Neuron, Vol. 27, 359–370, August, 2000, Copyright ©2000 by Cell Press

Site-Specific Retinoic Acid Production in the Brain of Adult Songbirds

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Summary

The song system of songbirds, a set of brain nuclei necessary for song learning and production, has distinctive morphological and functional properties. Utilizing differential display, we searched for molecular components involved in song system regulation. We identified a cDNA (*zRalDH*) that encodes a class 1 aldehyde dehydrogenase. zRalDH was highly expressed in various song nuclei and synthesized retinoic acid efficiently. Brain areas expressing zRalDH generated retinoic acid. Within song nucleus HVC, only projection neurons not undergoing adult neurogenesis expressed *zRalDH*. Blocking zRalDH activity in the HVC of juveniles interfered with normal song development. Our results provide conclusive evidence for localized retinoic acid synthesis in an adult vertebrate brain and indicate that the retinoic acid–generating system plays a significant role in the maturation of a learned behavior.

Introduction

Retinoids (vitamin A and derivatives) play essential roles in embryonic development, cellular differentiation, and homeostasis (Wolbach and Howe, 1925; Sporn et al., 1994). Their most potent naturally occurring metabolite—all-*trans* retinoic acid—is important for limb formation and regeneration (Thaller and Eichele, 1987; Smith et al., 1989; Bryant and Gardiner, 1992; Johnson and Scadding, 1992; Scadding and Maden, 1994), specification of the anterior–posterior primary axis, and CNS formation and patterning (Durston et al., 1989; Maden and Holder, 1991; Ruiz i Altaba and Jessell, 1991; Chen and Solursh, 1992; Sundin and Eichele, 1992; Blumberg et al., 1997). Retinoid signaling is also essential for the normal establishment of the olfactory pathway (Anchan et al., 1997), development of spinal cord (Colbert et al., 1993; McCaffery and Drager, 1994), early patterning of the vertebrate eye (Mey et al., 1997; Drager et al., 1998), and specification of motor neuron subtype identity in the spinal cord (Sockanathan and Jessell, 1998).

Retinoic acid is synthesized from retinaldehyde by aldehyde dehydrogenases (ALDHs). These enzymes catalyze the oxidation of various endogenous and exogenous substrates and differ in subcellular localization, substrate preference, charge, and inhibitor susceptibility (Napoli and Race, 1987; Posch et al., 1989; Napoli et al., 1992). Retinaldehyde is thought to be an important substrate of a class 1 (cytosolic) ALDH named ALDH1 in rats, AHD2 in mice, and E1 in humans (Greenfield and Pietruszko, 1977). However, this enzyme also oxidizes a broad range of aldehydes (Manthey et al., 1990). Another class 1 ALDH more efficient and selective in retinaldehyde oxidation has been described (Wang et al., 1996; Zhao et al., 1996) and named RalDH(II) in the rat and RALDH2 in the mouse. Data on RALDH2's tissue distribution during development and on gene knockouts indicate that it plays a major role in retinoic acid synthesis in embryonic tissues (Niederreither et al., 1997, 1999).

In contrast to the embryonic period, the localization, biosynthesis, and function of retinoids in adult neuronal tissues remain poorly established. Retinoic acid-synthesizing activity has been detected in crude homogenates of adult rabbit brain (Dev et al., 1993), though the enzymes involved and their selectivity and precise localization were not determined. Studies on the brain distribution of the retinoic acid binding proteins CRABP-I and CRABP-II (Bailey and Siu, 1988; Zetterstrom et al., 1994) and of retinoid receptors (Krezel et al., 1999; Zetterstrom et al., 1999) in rodents provide further suggestive evidence for the presence of retinoic acid in the adult vertebrate brain. However, neither the synthesis and presence of retinoic acid nor its role in a specific brain system or function has been demonstrated in adults.

We have been searching for molecular cues related to the functional properties of the song system in songbirds. This system consists of a set of interconnected brain nuclei essential for song learning and production (Nottebohm et al., 1976). The high vocal center (HVC) integrates auditory and motor activities and constitutes a nodal nucleus in the song system. HVC projects to the nucleus robustus archistriatalis (RA), which in turn projects to hypoglossal motor neurons that innervate the vocal organ, the syrinx. This pathway is the motor backbone of the song system and is necessary for production of learned song (Nottebohm et al., 1976, 1982; Yu and Margoliash, 1996; reviewed by Margoliash, 1997; Wild, 1997). HVC is also connected to RA indirectly, through an anterior forebrain pathway that sequentially connects HVC to area X of the paleostriatum, then to thalamic nucleus DLM, on to the lateral magnocellular nucleus of the anterior neostriatum (IMAN), and from there to RA (Okuhata and Saito, 1987; Bottjer et al., 1989). The anterior pathway is necessary for acquisition but not production of learned song (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991).

HVC shows marked growth during the first few months after hatching, when song is being learned, due to an increase in neuronal size and number (Bottjer et al., 1985; Alvarez-Buylla et al., 1992; reviewed by Nottebohm, 1989; Alvarez-Buylla and Kirn, 1997). The HVC neurons that project to RA, but not those that project to area X, continue to be produced and replaced during adult life (Alvarez-Buylla et al., 1990, 1992; Kirn et al., 1991; Alvarez-Buylla and Kirn, 1997). The identification of genes preferentially expressed in HVC could potentially lead to basic insights into the functional organization of the song system and help to explain the regulation of adult neurogenesis.

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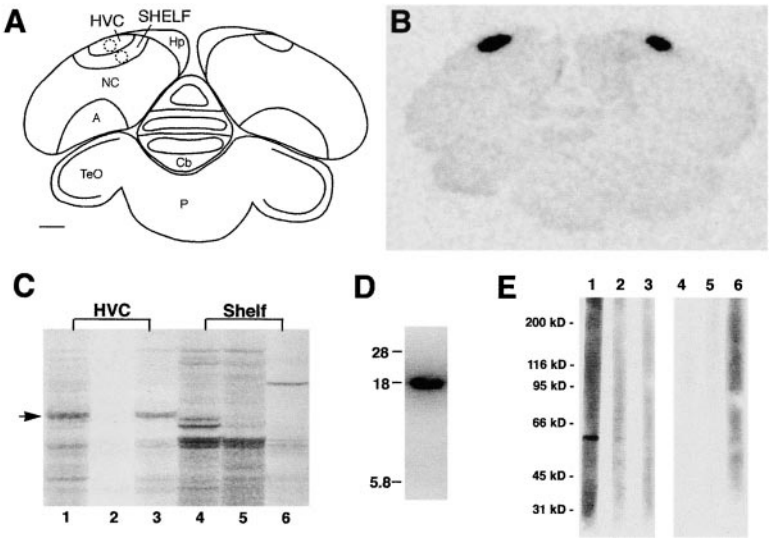

Figure 1. Isolation and Expression Analysis of *zRalDH* in Zebra Finches

(A) Diagram of frontal brain section through HVC; dashed circles show punch sites.
(B) Expression in in situ autoradiogram (same level as in [A]) is restricted to HVC.
(C) DD gel comparing HVC (1–3) and shelf (4–6) in three birds; arrow indicates a band (*zRalDH*) present in HVC but not shelf samples.
(D) A single band (~2.4 kb) is detected on a forebrain poly(A)$^+$ blot hybridized to *zRalDH*.
(E) Western blot with anti–class 1 (ALDH1; lanes 1–3) or anti–class 2 (msALDH; lanes 4–6) ALDH antibodies; compared are HVC (1 and 4), NC (2 and 5), and shelf (3 and 6). A single band is recognized in lane 1 but not in other lanes. Abbreviations: A, archistriatum; Cb, cerebellum; Hp, hippocampus; HVC, high vocal center; NC, caudal neostriatum; P, paleostriatum; TeO, optic tectum.
(A and B) Dorsal is up; scale bar, 0.5 mm.

We used differential display (DD) to screen for molecular markers of HVC in zebra finches and isolated a brain cDNA (*zRalDH*) encoding a class 1 ALDH whose expression is highly enriched in HVC. Within HVC, *zRalDH* expression localizes to the stable neuronal population that projects to area X. We show that zRalDH has a very high affinity for retinaldehyde and that brain areas expressing zRalDH generate retinoic acid. In addition, blocking zRalDH enzymatic activity in the HVC of juveniles disrupts the normal process of song maturation. Our results clearly demonstrate that retinoic acid production occurs in discrete sites of an adult vertebrate brain. They also indicate that retinoid-regulated processes in the CNS are not restricted to embryogenesis and can play a modulatory role in the maturation of a learned behavior.

Results

Isolation of *zRalDH*

To identify genes specifically expressed or enriched in song nuclei, we compared the mRNAs of HVC with those of the underlying neostriatal shelf in adult male zebra finches using DD (Figure 1A). These neighboring regions differ markedly in cytoarchitectonics, connectivity, and physiology (e.g., Nottebohm et al., 1976, 1982; Katz and Gurney, 1981; Fortune and Margoliash, 1995; Vates et al., 1996; Mello et al., 1998); steroid hormone binding (Arnold et al., 1976); and gene expression during vocal communication (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). In addition, adult neuronal replacement in HVC is seasonally and hormonally regulated (e.g., Kirn and Nottebohm, 1993; Rasika et al., 1999), whereas such events have not been described in the shelf.

A set of modifications were introduced to the standard DD protocol to increase its reliability, using in situ hybridization as a sensitive procedure to confirm differential expression (see Experimental Procedures for details). Several candidate DD bands showed differential expression between HVC and the shelf (data not shown). One of these had a very distinctive pattern (Figure 1C, arrow). It was expressed at high levels in HVC (Figure 1B) but absent in the shelf and all other structures at the same level of sectioning, including caudal neostriatum, archistriatum, hippocampus, brainstem, and cerebellum (Figure 1B). A single transcript was detected on Northern blots of brain tissue (Figure 1D).

A full-length cDNA with the same brain expression pattern and containing the sequence of the original DD fragment was obtained from the screening of an embryonic zebra finch library. This cDNA had two possible start codons, the first likely representing the initiation site. The open reading frame predicted a 55 kDa protein containing strictly conserved residues for ALDH (Hempel et al., 1993, 1997) and a cofactor binding motif (Figure 2A). Sequence analysis revealed homology to ALDHs. The amino acid sequence had highest homology (95% residue identity) to rat RalDH(II) and mouse RALDH2 (mAH2; Figure 2B), and lower homologies (72%–65%) to other class 1 (cytoplasmic) ALDHs (Figure 2B) and to class 2 (mitochondrial) ALDHs. We named our clone *zRalDH* (for zebra finch retinaldehyde-specific ALDH). Western analysis with an antibody that recognizes class 1 ALDHs revealed a single band in HVC but none in the shelf (Figure 1E, left lanes) of adult male zebra finches. No bands were detected in these samples with an antibody that recognizes class 2 (mitochondrial and microsomal) ALDHs (Figure 1E, right lanes).

Characterization of Recombinant zRalDH

The possibility that *zRalDH* might be involved in retinoic acid synthesis, as suggested by sequence analysis, was intriguing, as the presence of a retinoid-synthesizing enzyme in specific regions of the adult brain had not yet been demonstrated. We therefore cloned *zRalDH* into an expression vector (Figure 3A) and assayed the ability of the recombinant protein to enzymatically oxidize aldehydes. The results showed that zRalDH catalyzes the oxidation of all-*trans* retinaldehyde, the immediate precursor of all-*trans* retinoic acid, with very high affinity (low Km) for this substrate (Figure 3B; Table 1). In contrast, affinity for other aldehydes was either very low (500- to 1000-fold lower) or undetectable (Table 1). zRalDH's enzymatic oxidation of retinaldehyde was abolished in the presence of 10–50 μM disulfiram (DS; Figure 3C), a potent inhibitor of class 1 ALDHs (Vallari and Pietruszko, 1982; Veverka et al., 1997). Thus, zRalDH

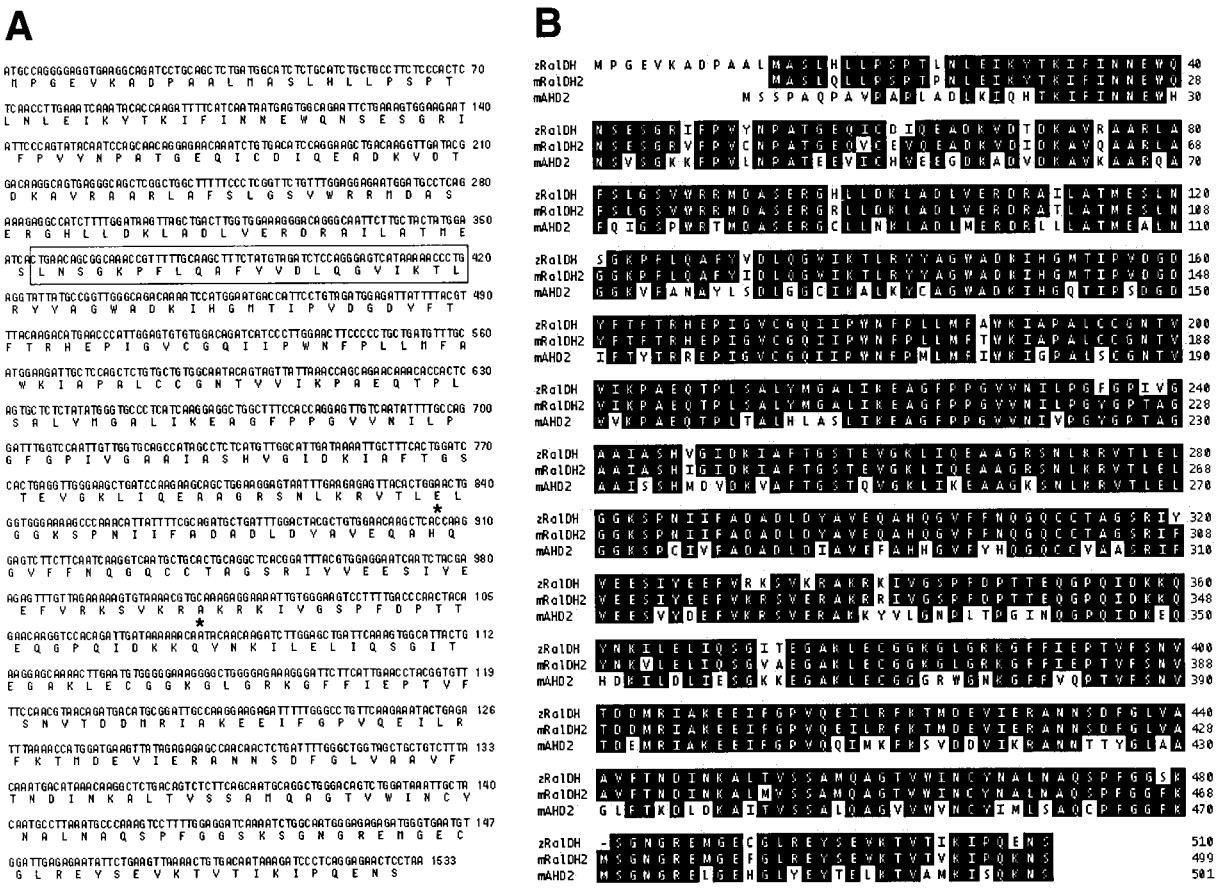

Figure 2. Sequence Analysis of *zRalDH*

(A) Open reading frame and deduced amino acid sequence. The catalytic domain is enclosed by a rectangle; other residues essential for enzymatic activity of class 1 ALDHs are indicated by asterisks.

(B) Alignment of zRalDH's amino acid sequence with those of mouse (m)RALDH2 and AHD2. Boxed regions indicate residue identity; extended amino terminus of zRalDH due to alternative start codon.

has a clear and marked preference for retinaldehyde as a substrate, indicating a specialization in retinoic acid synthesis.

Retinoic Acid Production in the Adult Brain

To test whether the brain of adult zebra finches in fact produces retinoic acid, we cocultured an F-9 cell line (Figure 4A), which carries a β-gal reporter gene under the control of a retinoic acid–sensitive promoter element (Wagner et al., 1992), with explants from HVC or shelf. HVC explants induced β-gal expression in the surrounding F-9 cells (Figures 4B and 4D), indicating the presence of retinoic acid in the explants. In microtiter plates, one or two HVC explants induced β-gal in all F-9 cells in a well. In contrast, explants from the shelf did not have any inductive effect (Figure 4C). The results above were consistent (n = 6 birds tested in the two incubation systems in triplicate). To test whether retinoic acid production in HVC explants was associated with zRalDH enzymatic activity, we incubated the explants in medium containing 10 μM DS prior to the coculture. This treatment significantly reduced or abolished the inductive effect of the HVC explants on F-9 cells (Figure 4E; n = 4 birds), whereas preincubation in medium without DS for the same period did not reduce their inductive effect.

To ensure that a retinoic acid-synthesizing activity and retinoic acid are in fact present in HVC, and not an artifact of the culture conditions, we prepared HVC and shelf homogenates immediately upon their dissection. We then performed an enzymatic assay (same as for recombinant zRalDH) with the soluble fraction of the HVC homogenate (combined from three birds), utilizing retinaldehyde as substrate. We detected a retinaldehyde-oxidizing activity that was abolished by 10–50 μM DS (Figure 3D), providing evidence that zRalDH is active in HVC. We then incubated F-9 monolayers with the fresh homogenates (in triplicate) and obtained the same results as with the intact tissue fragments, i.e., β-gal induction with HVC but not with shelf homogenates (data not shown but same as in Figures 4C and 4D), indicating that retinoic acid was present in HVC but not in shelf fragments.

To control for the specificity of DS, it was important to ensure that known DS targets other than zRalDH (Lam et al., 1997; Mays et al., 1998; Driscoll et al., 1999; Kharasch et al., 1999) were absent from HVC. Western analysis of tissue homogenates with antibodies that recognize the respective homologs in zebra finch tissues excluded the presence of class 2 mitochondrial ALDH2 (Figure 1E), cytochrome P450–2E1, and peptidylglycine α–hydroxylating monooxygenase in HVC (data not shown).

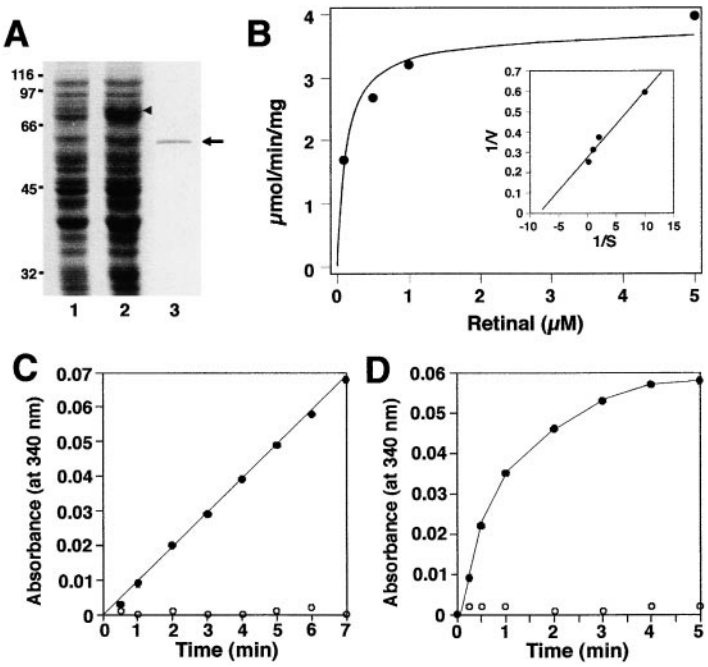

Figure 3. Biochemical Analysis of Recombinant zRalDH

(A) SDS–PAGE. 1, control bacterial lysate; 2, lysate expressing zRalDH-GST fusion protein (arrowhead); 3, zRalDH (arrow) after cleavage and purification.

(B) Kinetics of retinaldehyde oxidation by zRalDH. Initial reaction rates are plotted versus retinal concentration (in μM); inset shows double reciprocal plot. Abbreviations: S, substrate concentration; V, initial reaction rates.

(C) zRalDH's retinaldehyde-oxidizing activity is blocked by DS; values are average of duplicates at 10 μM substrate. Dots, no DS; circles, 10 μM DS.

(D) The retinaldehyde-oxidizing activity of HVC homogenates (500 μg of soluble protein) is blocked by DS; values are average of duplicates at 10 μM substrate. Dots, no DS; circles, 10 μM DS.

The former two are hepatic enzymes that participate in ethanol metabolism, the latter is an enzyme highly expressed in heart atrial tissue and involved in synthesis of biologically active peptides. We also performed the enzymatic assay for ALDH utilizing the HVC homogenate and acetaldehyde (the preferred substrate of ALDH2) but detected activity only at very high substrate concentrations (Km = 480 μM), confirming the absence of mitochondrial ALDH2 in HVC (ALDH2's Km for acetaldehyde is 1 μM; Johnson et al., 1987). These observations indicate that zRalDH is the most likely target for the DS action in HVC tissue fragments and homogenates. We conclude from the above that retinoic acid is present in adult HVC and that its production is linked to zRalDH expression.

Mapping *zRalDH* Expression and Retinoic Acid Production

We carried out a detailed in situ analysis of *zRalDH* expression to determine whether brain regions other than HVC produce retinoic acid (n = 6 adult zebra finches). High expression occurred in song nucleus lMAN (Figure 5B), localized in the large neurons (Figure 5G) known to concentrate androgens (Arnold et al., 1976). Expression was lower in the neostriatum (N) surrounding lMAN and in the rostral hyperstriatum accessorium, and absent from most of the rest of the brain.

Table 1. Michaelis-Menten Constants for Aldehyde Substrates Tested in the zRalDH Enzymatic Assay

Aldehyde	Km (μM)	Vmax (μmol/min/mg zRalDH)
Retinaldehyde	0.118	3.65
Benzaldehyde	67	2.79
Acetaldehyde	149	3.01
Citraldehyde[a]	–	–

[a] No activity detected at any concentration tested.

zRalDH expression along the rostro–caudal axis of N, a portion of the avian brain thought to correspond to parts of the mammalian cortex (Karten and Shimizu, 1989), formed a gradient that peaked in lMAN and decreased rostrally and caudally (Figure 5H). As determined by emulsion autoradiography, this was due to a change in the density of neurons expressing *zRalDH* (data not shown). Other cloned brain cDNAs are expressed uniformly in N and do not form gradients (e.g., Clayton et al., 1988). A *zRalDH* expression gradient may explain the debated question of how retinoic acid gradients are formed during development in the presence of uniform precursor levels (see Tabin, 1991).

We then performed the F-9 assay with brain explants from several areas where we characterized *zRalDH* expression (n = 6 male zebra finches). Regions expressing *zRalDH* (lMAN, rostral N, and HA) yielded the blue product indicative of retinoic acid presence, whereas regions negative for *zRalDH* expression, such as paleostriatum and caudoventral N, failed to do so (data not shown but essentially the same as for HVC and shelf in Figure 4). Thus, *zRalDH* expression is a good indicator of the presence of retinoic acid and can be used to map its production sites in the adult brain.

The expression pattern of *zRalDH* seen in adults did not change with age (20, 38, 50, and 60 days posthatch; n = 4 male zebra finches per group), except for in song nucleus RA (Figure 6A). Expression in RA was initially low but detectable, and it peaked by day 38 and decreased thereafter to reach undetectable levels (as in adults) by day 60. Emulsion autoradiography showed a large number of labeled RA neurons and high labeling density (>20 grains/cell) with clear somatic distribution during peak expression (Figure 6B). Later, both the number of labeled neurons and the labeling density/cell decreased and eventually disappeared (Figure 6C). The peak *zRalDH* expression in RA occurs at a time during the sensitive period for song acquisition when axonal fibers from HVC enter RA to establish a projection essential for normal song production (Konishi and Akutagawa, 1985).

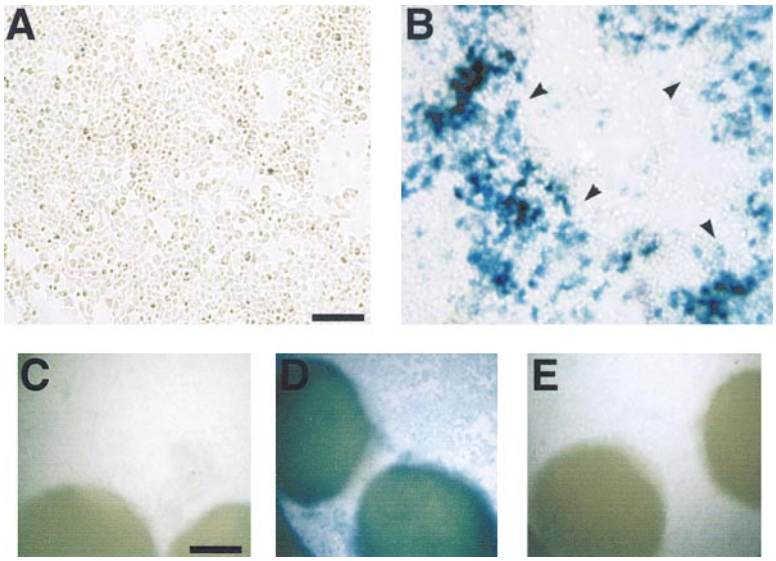

Figure 4. Detection of Retinoic Acid in Adult Brain Explants Cocultured with F-9 Retinoid-Sensitive Cells in Petri Dishes or Microtiter Plates

(A) Control monolayer without explants.
(B and D) HVC explants induced the blue reaction product in F-9 cells, indicative of the presence of retinoic acid; arrowheads indicate original explant placement.
(C) Shelf explants had no effect on F-9 cells.
(E) Preincubation of HVC explants with 50 μM DS prevented the inductive effect. The cultures were performed in petri dishes (A and B) or microtiter plates (C–F). Tissue fragments visible in (C) through (E).
Scale bars, 100 μm (A and B); 200 μm (C–E).

Neuronal Subtype Expressing zRalDH in HVC

zRalDH expression in the caudal neostriatum was confined within HVC boundaries (Figures 5C and 5D) but did not occur in all cells. HVC has large neurons that project to area X (Bottjer et al., 1989; Nottebohm et al., 1982) and smaller ones that project to nucleus RA (Nottebohm et al., 1982); neurons projecting to both targets have not been observed (Kirn et al., 1999). While area X–projecting neurons are formed before hatching and remain stable postnatally, RA–projecting neurons are continuously replaced throughout adulthood (Alvarez-Buylla et al., 1990). HVC neurons expressing *zRalDH* were large (Figure 5F, closed arrows) and often close to smaller unlabeled cells (Figure 5F, open arrows), and thus appeared to correspond to X-projecting neurons. Expression also occurred in paraHVC (5E, arrows),

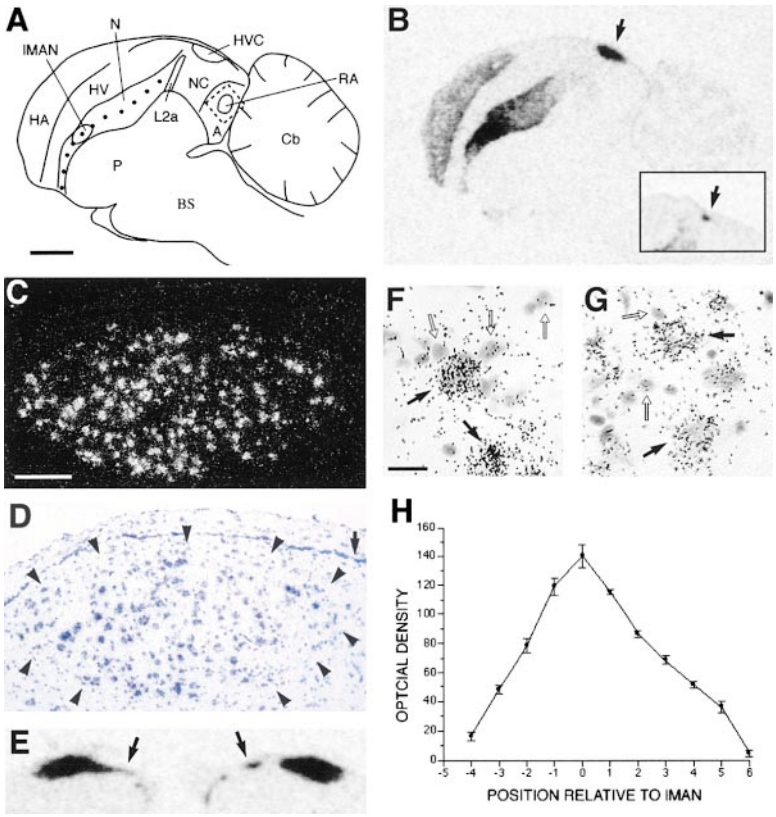

Figure 5. In Situ Analysis of *zRalDH* Expression in Adult Zebra Finch Brain

(A) Diagram of parasagittal section (2.2 mm lateral).
(B) Corresponding autoradiogram shows expression restricted to HVC (arrow), IMAN, adjacent N, and rostral HA; inset, expression in adult female HVC (arrow). The same pattern was seen in canaries, song sparrows, and black capped chickadees.
(C and D) Dark- and bright-field views of male HVC (same level as in [A]): expression (white-silver grains in [C]) is confined to the cytoarchitectonic boundaries of HVC (arrowheads in [D]); arrow depicts the ventricle.
(E) In situ autoradiogram of frontal section (caudal to Figure 1A) shows expression in paraHVC (arrows).
(F) Expression in HVC occurs in large neurons (arrows); smaller cells (open arrows) are unlabeled. (G) Expression in IMAN occurs in large neurons (arrows); smaller cells (open arrows) are unlabeled. (H) Densitometric measurements (OD ± SEM; n = 6 birds) along the rostro–caudal axis of N (dots in [A]; negatives values are rostral and positive ones caudal to IMAN); expression peaks in IMAN.
Abbreviations: BS, brain stem; HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; L2a, field L subdivision; IMAN, lateral magnocellular nucleus of the anterior neostriatum; N, neostriatum; P, paleostriatum; RA, nucleus robustus archistriatalis; for others, see Figure 1. Orientation, dorsal is up and anterior to the left in (A) through (D). Scale bars, 1 mm (A and B); 200 μm (C and D); 10 μm (F and G).

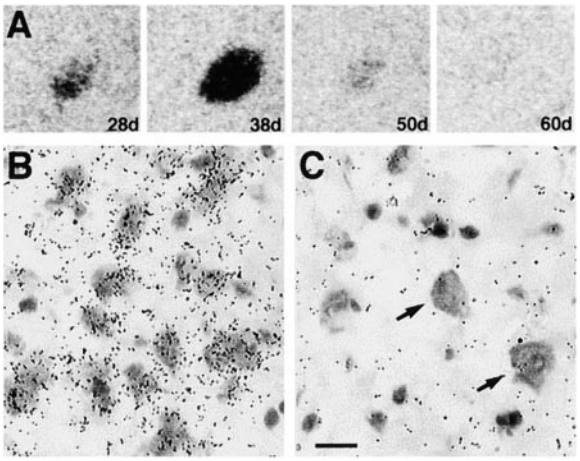

Figure 6. *zRalDH* Expression in Nucleus RA of Juvenile Male Zebra Finches

(A) Expression peaks at day 38 posthatch; area shown in autoradiograms corresponds to the dashed square around RA in Figure 5A. (B and C) Detailed views of RA after emulsion autoradiography. Notice the large number of labeled cells at day 38 (B) and the low cell density and large unlabeled neurons (arrows) typical of adult RA at day 60 posthatch. Scale bar, 10 μm.

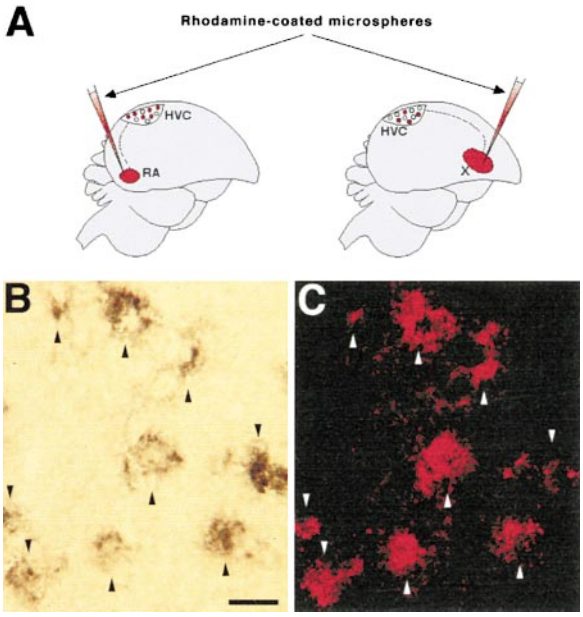

Figure 7. *zRalDH* Expression in HVC's X-Projecting Neurons

(A) Rhodamine-coated microspheres were placed into area X or RA in male zebra finches to retrogradely label HVC's projection neurons. (B) Bright-field view of *zRalDH*-expressing cells (black arrowheads) in HVC, as detected by nonradioactive in situ hybridization. (C) Retrogradely labeled X-projecting neurons visualized by fluorescence microscopy (same field as in [B]); all labeled cells (white arrowheads) express zRalDH. Scale bar, 10 μm.

a medial HVC extension that contains X- but not RA-projecting neurons (Foster et al., 1997). The same labeling pattern was observed in juveniles at all ages examined (data not shown).

To determine directly which HVC neurons express *zRalDH*, we injected rhodamine-coated latex microspheres into either area X or RA (n = 3 birds each; Figure 7A). After adequate survival, we performed in situ hybridization on sections containing HVC, using a digoxygenin-labeled *zRalDH* riboprobe. Retrogradely labeled somata resulting from area X injections (i.e., X-projecting neurons; Figure 7C) were *zRalDH* positive (Figure 7B); in contrast, none of the retrogradely labeled RA-projecting neurons were *zRalDH* positive (data not shown). These results demonstrate that *zRalDH* expression occurs in HVC's X-projecting neurons but not in RA-projecting ones (compare with Holzenberger et al., 1997).

zRalDH Activity in HVC Is Required for Normal Song Maturation

zRalDH's conspicuous expression pattern suggested that it might be involved in some aspect of birdsong behavior. We tested this by blocking zRalDH's enzymatic activity in HVC and assessing the effect on song behavior in male zebra finches. We utilized DS, which efficiently inhibits the retinaldehyde-oxidizing activity of recombinant zRalDH and of HVC homogenates, as well as retinoic acid synthesis in HVC explants (see above). HVC was selected as a target as it plays a central role in song learning and production, displays prominent zRalDH expression, and is close to the brain surface, facilitating surgical access.

We placed DS crystals embedded in agarose (DS/agarose) bilaterally above the ventricular surface of HVC (Figure 8, top middle diagram) in both juveniles during the sensitive period for song acquisition (30–35 days old) and in adults with crystallized song (>120 days old). Controls (Figure 8, top left and right diagrams) were juveniles implanted with either vehicle-containing agarose above HVC or DS/agarose at a distant location (1.0 mm lateral and 1.5–2.0 mm rostral to HVC; this placement resulted in brain damage and ventricular exposure comparable to those of HVC implants). After implants, the birds had unrestricted access to the songs of normal adult males. For juveniles, song was then recorded after the period it normally takes for song to crystallize (>90 days posthatch); for adults, song was recorded both prior to surgery and at various times (4 days, 1 week, 2 weeks, and 1 month) following implants. The recorded songs were carefully analyzed for song stereotypy (as in Scharff and Nottebohm, 1991) by computing the average duration of individual notes and intervals within the motifs and their variability, which gives a measure of the stability in the production and timing of the various notes. We also computed song consistency and linearity, which evaluate the occurrence of frequent versus infrequent transitions between notes and the stability in the placement of notes across repeated motifs.

The songs of both control groups (n = 3 for HVC vehicle/agarose implants and n = 5 for distant DS/agarose implants) showed the high stereotypy characteristic of stable zebra finch song (Figure 8A; Table 2), including fixed duration of notes and intervals, preservation of the number and order of notes across successive motif renditions, and stable morphology of individual notes. In contrast, juveniles that received DS implants over HVC (n = 7) had a consistent and marked detrimental effect on song maturation (Figure 8B), as evidenced by a 4- to 6-fold greater variability in note and interval duration across song renditions, although average note

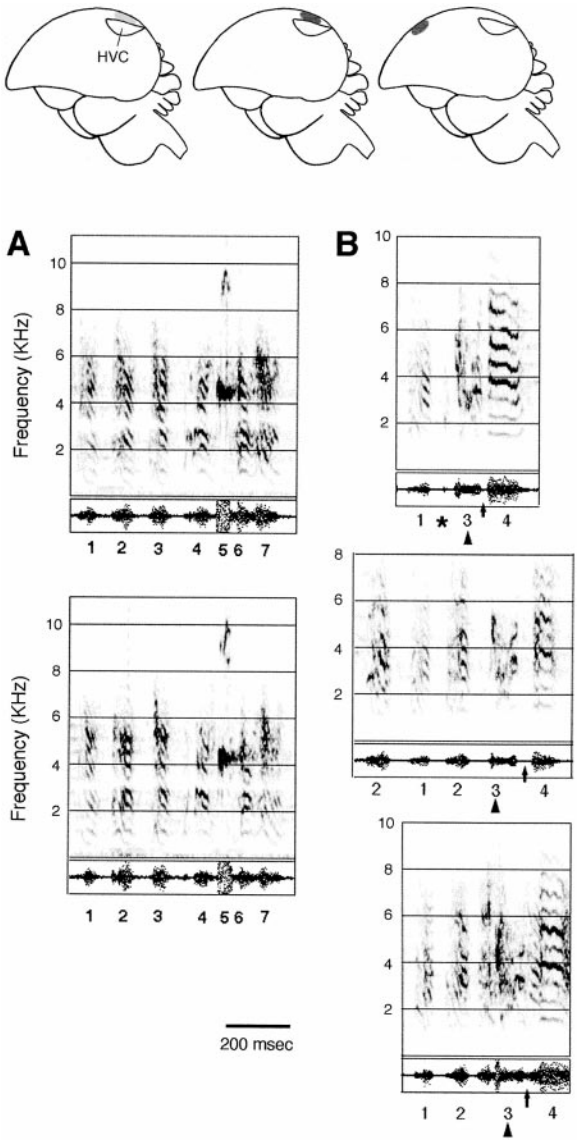

Figure 8. Disulfiram Disrupts Normal Song Development in Zebra Finches

Top diagrams: placement of vehicle (light gray, left) or DS (dark gray, middle) in HVC, or DS far from HVC (dark gray, right) in juvenile males. (A) and (B) show sonograms of consecutive motifs recorded at day 90 posthatch. Numbers indicate individual notes, motifs are aligned vertically at note 1. Songs from vehicle-treated controls (A) and from birds that received DS implants far from HVC (data not shown) were typical stereotyped song, with stable notes; fixed durations of notes and intervals; and preservation of number and order of notes from one motif rendition (top) to the next (bottom). Songs from birds that received DS implants in HVC (B) showed high variability in note and interval duration and in note morphology (e.g., follow arrowhead indicating note 3 and arrow indicating interval between notes 3 and 4 across panels), as well as disruptions of motif stereotypy due to note deletions (asterisk in top panel), changes in linearity (middle panel, note 2), and addition of extraneous notes (data not shown).

and interval durations were not affected (Table 2). DS-implanted birds also showed high instability in the performance of fast frequency modulations, resulting in poor note morphology (e.g., note 3 in Figure 4B, lower panel). In addition, these birds frequently omitted individual notes (e.g., Figure 4B, asterisk in upper panel), inverted note sequences (Figure 4B, middle panel), or inserted extraneous notes. This resulted in high variability in the structure of successive motifs, as reflected in low consistency and linearity values (Table 2). The songs of all adults ($n = 3$ for HVC DS/agarose implants, $n = 3$ for HVC vehicle/agarose implants) were not affected during the postsurgery period analyzed (data not shown).

No morphological abnormalities were apparent after the implants, the histological aspect of HVC and surrounding tissue in implanted birds being equivalent to that in untreated animals (Figure 5). Both area X– and RA-projecting neurons appear to be preserved, but quantitative morphometric analysis would be required to discard a subtle effect of DS on specific cell populations. As the DS crystals were still present in the implants upon histological examination, it is likely that DS was continuously available from surgery until sacrifice. The lack of an effect when DS was placed at a brain site distant from HVC argues against the possibility that DS placed in HVC acted at another brain site after diffusion. It also makes it unlikely that DS affected distant organs such as the liver after leaking into the bloodstream. In addition, no abnormalities were detected for general indicators such as weight, plumage condition, and beak coloration. Finally, DS-treated birds preserved normal perching and alert posture, reaction to the presence and vocalizations of females, tendency to direct song toward females, and ability to call and sing in response to the presence and/or vocalizations of other males. Combined, our results indicate that the retinaldehyde-oxidizing activity of zRalDH is required for normal song maturation.

Discussion

The production and physiological action of retinoic acid have been extensively investigated during embryogenesis. We show here that it is also produced at specific sites in the adult songbird brain, in association with expression of *zRalDH*, a retinaldehyde-specific ALDH. This provides conclusive evidence for localized retinoic acid synthesis in an adult vertebrate brain. In addition, z*RalDH* expression and retinoic acid synthesis are very prominent in HVC, a nucleus that controls the acquisition and production of learned song. We show that zRalDH's enzymatic activity in HVC is required for the song of juvenile zebra finches to become stereotyped. Below we discuss the evidence and possible implications.

zRalDH was identified by DD comparison of HVC and shelf from zebra finches. The closely related *RalDH(II)* and *RALDH2* were isolated respectively from rat testis (Wang et al., 1996) and a mouse teratocarcinoma cell line (Zhao et al., 1996). These cDNAs encode class 1 ALDHs highly efficient and selective in all-*trans* retinaldehyde oxidation, and more specifically involved in retinoic acid synthesis than other class 1 enzymes. Based on tissue distribution and data from gene knockouts (Niederreither et al., 1997, 1999), they are major contributors to retinoic acid synthesis during embryogenesis. Likewise, zRalDH has an ALDH activity with very high affinity for retinaldehyde and high sensitivity to DS. Although it is possible that zRalDH may oxidize other as yet unidentified endogenous aldehydes, our results indicate that its main physiological role is the conversion

Table 2. Effect of Disulfiram on Song Parameters

Animal Groups	Average Note Duration (ms)	Average Variability of Note Duration	Average Interval Duration (ms)	Average Variability of Interval Duration	Consistency	Linearity
Control 1 (n = 3)	106.59 ± 12.65	6.16 ± 1.08	34.16 ± 8.12	5.20 ± 0.54	0.95 ± 0.03	0.85 ± 0.10
Control 2 (n = 5)	113.80 ± 7.91	5.52 ± 0.45	47.36 ± 6.40	5.93 ± 0.84	0.96 ± 0.06	0.83 ± 0.04
Disulfiram-implanted juveniles (n = 7)	121.16 ± 13.28	24.89 ± 1.75*	51.65 ± 3.63	24.24 ± 3.54*	0.53 ± 0.07*	0.38 ± 0.06*
Disulfiram-implanted adults (n = 3)	129.62 ± 14.33	6.64 ± 1.01	34.23 ± 5.88	5.74 ± 0.51	0.93 ± 0.06	0.92 ± 0.06

All values represent mean ± SE; *$p < 0.05$

Juvenile and adult male zebra finches implanted with disulfiram in the vicinity of song nucleus HVC are compared with juveniles implanted with vehicle in the vicinity of HVC (Control 1) or with disulfiram at a site distant from HVC (Control 2). Several song renditions (typically, 15–20 per bird) were studied, and all notes and intervals encountered were included in the analysis. The songs were digitized and average note and interval durations were calculated (SoundEdit) by averaging the mean duration of notes and intervals of each individual bird. Variability of note and interval durations were obtained by first averaging the standard deviations of the means of the duration of notes and intervals of each individual bird and then averaging the data across each group. The stereotypy of song motifs was evaluated by calculating consistency and linearity indices. The consistency index reflects how often the most frequent note sequence occurs and is calculated by dividing the number of typical transitions between notes in a song by the number of all transitions rendered; typical transitions are the most frequent ones. The linearity index reflects the order of notes within a song and is calculated by dividing the number of different notes by the number of transitions between notes encountered in a song. For the last two indices, a value of 1 reflects maximal stereotypy, and a value of 0 reflects no stereotypy.

of retinaldehyde into retinoic acid. Taking into account the biochemical and sequence data, we conclude that *zRalDH*, rat *RalDH(II*), and mouse *RALDH2* are homologs and likely represent a class 1 ALDH specialized in retinaldehyde oxidation (also see Napoli, 1996).

The incubation experiments of fresh tissue homogenates and tissue explants with the reporter cell line, and the enzymatic assay with fresh homogenates, provided evidence that retinoic acid is present at specific sites of the adult songbird brain and that these areas are capable of producing retinoic acid. In fact, the brain distribution of *zRalDH* mRNA predicts sites of retinoic acid production. Importantly, Northern and Western analysis show that zRalDH is the only class 1 ALDH present in the HVC of adults. Moreover, the retinoic acid production in tissue explants and the retinaldehyde-oxidizing activity of both recombinant zRalDH and fresh HVC homogenates is inhibited by DS. We thus conclude that the enzymatic activity of zRalDH is responsible for the ability of specific zebra finch brain sites to synthesize retinoic acid.

The in vivo DS implant experiment provided functional evidence for a role of the retinoid-synthesizing activity of zRalDH in the maturation of song behavior. Birds that received DS by local administration in HVC as juveniles failed to present stable, stereotyped song as adults and produced vocalizations that resembled more closely plastic song, normally produced before song crystallization. The lack of an observable effect in the control groups indicates that the behavioral effect of DS was not due to nonspecific traumatic lesion to HVC and/or overlying hippocampus, or a result of DS diffusion to other brain areas or into the bloodstream. As discussed above, DS is highly effective in blocking zRalDH's retinaldehyde-oxidizing activity. As DS targets other than zRalDH are undetectable in HVC, we conclude that zRalDH's enzymatic activity in the HVC of juveniles is critically required for the normal development of stable adult song in zebra finches. Based on our biochemical evidence linking zRalDH activity to retinoic acid production in the brain, we also conclude that the behavioral effect of DS is likely related to a blockade of retinoic acid synthesis in HVC.

It is intriguing that DS implants had no effect in adults, as adult HVC shows high levels of zRalDH expression. It is possible, however, that longer treatment or follow-up periods are necessary before an effect on adult song can be detected, as required after deafening (Nordeen and Nordeen, 1992) or under a regimen of delayed auditory feedback (Leonardo and Konishi, 1999). The lack of an effect in adults indicates that DS does not immediately interfere with the birds' ability to produce crystallized song, suggesting that DS does not simply have a direct toxic effect on HVC cells. Interestingly, the selective ablation of HVC's X-projecting neurons, the same cells that express zRalDH, has no apparent effect on song when performed in adults (Scharff et al., 2000) but results in marked detrimental effects on song development when performed in juveniles (C. S., unpublished data), indicating that the integrity of X-projecting neurons in juveniles is required for normal song development. We thus suggest that the disruptive effect of DS on song maturation was likely due to an interference with the normal physiology of X-projecting neurons. Future experiments on the dynamics of retinoic acid production in HVC should provide further insights into the relationship between zRalDH function and song maturation.

Retinoic acid, upon binding to its receptor(s), is a potent regulator of gene expression. The fact that some of its known gene targets are expressed in song nuclei is consistent with a modulatory role for retinoic acid in the song system. For instance, insulin-like growth factor II (IGF-II), a retinoid target (Melino et al., 1993; Vincent et al., 1996), belongs to a peptide family implicated in neuronal survival during development and early postnatal life (Galli et al., 1995; Johnston et al., 1996) and is selectively expressed in HVC's X-projecting neurons (Holzenberger et al., 1997). Another retinoid target, *trkB* (Kaplan et al., 1993; Cheung et al., 1996), encodes the receptor for brain-derived neurotrophic factor (BDNF) and is preferentially expressed in the adult HVC, where BDNF has been implicated in neuronal survival (Rasika et al., 1999). Expression of the plasticity-associated phosphoprotein GAP-43 has a developmental profile in song nucleus RA (Sakaguchi and Saito, 1996) that parallels that of *zRalDH* expression in RA neurons, peaking

around the time when HVC fibers penetrate RA to establish the HVC to RA projection (Konishi and Akutagawa, 1985). By controlling the expression of IGF-II, TrkB, GAP-43, and other as yet unknown targets, retinoic acid could influence processes such as neuronal growth, survival, differentiation, and plasticity within song nuclei and thus play a modulatory role on the song system and on song behavior.

In summary, we presented definitive evidence for localized retinoic acid synthesis by a retinaldehyde-specific ALDH (zRalDH) in an adult vertebrate brain, as well as functional evidence for an involvement of the retinoid-generating activity of zRalDH in the maturation of song behavior. The main implication is that processes under retinoid control appear to extend beyond the embryonic period into adulthood and affect the organization of brain circuitry and behavior. Further studies in the songbird system will provide unique opportunities to investigate the relationship between retinoid signaling and the biology of a complex learned behavior.

Experimental Procedures

Tissue Preparation

Adult male zebra finches (*Taeniopygia guttata*) from the Rockefeller University Field Research Center (Millbrook, NY) were killed by decapitation. Brains were dissected, embedded in 1% agarose, and sliced sagittally (200 μm) with a tissue chopper. Sections were separated in sterile ice-cold phosphate-buffered saline (PBS). Regions of interest were visualized under dark-field illumination, punched conservatively with glass capillaries (0.5 mm diameter) to avoid cross-contamination, under RNase-free conditions, and quickly frozen for DD or Western blots. For in situ hybridization, birds were decapitated and the brains were dissected, embedded in TissueTek, and frozen with a dry ice/ethanol bath. For retinoic acid detection, birds were killed with Nembutal overdose and perfused with sterile saline to eliminate blood. The brains were embedded in agarose and sliced (100 μm) with a tissue chopper under sterile conditions; regions of interest were punched and used immediately.

Differential Display

We isolated total RNA (as in Chomczynski and Sacchi, 1987). We outline here several modifications to DD (Liang and Pardee, 1992) that were critical for the successful outcome of the screening. DNase treatment was omitted to minimize loss of material. Reverse transcription reactions containing 25–50 ng of RNA/sample and 5 U of reverse transcriptase (BRL) were incubated for 10–15 min at 37°C, followed by 2 min at 65°C. PCR reactions contained 3 μl of reverse transcription mix in a 10 μl volume. We used 40 cycles of 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s, followed by 72°C for 5 min. These conditions were crucial to reduce smearing of PCR products on sequencing gels and to solve ambiguities during selection of differential bands. The samples were run on standard sequencing gels, which were dried without fixation and exposed to X-ray film for 1–2 days. To minimize false positives due to low stringency PCR, genomic variability, and intrinsic PCR variability, we used triplicate samples and a stringent criterion (presence in at least two HVC but no shelf samples) for band selection. After elution and reamplification, bands of correct size were TA cloned (Invitrogen). Because of heterogeneity of DD fragments, several transformants were picked, checked for insert size, and analyzed by in situ hybridization as a confirmatory step. Otherwise, protocols, primers, and reagents were as in Liang and Pardee (1992). For alternative modifications of the DD protocol and a general discussion on DD, see Mello et al. (1997) and Mello and Jarvis (1999).

In Situ Hybridization

We used a previously described protocol (Mello and Clayton, 1994; Mello et al., 1997), with modifications. Riboprobes were prepared in both orientations using [^{35}S]UTP. For screening, probes were hybridized to 10 μm frozen parasagittal brain sections from adult male zebra finches. Conditions of hybridization and washes were optimized for each clone until signal was obtained for only one strand; optimal temperatures were in the 50°C–55°C range (at 0.1× SSPE). For characterization of *zRalDH* expression, antisense riboprobes from the full-length clone were hybridized to serial parasagittal and frontal sections from adult and juvenile zebra finches (male and female) at high stringency (65°C and 0.1× SSPE for final washes). Hybridized sections were exposed to a PhosphorImager screen and/or X-ray film, and/or dipped in autoradiographic emulsion (NTB2, Kodak) and stained with cresyl violet for identification of brain structures. Criteria for neuronal cells were based on morphology (size and shape of somata, presence of large, pale staining nucleus and clear nucleolus, Nissl substance) and tract tracing (see below). No signal was detected upon hybridization with sense probes.

Cloning of *zRalDH*

A cDNA library of total embryonic tissue from zebra finches was constructed using GIBCO–BRL's Superscript Plasmid System and screened by standard colony hybridization with a random primed probe. Three full-length and several partial clones were obtained; one was fully sequenced twice in both directions using Sequenase (USB). Analysis was done with DNASTAR.

Northern Blot

Three μg of poly(A)$^+$ RNA from whole forebrain was run on 1% MOPS/formaldehyde agarose gel, blotted onto nylon filter, and hybridized as described previously (Clayton et al., 1988; Mello et al., 1997), using ^{32}P-labeled riboprobes.

Western Blot

Tissue samples were homogenized (in 50 μl of 150 mM NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, and 1% Triton X-100), vortexed, sonicated, and boiled. After addition of β-mercaptoethanol, samples were run on 8% polyacrylamide gels and transferred onto nitrocellulose. The membranes were incubated for 1 hr in blocking solution (Blotto with 5% fat-free milk and 0.1% Triton X-100 in Tris-buffered saline) and then for 1.5 hr with primary antibodies (anti-ALDH1 or anti-msALDH; 1:500 dilution in Blotto; see Miyauchi et al., 1993; Godbout, 1992). The membranes were then washed three times for 5 min with Blotto, followed by 30 min incubation with a secondary antibody (for ALDH1, anti-rabbit horseradish peroxidase– (HRP-) conjugated IgG, 1:4000 dilution; for msALDH, anti-mouse HRP-conjugated IgG, 1:1000 dilution), three 5 min washes with Blotto, three rinses with PBS, and detection with the enhanced chemiluminescence system (Amersham). Incubations were at room temperature.

Recombinant zRalDH

zRalDH was PCR amplified with primers to the 5′ and 3′ ends of its open reading frame (21-mer-bearing SalI and NotI sites, respectively) and ligated into pGEX-4T3 to create an in-frame fusion to the carboxy-terminus of glutathione S-transferase (GST) with an intermediate thrombin cleavage site. After transformation of BL-21DE3 cells, positive clones were grown overnight and IPTG induced. Recombinant GST-zRalDH was purified from bacterial lysates using glutathione-agarose beads, and zRalDH was released by thrombin cleavage using one of two procedures. In the first procedure, glutathione-GST-zRalDH complexes were eluted by incubation with buffer containing 10 mM glutathione (Sigma) and dialyzed overnight at 4°C in 20 mM HEPES (pH 8.5) with 150 mM KCl, 1 mM EDTA, and 2 mM DTT. Cleavage was performed by adding thrombin (Sigma), followed by the inactivator hirudin (Sigma). In the second procedure, the beads were pelleted and resuspended in PBS, followed by a 15 min incubation at room temperature with thrombin; further details according to manufacturer (GST fusion system, Pharmacia). To obtain zRalDH protein virtually free of contaminants, FPLC gel filtration with a Superdex 75 column (Pharmacia) was performed with standard protocols.

Enzymatic Analysis

zRalDH released by thrombin cleavage was assayed spectrophotometrically by following reduction of NAD to NADH at 340 nm in solution containing 20 mM HEPES (pH 8.5), 1 mM EDTA, 150 mM potassium acetate, 2 mM DTT, 400 ng of recombinant zRalDH, and

varying concentrations of substrate (range, 0.1–500 μM) in a total volume of 0.5 ml. Reactions were initiated by addition of NAD (2 mM final concentration) and protein and were monitored for 12 min; controls were run in the absence of NAD or protein. Assays were done in duplicate or triplicate and initial reaction velocities (in mmol of NADH/min/μg of recombinant zRalDH) determined by calculating the slopes of the initial phase using linear regression analysis. Michaelis-Menten parameters were determined by nonlinear regression analysis (Macfit software) of plots of initial rates versus substrate concentration; initial estimates were from douple reciprocal plots. Except for all-*trans* retinaldehyde, all reagents were from Sigma. For homogenates, tissues from three adult zebra finches were obtained by punching, homogenized on ice in a suitable volume of 0.1 M PB (pH 8.0) containing 5 mM EDTA, and centrifuged for 20 min at 4°C. The protein content of the supernatant was determined by spectrophotometry. Reactions were initiated by adding substrate (retinaldehyde or acetaldehyde, to varying concentrations) and NAD (to 2 mM) to a tube containing buffer and soluble protein (500 μg). Duplicate or triplicate reactions were run for each concentration; controls were run without NAD or substrate. Analysis was performed as for zRalDH.

Retinoic Acid Detection

F-9 cell assays were performed as in Wagner et al. (1992). F-9 cells were grown to confluence in Petri dishes or microtiter plates; the latter were used to ensure close contact between explants and monolayer. Growth medium was replaced immediately prior to coculture by serum-free L15 medium containing 10 mg/ml BSA and 1 mg/ml glucose. Brain explants were placed on top of monolayers (typically six explants per petri dish and two per microtiter well), and excess medium was gently aspirated, leaving a thin layer. Cultures were placed at 37°C in a CO_2 incubator for 2 hr for explant attachment. Additional medium was slowly added and the cultures incubated for 24 hr and fixed with 1% glutaraldehyde. The dishes were then incubated with standard X-gal solution for 2–6 hr at 37°C. Two controls run in parallel behaved as expected: (1) monolayers not exposed to explants and incubated with X-gal (negative control) and (2) monolayers exposed to 5×10^{-8} M all-*trans* retinoic acid (positive control).

Tract Tracing for In Situ

We stereotaxically injected 50 nl of rhodamine-coated microspheres, a retrograde tracer, into area X or RA of adult male zebra finches. After 5 day survival, birds were decapitated and their brains quickly dissected and frozen. Cryostat sections through HVC were hybridized with digoxigenin-labeled riboprobes for *zRalDH*, as in Holzenberger et al. (1997).

Image Analysis

X-ray film autoradiograms of hybridized parasagittal brain sections were imaged with a CCD camera and NIH Image software. Optical density was determined at regular intervals along the rostro–caudal axis, at the middorsoventral position of N ($n = 6$ animals); measurements were within the linear range. For figure preparation, we used Photoshop software.

Disulfiram Administration In Vivo

Juvenile (30–35 days old) and adult male zebra finches were implanted as described below and then returned to their aviaries. Implants were prepared by embedding DS crystals in 1% agarose, which was cut into small fragments ($\sim 0.5 \times 0.3$ mm $\times$ 0.3 mm). These were inserted under anesthesia through a small cut to the hippocampus and placed upon the ventricular surface of HVC. Controls were implanted with vehicle(saline)/agarose fragments of similar size at the same location or with DS/agarose fragments at a distance from HVC. All implants were done bilaterally. The birds were monitored for general indicators of metabolic and hormonal state, as well as posture, singing, and other communicative behaviors. Recorded songs were digitized and analyzed with SoundEdit as in Scharff and Nottebohm (1991). For statistical analysis, we utilized ANOVA. After recordings, birds were sacrificed and their brains processed histologically for verification of implant placement.

Acknowledgments

We thank M. Wagner for the F-9 cell line, R. Godboud for the anti-ALDH1 antibody, Y. Tashiro for the anti-msALDH antibody, U. Drager for advice on retinoic acid detection and DS usage, X. Wang and J. L. Napoli for providing all-*trans* retinaldehyde, U. Nehrbass for advice on protein purification and critical reading of the manuscript, R. Malcher for help with preparing alignment of sequence data, M. Rivas for use of her laboratory space and equipment during the initial stages of the project, and the birdkeepers of the Rockefeller University Field Research Center for providing animal care. Support was provided by a graduate fellowship from Rockefeller University to N. I. D.-N. and National Institutes of Health grants MH18343 to F. N. and DC02853 to C. V. M.

Received August 2, 1999; revised June 15, 2000.

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GenBank Accession Number

The GenBank accession number for the *Taeniopygia guttatta* class 1 aldehyde dehydrogenase mRNA reported in this paper is AF162770.