

For Whom The Bird Sings: Context-Dependent Gene Expression

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Summary

Male zebra finches display two song behaviors: directed and undirected singing. The two differ little in the vocalizations produced but greatly in how song is delivered. "Directed" song is usually accompanied by a courtship dance and is addressed almost exclusively to females. "Undirected" song is not accompanied by the dance and is produced when the male is in the presence of other males, alone, or outside a nest occupied by its mate. Here, we show that the anterior forebrain vocal pathway contains medial and lateral "cortical-basal ganglia" subdivisions that have differential ZENK gene activation depending on whether the bird sings female-directed or undirected song. Differences also occur in the vocal output nucleus, RA. Thus, although these two vocal behaviors are very similar, their brain activation patterns are dramatically different.

Introduction

Songbirds, much like humans, learn their vocalizations by imitating adult conspecifics (Thorpe, 1961; Marler, 1970, 1997). In the most commonly studied songbird species, the zebra finch, only males sing. Each male learns to produce a unique song that begins with introductory notes followed by a motif of 5–11 different notes (Figure 1A). The male typically repeats this motif 1–6 times in quick succession, producing a song bout. Two ways of singing this motif have been identified, "directed" and "undirected" (Morris, 1954; Hall, 1962; Immelmann, 1962; Dunn and Zann, 1996a, 1996b, 1997). Directed song is given when a male faces a female and sings to her. It is usually accompanied by a courtship dance that includes cheek and nuchal feather erection and pivoting of the body while approaching the female. Undirected song appears not to be addressed to anyone in particular, as the male does not face another bird when singing. It is produced while the male is perched, often outside the nest of an incubating mate, or with other males, or alone (reviewed by Zann, 1996). Although the contextual use of directed and undirected song is very different, the vocalizations are nearly indistinguishable to the human ear. Sound-spectrographic analysis shows, however, that female-directed song is preceded by more introductory notes per bout, that it includes

more motifs per bout, and that each motif is delivered slightly faster (by 10–40 ms) than undirected song (Figure 1A; Sossinka and Böhner, 1980; Bischof et al., 1981; Caryl, 1981). The two behaviors also differ in hormone sensitivity: the amount of directed song increases with estrogen treatment, undirected with testosterone (Pröve 1974; Arnold, 1975b; Harding et al., 1983; Walters et al., 1991).

Though no separate neural circuits for directed and undirected song have been described, a great deal is known about the brain circuits that mediate song acquisition and production. Referred to collectively as "the song system," these circuits consist in male zebra finches of a posterior motor pathway necessary for song production and an anterior pathway necessary for song acquisition. Both pathways originate in the high vocal center (HVC) of the neostriatum. In the posterior pathway (Figure 1B, black arrows), neurons of one cell type in HVC project to the robust nucleus of the archistriatum (RA); RA in turn projects to the dorsomedial (DM) nucleus of the midbrain and to motoneurons (nXIIIs) that innervate muscles of the trachea and syrinx. In the anterior pathway (Figure 1B, gray arrows), neurons of a different cell type in HVC project to Area X of the paleostriatum; Area X in turn projects to the medial nucleus of the dorsolateral thalamus (DLM); DLM projects to the lateral magnocellular nucleus of the anterior neostriatum (MAN); and MAN projects both to RA and back to Area X (see Figure 1B legend for references). The posterior pathway is commonly called the direct vocal motor pathway; the anterior pathway is called the indirect pathway, as it is the long way for information to travel from HVC to RA. "Posterior pathway" is a terminology we introduce here to reflect a neutral and congruent nomenclature with the already established use of "anterior pathway."

Both the posterior and anterior pathways have mammalian correlates. The upper subdivisions of the avian forebrain, the hyperstriatum, neostriatum, and archistriatum (Figure 1B), are believed to be homologous to different layers of the mammalian cortex. The underlying paleostriatum is thought to be homologous to the basal ganglia, and the thalamus of birds is homologous to that of mammals (Figure 1B; Karten and Shimizu, 1989; Karten, 1991; Wild et al., 1993; Medina and Reiner, 1995; Veenman et al., 1995, 1997; Medina et al., 1997; Striedter, 1997). As in mammalian cortical-basal ganglia-thalamocortical loops (Alexander et al., 1986), the anterior vocal pathway of songbirds goes from several cortical regions (HVC and MAN) to striatum (Area X), to thalamus (DLM), and back to frontal cortex (MAN) (Okuhata and Saito, 1987; Bottjer et al., 1989; Bottjer and Johnson, 1997; Vates et al., 1997). In the songbird anterior vocal pathway, the striatum (i.e., paleostriatum augmentatum) projects directly to the thalamus, bypassing the globus pallidus (i.e., paleostriatum primitivum; Figure 1B). The posterior pathway is akin to the direct motor pathway of mammals.

Lesions of the mammalian motor cortex result in clear motor deficits, as is the case with lesions of HVC and RA (Nottebohm et al., 1976; Simpson and Vicario, 1990).

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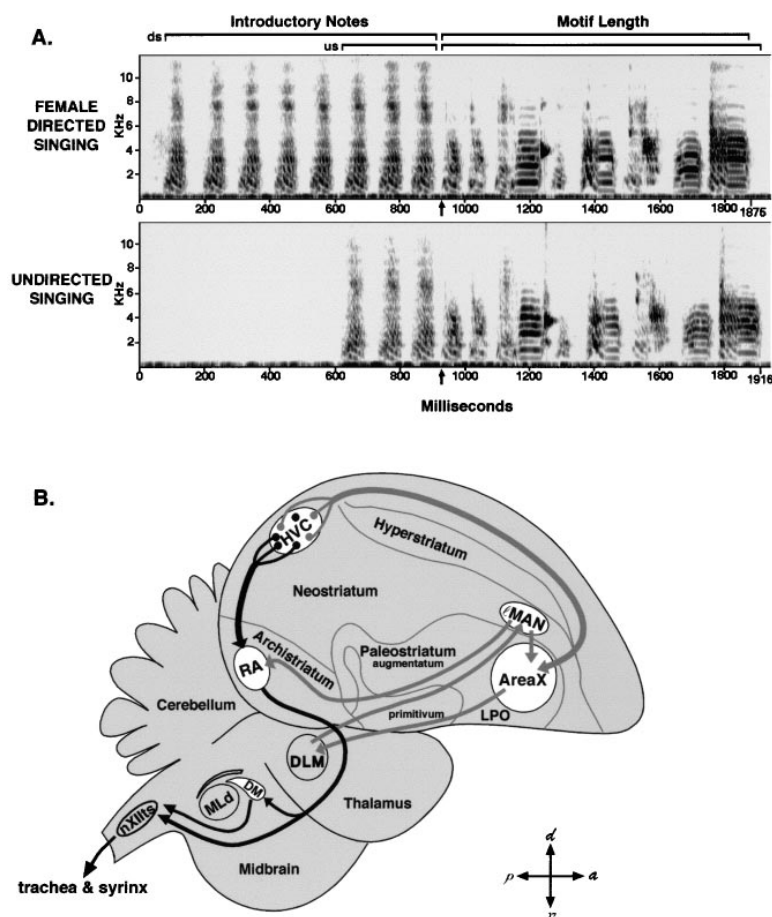


Figure 1. Examples of Undirected and Directed Song and Partial Diagram of Song System Pathways

(A) Sound spectrographs (frequency in kHz versus time in milliseconds) of song from an individual male zebra finch, recorded when singing toward a conspecific female (directed) or when singing alone (undirected). This represents the larger vocal difference seen between these two song types. Both songs are aligned at the beginning of the motif (arrow). The directed motif was produced 41 ms faster than the undirected (1916 versus 1875 ms). Lines above spectrographs delineate number of introductory notes and motif length for the directed (ds) and undirected (us) song bout.

(B) Anatomical diagram showing song nuclei connectivity relevant to this report. Black arrows indicate the posterior vocal pathway, gray arrows the anterior (Nottebohm et al., 1976, 1982; Okuhata and Saito, 1987; Bottjer et al., 1989; Vicario, 1993; Wild, 1993; Johnson et al., 1995; Nixdorf-Bergweiler et al., 1995; Vates and Nottebohm, 1995). Structures filled in white indicate song nuclei that show singing-induced expression of Fos (Kimbo and Doupe, 1997) and/or ZENK (Jarvis and Nottebohm, 1997). MLd (nucleus mesencephalicus lateralis pars dorsalis) is the midbrain's ascending auditory station (Vates et al., 1996) and is shown for reference to DM under the overlying ventricle. Abbreviations: LPO, lobus parolfactorius; a, anterior; d, dorsal; p, posterior; v, ventral; other abbreviations are as in the introduction.

Lesions of the adult mammalian striatum result in little or ambiguous motor deficits, whereas striatal lesions in younger animals have larger effects (reviewed by DeLong and Georgopoulos, 1981; Lidsky et al., 1985; Mink, 1996). Similarly, when Area X or MAN are removed in juvenile zebra finches, vocal learning is disrupted. When removed in adults, who have already mastered their songs, no effects on song production are detected (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). Yet, surprisingly, in adults the act of singing induces a strong immediate-early gene response in nuclei of both the anterior (Jarvis and Nottebohm, 1997) and posterior (Jarvis and Nottebohm, 1997; Kimbo and Doupe, 1997) pathways. Electrophysiological recordings during singing (Hessler and Doupe, 1997, Soc. Neurosci., abstract; Margoliash, 1997) and further gene expression studies (Jarvis et al., 1997; Jin and Clayton, 1997; Mello and Ribeiro, 1998) are consistent with these findings. Thus, though not necessary for singing, the anterior pathway is active during singing.

In this report, we refine the functional correlates of singing-induced gene activity in the song system. We used *in situ* hybridization and immunocytochemistry to identify brain regions showing vocally activated expression of the ZENK immediate-early gene. ZENK, an acronym for the same gene known in mammalian species as *zif268*, *egr1*, *NGF1-A*, and *Krox-24* (Mello et al., 1992),

is a transcription factor that binds to promoter regions of other genes and modulates their expression (reviewed by Chaudhuri, 1997). We show that the anterior pathway contains medial and lateral subdivisions that have differential ZENK activation depending on whether the bird sings female-directed or undirected song. Thus, vocally activated expression in the song system depends on to whom the bird sings.

Results

We noted that the social context in which we elicited singing affected the manner in which our birds sang, i.e., directed or undirected song. Thus, we first describe the effects of social context on ZENK expression.

Social Context

Figure 2 shows that the brain's ZENK activation pattern was dramatically different depending on the social context in which singing occurred. The largest difference was seen in lateral Area X (Area X) of the striatum, followed by lateral MAN (MAN) and RA, where ZENK expression was low when males sang in the presence of females (female context), high when they sang in the presence of other males (male context), and for some animals even higher when they sang by themselves (solo context) (Figures 3 and 4). A nonsignificant trend was seen for HVC. In contrast, ZENK induction in medial

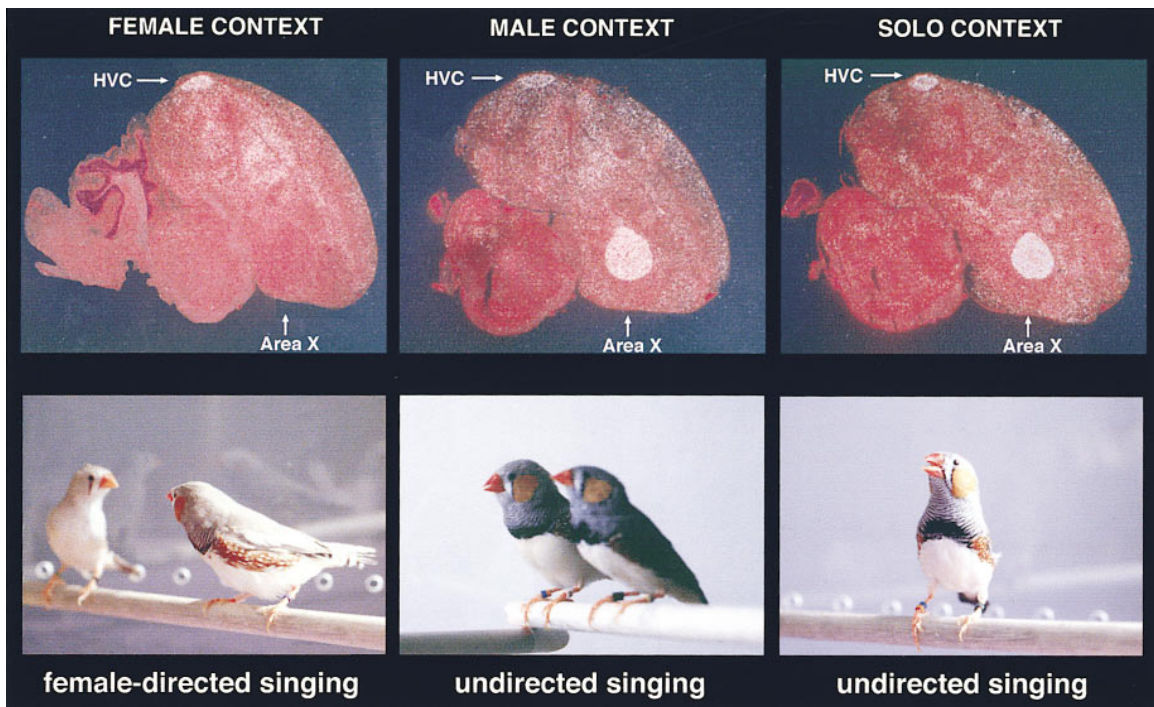


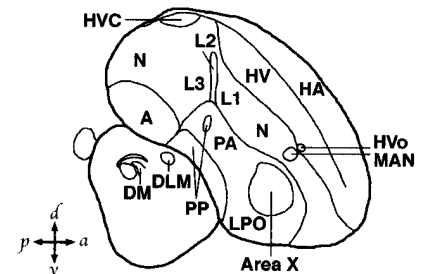
Figure 2. Singing-Induced ZENK Expression Differs in a Context-Dependent Manner

(Top) Darkfield view of cresyl violet-stained (red) parasagittal brain sections, ~ 2.3 mm from the midline, hybridized to an ^{35}S -labeled ZENK riboprobe (white-silver grains), from zebra finch males who sang either 30 min in the presence of females (left section, female context; 87 song bouts), in the presence of other males (center section, male context; 97 song bouts), or alone (right section, solo context; 91 song bouts). The bird in the female context sang all of his songs directed toward the females. The birds in the male and solo context sang all of their songs undirected. These examples are representative of the largest gene expression differences observed. In the female and male context, increased expression was seen in the auditory forebrain below HVC (fields L1 and L3, caudal HV), regions known to show ZENK induction due to hearing song independent of singing (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). However, this induction was minimal when males heard only themselves (solo context).

(Center) Examples of body postures males make when they sing in these three different contexts. Males can be recognized by their orange cheek patches, brown polka dot sides, and zebra-striped chests. Females are more uniform in their plumage. In the left photo, a male (right) and a female (left) are facing each other, and the male is singing as he hops toward her. In the center photo, two males are perched side by side, and the left one is singing without facing the male on the right. In the right photo, a male is shown singing by himself.

(Bottom right) Anatomical diagram of the above brain sections.

Abbreviations: A, archistriatum; HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; LPO, lobus parolfactorius; N, neostriatum; PA, paleostriatum augmentatum; PP, paleostriatum primitivum; a, anterior; d, dorsal; p, posterior; v, ventral; other abbreviations are as in the text.



portions of Area X (*mArea X*) and MAN (*mMAN*), in an oval nucleus located in the hyperstriatum ventrale (HVo) above MAN, in *mHVC*, and in a cap of cells posterodorsal to RA (cRA) did not differ between the three contexts (Figures 3 and 4). These changes were rapid (30 min), large (up to 40-fold), and linearly proportional to the amount of singing (Figure 4). These results lead us to propose that cRA, HVo, *lArea X*, and *mArea X* are functionally distinct structures in the songbird brain (see Anatomy in Experimental Procedures).

Directed versus Undirected Singing

The higher levels of singing-induced ZENK expression in *lArea X*, MAN, and RA of birds in the solo and male context relative to those in the female context (Figures 2 and 4) suggested to us that the male and solo context expression levels were a result of a common behavioral

feature: undirected singing. In the solo context, 100% of the songs produced were by definition undirected; in the male context, video analysis revealed that on average 81% of the songs produced were undirected. However, in the presence of females, only 13% were undirected; the remaining were directed toward the females (Figure 2). We noted that individuals who produced both directed and undirected song in the male or female contexts had ZENK expression levels intermediate between those of birds that produced 100% directed or 100% undirected song (Figure 5A).

To quantify this result, we sought a measurement that would eliminate the amount of singing as a variable. We found that dividing the *lArea X* expression value of each individual bird by its HVC value from the same brain section (equivalent to *mHVC*) resulted in a ZENK expression ratio (Figure 5A) that was not influenced by

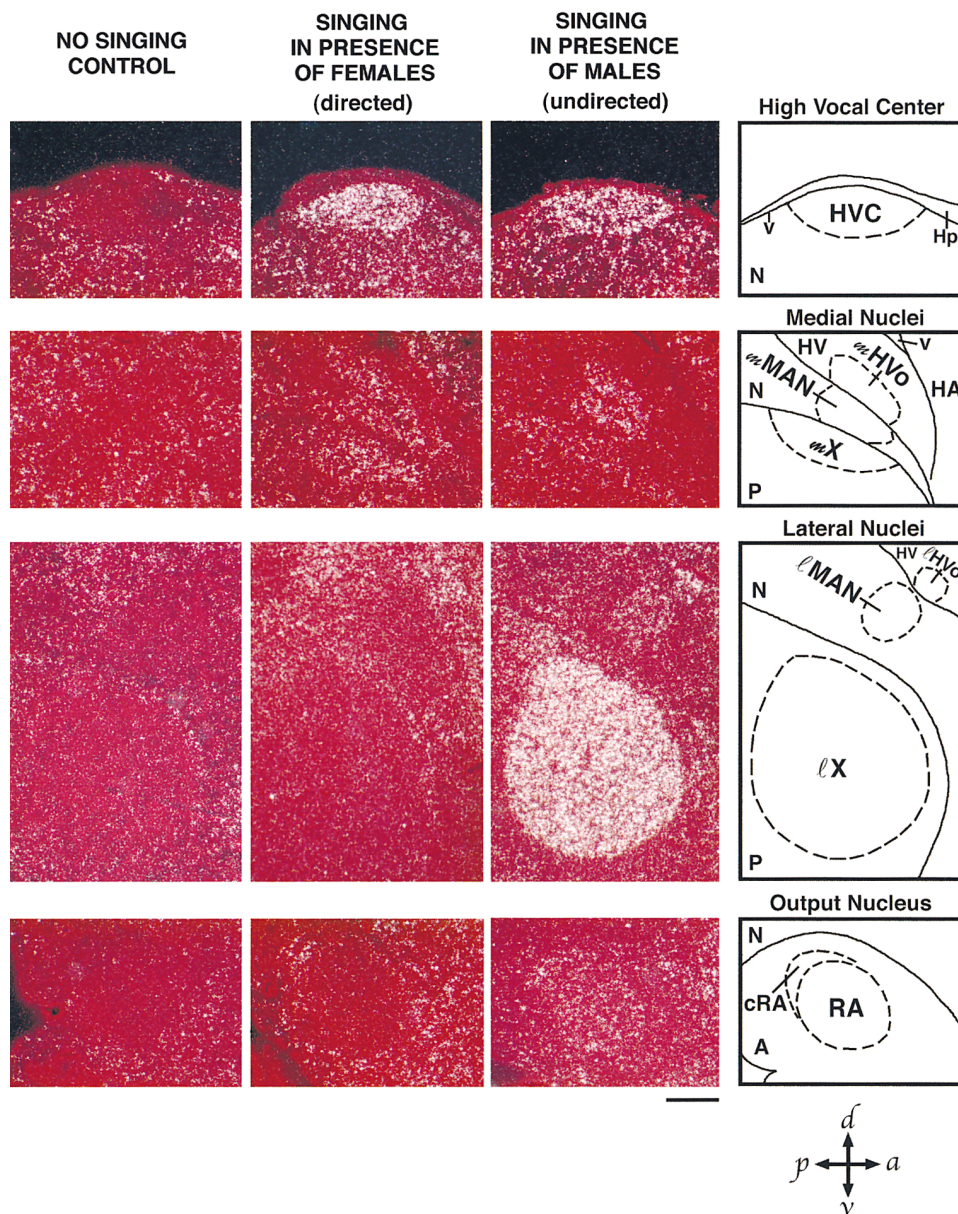


Figure 3. Examples of Song System Nuclei in which Singing-Induced ZENK mRNA Expression Is or Is Not Context Dependent; Darkfield High Magnification Parasagittal View

(Left) Song nuclei of a control male who did not sing while in the presence of females and singing males.

(Center) Male from Figure 1 who produced 87 bouts of female-directed song in the female context.

(Right) Male from Figure 1 who produced 97 bouts of undirected song in the male context.

(Anatomical diagrams) Dashed lines demarcate regions of singing-induced expression; solid lines demarcate cresyl-defined lamina. We define the cap region of RA (cRA) to include cells both within and outside RA's cresyl border (see Experimental Procedures). Medial nuclei, 0.2 mm; lateral nuclei and HVC (medial), 2.3 mm; RA, 3 mm from the midline. Abbreviations: A, archistriatum; HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; Hp, hippocampus; N, neostriatum; P, paleostriatum; v, ventricle; a, anterior; d, dorsal; p, posterior; v, ventral; other abbreviations are as in text.

Scale bar, 150 μ m.

the number of song bouts produced (compare graphs of Figure 4 with Figure 5B). These ratios differed between contexts, and the differences spanned three orders of magnitude, so that a logarithmic axis was necessary to show their spread (Figure 5B). No overlap was found in expression ratios between birds that sang 100% directed (ratios < 0.3) and 100% undirected (ratios >

0.6) song (Figure 5C). The ratios for birds that sang a combination of both song types fell between the two extremes of this distribution. That is, *Area X* expression was always lower than HVC (ratio < 1) when birds produced directed song and close to or higher than HVC (ratio \approx 1) when birds produced only undirected song. The more undirected song produced, the more *Area X*

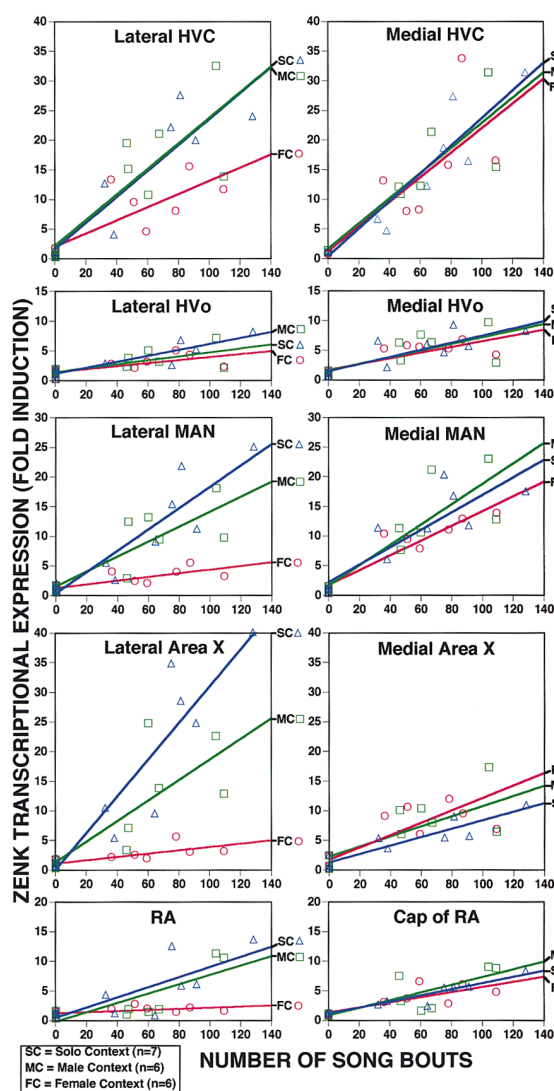


Figure 4. Quantitative Relation between ZENK Expression, Amount of Singing, and Context

Relationship between singing amount (bouts/30–40 min, x-axis) and ZENK expression (fold increase over silent controls, y-axis) in ten different brain regions (individual graphs) depending on three different social contexts (solo context, blue triangles; male context, green squares; female context, red circles). Simple linear regressions showed that singing amount was significantly correlated with ZENK expression in all nuclei across all contexts ($r = 0.817\text{--}0.868$, $p = 0.050\text{--}0.0001$), except for RA in the female context ($r = 0.451$, $p = 0.191$). Multiple regression showed that, in addition to singing amount, context was a significant factor for RA and several lateral structures, *MAN* and *Area X*, where expression was lower in the female than in the male or solo context ($r = 0.716\text{--}0.891$, $p = 0.004\text{--}0.047$); a nonsignificant trend was seen for *HVC* ($r = 0.867$, $p = 0.060$); medial structures *HVo* and *cRA* showed consistent singing-induced expression regardless of context ($r = 0.740\text{--}0.922$, $p = 0.116\text{--}0.92$); and expression in lateral structures was sometimes lower in the male than in the solo context, but these differences were not significant ($r = 0.740\text{--}0.922$, $p = 0.138\text{--}0.820$) and are due to additional variability of several males in the male context (see Figure 5). Values from different brain regions of the same animal can be cross-referenced by number of song bouts. For example, the bird who sang the most bouts, 128 in the solo context, is located as a triangle at the right end of each graph.

expression approached or exceeded the high HVC levels. Similar ratio differences, independent of singing amount, were found when comparing expression in *Area X:HVC*, *RA:cRA*, and *MAN:mMAN* (data not shown).

Number of Introductory Notes

Considerable *Area X:mHVC* ratio variability was still found among birds that sang 100% directed or 100% undirected songs (Figure 5C), suggesting the presence of an additional variable affecting ZENK expression. To search for this possible variable, we performed a detailed song analysis of those video- and audiotaped birds that produced >90% directed or undirected song. Three aspects of singing behavior were examined: motif speed, number of motifs per bout, and number of introductory notes before a bout (Sossinka and Böhner, 1980). Regardless of context, some birds showed a linear drift in motif length, speeding up or slowing down song delivery over a 30–40 min singing session. The slope (and direction) of this drift, however, did not correlate with *Area X:mHVC* expression ratio differences ($r = 0.185$, $p = 0.544$, $n = 11$ undirected and 5 directed singers; simple regression). The number of motifs produced per song bout also did not correlate with the variability seen ($r = 0.070$, $p = 0.782$; simple regression). In contrast, the average number of introductory notes produced before the first motif of each bout showed a significant negative linear correlation with expression ratios (Figure 5D). That is, the more introductory notes produced before a bout (typical for female-directed song), the lower the *Area X:mHVC* expression ratio.

Development and Song Choice

Song learning in male zebra finches occurs in three developmental phases: subsong, plastic song, and stable adult song. During subsong, young birds (posthatch days ~25–50) produce a diversity of variable undirected sounds (Arnold, 1975a; Zann, 1996) reminiscent of babbling in human infants (Thorpe, 1958; Marler and Peters, 1982). During plastic song (posthatch days ~50–80), vocal imitation is first recognizable and directed song first occurs. Once a model has been matched, song is stereotyped and the male is considered an adult (posthatch day ~90; Immelmann, 1969; Arnold, 1975a; Slater et al., 1988; Zann, 1996).

We wanted to determine if the onset of female-directed song in young males was accompanied by differential ZENK expression. Young males, adult males, and adult females were placed into individual cages adjacent to each other. In this context, males could choose to whom, if to any one, to direct their singing behavior. During subsong, juveniles never directed song toward females; all songs were undirected. As previously reported (Jin and Clayton, 1997), ZENK expression in RA of juveniles producing subsong was higher per bout of singing than in adults (Figure 6A). However, their *Area X:mHVC* (Figure 6B) and *RA:cRA* (Figure 6C) expression ratios were comparable to adults singing stable undirected song. Likewise, older juveniles singing plastic undirected song had an activation pattern comparable to adults singing stable undirected song. Among

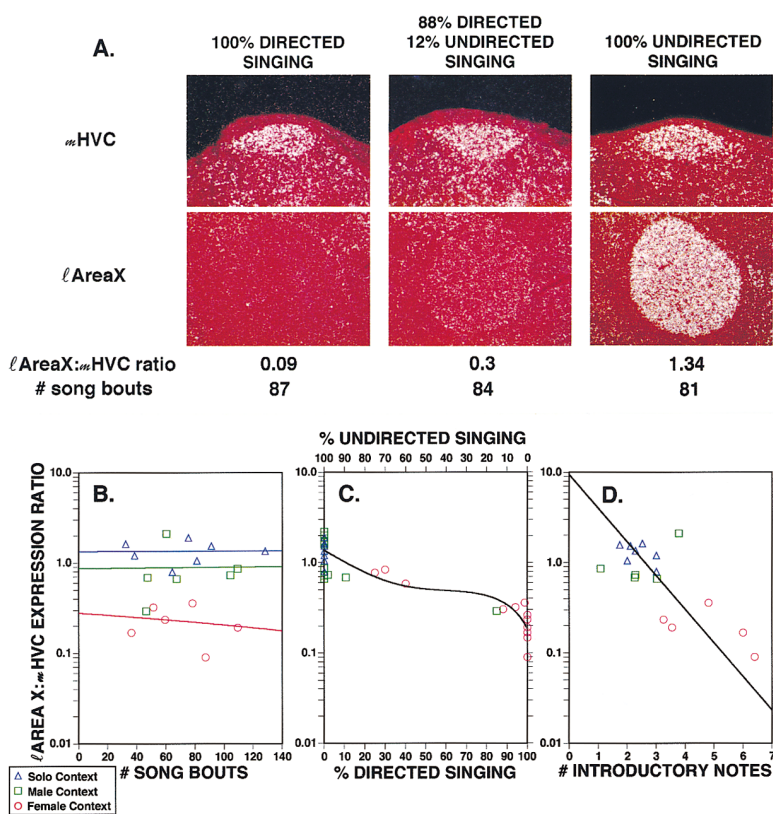


Figure 5. Ratio of *mHVC* to *lArea X* Expression Varies with the Proportion of Directed and Undirected Song Produced per Session

(A) Darkfield images of *mHVC* and *lArea X* (of the same brain sections) from individual animals singing proportionately different amounts of female-directed and/or undirected song. The female-directed singer (left) represents a large *lArea X:mHVC* difference (>10 -fold), where induced expression is barely detectable in *lArea X* when producing many introductory notes (see [D]). Scale bar, 150 μ m. (B–D) *lArea X:mHVC* expression ratios (y-axes) from males in the three singing contexts relative to the number of song bouts (B), the percentage of directed/undirected singing (C), and the number of introductory notes produced (D) (x-axes). The birds in (B) are from Figure 4. (C) includes additional animals from manual scoring (see Experimental Procedures). (D) includes only birds who sang $>90\%$ directed or undirected song from (B). Linear (B), third order polynomial (C), and logarithmic regression (D) curves are drawn as best fits to the data.

these older juveniles, some directed a majority of their songs to females and had expression patterns similar to adult males producing female-directed song (low *lArea X* and RA expression; Figures 6B and 6C). Thus, the absolute level of singing-induced ZENK expression is dependent on developmental age, but the pattern of expression is, as in adults, dependent on to whom the bird directs its singing.

When given the choice, most adult males in this more

inclusive context sang to the females. None of them directed song to the other males. To get a sizable sample of adult males that sang in an undirected manner, the experiment was repeated a number of times. The birds were then separated into two groups: those who sang $>50\%$ undirected songs and those who sang $>50\%$ female-directed songs. The *lArea X:mHVC* (Figure 6B) and RA:cRA (Figure 6C) expression ratios in these birds were similar to those seen in the male and female choice-

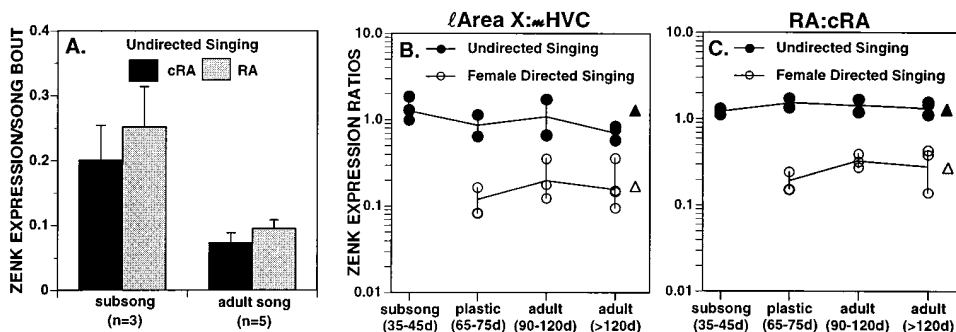


Figure 6. Singing-Induced ZENK Expression Is Higher in Juveniles than Adults, but in Both the Pattern of Expression Is Context Dependent

(A) ZENK expression in cRA and RA relative to singing amount in juveniles and adults producing undirected subsong and stable song, respectively. Total amount of expression depends on age. (B–C) ZENK expression ratios for *lArea X:mHVC* and RA:cRA of males who had a choice of whom to sing during different developmental ages in days (d). Closed circles represent values of individual birds who produced $>50\%$ undirected song. Open circles represent those who sang $>50\%$ female-directed song. Closed and open triangles represent averages obtained from birds who sang 100% undirected ($n = 10$) or 100% female-directed ($n = 5$) song in choice-limited context, respectively. Lines spanning values at each developmental age represent the range. Lines spanning ranges represent averages at each developmental age. Regardless of age, the pattern of singing-induced expression depends on to whom singing is directed.

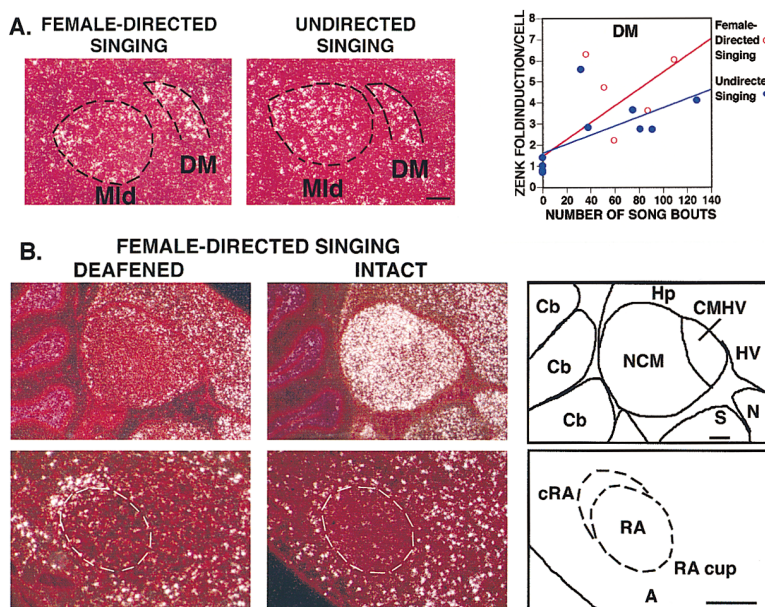


Figure 7. ZENK Expression in Midbrain Nuclei and Effects of Deafening on NCM and RA Cup but Not cRA

(A) DM showed ZENK induction with both female-directed (open red circles) and undirected (closed blue circles) singing, but its linear correlation with singing amount was weaker than in telencephalic nuclei ($r = 0.760$, $p = 0.018$ for directed; $r = 0.606$, $p = 0.063$ for undirected singing; simple regression). A trend for lower activation during undirected singing was not significant ($r = 0.674$, $p = 0.486$ for context; multiple regression). Expression in MLd is due to hearing song (Mello and Clayton, 1994).

(B) Deafening blocked ZENK induction in NCM and the RA cup but not in cRA during singing.

Scale bars, 50 μm (A); 150 μm (B). Abbreviations: Cb, cerebellum; CMHV, caudomedial hyperstriatum ventrale; NCM, caudomedial neostriatum; Hp, hippocampus; HV, hyperstriatum ventrale; S, septum.

limited contexts. Thus, hearing female calls or seeing females at close quarters does not alter the undirected ZENK expression pattern. Similarly, even when the social context includes other males, the female-directed expression pattern persists.

Subtelencephalic Nuclei

We wanted to determine if differential gene activation in telencephalic portions of the posterior and anterior pathways was accompanied by similar differences in their respective subtelencephalic targets: DM of the midbrain and DLM of the thalamus (Figure 1B). DM showed vocally induced ZENK expression that was comparable in directed and undirected singing (Figure 7A). DLM ZENK expression was not homogeneous and was not clearly linked to singing (Figure 2), as previously noted (Jarvis and Nottebohm, 1997; Mello and Ribeiro, 1998). ZENK expression in MLd, a midbrain auditory relay adjacent to DM (Figure 1A), occurred regardless of singing (Figure 7A) as MLd ZENK expression is known to be induced by hearing conspecific song (Mello and Clayton, 1994). There was no difference in MLd expression between males that sang directed and undirected song ($r = 0.348$, $p = 0.293$; multiple regression), and thus its expression in this paradigm does not appear to be directly related to courtship behavior as has been reported for MLd in Japanese quails (Ball et al., 1997). Singing-induced ZENK expression was also noted in the thalamic nucleus uvaeformis (Uva) and its telencephalic target nucleus interfascialis (Nif), both of which project to HVC (Nottebohm et al., 1982; Striedter and Yu, 1998). However, it was not possible to reliably determine if there was a quantifiable difference between directed and undirected singers, as these nuclei are small and embedded in adjacent auditory regions that express ZENK when birds hear song (such as field L for Nif; Mello and Clayton, 1994; Vates et al., 1996), making it difficult to determine their expression boundaries.

cRA, Hearing versus Singing

The region surrounding RA receives a prominent projection from the auditory region below HVC, the HVC shelf (Mello et al., 1998b). Thus, we wondered if ZENK activation in cRA could have resulted from hearing song. To address this issue, expression was examined in deafened males, after they sang to females. ZENK induction in song nuclei of deaf males was equivalent to that of intact birds (Figure 7B shows example for cRA). In contrast, ZENK induction in various parts of the ascending auditory pathway (the caudomedial neostriatum [NCM]) and descending pathway (the RA cup) (Mello and Clayton, 1994) was absent in deaf finches (Figure 7B), as observed in canaries (Jarvis and Nottebohm, 1997). Thus, differential ZENK expression resulting from female-directed song does not require auditory feedback.

Singing-Induced ZENK Expression in HVC's Two Projection Neurons

We wanted to determine whether differential ZENK expression in RA and Area X was due to differential activation of HVC's two types of projection neurons: those that project to RA and those that project to Area X (Figure 1B). To label HVC's two projection neuron populations, different retrograde fluorescent tracers were injected into RA and Area X. After males sang either female-directed or undirected song, we quantified how many of the retrogradely labeled projection neurons in HVC also expressed ZENK protein. For both directed and undirected singing, equal proportions of HVC's RA- and Area X-projecting neurons expressed ZENK protein (Figure 8). The percentages increased uniformly with singing amount. This suggests that (1) both HVC's RA- and Area X-projecting neurons are active during singing and (2) differential ZENK expression in downstream nuclei does not stem from differential activation of HVC's two projection neurons.

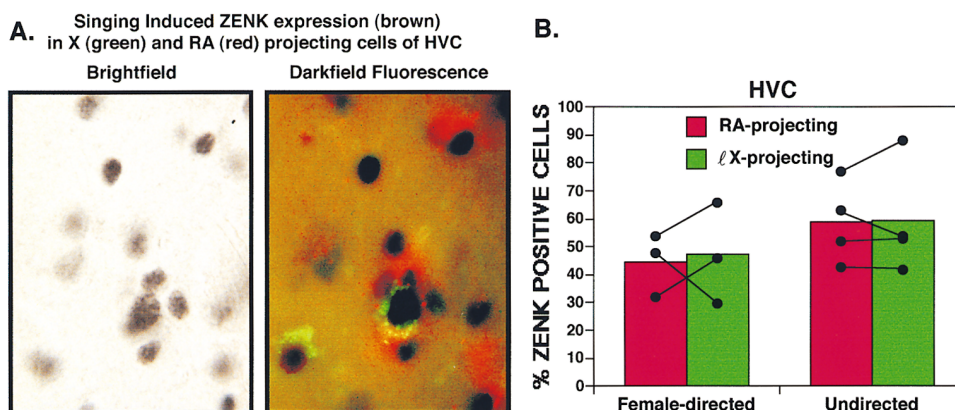


Figure 8. Singing Induces ZENK Protein in Both Types of HVC Projection Neurons

(A) Both RA- (red Dil cytoplasmic backfill) and Area X- (green fluorescein cytoplasmic backfill) projecting cells of HVC have singing-induced ZENK protein expression (brownish-black nuclear precipitate), here shown for undirected singing.

(B) Percentages of RA- and Area X-projecting neurons that express ZENK are similar for directed and undirected singing. Circles connected by lines represent the means for individual birds. The more a bird sang, the higher was the percentage of both cell types showing ZENK expression. Silent controls had very few ZENK-labeled cells in HVC (data not shown; see also Mello and Ribeiro, 1998).

Discussion

In this study, we show that female-directed singing results in low ZENK expression in large portions of the song system; undirected singing results in high levels throughout this system. This differential activation does not require auditory feedback from the female's vocal responses (i.e., calls), since the same expression pattern occurs in deaf males when they sing to females. Moreover, differential activation is coincident with the first signs of female-directed singing behavior in juvenile males. Below, we propose a potential neurobiological mechanism for the differential activation and then discuss possible functional consequences.

Potential Neurobiological Mechanism Evidence for Two Functional Subsystems

Figure 9A summarizes a three-dimensional perspective of the ZENK expression patterns seen after directed and undirected singing. We overlaid the patterns with lines and arrows to indicate the flow of information as inferred from the literature on song nuclei connectivity (see Figure 9A legend for references). Blue lines connect regions (dark gray) that show differential activation with directed and undirected singing. Red lines connect regions (white) that show similar activation with both singing behaviors. When examined in this view, the combination of differential gene activation and connectivity suggests a novel functional organization of the song system.

Two functional subdivisions of the anterior pathway are revealed: a medial and a lateral one. As schematized in Figure 9B, both subdivisions start in HVC. The lateral subdivision then forms a loop: lArea X to lThalamus to lMAN back to lArea X. In a parallel fashion, the medial subdivision forms analogous connections, except that a projection from mArea X to mThalamus has not been described. An mStriatum to mThalamus connection does exist in pigeons (Veenman et al., 1995, 1997), and we therefore suspect that songbird mArea X might show

it, too. If so, these subdivisions would comprise two parallel cortical-striatal-thalamocortical loops. They differ in that whereas the medial subdivision returns to its point of origin, HVC, the lateral one ends in RA. This way, the anterior pathway's medial and lateral components could separately influence activity, and thus ZENK activation, in HVC and RA.

Electrophysiological Relevance and the Anterior Pathway

ZENK is thought to respond to changes in electrophysiological activity (Stripling et al., 1997), as its mRNA expression is regulated by membrane depolarization (Chaudhuri, 1997). However, the converse is not so; not all electrophysiological activity results in ZENK expression (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). Thus, absence of expression may indicate either no electrophysiological activity, no changes in activity, or activity to which ZENK is not responsive.

Our previous report (Jarvis and Nottebohm, 1997) on singing-induced ZENK expression in lMAN and Area X was paradoxical, because prior studies (McCasland, 1987) had detected no electrophysiological activity in these regions during singing. Recent limited data indicate, however, that Area X neurons can be inhibited or excited during singing (Margoliash, 1997). Our ZENK expression data are compatible with the possibility that lArea X's tonic activity is inhibited during directed singing and upregulated during undirected singing. Preliminary electrophysiological data support this notion (Hessler and Doupe, 1997, Soc. Neurosci., abstract).

Instruction for differential activation does not appear to come from HVC, since comparable numbers of HVC's Area X-projecting neurons show ZENK activation with both directed and undirected singing. Instead, it is possible that HVC activity reaches Area X (Figure 9B, red arrows) in both contexts, but transmission and ZENK expression in lArea X is differentially modulated by catecholaminergic innervation from the midbrain's Area ventralis of Tsai (AVT) and the nucleus tegmenti pedunculo-pontinus pars compacta (TPc) (Lewis et al., 1981). The

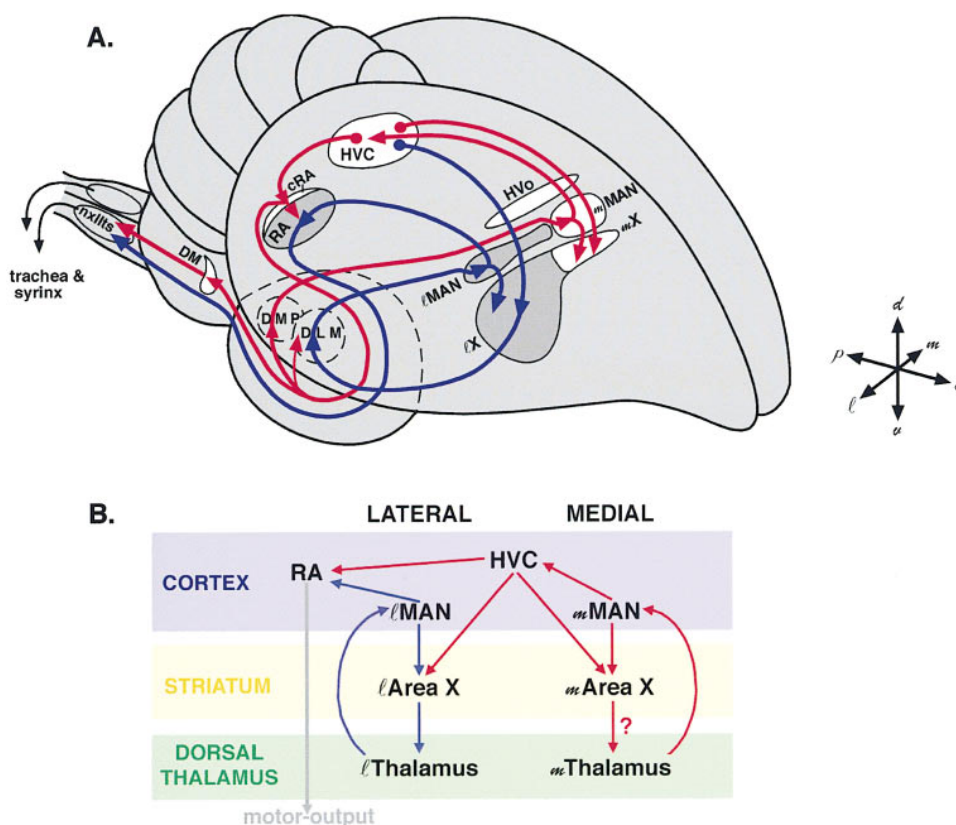


Figure 9. Subdivisions of Song System Circuitry Revealed by Context-Dependent ZENK Expression

(A) Semi-three-dimensional perspective of regions showing singing-induced ZENK expression. Lines and arrows indicate the flow of information as inferred from the literature on song nuclei connectivity (Nottebohm et al., 1976, 1982; Okuhata and Saito, 1987; Bottjer et al., 1989; Vicario, 1993; Wild, 1993; Johnson et al., 1995; Nixdorf-Bergweiler et al., 1995; Vates and Nottebohm, 1995; Foster et al., 1997; Vates et al., 1997). Blue lines connect regions (dark gray) that show low activation with female-directed singing and high activation with undirected singing. Red lines connect regions (white) that show similar activation with both singing behaviors. Exact medial-lateral expression boundaries have not yet been determined. Not shown are ascending projections to HVC from Uva of the thalamus and Nlf of the avian cortex (Nottebohm et al., 1982; Striedter and Yu, 1998) and catecholaminergic projections from AVT-TPc-LoC of the thalamus and VP of the striatum (Lewis et al., 1981; Li and Sakaguchi, 1997; Mello et al., 1998a).

(B) Two parallel subdivisions, lateral and medial, of the anterior pathway are shown. A connection from *mArea X* to *mThalamus* is proposed. In mammalian brain terminology, these subdivisions consist of cortical-striatal-thalamocortical loops whose output is to the vocal motor cortex. The lateral loop (blue lines) would influence activity in RA, the medial (red lines) in HVC.

AVT-TPc complex is the avian equivalent of the mammalian ventral tegmental area and substantia nigra pars compacta (VTA-SNpc; Medina and Reiner 1995) and is thought to modulate excitatory cortical inputs to the striatum by releasing dopamine onto striatal neurons (Smith and Bolam, 1990; Mink, 1996). The presence of catecholamines and their receptors in Area X has been shown by a number of studies (Bottjer et al., 1989; Casto and Ball, 1994, 1996; Soha et al., 1996), with dopamine higher relative to other catecholamines and other brain regions (Harding et al., 1998). Modulation of activity in *lArea X* via AVT could indirectly affect activity in *MAN* and RA (Figure 9B, blue arrows). Alternatively, modulation could occur directly through catecholaminergic innervation to RA and MAN (Bottjer et al., 1989; Casto and Ball, 1994, 1996; Soha et al., 1996; Harding et al., 1998) from the ventral paleostriatum (VP) and/or locus coeruleus (LoC) (Li and Sakaguchi, 1997; Mello et al., 1998a). Interestingly, α 2-adrenergic receptor density

has been shown to be higher in *mMAN* than in *MAN* (Casto and Ball, 1996).

Electrophysiological Relevance and the Posterior Pathway

Absence of robust ZENK induction in the body of RA during female-directed singing is provocative. Lesion experiments (Nottebohm et al., 1976; Simpson and Vicario, 1990), electrophysiological recordings (Yu and Margoliash, 1996), and stimulation (Vu et al., 1994) suggest that the bulk of cells in RA (not just those of the dorsal cap, cRA) are involved in song production. Moreover, Fos expression is found throughout RA in males induced to sing by presentation of females (Kimbo and Doupe, 1997), and these males presumably sang directed song. Since Fos, like ZENK, responds to electrophysiological activity, it suggests that even in the presence of membrane depolarization an additional factor is required for ZENK expression in RA to occur. We propose that the lateral anterior pathway provides this

additional factor, modulating some aspect of RA's activity differently during directed singing, and that ZENK, but not Fos, is sensitive to this modulation. In contrast to the situation in RA, under equivalent behavioral conditions, Fos—but not ZENK—expression is absent in the anterior pathway and in HVC's Area X-projecting cells (Kimbo and Doupe, 1997). However, HVC's RA-projecting cells show singing-induced expression of both genes. Perhaps not surprisingly, singing apparently requires or causes differential cellular activity in the various regions of the song system, which in turn induces either Fos expression, or ZENK expression, or both.

Previous reports on singing-induced expression (Jarvis and Nottebohm, 1997; Jin and Clayton, 1997; Kimbo and Doupe, 1997; Mello and Ribeiro, 1998) did not address the type of song produced. Some differences between these reports and the present findings are also likely due to this variable. For instance, Jin and Clayton (1997) report little singing-induced ZENK expression in the main portion of adult RA and high levels in a posterior region, similar to what we define as cRA; they also report consistently high levels throughout juvenile RA. They suggest that this age difference is related to the vocal plasticity required for song learning in juveniles and the absence of such plasticity in adults. In contrast, we find that the pattern of ZENK expression in RA is more closely associated with manner of singing than with age.

Possible Functional Consequences *Internal State*

Undirected singing in adults occurs in a context that is reminiscent of song learning, when there is no obvious audience and the main purpose of singing seems to be to practice. The anterior pathway is required for song learning in young animals (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Song of adult male zebra finches deteriorates after deafening (Nordeen and Nordeen, 1993) or syringeal denervation (Williams and McKibben, 1992). Lesions of MAN prevent this deterioration (Williams and Mehta, 1995, Soc. Neurosci., abstract; Brainard and Doupe, 1997, Soc. Neurosci., abstract). We infer from this that maintenance of learned song is a process of continued learning in adults and that the anterior pathway is involved in this process. ZENK expression in the lateral subdivision of this pathway occurs when juvenile and adult males sing undirected song. In this vein, undirected singing by adults may be a process of continuous action-based learning (Marler and Nelson, 1993; Marler, 1997) and synaptic strengthening, to maintain what is already known (Jarvis and Nottebohm, 1997). During female-directed song, the lateral anterior pathway may be shut down as attention is focused, not on song rehearsal, but on courtship.

This as well as our previous study (Jarvis and Nottebohm, 1997) highlight motor, as opposed to auditory, aspects (Williams, 1989; Doupe and Solis, 1997; Scharff et al., 1998) of the adult anterior pathway. The identical ZENK expression patterns in Area X and MAN of intact and deafened animals (Jarvis and Nottebohm, 1997), as well as the absence of auditory activity in the anterior pathway in awake animals (Hessler and Doupe, 1997, Soc. Neurosci., abstract; Margoliash, 1997), make a primarily sensory role for this pathway in adults highly

unlikely. However, it remains possible that the anterior vocal pathway processes auditory information only when the bird sings. This would be compatible with one of the proposed roles of the mammalian cortical-basal ganglia loop, i.e., to serve as a sensory analyzer for motor systems during the performance of motor behaviors (Lidsky et al., 1985).

Motor Output

Finally, differential activation of the song system during directed and undirected singing could cause the observed differences in behaviors, such as song speed, the number of introductory notes, or even the dance that accompanies directed singing. If so, one would have to postulate selective inhibition of the lateral loop and excitation of the medial loop in order for these behaviors to manifest themselves, as has also been proposed for the mammalian cortical-basal ganglia loop (Mink, 1996). Although not known, male and female zebra finches may detect the small "vocal" differences between directed and undirected song as important. A finer lesion analysis is in order.

Conclusion

The anterior forebrain vocal pathway of songbirds can no longer be thought of as a pathway whose function is restricted to early stages of song learning. It is fully functional in the adult and appears to be involved in singing, in a context-dependent manner. Context-dependent use of vocalizations has now been described in many songbird species (Catchpole and Slater, 1995), and thus differential activation of this pathway may be common. Furthermore, context-dependent neuronal activity occurs in the mammalian striatum and supplementary motor area of the frontal cortex that projects to the striatum (Lidsky et al., 1985; Romo and Schultz, 1992; Schultz et al., 1995; Mink, 1996; Aldridge and Beridge, 1998), avian homologs of regions that contain the anterior vocal pathway. Thus, the context-dependent function of this circuit may be a basic feature of the vertebrate brain. In general, the results of our study emphasize that paying close attention to the animal's behavior, and in this case to whom the bird sings, is of paramount importance for drawing accurate conclusions about how the brain works. The complexities we have uncovered suggest that if molecular biology is to achieve its full impact on the understanding of brain function, it will have to devote as much scrutiny to the behaviors it tries to explain as to the molecules that are involved.

Experimental Procedures

Animals

We used male and female zebra finches from our breeding colonies at the Rockefeller University Field Research Center, in Millbrook, NY. Unless otherwise indicated, adult birds ranged in age from 90 days to 1 year.

Singing Behavior

Birds were kept under silent conditions for at least 2 hr before each singing session. This was accomplished either by the investigator sitting next to their cage or by starting singing sessions at dawn, right after the birds woke up. A 2 hr silent period is known to reduce ZENK mRNA expression in auditory and vocal pathways to basal levels (Jarvis and Nottebohm, 1997). When the bird started singing,

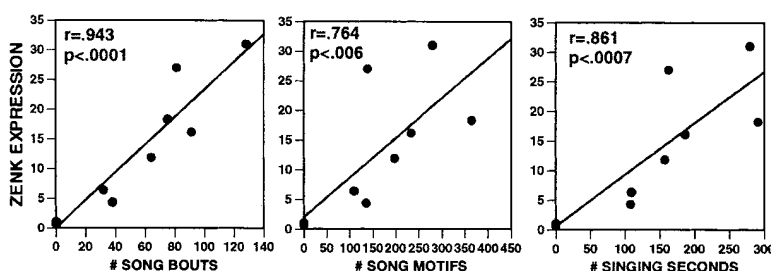


Figure 10. Correlation between ZENK Expression and Singing Is Strongest with the Number of Song Bouts

Correlations between ZENK expression (fold induction, y-axis) with three different measures of singing amount (x-axis), shown for *mHVC* after undirected singing as a representative example. Weakest to strongest correlations are with number of song bouts, number of seconds singing, and number of song motifs ($n = 7$ singers and 4 controls; simple regression).

song behavior was scored continuously for 30–40 min in one of the following contexts.

Female Context ($n = 12$)

A single male in a cage was kept alone in a room. A cage with 2 females was then introduced next to his cage. We found that 2 females were better at eliciting song than 1 female. The females in their cage usually stood as close as possible to the male's cage and at times fought over that space. Males tended to sing vigorously to the females at first, but then singing declined. Thus, to encourage consistent singing throughout the session, a cage with new females was introduced approximately every 10 min throughout the 30–40 min session. This experiment was repeated until we had a sample of 12 males who sang 20 or more song bouts during a session. Of these 12 birds, 9 began singing at first introduction of the females, and 3 began singing within the first 2–10 min.

Male Context ($n = 12$)

Three or four cages containing 1 male each were placed next to each other. Singing was further encouraged by playing tape recorded song of another male. This experiment was repeated until we had a sample of 12 males who sang 20 or more song bouts during a session. Of these 12 birds, 4 began singing right away, 7 began singing within the first 2–10 min, and 1 began singing at 20 min.

Solo Context ($n = 12$)

A male in a cage was isolated in a secluded room or in a soundproof box. After the silent period, the investigator then waited for the bird to sing. Most birds sang after we waited 30 min to 2 hr. Males that sang 20 or more song bouts within a 30–40 min period were then sacrificed. Differences in the latency to begin singing in this and the above two contexts did not effect the ZENK expression patterns.

Development and Free Choice Context ($n = 12$ Adults and 7 Juveniles)

Several cages containing 1 male each were placed next to each other and next to cages containing females. In some cases, individually caged juvenile males were added for a developmental study. Singing was encouraged by playing tape-recorded song. The age of the juveniles ranged from 35 to 75 days; the age of the adults ranged from 90 days to 4.5 years.

Silent Controls ($n = 4$ Adults and 4 Juveniles)

Some of the males in the above free choice group who did not sing and called infrequently were used as silent controls.

Deafened Males ($n = 3$)

Males were deafened by cochlear removal as described (Konishi, 1964). Female-directed singing was elicited as described above, and these birds also sang 20 or more bouts in 30 min.

Behavior Analysis

Equipment

We used a Sony DCR-VX1000 digital video camera connected to a TV monitor and a Sennheiser ME62 omnidirectional microphone to record singing behavior for 6 of the 12 birds in the male and female contexts. A Marantz PMD222 tape recorder was used to record singing for 7 of the 12 solo singers. Sonograms were generated using SoundEdit 16 (Macromedia, San Francisco) or Canary 1.2.1 (Cornell Bioacoustics Workstation, Ithaca, NY) for Macintosh computers. The remaining birds were scored manually during the behavior observation.

Song Analysis

Song was scored as "female directed" when two of three typical behaviors (Zann, 1996) occurred together: (1) the singer faced the

female, (2) the plumage of ear-coverts, abdomen, and flanks was fluffed but forehead feathers were flattened, and (3) the singer hopped toward the female during singing. Song was considered "male directed" when the singer exhibited the above behaviors oriented toward a male. In the presence of other birds, song was considered "undirected" when the singer did not orient toward another bird, and plumage display as well as hopping were absent. Song of birds in the solo context was by definition undirected.

To quantitate the amount of singing, three different parameters were analyzed: number of song bouts, number of motifs, and total time spent singing (number of motifs multiplied by motif length in seconds). A bout was defined as a succession of motifs separated by less than 2 s. A comparative analysis of the three measures showed that the number of bouts was the best predictor of singing-induced ZENK expression, followed by the total number of seconds singing and then by the number of motifs (Figure 10). For this reason, we used the number of bouts as the measure of singing activity throughout the study. Video- and tape-recorded samples gave the most reliable measures, and we used them for most of our analysis. However, in some cases (Figures 5C and 6), we combined results from video and manual scoring to enlarge sample sizes. Manual scoring yielded more variable but comparable results to those obtained from the video material.

Anatomy

We introduce nomenclature for four anatomical regions not previously defined as distinct regions in the songbird brain: *mArea X*, *lArea X*, *cRA*, and *HVo*. Area X has been referred to as a unitary structure, but we observed that singing-induced ZENK expression in medial (0.2 mm from midline) and lateral (2.3 mm from midline) Area X differed depending on context. We do not know yet if there is a gradual or abrupt boundary between medial and lateral Area X because coronal sections have not yet been done. However, we did note that Area X differential expression followed that of medial and lateral MAN above it. That is, whenever *mMAN* had high expression so did *mArea X* below it, and whenever *MAN* was differentially regulated so was *lArea X* below it. Thus, we define *mArea X* as the region that sits directly below *mMAN* abutting the dorsal LPO border and *lArea X* as the larger region in LPO that sits below *MAN* (see Figure 9). Support of a functional medial-lateral subdivision of Area X comes from recent evidence showing that *mMAN* projects to *mArea X* but not *lArea X* (Foster et al., 1997). Comparison of that study (Foster et al., 1997) with others (Nixdorf-Bergweiler et al., 1995; Vates and Nottebohm, 1995) suggests that there is a topographic mapping of medial and lateral MAN onto medial and lateral Area X, respectively. Interestingly, medial and lateral LPO in pigeons is distinctly topographic in its connectivity (Veenman et al., 1995).

The cap of RA (*cRA*), as defined by singing-induced ZENK expression, corresponds to a tier of cells that straddle the posterodorsal boundary of RA (Figure 9). A region that coincides in part or fully with this description has been described in various studies and appears to include cells within RA that project to the thalamic nucleus DMP and midbrain nucleus DM (Vicario, 1993; Foster et al., 1997; Vates et al., 1997) and cells posterodorsal to RA, which also project to DMP (Foster et al., 1997). Input to the RA portion comes from the medial-most part of MAN, as shown by Johnson et al. (1995) who called this area the "cap region of RA"; input to the posterodorsal region outside of RA comes from the HVC shelf (Mello et al., 1998b). Jin and Clayton (1995) found a similar region of singing-induced ZENK expression that they called posterior RA (RAP),

which overlaps partly with the expression pattern we present here as cRA. In general, cRA is difficult to define and should be taken with caution when comparing across studies. However, the growing body of evidence, including this study, indicates that it is a distinct anatomical region. The RA cap is not to be confused with the RA cup, which is anteroventral to RA (Mello et al., 1998b).

HVo is a small, newly identified, oval region directly dorsal to medial and lateral MAN. At its biggest, it spans 180 μ m in anteroposterior and 80 μ m in dorsoventral dimensions. It came to our attention because of its consistent singing-induced ZENK expression. It is presumably the songbird homolog of the parrot oval nucleus of the hyperstriatum ventrale (HVo), which is in a similar position dorsal to an anterior neostriatal vocal control nucleus (Striedter, 1994; Durand et al., 1997). The parrot HVo also shows singing-induced ZENK expression (Jarvis et al., 1997, Soc. Neurosci., abstract). A detailed report on HVo will be published elsewhere.

Gene Expression and Quantification:

In Situ Hybridization

All birds were killed 30–40 min after onset of singing, when ZENK mRNA expression is known to peak (Jarvis and Nottebohm, 1997). When 2–3 birds were observed at the same time, ~10 min would elapse between the first and last birds killed. Brains were removed and processed for in situ hybridization as described (Mello et al., 1997), and relative fold increase per cell was quantified as described (Jarvis and Nottebohm, 1997). In brief, sections were hybridized to an ³⁵S-labeled riboprobe made from a canary ZENK cDNA. For quantitative comparison, sections from all 66 birds were hybridized at the same time to minimize procedural variability. Slides were dipped in photosensitive emulsion, dried upright, and exposed for 3 weeks to prevent emulsion saturation. Slides treated in this manner tend to have a thicker emulsion layer at the bottom than at the top, and for this reason all comparisons were based on sections from the bottom part of the slide. After developing, sections were counterstained with cresyl violet acetate (Sigma). For each nucleus sampled, we counted the total number of silver grains, and the total number of cells, in two representative 130 μ m \times 130 μ m fields (one caudal, one rostral) per bird, using a 60 \times objective and NIH image computer analysis software. Values for silver grains from the two fields were averaged, as were values for cell counts. The number of grains for each nucleus was then divided by the number of cells (ranging between 70 and 100) per field. The resulting value was normalized by dividing it by the number of grains per cell for the corresponding region in nonsinging controls. The final value represented "fold" ZENK mRNA induction per cell of a given region for a given bird. The medial-lateral subdivisions of the regions quantified are those described in Figure 3. "Lateral" HVC refers to the part of HVC that is in the same medial-lateral plane as RA but not Area X.

In this and a previous report (Jarvis and Nottebohm, 1997), we show large differences, >20-fold, in the singing-induced ZENK expression in some song nuclei. However, Jin and Clayton (1997) and Mello and Ribeiro (1998) showed smaller differences, i.e., <10-fold. The discrepancy probably results from what is being quantified. We measured the average ZENK mRNA induction per cell. Jin and Clayton (1997) and Mello and Ribeiro (1998) measured the average number of cells showing ZENK mRNA and protein induction, respectively. Apparently, increased expression per cell yields a relatively higher value than number of cells.

Immunocytochemistry and Double Labeling

with Retrograde Tracers

Surgery

Birds were anesthetized with 0.05 ml of a 1:5 Nembutal dilution, placed in a stereotaxic holder, and injected with four injections of 15 nl each into RA and six injections into Area X of either rhodamine (red) or fluoresceine (green; Lumafuor, New York) microspheres or Dil (red; Molecular Probes, Eugene, OR). In each bird, one tracer was injected into RA and another into Area X, to unambiguously identify ZENK-labeled HVC's RA- and Area X-projecting neurons. Only one hemisphere per bird was injected, for two reasons: birds showed a higher probability of singing when injected only unilaterally, and the uninjected side served as a control for potential tracer

effects on ZENK expression. Tracers were alternated between nuclei in different birds to avoid creating bias in quantitative analysis. Dil was prepared by dissolving 5 mg in 10 μ l dimethylformamide (Sigma), heated to 65°C for 30 min, and then diluted in 80 μ l corn oil (Wesson) preheated to 50°C, resulting in a 5% final concentration (Lois and Alvarez-Buylla, 1994). Microspheres were used as supplied.

Behavior and ICC

After allowing 3–4 days for dye transport, males were presented with females to solicit directed song or kept alone to solicit undirected song. Experiments began soon after birds woke up. This ensured a minimum of 3 hr during which the birds neither sang nor heard song preceding the singing session to assure basal levels of ZENK protein expression (Mello and Ribeiro, 1998). Singing amount was recorded manually, and males who sang 30 or more song bouts during 45–60 min were perfused under deep Nembutal anesthesia with 60 ml of PBS, followed by 60 ml of 4% paraformaldehyde in PBS. Brains were removed and cryoprotected in 30% sucrose overnight at 4°C. Sagittal sections (14 μ m) of both injected and uninjected hemispheres were cut on a freezing sliding microtome. Sections were processed for immunocytochemistry using a polyclonal antibody against mouse egr-1 protein (Santa Cruz Biotech, Santa Cruz, CA), which recognizes the avian ZENK protein (Mello and Ribeiro, 1998).

Briefly, nonspecific binding was blocked by 3 \times 10 min washes in PBS (pH 7.0) with 10% skim milk (PBSM). Sections were then incubated overnight at 4°C with a 1:1000 dilution of the egr-1 antibody in PBSM, followed by 3 \times 10 min PBSM washes and incubation in a 1:250 dilution of biotinylated secondary anti-rabbit IgG (Sigma; St. Louis, MO) for 1 hr at room temperature. After 3 \times 10 min PBSM and amplification with avidin-biotin complex (ABC, Sigma) for 1 hr, 2–3 min of incubation with a nickel-intensified diaminobenzidine (DAB) solution (1 g nickel ammonium sulfate, 80 mg dextrose, 16 mg ammonium chloride, 25 units glucose oxidase [Sigma], 20 ml acetate buffer [0.2 M, pH 6.0], and 20 ml dH₂O) resulted in a brownish-black reaction product. After 3 PBS washes, sections were mounted with 0.3% gelatin in dH₂O onto TESPA- (triethoxysilane amino-propyl acid, Sigma) coated slides and quickly coverslipped with Anti-Fade Light (Molecular Probes, Eugene, OR) for brains containing Dil, or they were air dried and coverslipped with mineral oil for brains containing only microspheres. Reactions did not include Triton, glycerol, or organic solutions to avoid loss of tracers. Analysis was performed within 1–2 weeks after the reaction was completed. Under aqueous conditions, the DAB precipitate partially faded after 1–2 months at 4°C. Microspheres and Dil did not affect ZENK expression at the injection site, beyond the unavoidable damage due to pipette insertion. Fluorogold, however, prevented ZENK induction at and around the site of injection and thus was not used (data not shown).

Quantification

Using a computer yoked mapping system (Alvarez-Buylla and Vicario, 1988), we quantified five categories of labeled cells in HVC: the number of retrogradely labeled RA and Area X projection neurons with and without ZENK immunoreactivity and the number of ZENK-immunopositive cells without retrograde label in 2–5 (mean, 3.1) sections per bird ($n = 4$ directed and 6 undirected singers). Values of all sections per bird were summed. The percentage of ZENK-positive RA-projecting neurons was calculated by dividing the number of ZENK-labeled RA projection neurons by the total number of RA projection neurons, multiplied by 100. An analogous calculation was made for Area X projection neurons.

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