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Song presentation induces gene expression in the songbird forebrain

(immediate-early gene/natural stimulus/auditory/avian neostriatum/species recognition)

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ABSTRACT **We investigated the participation of genomic regulatory events in the response of the songbird brain to a natural auditory stimulus of known physiological and behavioral relevance, birdsong. Using *in situ* hybridization, we detected a rapid increase in forebrain mRNA levels of an immediate-early gene encoding a transcriptional regulator (ZENK; also known as zif-268, egr-1, NGFI-A, or Krox-24) following presentation of tape-recorded songs to canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*). ZENK induction is most marked in a forebrain region believed to participate in auditory processing and is greatest when birds hear the song of their own species. A significantly lower level of induction occurs when birds hear the song of a different species and no induction is seen after exposure to tone bursts. Cellular analysis indicates that the level of induction reflects the proportion of neurons recruited to express the gene. These results suggest a role for genomic responses in neural processes linked to song pattern recognition, discrimination, or the formation of auditory associations.**

Songbirds hear the song of other individuals of their species and respond by modifying their own vocal and social behavior (1–5). Auditory experience is an essential component of song learning: young birds learn their songs by imitating models that they hear. In adults, vocal communication through song plays a central role in reproduction and territoriality and presumably requires the formation and storage of auditory associations that involve other sensory modalities as well, such as visual representations of other birds and of the environment.

Study of brain areas related to normal singing behavior has revealed a specialized brain circuit that includes a well-defined series of sexually dimorphic nuclei essential for song production (6–8). This circuit ultimately controls the output of motoneurons of the XII nerve, which then project to muscle fibers of the syrinx, the vocal organ of songbirds. Compared to areas involved in the motor control of song, however, brain areas related to perceptual aspects such as analysis and storage of complex auditory patterns are still poorly defined. A primary auditory area of the avian forebrain, field L, has been described based on the projection from the thalamic auditory relay, nucleus ovoidalis (9, 10). In songbirds, conspecific song is known to be an effective stimulus for neurons in field L and is the preferred stimulus in parts of the song control circuit (11–15). However, the detailed connectivity of auditory areas and the pathway(s) that conveys information from primary auditory areas to song-selective brain areas, such as parts of the song control circuit (13–15), remains to be determined. Similarly, the molecular and cellular consequences of exposure to song are still largely unknown as is the mechanism(s) by which these responses may ultimately translate into physiological and behavioral changes.

We have begun to investigate these issues by focusing on genes that respond rapidly to various signals associated with neural activity and growth (16–20). Many of these immediate-early genes (IEGs) encode transcriptional regulators and have been postulated as mediators of long-term effects of growth factors and membrane-depolarizing signals on neural activity (21). To study whether genomic regulatory events are part of the brain's response to birdsong, we examined the effect of exposure to song on expression of an IEG known as zif-268, egr-1, NGFI-A, or Krox-24 (22–25), here referred to by the acronym "ZENK".

METHODS

Song Exposure. Twenty-four adult male songbirds of two different species, zebra finches (*Taeniopygia guttata*) and canaries (*Serinus canaria*) in the spring, were obtained from Canary Bird Farms (Englishtown, NJ) and from closed colonies maintained at the Rockefeller University Field Research Center (Millbrook, NY). Each bird was placed in a neutral acoustic environment isolated from other birds for 24 hr and was then exposed for 45 min to a tape-recorded sound stimulus: either conspecific birdsong, heterospecific song, a non-song auditory stimulus, or no auditory stimulus. The conspecific song tapes consisted of recorded bouts of song from three different individuals of the same species, including the bird to which the stimulus was presented. The song stimuli lasted for ≈20–30 sec and were presented every minute during the stimulation period. For heterospecific song stimulation, canaries heard zebra finch song and vice versa. The non-song auditory stimulus consisted of a tape of tone bursts at frequencies from 1 to 5.5 kHz, similar to the frequency range of natural bird song; their duration varied from 50 to 400 msec and they were presented in a random sequence either singly or in groups of four ascending or descending frequencies, separated by intervals of 5 sec. In all cases the speakers were placed 35 cm from the center of a 47 cm × 26 cm × 23 cm cage and the average sound intensity was adjusted to 70 decibels at the center of the cage. A microphone was placed near the cage and the stimulus presentation and the bird's vocal response were recorded. All birds used in this experiment were in similar hormonal condition, as judged by the amount of singing activity and by beak color (zebra finches). The stimulus presentation was always performed between noon and 3:00 p.m.

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Abbreviations: IEG, intermediate-early gene; HVC, higher vocal center; NCM, medial caudal neostriatum; HV, hyperstriatum ventrale; ZENK, an IEG known as zif-268, erg-1, NGFI-A, or Krox-24.

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Tissue Preparation and *in Situ* Hybridization. At the end of the stimulus presentation, the birds were sacrificed by decapitation. Frozen brain sections (10 μm) were hybridized essentially as described by Clayton *et al.* (26) with ^{35}S-labeled antisense riboprobes derived from a canary ZENK cDNA clone (see below) and exposed to x-ray films for 1–2 weeks or dipped in NTB2 emulsion (Kodak) and exposed for 3–6 weeks. Adjacent sections were also hybridized to riboprobes derived from the sense strand of the ZENK cDNA clone and to another forebrain-enriched canary probe, pCF-2 (26), which was found not to change significantly after exposure to song and therefore was used as standard for the hybridization (below). To minimize variability, all sections were hybridized in a single experiment and exposed simultaneously.

Isolation of Canary ZENK cDNA. The canary cDNA homologue of ZENK was cloned by a low-stringency screening of a λgt10 cDNA library representing canary brain song nucleus higher vocal center (HVC) and associated neo- and hyperstriatum using rodent probes kindly provided by V. P. Sukhatme and J. Milbrandt and subcloned in Bluescript (Stratagene). The identity of the clone was confirmed by sequence analysis, and riboprobes synthesized from it hybridized cleanly to the appropriate sized band on blots of canary and zebra finch brain total RNA (C.V.M. and D.F.C., unpublished data).

Image Analysis. Autoradiograms of canary and zebra finch brain sections hybridized to ZENK RNA probes (2-weeks exposure) were analyzed using an Eyecom II image processing system. Before taking measurements, a calibration curve was obtained using optical density standards and measurements were done within the linear range. For each bird, the average optical density over the medial caudal neostriatum (NCM) was determined in two adjacent sections taken in a parasagittal plane 200–300 μm from the midline, excluding a negative area believed to correspond to part of field L (see Fig. 1). To control for variations in backgrounds, hybridization efficiency, or section thickness, the initial density values obtained with ZENK for each bird (experimentals and controls) were divided by the values obtained from the next two adjacent sections hybridized with pCF-2. All resulting NCM densities were then divided by the mean NCM signal obtained in unstimulated control birds of each species to create a normalized scale (as a result of this normalization, mean unstimulated = 1 on this scale).

RESULTS

Substantial increases in ZENK mRNA levels occurred within the forebrain of all birds that were exposed to song compared to the groups that heard no auditory stimulus (Figs. 1 and 2) or tone bursts (Fig. 2). The area showing the most marked induction (Fig. 1*a*) has well-defined boundaries and corresponds to the most medial part of the caudal neostriatum (NCM, Fig. 1 *c* and *d*). In a series of sagittal sections, NCM begins next to the midline as a small circular area. In more lateral sections it becomes gradually larger, assuming a drop-like shape, and eventually becomes continuous with the rostral parts of the neostriatum. High ZENK levels can be seen in NCM in serial sagittal sections from the midline up to about 500 μm lateral to the midline, when the signal becomes patchy and less homogeneous (not shown). A high ZENK induction is also seen in the most medial part of the HV immediately adjacent to NCM (Fig. 1 *c* and *d*). At approximately 300 μm from the midline, the two areas combined (NCM and the very medial HV) resemble a lobular structure presenting a characteristic ovoid or drop-like shape (Fig. 1*a*, arrow, and *d*). Lower levels of ZENK RNA induction are seen in more anterior and lateral parts of the telencephalon and in some discrete nuclei in the midbrain (not shown; a detailed description of other brain areas showing ZENK induction following song will be presented elsewhere). Little or no ZENK induction can be detected in the nuclei that

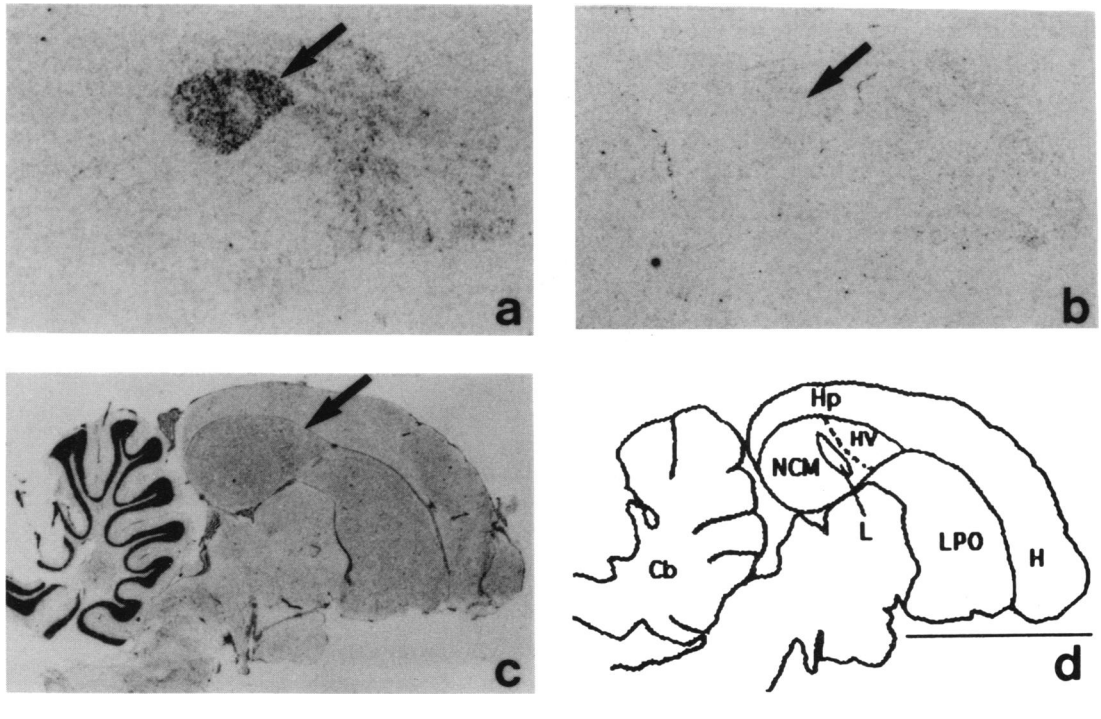

FIG. 1. Induction of ZENK mRNA levels in the songbird forebrain following exposure to song. *In situ* hybridization autoradiograms of sections corresponding to the parasagittal plane 250 μm lateral to the medial surface of the brain are shown. (*a*) Adult male zebra finch exposed for 45 min to recorded conspecific song. (*b*) Unstimulated control. (*c*) Cresyl violet staining of the section whose autoradiogram is shown in *a*. (*d*) Camera lucida drawing of the section shown in *c*. The arrows in all panels point to the area of high ZENK induction that includes NCM and a portion of hyperstriatum ventrale (HV). Cb, cerebellum; H, hyperstriatum; Hp, hippocampus; LPO, paraolfactory lobe; L, field L. (Bar = 4 mm.)

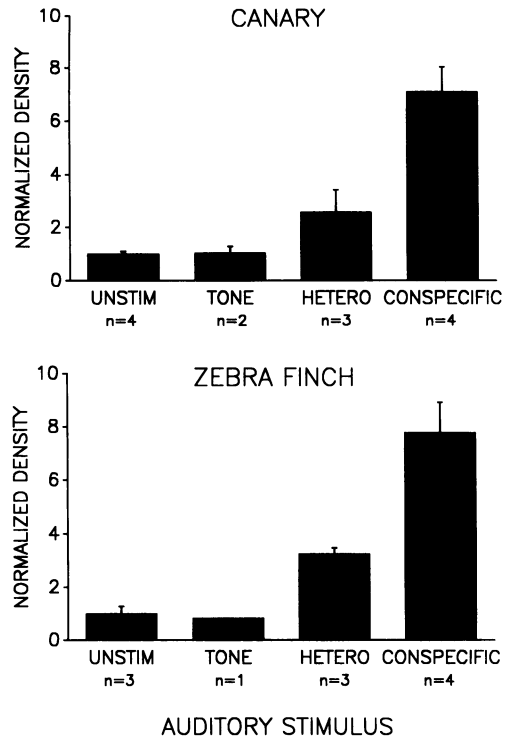

FIG. 2. Relative ZENK induction in NCM in birds presented with various auditory stimuli: conspecific song, heterospecific (HETERO) song, tone (TONE) bursts, or no auditory stimulus (UNSTIM). *In situ* hybridization autoradiograms (as in Fig. 1) were analyzed by densitometry. For each group, the mean ± SE of the normalized optical densities in NCM is shown.

comprise the motor pathway for song production, including HVC, RA (robust nucleus of the archistriatalis), DM (dorsomedial nucleus of the intercollicular nucleus), X (area X), MAN (magnocellular nucleus of the anterior neostriatum), DLM (medial portion of the dorsolateral nucleus of the thalamus) (refs. 6–8; data not shown), or in an area in the medial neostriatum that contains small cells with darkly staining nuclei and is thought to represent the primary auditory projection area of the forebrain, field L (9, 10, 27); in Fig. 1, this corresponds to the small area of low signal within NCM. No signal was detected in sections hybridized to sense-strand control probes (not shown).

We then concentrated on NCM, the area showing the most robust ZENK induction. Densitometric analysis of ZENK levels in NCM (Fig. 2) revealed that both classes of song (conspecific and heterospecific) caused an increase in ZENK above control levels in both species; no induction was observed following stimulation with tone bursts. The ranges of observed ZENK induction above unstimulated birds were 4.5–9 times in canaries and 5–10 times in zebra finches for conspecific song and 2–4 times in canaries and 3–4 times in zebra finches for heterospecific song (standard errors calculated from the data are shown in Fig. 2). For a conservative statistical test of these differences, individual normalized density values from combined controls (unstimulated plus tone-stimulated) were compared with individual values from the song-stimulated groups, and values from the conspecific groups were compared with values from the heterospecific groups [Mann–Whitney U test (28), criterion of $P < 0.05$, two-tailed probabilities]. In canaries, significant differences were seen between heterospecific ($n = 3$, $U = 0$, $P = 0.024$) and conspecific ($n = 4$, $U = 0$, $P = 0.005$) groups compared with combined controls ($n = 6$). In zebra finches, a significant difference from combined controls was seen for the conspecific group ($n = 4$, $U = 0$, $P = 0.028$), whereas the induction for the heterospecific group was only suggestive ($n = 3$, $U = 0$, $P = 0.056$), due to the low number of animals. Combining data from both species, differences in induction for birds that heard conspecific vs. heterospecific song were highly significant ($n = 8$, $n = 6$, $U = 0$, $P < 0.001$).

To investigate the induction in NCM at the cellular level, hybridized sections were also prepared for emulsion autoradiography (Fig. 3). Within the NCM of song-stimulated birds, a large number of cells with neuronal appearance (larger, with paler staining nuclei) are labeled with high concentrations of autoradiographic silver grains (Fig. 3 *a* and *b*). This high level of induction is confined to NCM and adjacent HV and respects the boundaries with the overlying hippocampus and parahippocampal area (Fig. 3*a*). In the NCM of unstimulated birds, most of the cells are unlabeled (Fig. 3*c*). However, even in these brains a few cells in NCM (<5%) are labeled as highly as most cells in the song-stimulated brain (Fig. 3*c*, arrow). Thus some expression of the ZENK gene occurs in

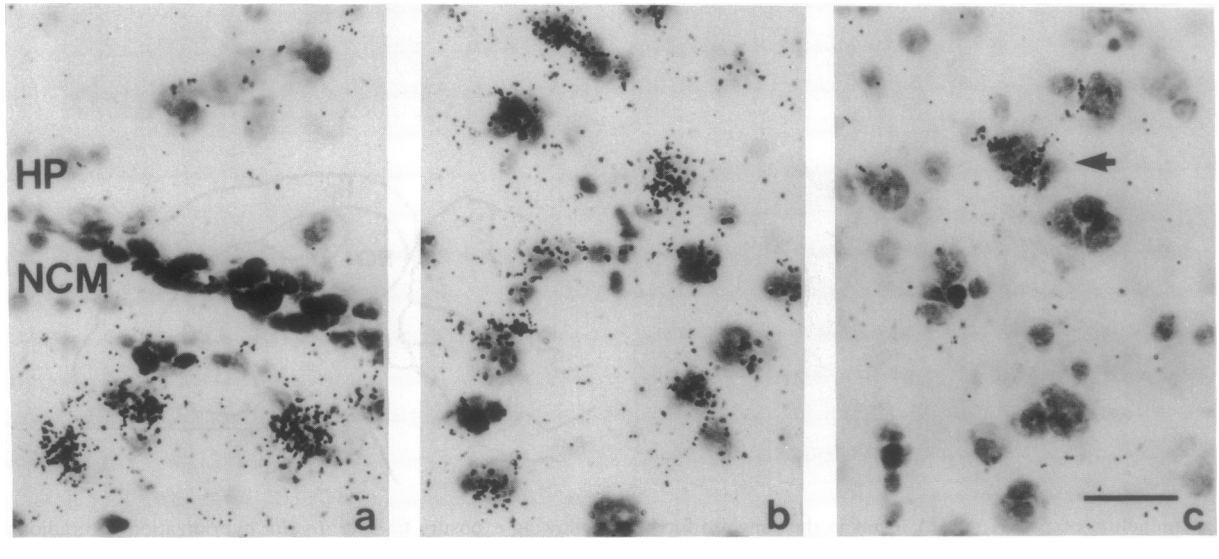

FIG. 3. High-power bright-field view of NCM in sections dipped with autoradiographic emulsion. (*a*) Conspecific song-stimulated zebra finch, boundary between hippocampus (HP) and NCM. Large numbers of silver grains are seen on cells in NCM but not HP. The boundary between NCM and HP is defined by the darkly staining tightly-packed cells of the ventricular zone. (*b* and *c*) Comparison of NCM between conspecific song-stimulated (*b*) and unstimulated (*c*) birds. The arrow indicates a labeled cell in control NCM. (Bar = 25 μm.)

NCM of birds not exposed to song, and the difference in overall RNA levels between the control and experimental animals (Fig. 2) appears to be due primarily to an increase in the number of cells expressing the gene at high levels.

DISCUSSION

We have presented evidence demonstrating that the brains of songbirds respond to song playbacks with a large and rapid increase in ZENK mRNA levels. ZENK increase was most marked within the neostriatum, a main subdivision of the avian forebrain considered analogous to portions of the mammalian sensory and association neocortex (29–32). In both species studied, conspecific song elicited the highest response, whereas other classes of auditory stimuli were less effective or ineffective. These results demonstrate that a meaningful natural stimulus may quickly induce IEGs to high levels in the intact vertebrate telencephalon, without the need for electrical or noxious stimulation or pharmacological manipulation.

We have used NCM to designate the large area of the caudo-medial neostriatum with an especially robust ZENK induction in response to song presentation. This area has no known connections with the song control circuit and has not yet been assigned a functional role in the processes of song production or perception. However, NCM lies in close apposition to the primary auditory area of the forebrain, field L, and possibly contains a significant proportion of units that selectively respond to complex auditory stimuli, as has been reported for a neostriatal area adjacent to field L that may correspond to NCM (11). Studies using Golgi staining (29) and *Phaseolus vulgaris* Leucoagglutinin (PHA-L) and Fluorogold as anatomical tracers (C.V.M., unpublished data) have demonstrated that the caudo-medial neostriatum receives an input from field L; it is also reciprocally connected with the medial part of the HV, where selective responses to complex sounds have been recorded (11) and a significant ZENK induction also occurred. ZENK induction has thus revealed that some specific brain areas that are intimately connected with the primary auditory area are highly activated by song and may represent auditory processing areas of the avian brain. In this regard, it is interesting to note that a homology has been suggested between the mammalian auditory neocortex and a large portion of the avian caudal forebrain, including field L, the surrounding neostriatum, and the adjacent HV (29, 31, 32).

Conspecific song elicited a greater response in NCM than heterospecific song, and tone bursts within the same range of frequencies as birdsong failed to elicit a significant response. Units within NCM thus seem to be performing tasks related to feature detection of auditory stimuli. This suggests that levels of ZENK induction in NCM may reflect processes of auditory discrimination in the forebrain and are higher after stimuli that are of greater behavioral significance to the bird. We do not yet know whether the preference for conspecific song is innate or dependent upon previous auditory experience. Using electrophysiological techniques, a preference for neuronal activation by conspecific song, especially the bird's own song, was also described in HVC, a main component of the motor pathway for song production (15). It is, however, unclear how auditory information reaches this motor pathway. It will be interesting to study in detail the anatomical connections of areas revealed by ZENK induction and whether NCM function is related to the selective activation of HVC by specific sounds.

Although our results suggest that the ZENK induction we observed in NCM is related to auditory processing, other aspects of the bird's behavior that can be triggered by song presentation should also be considered, such as song production or nonspecific social arousal. These other possibilities seem to us unlikely explanations for the gene induction because of the following: (*i*) most birds did not sing during the song playbacks under our experimental conditions; (*ii*) similar ZENK induction was seen in female zebra finches who never sing (data not shown); (*iii*) no appreciable induction was seen in one male zebra finch who was presented with a female and sang vigorously to her during the presentation period but was not exposed to song tapes. This seems to preclude a significant effect of active singing behavior or motivation to sing on ZENK induction in NCM, although an arousal or motivation effect caused by song presentation cannot be completely excluded. To further distinguish among these possibilities experimentally, we plan to manipulate the features and context of the song stimulus and the experiential background of the subjects and assay the effect on the proportion of cells labeled above threshold in NCM.

ZENK encodes a "zinc-finger" protein that binds specifically to a DNA sequence found in the promoters of several identified genes (33), and its RNA levels have been shown to increase during nerve growth factor-induced neuronal differentiation (19, 20, 24) and induction of long-term potentiation (34, 35). Thus, the induction of ZENK could reflect the activation of a cellular program that modulates long-lasting changes in response to particular patterns of sensory stimulation. Changes in IEGs including ZENK have also been recently observed in the rodent supra-chiasmatic nucleus after presentation of light stimuli that shift the biological clock in these animals (36). Further studies are necessary to determine what ZENK induction patterns occur during the periods when song is learned (juveniles) or modified (fall canaries) dependent upon auditory input, when substantial levels of neuritic and synaptic growth take place within the song circuit (37–41), and whether changes in ZENK RNA result in increased levels of the specific encoded protein.

Alternatively, ZENK induction could be part of a general homeostatic or signal-response mechanism that occurs whenever cells are physiologically activated. This interpretation, however, is not supported by the observation that ZENK was not induced to significant levels in field L or in the motor pathway for song production in any of our birds. In studies using electrophysiological and biochemical techniques (such as 2-deoxyglucose), both of these areas were shown to be physiologically activated by sound stimuli, especially by conspecific song (11–15). Since auditory information probably reaches NCM by way of field L, this seems to indicate that cells in field L can be electrically activated without showing a concomitant ZENK induction. (We cannot yet completely exclude the possibility of some overlap between the terminal field of fibers from thalamic nucleus ovoidalis and areas of high ZENK signal as seen in sagittal sections.) Similarly, tone bursts were incapable of inducing ZENK RNA in NCM, although they have been shown to elicit electrophysiological activity there (11, 12). This suggests that activity is necessary but probably not sufficient for ZENK gene induction.

The study of songbird brains has provided suggestive evidence on the role of cellular plasticity in behavioral learning (8, 40). Our experiments provide direct evidence for the involvement of a genomic regulatory response in the biology of song and implicate distinct forebrain areas in the processing of species-specific vocalizations. Analysis of this response in the context of the behavior and neurophysiology of songbirds may provide unique insights into the relationship between specific gene regulation and processes of behavioral and neuroanatomical change.

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