orientation: Dorsal is up, and anterior is to the left. L1–L3, subfields of field L; PC, caudal paleostriatum. Scale bar = 100 μm.

in unstimulated controls; thus, ZENK protein appears not to be regulated by activity in this area.

In all other brain areas, ZENK-labeled cell nuclei were either absent, as in most of the brainstem, the ventricular zone, meninges, choroid plexus, and telencephalic fibrous laminae, or variable, as in the vicinity of thalamic nucleus ovoidalis and in the cerebellum (not shown). The latter was usually negative, but, occasionally, a few labeled cells were seen in the granule cell layer. Individual structures within the hypothalamus are not easily identified in parasagittal sections and were not studied in detail.

Distribution of ZENK protein after metrazole

This group served as a positive induction control to optimize the ICC protocol for ZENK protein detection, and it provided an independent assessment of ZENK protein expression by brain depolarization in songbirds. The distribution of ZENK protein throughout the brain was similar to that described previously for ZENK mRNA after metrazole administration (Mello and Clayton, 1995). Most forebrain subdivisions showed homogeneous increases in the number of ZENK-labeled nuclei, particularly the hyperstriatum ventrale and the paleostriatum (including area X). Fewer labeled nuclei were seen within song nuclei HVC, lMAN, and RA (not shown). In contrast, very few or practically no ZENK-labeled cell nuclei were seen in field L2, nucleus basalis, and the ectostriatum (Fig. 10).

DISCUSSION

We have presented a detailed description of the regulation of ZENK protein by song in the brain of zebra finches. Birds that hear conspecific song without vocalizing express ZENK protein in several structures of the central auditory pathways. Birds that sing upon hearing song, in addition, show ZENK protein expression in several song control nuclei. The present results are in agreement with the patterns of ZENK mRNA expression after song stimulation that were described previously (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997), and they provide a direct demonstration of the time and brain sites where

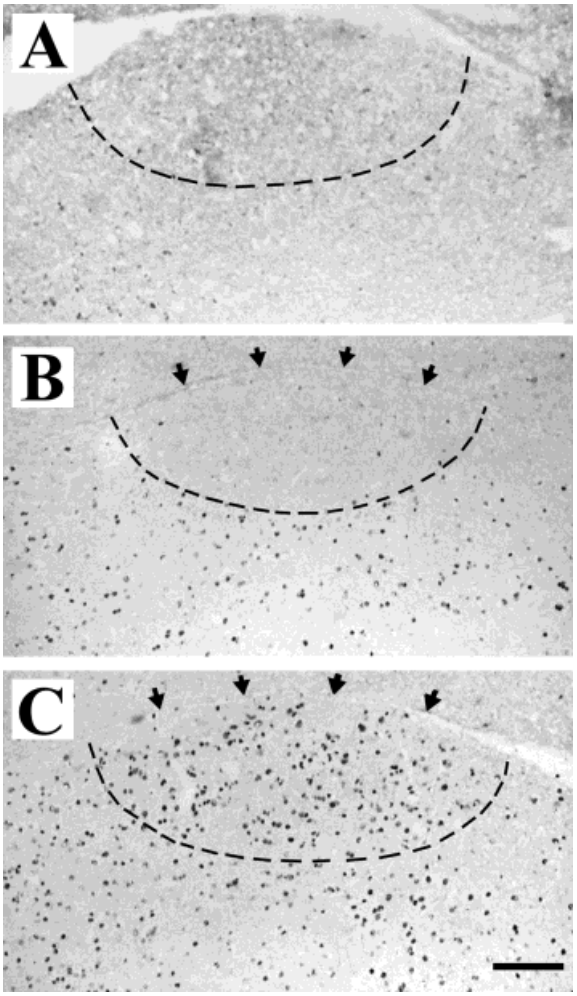

Fig. 6. ZENK expression in the caudodorsal neostriatum. The area shown in photomicrographs **A–C** corresponds to the area enclosed by the upper dashed rectangle in Figure 3E (2.2 mm parasagittal plane) and includes nucleus high vocal center (HVC), the HVC, shelf and the hippocampus. The dashed lines delimit the ventral boundary of HVC and the arrows in B and C indicate the dorsal boundary of HVC, i.e., the ventricular zone. The latter is expanded in A due to detachment of the overlying hippocampus. Notice the increased number of ZENK-labeled nuclei only in the HVC shelf of a stimulated bird from the hearing-only group (B) and in both HVC and the shelf in a stimulated bird from the "hearing-and-singing" group (C) compared with an unstimulated control (A). For orientation, see Figure 3. Scale bar = 100 μm.

ZENK protein is present and exerts its action(s) on downstream genes.

ZENK mRNA induction by song in NCM is known to be quite rapid, peaking at 30 minutes and returning to control levels 60–90 minutes after stimulus onset (Mello and Clayton, 1994; Mello et al., 1995). The present report shows that ZENK protein expression is more protracted: The number of labeled nuclei peaks at 1–2 hours after stimulus onset. However, the fact that an increase in the number of ZENK-labeled cell nuclei can be seen as soon as 15 minutes after stimulus onset indicates that some cells have a rather fast rate of ZENK protein synthesis and transport into the nucleus.

This time course of expression in NCM is compatible with ZENK protein playing an active role as a transcrip-

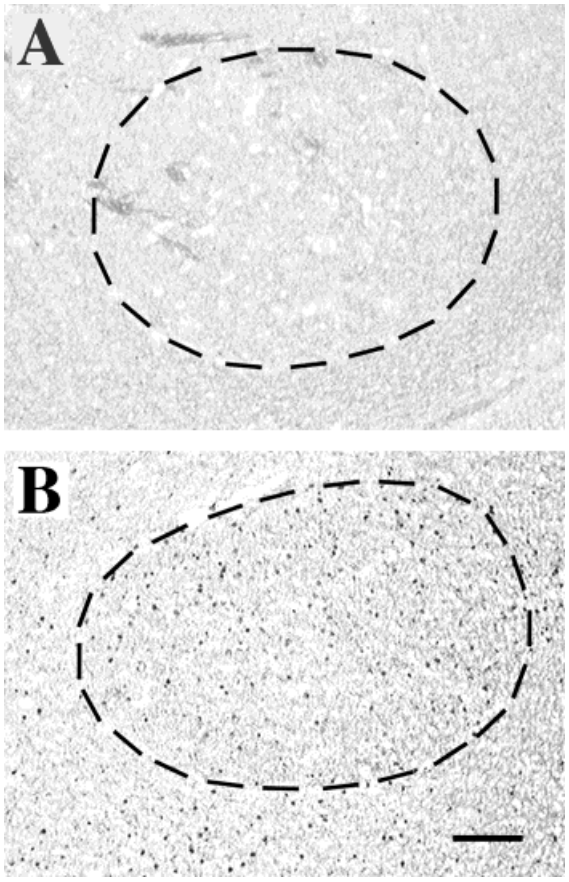

Fig. 7. ZENK expression in the intermediate archistriatum. The area shown in photomicrographs **A** and **B** corresponds to the area enclosed by the lower rectangle in Figure 3E (2.2 mm parasagittal plane) and contains nucleus robust nucleus of the archistriatum (RA; borders delimited by the dashed lines) and its surroundings. Notice the marked increase in ZENK-positive nuclei both within RA and in the surrounding archistriatum, particularly rostroventrally to RA (RA cup), in a bird from the singing and hearing group (B) compared with an unstimulated control (A). For orientation, see Figure 3. Scale bar = 100 μm.

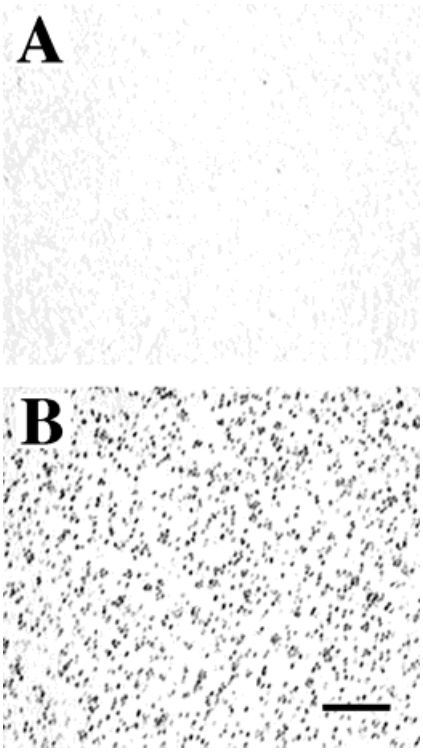

Fig. 8. ZENK expression in area X. The area shown in photomicrographs **A** and **B** corresponds to the area enclosed by the dashed rectangle in Figure 3C (1.0 mm parasagittal plane). Notice the absence of ZENK-positive nuclei in a stimulated bird from the hearing-only group (A) and the great increase in their number in a stimulated bird from the hearing-and-singing group (B); ZENK-labeled nuclei were not observed in unstimulated controls (not shown). Orientation: Dorsal is up, and anterior is to the left. Scale bar = 100 μm.

tional regulator during the first window of gene expression that follows NCM activation by song and that is necessary for long-term habituation (Chew et al., 1995, 1996). Genes whose transcription is regulated directly by ZENK are also likely to fall within this window, particularly its late phase, which extends to about 2.5 hours after stimulus onset. ZENK-regulated genes potentially include other IEGs and the ZENK gene itself, upon whose transcription ZENK protein could exert an inhibitory action, as described for c-fos (Sassone-Corsi et al., 1988). In contrast, neither ZENK mRNA nor ZENK protein (studied up to 6 hours and 7 hours after stimulus onset, respectively; Mello et al., 1995; present report) is present during the second window of gene expression (5.5–7.5 hours after stimulus onset). Thus, this second window appears not to represent a reinduction of all of the same activity-dependent genes as those of the first window. This argues, albeit indirectly, against an electrophysiological reactivation of NCM neurons during the second window, a mechanism that has been suggested in connection with memory consolidation in other systems (Grecksch and Matthies, 1980; Freeman et al., 1995). Alternatively, NCM cells could be reactivated without resulting in ZENK reinduction if the machinery that couples IEG induction to depolarization (Morgan and Curran, 1989) has been desensitized during this second window. Whether even later windows (Chew et al., 1996) also differ qualitatively from the first window for gene expression remains to be investigated.

The primary telencephalic auditory area, field L2, was known not to increase its ZENK mRNA levels after either song stimulation or seizure activity (Mello and Clayton, 1994, 1995). Our present results confirm that the ZENK gene is not inducible in L2 by the depolarizing activity associated with either hearing song or metrazole administration and argues against the possibility that ZENK protein might be high and might have a long half-life in L2, being maintained by low mRNA levels. Similarly, ZENK protein induction after metrazole was practically absent in the ectostriatum (visual) and in the nucleus basalis (somatosensory), providing further evidence for down-regulation of ZENK gene expression in these areas (Mello and Clayton, 1995). This is consistent with a hypothesized need to preserve primary sensory telencephalic maps, such as tonotopic and retinotopic representations, from activity-induced change (Mello and Clayton, 1995).

ZENK protein expression in song control nuclei reflected the distribution of ZENK mRNA (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997; Jarvis et al., 1997) and occurred in birds that vocalized, but not in those that did not vocalize, in response to the playbacks. The fact that

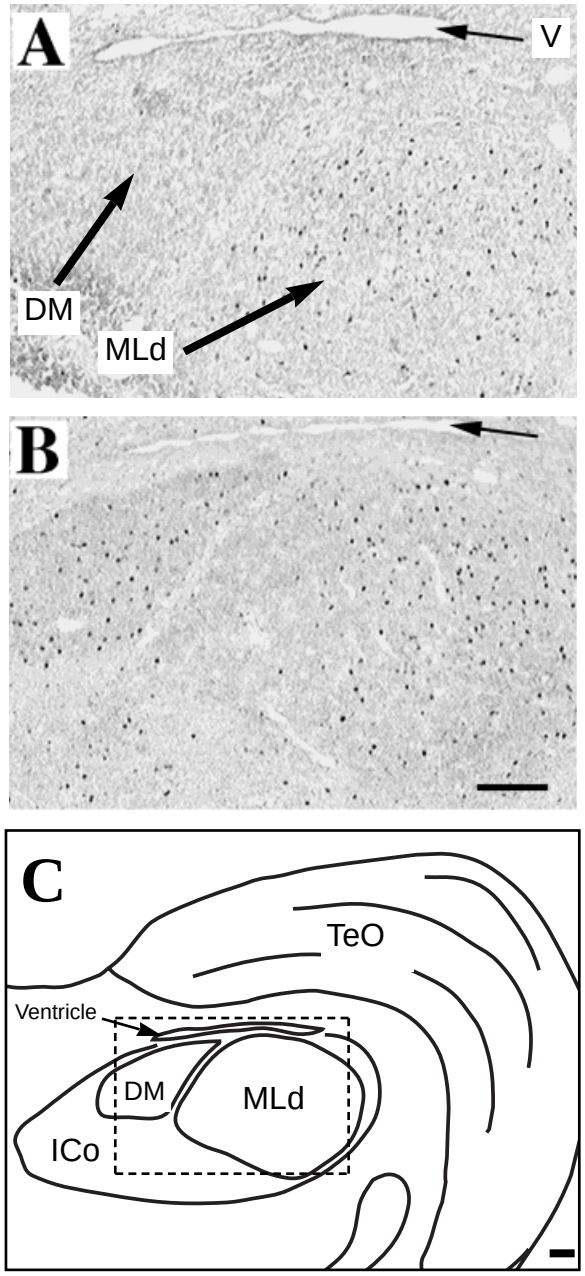

Fig. 9. ZENK expression in the caudodorsal midbrain. The area depicted in photomicrographs **A** and **B** corresponds to the area enclosed by the dashed rectangle in the camera lucida drawing in **C** (2.8 mm parasagittal plane) and contains auditory nucleus MLd and song motor nucleus DM (indicated by large arrows); for location and orientation of C, see Figure 3F. The small arrow indicates the ventricle. Notice that numerous ZENK-positive cells are present only in MLd in a stimulated bird from the hearing-only group (A) but are present in both MLd and DM in a stimulated bird from the hearing-and-singing group (B). Some labeled cells were sometimes seen in the intercollicular (ICo) region outside of DM and MLd (B), but their occurrence was not consistent across animals. The delineation of structures in C is based on their cytoarchitectonic appearance after Nissl staining and on differential background staining after ZENK ICC. The boundaries of DM correspond closely to the boundaries defined by connectivity studies (compare with Figs. 2A and 7 in Vicario, 1991, and with Figs. 1B and 10B in Wild et al., 1997). For detailed discussions on definition and connectivity of DM, see Puelles et al. (1994) and Wild et al. (1997). Orientation: Dorsal is up, and anterior is to the left. DM, dorsomedial nucleus of the intercollicular complex; MLd, nucleus mesencephalicus lateralis, pars dorsalis. Scale bar = 100 μm.

ZENK mRNA activation in the song system in response to singing occurs even after deafening (Jarvis and Nottebohm, 1997) has led to the conclusion that song motor activity is the main factor determining ZENK expression in song nuclei when birds sing, ruling out the auditory feedback known to occur in these areas as a factor (Katz and Gurney, 1981; Margoliash, 1983, 1986; Williams and Nottebohm, 1985; Doupe and Konishi, 1991; Margoliash and Fortune, 1992; Nordeen and Nordeen, 1992; Doupe, 1993; Vicario and Yohay, 1993, Vicario, 1994; Volman, 1996). In contrast, the absence of ZENK activation in song nuclei when a bird hears song indicates that distinct neuronal activation patterns within song nuclei are associated with these two behavioral situations, as suggested previously (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997). Mechanistically, this could result from the activation of different input pathways converging onto HVC (Nottebohm et al., 1982; Fortune and Margoliash, 1995; Vates et al., 1996) and possibly expressing different classes of neurotransmitters.

Our present work differs from previous studies in the following regards: 1) Relatively high numbers of cells labeled for ZENK protein were seen in the rostromedial forebrain (HA and HD), possibly overlapping with visual representation areas (Karten et al., 1973). The presence of ZENK-labeled nuclei in these areas was dependent neither on song presentation, because it occurred equally in both stimulated birds and unstimulated controls, nor on presentation of a specific visual stimulus, because all birds were kept inside isolation chambers, and they were presented with playbacks through speakers installed inside the chambers. A possibility to be further investigated is related to the longer half-life of ZENK protein compared with that of its mRNA. ZENK protein levels at any given time could represent an integration of the animal's activity resulting from its wakefulness or arousal state, as described in Cirelli et al. (1996). Interestingly, activity in HA and HD, as revealed by 2-deoxyglucose uptake, is known to be modulated by arousal (Bischof and Herrmann, 1986; Rollenhagen and Bischof, 1996). 2) ZENK protein expression in nuclei of the anterior forebrain pathway (area X and lMAN) related to singing was quite variable, indicating the influence of an unidentified factor. Expression in these nuclei in response to singing remains a puzzle, considering the current view that these areas are not necessary for normal singing behavior in adult songbirds (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). 3) A significant ZENK protein induction in birds that sang, but not in birds that only heard song, was seen in DM of the ICo, a song control nucleus that was not examined previously for ZENK induction during singing. ZENK expression in DM during singing is consistent with the labeling observed in HVC and RA, the latter being the main input to DM (Vicario, 1991; Wild et al., 1997). It remains to be determined whether motor-driven gene expression is present even farther downstream in the song motor pathway.

To gain further insight into the significance of ZENK induction, it is important to compare its expression with that of other IEGs representing different programs of gene regulation, i.e., transcription factors with DNA-binding motifs other than that of ZENK. For instance, c-fos upregulation in song nuclei has been observed in zebra finches that sang in response to conspecific song playbacks, but no significant expression was seen in NCM (Kimpo and Doupe, 1997). In contrast, a small but signifi-

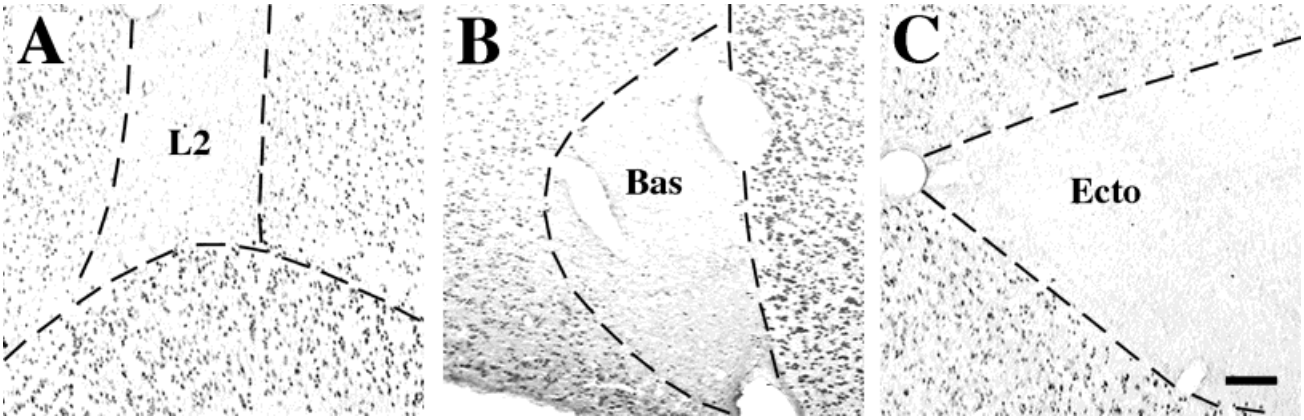

Fig. 10. ZENK expression in the zebra finch brain after metrazole administration. The areas shown in photomicrographs **A–C** are depicted by the rectangles in Figure 3D,F (left rectangle) and Figure 3F (middle rectangle), respectively; the dashed lines indicate the cytoarchitectonic boundaries as seen after Nissl staining. Notice that the number of ZENK-labeled nuclei is very low or absent in three primary thalamorecipient telencephalic zones, the auditory field L2 (A), the somatosensory nucleus basalis (B), and the visual ectostriatum (C), as opposed to the surrounding brain regions. For orientation, see Figure 3. Bas, nucleus basalis; Ecto, ectostriatum; L2, L2 subfield of field L. Scale bar = 100 µm.

cant increase in the number of c-fos-positive cells in the caudal neostriatum has been seen recently in starlings in response to playbacks of conspecific sounds (Wronski et al., 1997). Thus, it is possible that c-fos is less sensitive to the activation of NCM neurons and that it reveals only a subset of the cell population expressing ZENK. Consistent with this idea is the fact that c-jun, an IEG that is often coactivacted with c-fos, is also induced to low levels in NCM by song presentation (Nastiuk et al., 1994). Alternatively, cells that express c-fos in response to their activation could constitute a distinct population with phenotypic characteristics different from those of ZENK-expressing cells.

For the purpose of functional brain mapping, ZENK ICC has some clear advantages over mRNA detection with in situ hybridization, in particular, better tissue preservation and improved cellular resolution. Such properties should prove invaluable for studying the neurochemical identity of cells expressing ZENK in association with song behavior. ICC protocols are also more accessible than in situ methods to most neurobiologists, facilitating the use of ZENK for brain mapping of other naturally occurring behaviors in birds and other vertebrates. Indeed, ZENK protein regulation has been described recently in the brains of male Japanese quail undergoing sexual interactions (Ball et al., 1997). ZENK expression was seen in discrete brain areas, including the nucleus of the stria terminalis, the nucleus intercollicularis, and the medial and anterior hyperstriatum. Aside from indicating that ZENK is induced in the brain in the context of sexual behavior, that study demonstrates the feasibility of using ZENK for functional mapping in nonoscine avian species.

In summary, we provide here a detailed description of the regulation of the ZENK gene at the protein level in response to song presentation. The results give some insights into the nature of the waves of gene expression that follow the activation of NCM neurons by song presentation, and they provide a framework in which to plan searching strategies for other song-regulated genes. Our study also helps to establish ZENK ICC as one of the best methods available for functional brain mapping of vocal communication and, possibly, of other naturally occurring behaviors in vertebrates.

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