From molecules to behavior

**The neurobiological basis of song learning in songbirds:**

# The role of retinoid signaling in the song control system of the zebra finch.

Dissertation zur Erlangung des akademischen Grades des Doktors der Naturwissenschaften (Dr. rer. nat.)

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# Eidesstattliche Erklärung

Hiermit erkläre ich, die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe angefertigt zu haben und alle Hilfsmittel und Inhalte aus anderen Quellen als solche kenntlich gemacht zu haben. Des weiteren versichere ich, dass die vorliegende Arbeit nie Gegenstand eines früheren Promotionsverfahrens war.

Die dem Verfahren zugrunde liegende Promotionsordnung ist mir bekannt.

20. Juni 2009

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Unterschrift

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# Abstract

Retinoic acid (RAc), a vitamin A derivative, is a powerful signaling molecule known for regulating large-scale morphogenetic processes during vertebrate embryonic development. More recently, RAc has been shown to also play a postdevelopmental role in the control of learning related neuronal plasticity. For example, RAc is involved in rodents' hippocampal dependent learning performance as well as its age-dependent decline, in short and long term spatial memory, hippocampal LTP and LTD.

A study by Denisenko-Nehrbass et al. (2000) shows that a learning-related role for RAc exists also in birds, where RAc is involved in the acquisition of song, a particularly complex type of fast sequence learning. Male songbirds learn their song during a sensitive period early in life by imitating the song of a tutor (usually their father). They use a highly specialized neural system of interconnected brain nuclei for sensorimotor integration to gradually match their own song output with the tutor's song. Denisenko-Nehrbass and colleagues showed that the expression of a RAc synthesis enzyme, RalDH, is confined to parts of this song control system, and song acquisition becomes impaired by interfering with RAc signaling in this system.

Following up on these findings, this thesis aims at contributing to the understanding of RAc signaling in the zebra finch song system. We first focused on localizing the sites of actual retinoid signaling in the songbird brain by describing the distribution of the molecular retinoid pathway players, which include several receptors, synthesizing, and degrading enzymes. We completed piecemeal knowledge about their expression in the zebra finch brain by identifying the transcription patterns of two receptors, the retinoid X receptors (RXR) α and γ, and the three RAc degrading cytochromes CYP26A1, CYP26B1, and CYP26C1, as well as the distribution of the RAc synthesizing enzyme RalDH, and RAc itself. We localized these molecules by means of *in situ* hybridization, immunohistochemistry, and a reporter cell assay which we modified to detect RAc in entire brain sections. Our results lead to three main conclusions: (1) Area X of the zebra finch brain, a learning-related song nucleus belonging to the avian basal ganglia, is a particular interesting target of RAc signaling, as its retinoid receptor expression profile stands out by expressing RXRγ, which is not expressed in any other telencephalic structure. (2) Not only the song system, but also higher auditory areas of the zebra finch brain are a target of complex, gradient-like RAc signaling, as they exhibit a complex distribution of RAc synthesizing and degrading enzyme transcripts. (3) RAc distribution in the brain is considerably broader than suggested by the mRNA expression pattern of its synthesizing enzyme RalDH and involves long-range transport processes between different nuclei of the song control system.

We finally took a functional approach to RAc signaling, exploring neurogenesis control as a potential function of RAc signaling. It is well known that new neurons continue to be incorporated into the song system throughout a songbird's life, a striking example of sustained brain plasticity. After pharmacologically blocking RAc synthesis in song nucleus HVC, we observed decreased rates of new neuron incorporation and/or survival. Our data would be consistent with RAc being involved in controlling these processes. However, further research is necessary to exclude toxicity of the experimental treatment as a cause for decreased neurogenesis.

# Zusammenfassung

Das Vitamin-A-Derivat Retinsäure (RS) ist ein Signalmolekül, das vor allem für seine zentrale Rolle bei Morphogenese und Musterbildung im Vertebratenembryo bekannt ist. In jüngerer Zeit konnte gezeigt werden, dass RS nach Abschluss der Embryonalentwicklung für die neuronale Plastizität, die Lernen und Gedächtnis zugrunde liegt, wichtig bleibt. Bei Nagern wurde RS sowohl mit hippokampusabhängigen Lernleistungen als auch mit deren altersbedingtem Verfall in Zusammenhang gebracht; ebenso mit räumlichem Kurz- und Langzeitgedächtnis und hippokampaler Langzeitpotenzierung und -Depression.

Dass RS auch bei Vögeln eine Rolle fürs Lernen spielt, zeigten Denisenko-Nehrbass et al. (2000). RS ist nötig zum Gesangserwerb, einer besonders komplexen Form des Erlernens schneller motorischer Sequenzen. Singvogelmännchen erlernen ihren Gesang während einer sensiblen Phase in jungem Alter, indem sie den Gesang eines Tutors – meist ihres Vaters – imitieren. Ein hochspezialisiertes Gesangskontrollsystem aus miteinander verbundenen Gehirnkernen dient ihnen dazu, sensorische und motorische Informationen so zu integrieren, dass die eigenen Lautäußerungen nach und nach dem Tutorgesang angepasst werden. Denisenko-Nehrbass und Kollegen zeigten, dass das RS-Synthese-Enzym RalDH insbesondere in einigen Teilen des Gesangskontrollsystems exprimiert wird und dass darüber hinaus eine Störung des RS-Signalwegs innerhalb dieses Systems den Gesangserwerb beeinträchtigt.

Anknüpfend an diese Befunde soll diese Dissertation zum besseren Verständnis von RS im Gesangskontrollsystem des Zebrafinken beitragen. Wir haben uns zunächst auf die Lokalisierung der Orte im Singvogelgehirn konzentriert, an denen RS tatsächlich wirksam wird. Dazu bestimmten wir die Verteilung der molekularen Mitspieler des RS-Signalwegs (Rezeptoren, Synthetisierungs- und Degradierungsenzyme). Bruchstückhafte Kenntnisse über die Transkriptionsorte einiger Rezeptoren und des Synthese-Enzyms RalDH vervollständigten wir mit den Transkriptionsmustern zweier weiterer Rezeptoren, der Retinoid-X-Rezeptoren α und γ und dreier RS-degradierender Enzyme (CYP26s) sowie den Verteilungsmustern des RS-Synthese-Proteins RalDH und der RS selbst. Ihre Lokalisierung erfolgte über *In-situ*-Hybridisierung, Immunhistochemie und einen RS-Reporterzell-Assay, den wir so modifizierten, dass wir RS in ganzen Gehirnschnitten detektieren konnten. Unsere Resultate legen drei Schlussfolgerungen nahe: (1) Der für den Gesangserwerb wichtige Gehirnkern Area X, der zu den Basalganglien des Vogelgehirns gehört, ist eine besonders interessante RS-Zielregion, da er durch sein Rezeptorexpressionsprofil herausstach: Er war der einzige Ort im Telencephalon, an dem RXRγ exprimiert wurde. (2) RS ist nicht nur im Gesangssystem des Zebrafinkengehirns aktiv, sondern auch in höheren auditorischen Zentren, wo wir komplexe Transkriptionsgradienten von RS-Synthese- und Degradierungsenzymen vorfanden. (3) Die Verteilung von RS über das Gehirn geht wesentlich über die Transkriptionsorte des RalDH-Gens hinaus und erfordert weitreichende Transportprozesse im Gesangskontrollsystem.

Die Dissertation schließt ab mit einer funktionalen Studie, in der wir testeten, ob RS den Einbau neuer Neurone ins Gesangskontrollsystem kontrolliert. Ins Gesangssystem werden lebenslang neue Neurone eingebaut – ein Beispiel bemerkenswerter neuronaler Plastizität. Wir blockierten die RS-Synthese pharmakologisch im Gesangskern HVC, was die Neurogeneseraten tatsächlich verminderte. Obwohl dies darauf hinweisen könnte, dass RS an der Kontrolle dieser Prozesse wirklich beteiligt ist, sind weitere Untersuchungen nötig, um auszuschließen, dass die Neurogeneseraten lediglich auf Grund toxischer Nebenwirkungen der experimentellen Behandlung zurückgingen.

# I Introduction

## *1.1 Introduction to the retinoic acid signaling pathway in the central nervous system*

Retinoic acid (RAc), a derivative of vitamin A which vertebrates take up with their diet, acts through one of the most powerful and pervasive biological systems that exist for the regulation of gene transcription (Mark and Chambon, 2003). A large number of proteins ensure the uptake, systemic transport, metabolic conversion, storage, and intracellular availability of retinonids, and allow for RAc's exertion of transcriptional control of its target genes (for a review, see Blomhoff and Blomhoff, 2006). The best-described function of the RAc pathway is the regulation of large-scale morphogenetic and pattern formation processes in the vertebrate embryo, including the development of limbs, heart, hindbrain, and neural tube (reviewed by Ross et al., 2000, Maden, 2001).

Cells of the central nervous system synthesize RAc from all-trans retinol, the form of vitamin A circulating in the blood plasma (see fig. 1). All-trans retinoic acid (here called retinoic acid/RAc) is normally synthesized from all-trans retinol via a two-step reaction, first the oxidation of retinol to retinal by alcohol dehydrogenases (ADHs) and second the oxidation of retinal to retinoic acid by retinaldehyde dehydrogenases (RalDHs). While many different RalDHs exist and have discrete domains of expression in the vertebrate embryo (recently reviewed by Maden, 2007), only one RalDH – RalDH2 (here simply called RalDH) – is present in the posthatch zebra finch brain (Denisenko-Nehrbass et al., 2000, Denisenko-Nehrbass & Mello, 2001).

The major function of RAc is to regulate gene transcription by activating retinoic acid receptors (RARs), a class of nuclear receptors belonging to the superfamily of steroid/thyroid hormone receptors (Giguere et al., 1987, Petkovich et al., 1987). These ligand-dependent transcription factors regulate gene expression by binding to short DNA sequences in the vicinities of their target genes (fig. 1). There is growing evidence that RAc also has transcription-independent ways of action, such as control of translation of locally stored mRNAs (Chen and Napoli, 2008, Chen et al., 2008). We focus here on RAc transcriptional control, the best-described mode of RAc action, but alternative mechanisms will be discussed in section 4.3 of Chapter III.

In order to be able to initiate transcription, the RARs dimerize with another group of nuclear steroid/thyroid hormone receptors, the retinoid X receptors (RXRs) (Hermann et al., 1992, Heery et al., 1993, Chambon, 1994, Xiao et al., 1995, Kastner et al., 1997), and bind to short DNA sequences called retinoic acid response elements (RAREs) in the vicinity of their target genes. Upon binding of RAc, the DNA-associated receptor complex recruits coactivators (which are replacing corepressors, see fig. 1) ultimately allowing for gene transcription. Since in mammals, each of the two nuclear receptor classes comprises three proteins (RARα/β/γ and RXRα/β/γ) with several isoforms (Kastner et al., 1990, Kastner et al., 1994, Mendelsohn et al., 1994, Chambon, 1995, Chambon, 1996, Zelent, 1998, Mark et al., 2006), the possibilities of heterodimerization are numerous. Targets and activational capacity may be specific to some extent for each heterodimer, which is also suggested by the considerably varying expression patterns of the different receptor types in the mammalian brain (Krezel et al., 1999, Zetterstrom et al., 1999); however, there seems to be a considerable amount of functional redundancy as well (Kastner et al., 1995, Krezel et al., 1996, Mark et al., 1999). Still, the combinatorial logic of receptor dimerization is the basis of a highly complex system of RAc controlled gene transcription. Numerous RAc target genes are known (see Blomhoff and Blomhoff, 2006 for a review), the best-described example probably being the various *Hox* genes which RAc activates in the vertebrate embryonic hindbrain (reviewed by Glover et al., 2006): Its patterning crucially depends on the regionalized induction of *Hox* genes which are activated by RAc gradients in a concentration-dependent manner. The *Hox* genes determine the anterior-posterior axis of the hindbrain by setting the boundaries of the rhombomeres of which it consists.

The catabolism of RAc is an important mechanism for controlling RAc levels in cells and tissues. Cytochromes of the P450 family, CYP26A1, CYP26B1, and CYP26C1, degrade RAc to polar metabolites, such as 4-hydroxy RAc, 4-oxo RAc, 18-hydroxy RAc, or 5,6-epoxy RAc (see Blomhoff and Blomhoff, 2006 for a review). RalDHs and CYP26s are the two components of a system which shape the complex pattern of local RAc availability in the vertebrate embryo that is crucial for normal pattern formation (Fujii et al., 1997, Gavalas, 2002, Reijntjes et al., 2005, Hernandez et al., 2007, White et al., 2007): RalDH acts as on-switch to build up local RAc sources, and CYP26s as off-switch to build up local RAc sinks (Reijntjes et al., 2003, Reijntjes et al., 2004, Reijntjes et al., 2005, Glover et al., 2006, Duester, 2007). The three RAc degrading enzymes CYP26A1, CYP26B1, and CYP26C1 are

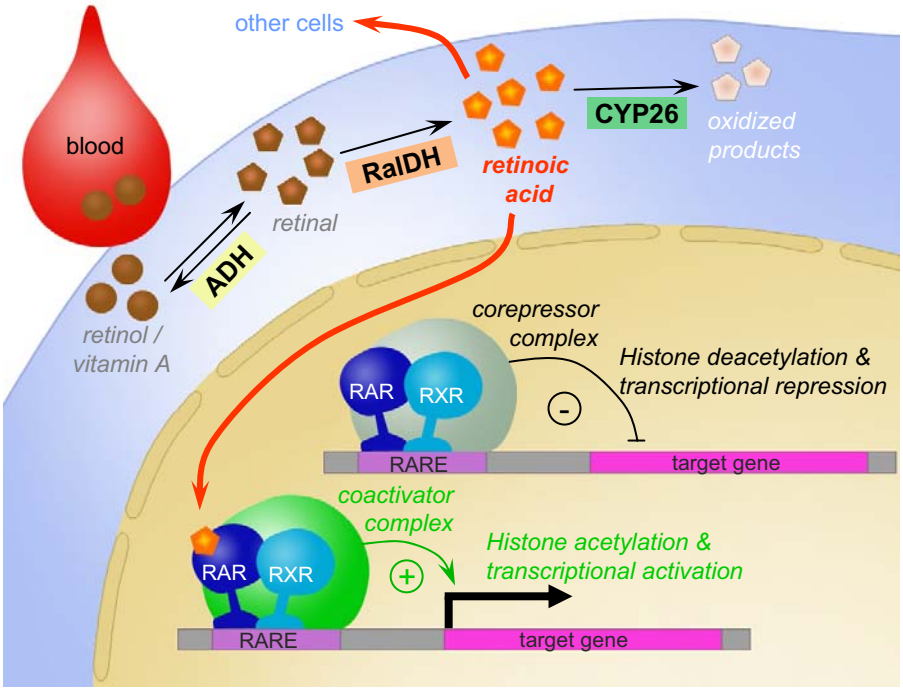


**Figure 1: Very simplified schematic of the retinoic acid (RAc) signaling pathway.** RAc biosynthesis from retinol, the form of vitamin A dissolved in the blood plasma, is a two-step oxidation process which takes place in the cytoplasm (for more details, see text). RAc regulates transcription of its target genes by activating nuclear receptors which function as transcription factors. To induce transcription, both retinoic acid receptors (RAR) and retinoid X receptors (RXR) have to be bound to a retinoic acid response element (RARE) in the vicinity of the target gene, and to be activated by RAc binding to the RAR. Due to its small size, hydro- and lipophilic properties, RAc can cross cell membranes and reach other cells by diffusion.

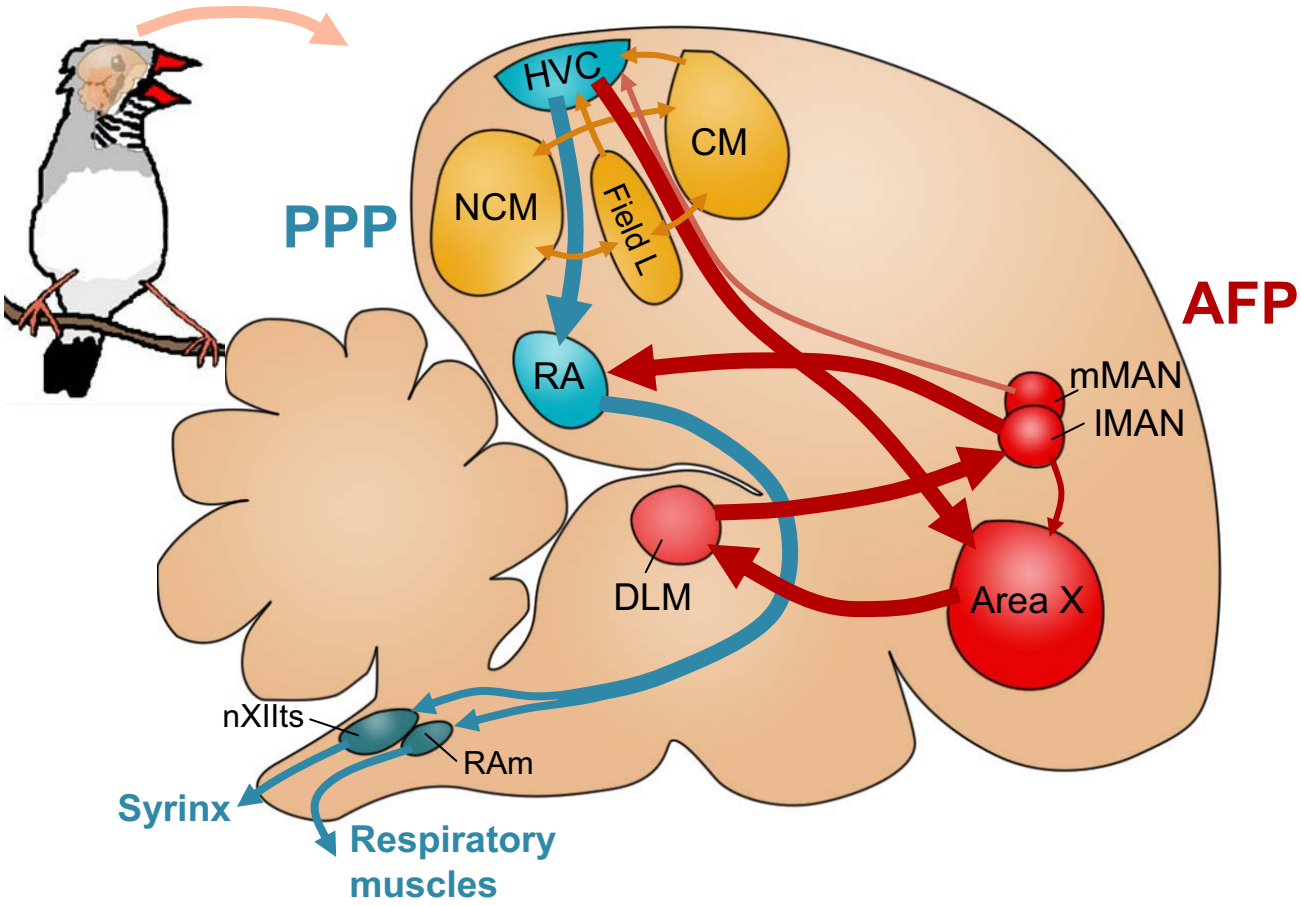


**Figure 2: Neural substrates for singing and song learning.** The drawing is a simplified schematic view of the song control system and song relevant auditory structures. The posterior premotor pathway (PPP, blue) is essential for normal song production after the early phase of juvenile subsong. It includes telencephalic song nuclei HVC and RA (for full names, see text), which projects ot the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) and nucleus retroambigualis (RAm) that control the birds vocal organ (syrinx) and the respiratory muscles, respectively. HVC is also the origin of the anterior forebrain pathway (AFP, red) which includes Area X, a basal ganglia structure, the thalamic nucleus DLM, and the pallial nucleus lMAN. LMAN sends projections back into the PPP at the level of RA, as well as projections back to Area X. Auditory inputs (yellow) enter the song system at the level of HVC. They originate in the Field L complex, the bird's primary forebrain auditory area, and CM. CM is, like NCM, one of Field L's higher auditory target areas which are highly interconnected with each other and with Field L.

expressed in a non-overlapping manner in the chicken embryo, suggesting individual roles for each of the CYP26 enzymes in RAc catabolism (Reijntjes et al., 2003, Reijntjes et al., 2004, Reijntjes et al., 2005).

RAc signaling is further complicated by the observation that some of the RAc metabolites seem to be also biologically active, but their activity has been only poorly investigated (Barua and Sidell, 2004, Reijntjes et al., 2005).

An important property of RAc is its ability to diffuse within tissue: Being a small molecule with lipophilic properties and water solubility at the same time, RAc is able to diffuse as efficiently through membranes as through watery phases, a property that makes it so suitable as a morphogen. Thus, RAc can exert effects on cells and regions which do not produce the molecule itself.

## *1.2 Introduction to the song control system in the songbird brain*

Young male songbirds imitate their adult tutor's song during a sensitive phase for sensorimotor learning (Thorpe, 1961, Konishi, 1965, Marler, 1970). Zebra finches start building up an auditory memory of it from around 25 days after hatching (post-hatch day; PHD), and around the same time, they produce their first vocalizations, the highly variable and poorly structured "subsong" (Roper and Zann, 2005). In the following, their song output becomes more and more structured and approaches their tutor's song ("plastic song"), until around PHD 90 the song has become a highly stereotyped, more or less exact copy of the tutor's song ("crystallized song"), which remains essentially stable during adulthood.

An elaborate and well-described system of interconnected brain nuclei extending over forebrain, midbrain, thalamus, and brainstem (see fig. 2) allows for the integration of sensory and motor activity which enables the juvenile male songbird to imitate his father's song.

On the basis of lesion, neurophysiological, neuroanatomical and molecular studies, this neural system controlling vocal learning and song has been divided into three pathways (see fig. 2): The first one is a descending (pre)motor pathway that controls song production through projections originating from telencephalic brain structures and target brainstem centers which serve vocal and respiratory function (Nottebohm and Arnold, 1976, Vicario, 1991, Wild, 1997). The second one is called anterior forebrain pathway (AFP), and is associated with the learning and maintenance of the bird's own song, but not directly involved in the production of already learned song (Sohrabji et al., 1990, Scharff and Nottebohm, 1991, see also reviews

by Brainard, 2004, Doupe et al., 2004). Both pathways show song-driven electrophysiological responses which, during the sensitive period of vocal learning, become increasingly selective to the bird's own song (Williams and Nottebohm, 1985, Doupe, 1993, Doupe, 1997, Margoliash, 1997, Solis and Doupe, 1997). The third pathway is an ascending auditory pathway which is not traditionally considered as being part of the song control system, but is rather specific to song (some of its neurons being specifically tuned to familiar or behaviorally relevant song) and probably involved in song memorization (for reviews, see Gentner, 2004, Mello et al., 2004, Theunissen et al., 2004, Bolhuis and Gahr, 2006). The auditory pathway converges on the telencephalic field L, the primary auditory projection region in the avian forebrain, and its target areas caudomedial nido- (NCM) and caudomedial mesopallium (CM). The nidopallial song nucleus HVC (a letter-based name) represents a connection point for all three pathways, and is the origin of the AFP as well as the posterior premotor pathway. While the premotor pathway proceeds in straightforward descending projections via the robust nucleus of the arcopallium (RA) to brainstem centers innervating respiratory centers and muscles of the vocal organ (syrinx), the AFP forms a pallial—basal-ganglia—thalamo—pallial loop encompassing projections to the striatal nucleus Area X (a basal ganglia structure), over the medial part of the dorsolateral thalamic nucleus (DLM), from here back to the lateral magnocellular nucleus of the anterior nidopallium (lMAN) and finally to RA of the posterior pathway.

During the phase of song learning, the song control nuclei undergo massive growth (or, in the case of lMAN, shrinking) processes, which include the addition and turnover of neurons (reviewed by Bottjer and Arnold, 1997). Some neuronal populations then continue being replaced in the mature bird, albeit at slower rates: The projection neurons from HVC to RA, a population that exhibits sparse firing precisely time-locked to the song output (Yu and Margoliash, 1996, Hahnloser et al., 2002), and a population of medium spiny neurons in Area X are two examples (Kirn and Nottebohm, 1993, Scharff et al., 2000, Nottebohm, 2004, Rochefort et al., 2007). As recruitment rates of HVC>RA projection neurons are particularly high during vocal development, and increase again in seasonal songbird species as canaries during seasonal changes in the song repertoire, adult HVC>RA neurogenesis is thought to be linked to vocal plasticity and learning (Kirn et al., 1994, see also review by Nottebohm, 2004).

## *1.3 Aim of this thesis*

This thesis aims at deepening our understanding of RAc signaling in the songbird brain. It follows up on findings by Denisenko-Nehrbass et al. (2000): This study combined an expression analysis of the RAc producing enzyme RalDH, which is known to create high RAc levels locally (McCaffery and Drager, 1994b, Niederreither et al., 1999), with a local pharmacological blocking approach. RalDH expression occurred in song nuclei HVC and lMAN (as well as in RA of juvenile birds), and locally blocking RAc production in HVC lead to impaired song acquisition. Thus, local RalDH activity in the song system was shown to influence song learning. However, it remained unknown where in the song system RAc acts: RAc can cross membranes and diffuse through tissues, so that the actual target sites of RAc signaling remain elusive as long as only RalDH transcription sites are known. A good additional indicator for RAc target sites are the expression patterns of the receptors which mediate the effects of RAc (Krezel et al., 1999, Zetterstrom et al., 1999). However, when the distribution of three of these receptors, RARα, β, and γ, was determined by *in situ* hybridization (Jeong et al., 2005), their expression turned out to be extremely widespread. They were found in parts of the song control system which were at several millimeters distance from RalDH transcription sites, raising the question of whether RAc could get there at all, or whether those receptors rather serve other purposes. In Chapters II and III, we describe the distribution patterns of other retinoid pathway molecules in the brains of juvenile and adult zebra finches, to clarify at which sites RAc signaling takes actually place. We first identified the remaining two zebra finch retinoid receptors which were yet undescribed, RXRα and RXRγ, and described their transcript distribution (Chapter II), as well as that of the RAc degrading cytochromes, which might generate local sinks of RAc (Chapter III). We made two interesting findings: (1) One receptor, RXRγ, exhibited an extremely confined expression limited to song nucleus Area X, revealing this nucleus as an interesting target area for RAc signaling. (2) The expression pattern of the RAc degrading enzyme CYP26B1 suggested that higher auditory areas of the zebra finch brain are a target of complex, gradient-like RAc signaling. However, both receptor and degrading enzyme distribution rather deepened than dispelled the gap to the narrow RalDH expression: Like the RARs, both retinoid X receptors were expressed far from RalDH expression sites, and the degrading cytochrome distribution was too sparse to generate noteworthy RAc sinks. In particular, it was still unclear whether and how RAc signaling could reach song nuclei Area X and RA, the two song nuclei which express receptors but lack RalDH expression.

Only through localization of the signaling molecule RAc itself could we finally show that RAc signaling takes indeed place at sites far from RalDH expression (Chapter III), including song nuclei Area X and RA. Localizing RAc in tissues has always been a difficult task, due to the extremely low physiological concentrations of the signaling molecule (see Gundersen, 2006 for a review). We could visualize RAc presence in entire brain sections of non-transgenic animals for the first time by modifying a RAc reporter cell assay that has been used before to detect RAc in small tissue samples (Wagner et al., 1992). Using this assay, we could show that long-range transport processes between song nuclei affect retinoid distribution. Using immunohistochemical labeling of RalDH protein, we further showed that the molecule undergoing the transport is the RalDH enzyme rather than RAc itself. RalDH enzyme transports largely reconcile between the sparse RalDH and the widespread receptor transcription, as they provided all those song control nuclei with RAc which did transcribe receptor mRNA but not RalDH mRNA.

In Chapter IV, we finally took a functional approach to RAc signaling in a specific site of the song control system: We explored neurogenesis control as a potential function of RAc signaling, asking whether RAc is necessary for the postdevelopmental incorporation of a specific neuronal population into nucleus HVC. We observed that local blocking of RalDH enzyme significantly decreased incorporation and/or survival of these neurons. Our data would therefore be consistent with RAc being involved in controlling these processes. However, the results suffered from an unexpected experimental difficulty, the RalDH blocking treatment having a toxic effect on brain tissue in some cases. We therefore cannot accept unreservedly the hypothesis of RAc regulating neurogenesis in the songbird brain before more experiments are done.

In sum, we show here that the telencephalic song control system is a major site of posthatch RAc signaling. Our results strongly suggest that RAc signaling, by allowing for a sort of molecular telecontrol of two song nuclei through two others, plays an important and complex role for song behavior. As RAc has been shown to be involved in different aspects of plasticity in the postdevelopmental nervous system, its complex trafficking within the zebra finch song system could mediate the plasticity underlying song acquisition in the juvenile bird as well as the dynamic maintenance of adult song. One of the processes controlled by RAc signaling seems to be the incorporation of new neurons into the song system, but further research is needed to confirm this possibility.

## *1.4 Abbreviations for anatomical brain regions*

| | | | |
|---|---|---|---|
| A | arcopallium | lMAN | lateral magnocellular nucleus of anterior nidopallium |
| Area X | Area X of the medial striatum | MLd | dorsal part of nucleus mesencephalicus lateralis |
| Cb | cerebellum | N | nidopallium |
| CLM | caudolateral mesopallium | NC | caudal nidopallium |
| CM | caudal mesopallium | NCM | caudomedial nidopallium |
| CMM | caudomedial mesopallium | NIII | oculomotor nerve |
| DLM | dorsolateral medial nucleus of the thalamus | M | mesopallium |
| DMP | nucleus dorsomedialis posterior of the thalamus | GP | globus pallidus |
| E | entopallium | Ov | nucleus ovoidalis |
| GP | globus pallidus | pHVC | para-HVC |
| H | hyperpallium | PMI | nucleus paramedianus internus of the thalamus |
| Hbm | nucleus habenularis medialis | RA | robust nucleus of the arcopallium |
| HA | hyperpallium apicale | S | septum |
| HD | hyperpallium densocellulare | SNC | compact part of substantia nigra |
| Hp | hippocampus | SpL | nucleus spiriformis lateralis |
| HVC | nucleus HVC of the nidopallium | SpM | nucleus spiriformis medialis |
| ICo | nucleus intercollicularis | St | striatum |
| IM | magnocellular part of nucleus isthmi | TeO | optic tectum |
| IPC | parvocellular part of nucleus isthmi | TrO | tractus opticus |
| LAD | lamina of the dorsal arcopallium | V | ventricle |
| L2a | subfield L2a of field L | VTA | ventral tegmental area |

# II Cloning and expression analysis of RXR receptors in the zebra finch brain

## 1 Introduction

An increasing body of evidence is showing that RAc, first known for its powerful role in embryonic pattern formation, in particular in the developing nervous system, continues to play a role in the postembryonic brain (see Maden, 2007 for a recent review). The detection of RAc binding in the adult brain with a radio-ligand binding assay (Bailey and Siu, 1990) led over to the localization of its binding proteins and receptors in the rodent brain (Stumpf et al., 1991, Mano et al., 1993, Krezel et al., 1999, Zetterstrom et al., 1999), identifying potential RAc responsive regions. Functional studies followed which focused the hippocampus as a main target region of RAc signaling, and revealed that RAc signaling is involved in neuronal plasticity, learning and memory, and other aspects of adult brain function (Chiang et al., 1998, Krezel et al., 1998, Alfos et al., 2001, Etchamendy et al., 2001, Etchamendy et al., 2003, Wietrzych et al., 2005, Mingaud et al., 2008).

Learning related RAc function in the song control system of the songbird brain has first been investigated in 2000 (Denisenko-Nehrbass et al.). The song control system is one of the best understood neural systems involved with complex sensorimotor learning (for reviews, see Doupe et al. (2004), Brenowitz and Beecher (2005), Bolhuis and Gahr (2006)). The cloning and expression analysis of the zebra finch retinaldehyde dehydrogenase (RalDH) by Denisenko-Nehrbass and colleagues revealed that this RAc producing enzyme is expressed in discrete regions of the juvenile and adult brain, with highest RalDH mRNA concentration in some song nuclei. Moreover, a RalDH blocking experiment showed that RAc production in song nucleus HVC is required for normal song acquisition (Denisenko-Nehrbass et al., 2000, Denisenko-Nehrbass and Mello, 2001). In addition, the mRNA distribution of the three retinoic acid receptors, RARα, β, and γ, in the zebra finch brain provides essential clues about where in the songbird brain RAc might exert its effects (Jeong et al., 2005). However, the actual sites of RAc action in the zebra finch brain are not certain: First, mRNA distribution is not necessarily equivalent to the distribution of functional proteins, which might be transported. Second, RAc itself can – due to its hydro-lipophilic properties – cross membranes and build up diffusion gradients, so that it may reach brain sites of some distance from its production sites (which can probably be in the range of several hundred microns; see Smith

(2001), Molotkov (2005)). And third, RARs are not functional on their own, but need to form heterodimers with retinoid x receptors (RXRs) to be able to act as transcriptional regulators upon binding of RAc (Hermann et al., 1992, Heery et al., 1993, Chambon, 1994, Xiao et al., 1995, Kastner et al., 1997). Thus, RXR distribution is as important as RAR distribution for putting forward hypotheses about possible target sites of RAc signaling. The existence of several types of RARs and RXRs, as well as different isoforms of a number of them, leads to a high number of resulting receptor dimers, with probably each dimer showing its own DNA binding and target gene specificity (even though the receptors may also exhibit functional redundancy to some degree; see Krezel et al., 1998, Mark and Chambon, 2003). It is this combinatorial logic of RAR-RXR heterodimerization (and in addition possibly RXR homodimerization, see Ijpenberg et al. (2004)) which mediates the extremely pleiotropic effects of RAc and makes the system of retinoid signaling so powerful and pervasive in vertebrates (Mark et al., 2006). However, the multitude of receptor combinations is a challenge for the identification of candidate sites of RAc signaling. Knowledge about the localization of each receptor is required to discriminate between regions with different receptor dimer availability (and therefore possibly different effects of RAc). To complete the picture of retinoid related receptor distribution, we cloned the zebra finch RXRs and determined their brain distribution by *in situ* hybridization in juvenile and adult male zebra finches.

A particular motivation to determine RXR distribution in the zebra finch brain arose from a puzzling discrepancy between RalDH and RAR distribution. RalDH has an extremely conspicuous and confined expression pattern with high expression in song nuclei HVC and lMAN and parts of nido- and hyperpallium only, while all other regions are devoid of RalDH transcript (Denisenko-Nehrbass et al., 2000; see also fig. 23, right panels). All three RARs, in contrast, show a widespread distribution of transcript, covering practically the entire pallium (RARβ) or both the pallium and the striatum (RARα, RARγ) (Jeong et al., 2005). RAR transcripts are therefore present in numerous regions that are more or less distant from RalDH expression, including striatal song nucleus Area X and arcopallial song nucleus RA. Are the RARs able to build functional receptor heterodimers in these regions? In other words, could RXRs possibly constrain regions of potential RAc signaling by showing a narrower distribution than the widespread RARs? In rodents, this might actually be the case, at least to some extent: RXR brain expression is reported to be somewhat more confined than RAR transcript distribution (Krezel et al., 1999), although the available data is not fully consistent (Zetterstrom et al. (1999) show partly contrary results). However, the conundrum of confined

RalDH versus widespread RAR expression is also known from embryonic development and remains as yet unsolved in this context (Drager, 2006). Thus, one motivation to determine the RXR expression patterns in the zebra finch brain was to find out whether the RXR distribution would be narrow enough to reconcile wide RAR with confined RalDH expression in this system.

Our results were mixed: RXRα showed an expression pattern that was just as broad as the RAR distribution; RXRγ, in contrast, turned out to have an extremely sparse and specific expression pattern restricted to some cells in a song nucleus, Area X. (Based on a genomic sequence search, we suggest that zebra finches possess no RXRβ, like chickens.) While RAc effects specifically mediated by RXRγ containing receptor dimers actually seem to be spatially confined, this does not seem to be true for RAc effects mediated through RXRα, as this receptor is so widely available. The RXRα mRNA distribution rather suggests that basically all RAR positive regions qualify for (RXRα mediated) RAc signaling. Therefore, the question of whether the receptors present at sites far from RalDH expression in fact mediate RAc effects remained unsolved. However, RXR expression strongly underpins the potential significance of RAc signaling in the context of song: First, RXRγ was exclusively available in a specific song nucleus, Area X; second, RXRα expression appeared to be regulated in an age-dependent manner in another song nucleus, lMAN. Third, putting together the now available expression data for all five retinoid receptors, it turns out that each of the telencephalic song nuclei HVC, RA, lMAN, and Area X exhibits a unique receptor expression profile, different from the other song nuclei and/or the surrounding tissue.

In sum, our data on RXR transcript distribution in the zebra finch brain completes existent data about RAR and RalDH expression, so that a detailed picture is now available about where functional receptor dimers of any possible combination may form, and possibly mediate RAc signaling. The distribution patterns we find are consistent with a specific role of RAc signaling within the song control system and for song behavior.

# 2 Materials & Methods

## *2.1 Animals*

Male zebra finches (*Taeniopygia guttata*) were obtained from breeding colonies at the Free University and the Max Planck Institute for Molecular Genetics, Berlin. Birds were housed in family or group cages in a breeding room with a 12:12h light-dark cycle.

In total, brains of 34 male birds and one zebra finch embryo were subjected to RXRα and/or RXRγ *in situ* hybridizations. 26 of the birds were adult males, two of them young juveniles of 20 days, two were 41-49 days old, and four 64-68 days old.

The embryo was used for comparison to RXR expression data in the chicken embryo. It was obtained by choosing a zebra finch clutch from which chicks had recently hatched, and picking an egg in which an embryo could be seen when inspected against a light bulb. The egg shell was cracked and the embryo was washed in PBS before over night fixation in 4% paraformaldehyde (Sigma)/phosphate buffer (PB) at 4°C. The embryo was then placed into PBS (phosphate-buffered saline) for one more night at 4°C, embedded in O.C.T. compound (Leica), frozen, cut on a Leica cryostat in sagittal sections of 10µm thickness, and stored at -75°C. Post-hoc comparison of the embryonic sections to the Hamburger-Hamilton chicken embryo atlas showed that the zebra finch embryo roughly corresponded to a stage 20 chicken embryo (Hamburger and Hamilton, 1992).

To determine singing-associated RXRα expression, adult male birds (ages between one and two years) were either presented with females for directed singing (3 birds), or sang alone (3 birds), and were killed by decapitation within 30min after they had been singing during a period of at least 30min (maximal duration of singing was 2h). Different females were presented to the directed singers every 3-15min, depending on how long the male sang towards the female. Song bouts of the directed singers were counted manually. Undirected singers were housed individually in sound-attenuation boxes for one day before the day of sacrifice, to acclimate to the novel environment, and their singing behavior was recorded using the Sound Analysis Live program (Tchernichovski et al., 2000). Of these birds, those individuals that had not been singing at all for at least 24h before being sacrificed were used as a non-singing control group (5 birds; two of these were only 10 months old, the remaining ones were between one and two years old, as the singers). Among the singers, the amount of singing varied between 9 and more than 100 song bouts.

To determine RXRα expression associated with time of the day, we sacrificed a group of adult male birds early in the morning within the first 20 minutes after lights turned on and before singing took place (3 birds), and another group in the evening between 6:30 p.m. and 7 p.m., approximately 1-1.5h before lights turned off (2 birds). Birds representing day time condition (12 birds) were sacrificed at least 2h after the lights turned on in the morning, to at least 2h before they turned off in the evening. On the day of sacrifice, they were housed individually in sound-attenuation boxes, their vocalizations being monitored with the Sound Analysis Live program. Only birds that had not been singing during the day of sacrifice were used to represent day time condition. Ages of these birds ranged from 13 to 15 months.

Experiments were approved by the responsible commission (*Landesamt für Arbeitsschutz, Gesundheit und technische Sicherheit Berlin*).

## 2.2 *Cloning of fragments for zebra finch RXRs for subsequent usage as in situ hybridization probes*

With the exception of one zebra finch RXRα fragment which we received from the ESTIMA database of zebra finch brain ESTs (Songbird Neurogenomics Initiative, http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=songbird; clone number of the RXRα fragment is SB03001B2G04.f1), we obtained fragments of the zebra finch homologs of RXRα and RXRγ that we used as probes for *in situ* hybridization by cloning from zebra finch total brain cDNA. The zebra finch RXRγ gene was not found by BLASTing either the ESTIMA database or the Songbird Brain Transcriptome database (http://songbirdtranscriptome.net/) with the GenBank sequences of other species' genes. To clone fragments of the zebra finch RXRγ homologue as well as a further RXRα fragment, we first isolated total RNA from adult zebra finch brain using TRIzol reagent (Invitrogen) according to the manufacturer's protocol (description see below, section 2.2.1). The mRNA was then converted to first-strand cDNA with Superscript II reverse transcriptase and Oligo(dT) primers (both from Invitrogen; description of the protocol see below, section 2.2.2). The cDNA served as template for PCR with fragment specific primers. Primer design was based on conserved regions identified with the aid of the UCSC genome browser and GenBank data of other species' mRNA sequences. Primer sequences for the RXRγ probe were 5'-GGGAAGCACTATGGGGTGTA-3' (forward) and 5'-CTGATCGACAAGCGCCA-GCG-3' (reverse) with a resulting fragment length of 799 bp. This probe was hybridized at 53°C, followed by RNAse treatment (see section 2.5, *Radioactive and non-radioactive in situ*

*hybridization*). Primer sequences for the additional RXRα probe (that turned out to be less suitable as a probe than the ESTIMA clone) were 5'-CCAAGCACATATGTGCCATC-3' (forward) and 5'-CGCTGGCGCTTGTCGATGAG-3' (reverse) with a resulting fragment length of 160 bp; hybridization temperature was 53°C (*in situ* hybridization with this probe also included an RNAse treamtent). All PCR products were examined on agarose gels, cleaned from nucleotides with the Qiaquick PCR purification kit (Qiagen), inserted into pGEM-T easy vectors (Promega), sequenced for identification, and stored at -75°C as bacterial cell stocks.

### *2.2.1 Isolation of total RNA from adult zebra finch brain using TRIzol*

Adult male zebra finches were anesthetized with an overdose of ketamine (75mg/kg) and xylazine (15mg/kg) in sterile saline solution, injected intramuscularly. They were then killed by decapitation, and brains were quickly dissected. A mortar was treated with RNase-AWAY (Roth) and chilled with liquid nitrogen, and whole brains were pestled in liquid nitrogen for several minutes until smoothly pulverized. The homogenized tissue was lysed in TRIzol (Invitrogen) by passing the mixture several times through a pipette. Samples were then incubated at room temperature (RT) for 5min to allow for dissociation of nucleoprotein complexes. To separate the solution into an aqueous phase containing the RNA and an organic phase, chloroform (Sigma) was added and tubes were shaken vigourously, incubated at RT for 3min, and centrifuged at 12,000 × g for 15min at 4°C. RNA was recovered by precipitation with isopropyl alcohol (Sigma). To this end, samples were incubated with isopropyl alcohol at RT for 10min and then centrifuged at 12,000 × g for 10min at 4°C. The RNA pellet was washed in 75% ethanol, allowed to air dry, and redissolved in RNAse free water. The RNA concentration was measured using a Nanodrop fluorospectrometer.

### *2.2.2 Conversion of total brain RNA to first-strand cDNA*

Total brain RNA obtained using TRIzol (see previous section) was converted to first-strand cDNA with the reverse transcriptase (RT) SuperScript II (Invitrogen). Reactions contained 25µg/ml Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen), 0.5mM of each dNTP (Roche), 20% (v/v) 5X First-Strand Buffer (Invitrogen), 10mM DTT, 2 units/µl RNaseOUT enzyme (Invitrogen), 10 units/µl of SuperScript II RT, and 0.1µg/µl of total brain RNA. Reverse transcription was performed according to the manufacturer's protocol. Briefly, Oligo(dT) primers, RNA, and dNTP mix were filled up with water to 60% of the reaction volume, before heating to 65°C

for 5min to melt secondary RNA structures. The mixture was then quickly chilled on ice before adding buffer, DTT, and RNaseOUT, and then incubated at 42°C for 2min to adjust temperature for the reverse transcriptase. Reverse transcription was started by adding SuperScript II RT, and reactions were incubated at 42°C for 50min. After this, the reaction was inactivated by heating (70°C for 15min). To remove RNA complementary to the cDNA, *E. coli* RNase H (NEB) was added to a final concentration of 0.1units/µl, and reactions were incubated at 37°C for another 20min. This first-strand reaction was used as a template in PCR reactions at a concentration of 4% (v/v) of the PCR reaction volume.

### *2.3 Generation of full ORF clones of zebra finch RXRs*

Starting from cDNA fragments obtained as described above, we subsequently cloned the full-length sequences of the zebra finch RXRs. To obtain 5' and 3' ends, we again used zebra finch whole brain cDNA as template, and primers one of which was positioned within the fragment we had already cloned and sequenced, whereas the other primer was positioned at the 3' or 5' end, respectively. Primer sequences were chosen on the basis of the trace archive of zebra finch whole genome shotgun (WGS) sequences (accessible via NCBI BLAST), as the whole zebra finch genome was not available yet at the time of primer design. Appropriate WGS sequences could be found by BLASTing with chicken, human, and mouse 3' and 5' sequences. 5' and 3' end primers were as follows:

- for RXRα: forward 5'-GTCGCAGACATGGACACCAAA-3', reverse 5'-TCTGTAAGTCATTTGGTGCGG-3'
- for RXRγ: forward 5'-GAGCGATGAACTGAGGATTA-3', reverse 5'-GACCCTCA(AG)GTGA(CT)CTGCAG(AGCT)GG-3'

PCR, gel purification, cloning and sequencing was done as described for the initially cloned fragments.

### *2.4 Labeling of riboprobes*

Plasmids containing fragments of interest were isolated from bacteria using Qiagen's miniprep kit (Qiagen). Templates for *in vitro* transcription were PCR products of the fragments of interest: The plasmids we used (pGEM-T easy and pSport1 (both Promega)) were designed in such way that if M13 primers are used for amplification, the fragments

comprised both RNA polymerase promoters necessary for transcription of sense and antisense strand. The PCR products could thus serve as templates for sense and antisense riboprobe production. They were obtained using the following PCR protocol: Reactions contained

- 10% (v/v) 10x PCR buffer with $MgCl_2$ (Sigma)
- dNTPs, 50µM each (Roche)
- M13 forward and reverse primers, 0.5µM each (MWG)
- template DNA, 50ng (plasmids with inserts of interest)
- Taq polymerase, 1.25u/50µl (Sigma)

Amplification was achieved in a thermocycler (Biozym) using the following protocol:

- 95ºC for 5min (initial melting of template)
- 30 cycles of
  - a - 94ºC for 30s (denaturation)
  - b - 52ºC for 30s (annealing)
  - c - 72ºC for 1min (extension)
- 72ºC for 5min (clean-up extension)

PCR products were purified using Qiagen's PCR purification kit and used for *in vitro* transcription.

Sense and antisense $^{33}P$ or digoxigenin-labeled riboprobes were generated using SP6, T3, or T7 RNA polymerases (all from Promega): The promoters/polymerases used for pGEM-T easy (Promega) were SP6 and T7, and for the pSport1 plasmid [Promega; GenBank Accession No. U12390], the cloning vector of the RXRα ESTIMA clone, promoters were T3 and T7.

For radioactive $^{33}P$-labeled riboprobes, the transcription buffer contained 50 mM DTT (Roche), 200 mM Tris-HCl (Roth), 30 mM $MgCl_2$ (Roth), 50 mM NaCl (Roth), and 10 mM spermidine (Roche). For transcription, we added template PCR product to a final concentration of 40ng/µl; rATP, rCTP, and rGTP (all from Roche) to a final concentration of 0.5mM each; 12µM rUTP (Roche), 2µCi/µl $^{33}P$-UTP (Amersham), 1µg/µl BSA (bovine serum albumin; Sigma), 5% (v/v) RNAse inhibitor (Amersham), and 10% (v/v) of the respective polymerase. For digoxigenin-labeled riboprobes, *in vitro* transcription was done with Roche transcription buffer, 0.4mM of each rNTP (Roche) where 36% of the total UTP was digoxigenin labeled digUTP (Roche), 10mM DTT (Roche), and RNAse inhibitor and polymerase as for radioactive *in vitro* transcription. Transcription took place in a heating block (Eppendorf) during 2h at 37ºC, except for SP6 reactions which required a temperature of 40ºC.

For non-radioactive probes, transcription was followed by purification in ProbeQuant G-50 Micro Columns (GE Healthcare). To this end, reactions were brought to 50µl with STE

buffer, and spun down for 1min at 735g using an Eppendorf centrifuge. The purified probes were used for non-radioactive *in situ* hybridization.

Radioactive probes were purified using custom-made columns consisting of a 1ml plastic syringe whose opening was blocked with fiber glass (Roth) stuffed into the syringe before it was filled with Sephadex beads (G50 fine, Amersham). Columns were washed twice with 100µl of washing buffer (10mM Tris-HCl pH 7.5 (Roth), 0.15M NaCl (Roth), 0.1mM EDTA, pH 8 (Sigma), 50µg/µl tRNA (Sigma), 0.1% SDS (Roth)) in an Eppendorf centrifuge for 2min at 1000rpm, then blocked four to five times with 100µl of a blocking buffer largely similar to the probe solution (10mM Tris-HCl (Roth), 50mM NaCl (Roth), 0.1mM EDTA, pH 8 (Sigma)) at 1000rpm for 2min. Three more rounds of blocking with 50µl blocking buffer were performed directly prior to probe purification (all done at 2000rpm for 3min). The purified probes were used for radioactive *in situ* hybridization.

### *2.5 Radioactive and non-radioactive in situ hybridization (ISH)*

Adult and juvenile male zebra finches were decapitated and their brains were quickly dissected and frozen over liquid nitrogen. Brains were cut either frontally (for some RXRα ISHs) or sagittally (for all other ISHs) at 14 or 16 µm on a cryostat (Leica) and stored at –75°C.

For $^{33}$P ISH, we followed a previously described protocol (Mello and Clayton, 1994; Mello et al., 1997), with slight modifications. Briefly, after postfixation for 5 minutes at RT in 3% paraformaldehyde (Sigma)/0.1M PBS and dehydration through an alcohol series (50%, 75%, and 100%, 2min each), the slides were acetylated for 10 minutes at RT in 0.0135% triethanolamine (Merck) and 0.0025% acetic anhydride (Fluka) in water. After dehydration in another alcohol series, the sections were hybridized overnight at the appropriate hybridization temperature for each probe (65°C for the RXRα-ESTIMA probe; 53°C for the second, shorter RXRα probe and the RXRγ probe, where hybridization was followed by RNAse treatment) in hybridization buffer (50% formamide (Fluka), 1µl/µl BSA (Sigma), 1µl/µl Poly A (Sigma), 2µg/µl tRNA (Sigma) in 2X SSPE) containing sense or antisense riboprobes (5 x $10^5$ cpm per section). Slides were carefully coverslipped, and hybridization was performed over night in a mineral oil bath (Roth) to keep a homogeneous temperature while preventing evaporation of hybridization solution. The slides were then freed from oil by washing twice in chloroform (Sigma), decoverslipped by dipping in 2X SSPE, and washed sequentially in 2X SSPE buffer

(1 hour at RT) and 2X SSPE/50% formamide (Fluka; 1h at hybridization temperature), followed by two high-stringency washes in 0.1X SSPE (30min at hybridization temperature). The sections were then dehydrated in an alcohol series and subjected to phosphorimager autoradiography. Exposure to a phosphorimager screen (GE Healthcare) was six to ten days. Radiographic signal was measured by a Storm PhosphorImager (Molecular Dynamics), and a GEL image file was produced. This file was analyzed and converted to a TIF file using the ImageQuant analysis software (Molecular Dynamics).

Digoxigenin ISH was done in the same manner with the following modifications: It included an additional 1 hour prehybridization step at 65°C in hybridization solution without probe (this hybridization solution differed from the one used for radioactive ISH and contained 2% (w/v) SDS (Roth), 2% (v/v) Blocking Reagent (Roche), 250µg/ml tRNA (Gibco), 100µg/ml heparin (Polyscience Europe), and 50% formamide (Fluka) in 5X SSC pH 4.5). Prehybridization was done in a wet chamber containing a paper towel soaked with 5XSSC/50% formamide, placed in an oven (Binder). Hybridization solution with 1-5% (v/v) of probe was then heated to 85°C for 5min to melt secondary structures and applied to the slides. After over night hybridization, slides were freed from oil and decoverslipped as described above, and rinsed in 5X SSC. This was followed by four washes at hybridization temperature: The first wash was performed in 1X SSC/50% formamide for 30min, the second one in 2X SSC for 20min, and the third and fourth one in 0.2X SSC for 20min each. Digoxigenin labeling was detected immunohistochemically. Two 5min washes in MABT (maleic acid buffer containing 100mM Maleic acid (Fluka), 150mM NaCl, 0.1% Tween-20 (Roth)) were followed by 1 hour of blocking in MABT/20% lamb serum (Invitrogen), and overnight incubation with Anti-DIG-AP (1:2500, Roche) in 5% serum/MABT at 4°C. Excess antibody was washed off with 3 washes in MABT, 5min each. For staining, we used standard NBT/BCIP staining (NBT=Nitro-Blue Tetrazolium Chloride; BCIP=5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt). Briefly, sections were prepared for the staining by incubation in NTMT pH9.5 for 10min (NTMT: 100mM NaCl, 100mM Tris-HCl pH9.5, 50mM $MgCl_2$, 0.1% Tween-20 (Sigma)). NBT/BCIP staining solution was prepared freshly by mixing 10ml NTMT pH9.5 with 45µl NBT (BioChemika) stock solution (75mg/ml in 70% dimethylformamide (Sigma)) and 35µl BCIP (BioChemika) stock solution (50mg/ml in 100% dimethylformamide). The staining solution was applied to the slides, slides were coverslipped with Parafilm, and incubated in the dark at RT (or at 4°C for the RXRγ probe) for several days (duration depending on probe and laboratory temperature). Fresh staining solution was

applied every day. After staining was completed, slides were rinsed in NTMT and washed twice in PBS for 5min.

This ISH protocol was slightly modified for sections where Area X>DLM projections had been labeled with a retrograde tracer (see below). The tracer, cholera toxin subunit B conjugated to Alexa-488, is intolerant to alcohol, and turned out to be sensitive to prolonged exposure to high temperatures. We therefore replaced dehydration in alcohol series by air drying, reduced prehybridization time to 15min at 55°C and hybridization time to about 5h at the same temperature. Also, instead of in the oil bath, hybridization was done in a wet chamber, containing a towel soaked with 5XSSC/50% formamide and placed in an oven (Binder), as we were not sure that the vapors of the chloroform used for removing excess oil could affect the tracer. However, we were not able to successfully establish ISH on the backfilled sections. While we could make the tracer outlast the procedure with the above modifications, we weren't able to get any specific ISH signal with the probes we used. Whether different probes with a lower optimal hybridization temperature might work is left for future experiments.

For comparison of expression patterns, some selected sections were hybridized with $^{33}$P and digoxigenin-labeled riboprobes for the RAc synthesizing enzyme RalDH (as detailed in Denisenko-Nehrbass et al., 2000). The RalDH probe was a kind gift from Claudio Mello, Ph.D., and Tarciso Velho from the Neurological Science Institute at Oregon Health and Science University, Beaverton, OR. It was the same probe as used in Denisenko-Nehrbass et al. (2000) and required a hybridization termperature of 60°C.

The probe for RXRγ and the smaller of our two RXRα probes required an additional RNAse A treatment after the wash in 50% formamide. The RNAse A treatment consisted in 10min incubation in TNE buffer (10mM Tris-HCl pH7.5 (Roth), 500mM NaCl, 1mM EDTA (Sigma)), followed by 20µg/ml RNAse A (Roche) in TNE for 30min at RT, and another 10min wash in TNE buffer. After RNAse treatment, washes were resumed as specified above.

### *2.6 Stereotactic injections*

To label DLM-projecting neurons in Area X, we stereotaxically injected cholera toxin subunit B, conjugated to Alexa Fluor 488 (Molecular Probes), as a retrograde neuronal tracer into song nucleus DLM. The surgery was performed in four adult males under complete anesthesia resulting from an intramuscular injection of ketamine (50mg/kg) and xylazine (10mg/kg) in

sterile saline solution. The birds' heads were fixed with the help of a stereotaxic apparatus (MyNeurolab) and the retrograde tracer was injected into song nucleus DLM with a hydraulic micromanipulator (Narishige). Stereotaxic coordinates relative to the 0-point at the bifurcation of the midsagittal sinus were: medial/lateral 1.3mm, anterior/posterior 1.2mm, dorsal/ventral -4.5mm. The amount of tracer injected was approximately 400nl. Birds received painkiller (Meloxidyl; active is meloxicam, dose 0.1 mg/kg) half an hour before anesthesia and and once per day for three days post surgery. After receiving injections, birds were allowed to survive for at least five more days and then killed by decapitation. Brains were quickly dissected, frozen immediately over liquid nitrogen and stored at -75°C until processed. Cutting was performed with a cryostat (Leica) in the sagittal plane at 14µm thickness.

### *2.7 Quantification of RXRα expression strength in song nuclei*

To quantify RXRα expression strength in song nuclei relative to the surrounding tissue, we analyzed photographs of non-radioactively *in situ* hybridized brain sections using Photoshop's brightness values for gray-scale pictures. The NBT/BCIP reaction used to visualize the digoxigenin-labeled probe is a colorimetric reaction which proceeds at a steady rate, allowing quantification of probe hybridization by color intensity. Trying to compare different ISHs (i.e., hybridizations performed at different times and thus underlying slightly different conditions, such as varying room temperature, duration of (pre-)hybridization, number of sections hybridized in one passage, etc.), we encountered the problem of a slightly variable overall labeling strength. This made the comparison of absolute brightness values between different hybridizations inappropriate to assess expression strength. We circumvented this variability by a normalization procedure: We mapped the brightness space covered by each section on a scale between 0 and 1, where 0 corresponds to the *average brightness of the area of least labeling minus one standard deviation*, and 1 to the *average brightness of the area with strongest labeling*. These respective bright and dark reference areas each covered 25-40% of the section (depending on its mediolateral plane; each slide did contain non-labeled as well as strongly labeled regions, and we made sure that bright reference areas only comprised non-labeled, while dark reference regions only comprised strongly labeled areas). The dark reference area always comprised parts of the frontal hyper- and meso-, and the posterior nidopallium, whereas the bright reference area always included the non-labeled parts of the thalamus, midbrain, and cerebellum, as well as non-labeled parts of the striatum. Using so large areas as bright and dark reference for normalization turned out to be less prone to

variability due to artifacts than, for instance, the brightest and the darkest spot of the section. Brightness values of all other regions were mapped to the normalized scale from brightest to darkest labeling in the section of interest. Note that using this method, in particular cases, a given region can be assigned a brightness value below 0 or above 1, if its labeling exceeds that of the (averaged) dark or bright reference area, respectively.

To determine the labeling difference between a song nucleus and the surrounding tissue, we used the mathematic *difference* between their normalized averaged brightness values. By means of this measure – in contrast to the *ratio* between labeling of nucleus and surrounding – the nucleus/surrounding-difference value remains related to the overall brightness space covered by the labeling of the section. This means, a labeling difference of 0.1 between a nucleus and its surrounding reflects a brightness difference of 10% of the entire section brightness range. This is irrespective of whether both regions show strong or weak labeling. Using the *ratio* as a difference measure, in contrast, would inflate a slight difference between two bright (weakly labeled) areas, and diminish the difference between two dark (strongly labeled) areas.

To test for effects of age, singing, or singing context on RXRα expression in the telencephalic song nuclei, we first performed regression analyses to see whether the continuous factors *age* and *amount of singing* were correlated with RXRα expression in lMAN, Area X, and RA. In addition, we performed ANOVAs with RXRα expression values in the same song nuclei as dependent variables, and age (juveniles vs. first-year adults vs. older adults), singing vs. non-singing, and singing context (directed and undirected) as independent variables. P-values smaller than .05 were considered significant. All statistics were done using Excel.

### *2.8 Immunohistochemistry*

Neuronal identity of digoxigenin labeled cells was confirmed by immunofluorescent staining with mouse anti-HuC/D antibody (Chemicon), Hu being a pan-neuronal marker (Barami et al., 1995). The antibody was applied to the digoxigenin labeled sections after they had been washed twice in PBS, permeabilized with PBST (0.2% triton/PBS, 30min), and saturated with 4% BSA (Roth) in PBST (30min). Anti-HuC/D concentration used was 1:200 (in PBST with 4% BSA); incubation time was 48h at 4°C. Excess primary antibody was washed off with PBS on a shaker (3 washes, 10min each). The primary antibody was detected with Alexa 568-labeled anti-mouse secondary antibody (Molecular Probes; 1:500 in PBST/4% BSA).

Incubation for the secondary antibody was 1-2h at room temperature in the dark, to prevent fluorescence from bleaching. Excess secondary antibody was washed off with PBS (3 washes, 10min each), sections were counterstained with 1µg/ml DAPI (4',6-Diamidino-2-phenylindole·2HCl·$H_2O$; Serva) in PBS, fixed for 10min in 4% paraformaldehyde/PB, and coverslipped with Mowiol (Roth).

Immunolabeling of RalDH protein, which we present in supplementary fig. 3, is described in detail in the *Materials & Methods* part of Chapter III, where we comprehensively describe immunohistochemical data on RalDH in the zebra finch brain.

# 3 Results

## *3.1 Cloning of zebra finch Retinoid X Receptor (RXR) genes*

The distribution of the RARs in the zebra finch brain is known to be relatively widespread and to exhibit much less spatial specificity than the distribution of the RAc synthesizing RalDH (Jeong et al., 2005): RARα and γ are present in the entire telencephalon, and RARβ in almost all pallial regions of the telencephalon. Since the RARs depend on RXRs to control the transcription of their target genes (Hermann et al., 1992, Heery et al., 1993, Chambon, 1994, Xiao et al., 1995, Kastner et al., 1997), the RXRs' distribution patterns need to be determined as well to identify possible target regions of retinoic acid signaling. Therefore, we cloned the zebra finch RXRs for subsequent *in situ* hybridization on brain sections. In contrast to mammals which have three RXR genes (RXRα, RXRβ, and RXRγ), zebra finches possess only two RXRs (see below). We first cloned fragments of these genes suited as probes for *in situ* hybridization, and used these fragments to then fish the complete open reading frames (for technical details, see *Materials & Methods*). A characterization of the zebra finch RXR genes and the probes we used for *in situ* hybridization is given in the following.

### *3.1.1 Chicken and zebra finches do not seem to have an RXRβ gene*

Unlike mammals, chicken and zebra finches seem to have two instead of three different retinoid X receptor genes.

At the time when this analysis was done, the zebra finch genome was not available yet. We therefore used the genome of the chicken as the closest relative available, as well as the trace archive of zebra finch whole genome shotgun sequences (zfWGS) to find candidate sequences for the birds' RXR genes, which we describe in the following.

If aligned to the chicken genome by BLAST, the coding sequence of the human RXRα mRNA showed noteworthy alignment scores at only two sites of the chicken genome (see fig. 3). The first, better match was located on chicken chromosome 17 (bp 8010708-8033867; identity 88.2% in 791 out of the 1389 bp human sequence), whereas the second one was on chromosome 8 (bp 5353669-5357979, identity 85.7% in 545 out of the 1389 bp). A predicted chicken RXRα mRNA (XR_027135.1) aligned to the first location, suggesting that this site on chromosome 17 is indeed where the chicken RXRα is coded.

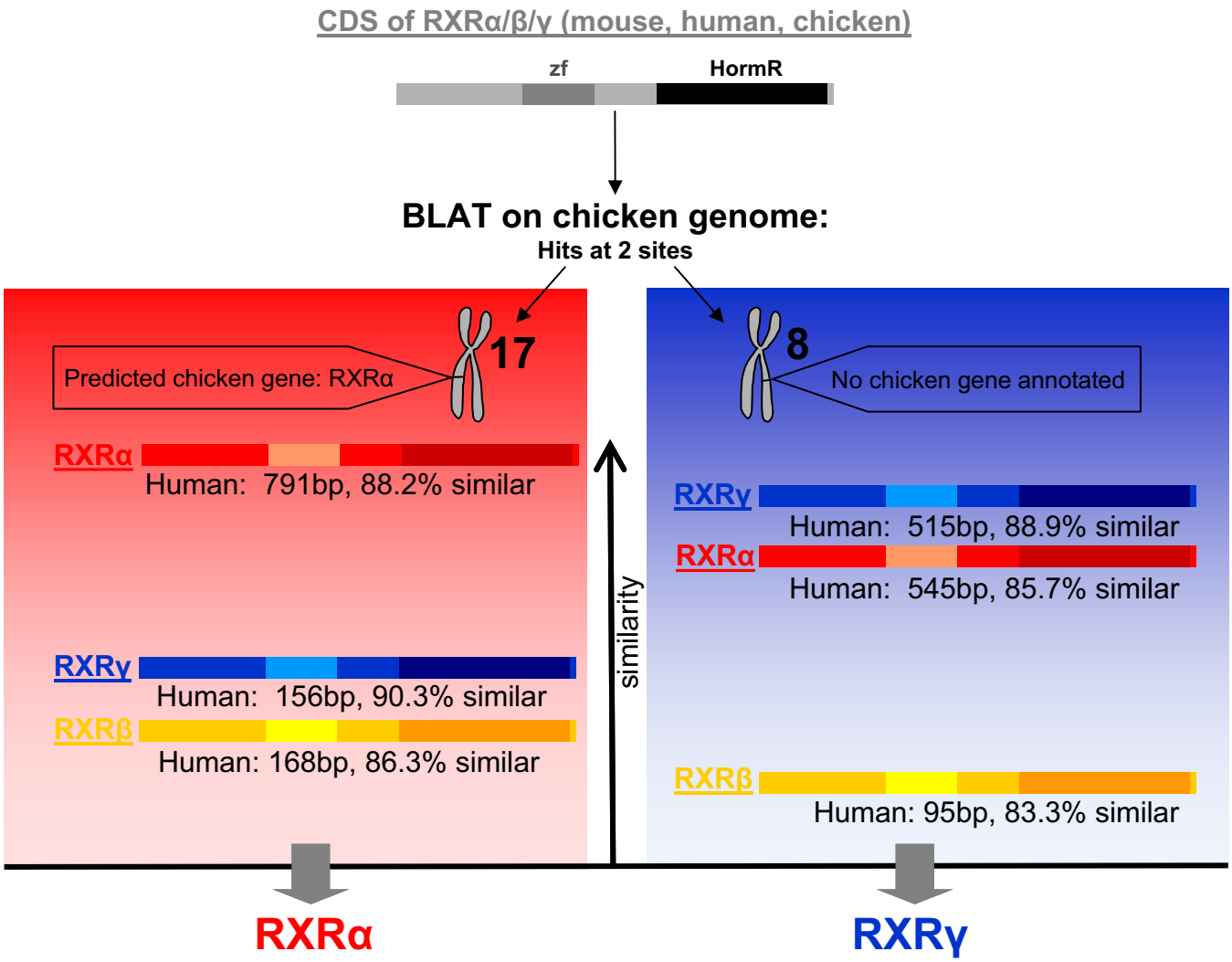


**Figure 3: BLATs with human or mouse RXR coding sequences on the chicken genome show that chicken possess only two RXRs which correspond best to mammalian RXRα and RXRγ.** Gray gene bar on top represents human and mouse coding sequences (CDS) of RXRα, RXRβ, and RXRγ gene, which all contain a zinc finger (zf) and a hormone receptor specific domain (HormR) coding for the ligand binding region of the protein. If used for BLAT on the chicken genome, these sequences produce noteworthy hits at only two sites which are located on chicken chromosomes 17 and 8. At the chromosome 17 site, a predicted chicken RXRα gene aligns, while no annotation is given at the chromosome 8 site. In the box, alignment scores of the human sequences to the chicken chromosome 17 and 8 sites are shown as examples (mouse sequences show comparable results). Bars representing the human genes are arranged according to their similarity to the chicken sequences (most similar sequences are shown on top). Human RXRα aligns best to the chicken chromosome 17 site, while RXRγ/b align less well to this site, suggesting that the chicken RXRα is actually located here. The chicken chromosome 8 site is less clearly identifiable: Its similarity to human RXRα and RXRγ is almost the same (in contrast, human RXRβ has only little similarity with this site). An alignment on protein level clarified this issue: Human RXRγ protein shares 84.4%, RXRα protein only 70.4% identical amino acids with the putative chicken RXR protein coded at the chromosome 8 site. We conclude that chicken RXRα is coded on chromosome 17, chicken RXRγ on chromosome 8, and that chicken have no RXRβ.

In turn, the coding sequence of the human RXRγ which also matched chicken genome sites on chromosomes 17 and 8 only, scored higher on the chromosome 8 site (88.9% identity in 515 out of the 1392 bp human sequence versus 90.3% in only 156 bp on chromosome 17) where the chicken RXRγ gene was annotated. No more sites of noteworthy alignment within the chicken genome were found with RXRα or RXRγ transcripts of either human or mouse.

To rule out that the chicken RXR site on chromosome 17 where no gene is annotated yet rather represents a homologue to the mammals' RXRβ gene instead of RXRα, a BLAST with the human and murine RXRβ coding sequences was performed on the chicken genome. As expected, alignment scores were considerably worse than with RXRα and RXRγ coding sequences (only a 168 bp score out of the human 1602 bp sequence with 86.3% identity at the chromosome 17 site, and at the chromosome 8 site no more than 95 bp with 83.3% identity, as compared to identity scores indicated above; see fig. 3). This suggested that neither the chicken chromosome 17 nor the chromosome 8 site code for a RXRβ gene.

BLASTing the zfWGS with human and chicken RXR coding sequences (CDS) suggested that zebra finches, as chicken and possibly all birds, have only two RXRs instead of three, either. BLASTing with both the chicken and human RXRα coding sequence, respectively, resulted in the same set of contiguous zfWGS sequences. Similarities between chicken and zebra finch in this set of sequences were 90-95%, leading us to the conclusion that these sequences indeed represent the putative zebra finch RXRα coding sequence.

The set of zfWGS sequences resulting from BLASTs with human and chicken RXRγ CDS was different from and did not overlap with the one yielded by RXRα BLASTs. This suggested that both the RXRγ and the RXRα gene do exist in zebra finches, and that they differ more from each other than from their human and chicken homologues.

BLASTing with human and mouse RXRβ coding sequences, in contrast, did not result in a contiguous set of zfWGS sequences spanning the entire query CDS. RXRβ query sequence parts coding for conserved domains as the C4 zinc finger and the ligand binding domain of nuclear hormone receptors did show some similarity to the same zfWGS sequences that had been brought up by the RXRα and RXRγ BLASTs, but similarities to RXRβ were always lower. In addition, an alignment of protein sequences confirmed that the two zebra finch RXRs correspond to RXRα and RXRγ, and not to RXRβ: Protein alignments (for zebra finch sequences, we used virtual protein sequences translated from genomic/WGS sequences found by BLAST) suggested that RXRα and RXRγ are relatively conserved across zebra finch, mouse, and human (93.8% and 84.6% identical amino acids, respectively). The mammals'

RXRβ protein does, in contrast, not align well with either zebra finch RXR (less than 60% identical amino acids).

A post-hoc BLAST analysis of the now available zebra finch genome database (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=59729) confirmed the above results. According to this database, the zebra finch RXRα gene is, as the chicken genes, located on chromosome 17, while the RXRγ gene resides on chromosome 8. No further location of noteworthy similarity with these two RXR genes existed throughout the zebra finch genome.

Altogether, the results of BLASTs on chicken and zebra finch genome as well as zfWGS library suggested that neither chicken nor zebra finches possess an RXRβ gene, whereas both of them do have RXRβ and RXRγ genes.

### *3.1.2 Zebra finch RXRα and RXRγ gene structure and probe design*

We were able to clone full ORFs of the zebra finch RXRα and RXRγ genes (fig. 4). The RXR genes, like all nuclear receptors, are characterized by two functional domains, a DNA binding and a ligand binding domain. The DNA binding domain is a zinc finger in the 5' half of the gene, whereas the hormone receptor binding domain is located towards its 3' end.

In contrast to the RARs where numerous transcriptional variants are known, the only variation known for RXRα/γ are two chicken RXRγ variants which differ at their 5' end (fig. 4.B; Seleiro et al. (1994)). The zebra finch RXRγ ORF sequence we identified corresponds to the shorter chicken RXRγ variant (which is also the one corresponding to the only known RXRγ variant in mammals, such as human, mouse, and rat). We also tried to amplify the longer variant from zebra finch brain cDNA with an appropriate 5' end primer, but were not successful. Therefore, we assume that the long RXRγ variant is either specific for chicken, or exists in zebra finches but is not expressed in adult brains which we used for cDNA production[1]. However, the fact that we were not able to clone this transcriptional variant from whole brain cDNA does not prove that it does not exist in zebra finch brains.

On the protein level, the conservation of RXRα is high between species (93.8% of amino acids are identical between zebra finch and human or mouse). Differences are mainly found in

[1] Post-hatch brain expression of this transcript is unknown for chicken either; its expression is only documented for the embryonic dorsal root ganglion (Seleiro et al., 1994).

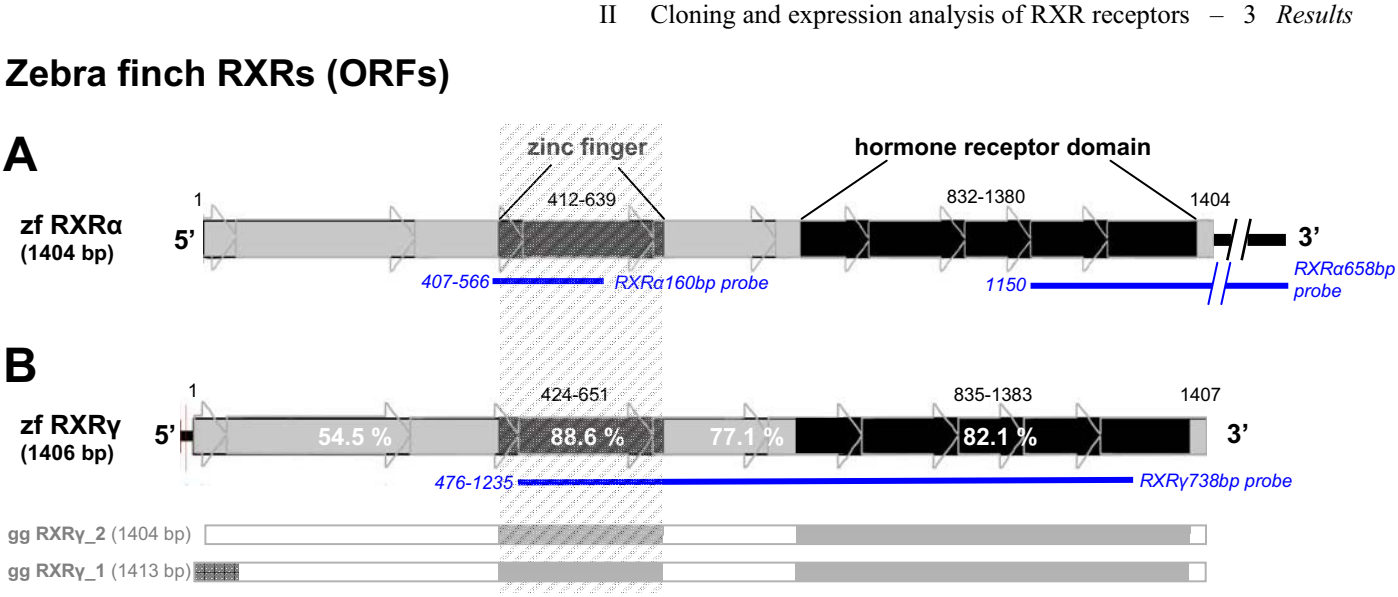


**Figure 4: Zebra finch RXRα and RXRγ domain structure and position of probes used for *in situ* hybridization (ISH).** The thick light gray bars represent the ORF, darker segments represent functional domains, arrows symbolize exons. Like all RXRs, zebra finch RXRs are characterized by a zinc finger in the 5' half of the gene, and a hormone receptor domain towards its 3' end. Start and end nucleotides of ORF and domains are indicated by numbers. The zinc finger domains of the two genes are aligned vertically. Blue lines represent the probes we used for ISH (aligned with the genes). **A:** We tested two probes for ISH; a 160bp probe overlapping with the zinc finger, and a 658bp probe overlapping with the 3' UTR. All RXRα ISHs documented in this study were done with the RXRα658bp probe, since the RXRα160bp probe yielded a bad signal-to-noise-ratio due to its positioning in the relatively conserved zinc finger. **B:** For RXRγ, identity to RXRα is indicated in percent for each part of the gene. In contrast to RXRα where only one transcriptional variant is known, two different transcriptional variants are reported for RXRγ in chicken. The ORFs of these different variants are symbolized by the narrow, light bars beneath the zebra finch RXRγ bar. We were not able to clone a zebra finch version of the longer chicken RXRγ variant; the only zebra finch RXRγ we found corresponds to the shorter variant. However, we cannot exclude that the other transcriptional variant also exists in zebra finches. Note that with our probe, we were not able to distinguish between the two variants which only differ in their 5' ends. The RXRγ738 probe yielded a distinct expression pattern different from RXRα even though it partly spans relatively conserved domains.

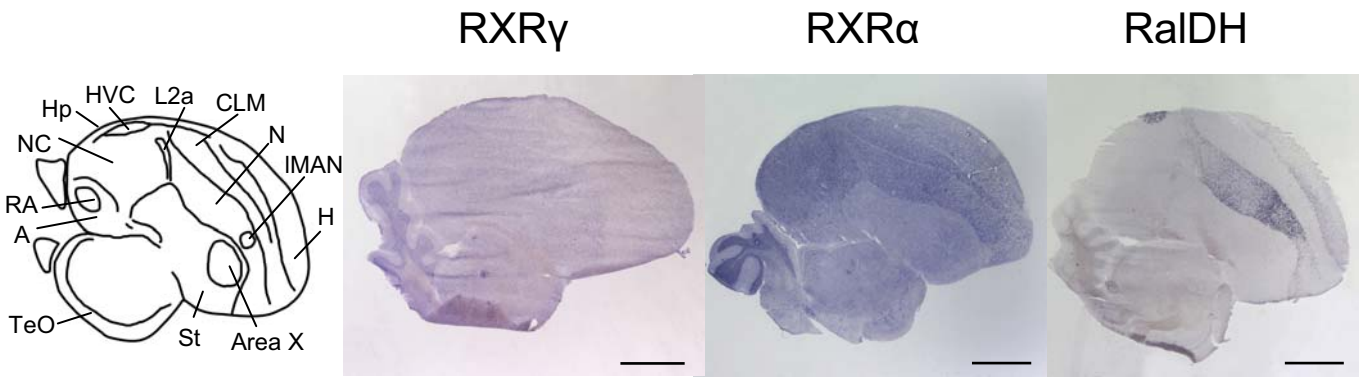


**Figure 5: Brain expression of RXRs in comparison to RalDH.** The diagram on the left depicts specific brain areas and nuclei approximately corresponding of the parasagittal sections shown right. The three right panels are *in situ* hybridizations of parasagittal brain sections hybridized with digoxigenin labeled antisense riboprobe for RXRγ, RXRα, and RalDH, depicting the general brain distribution and relative abundance of the transcripts. The brain levels shown are approximately equivalent. Expression of RXRα was abundant and more widespread than zRalDH expression, whereas RXRγ was expressed to very low levels only. Irregularities in the RXRγ photo are due to folds in the section. For abbreviations, see introduction. Scale bars = 2mm.

the first part of the protein near its N-terminus, upstream of the zinc finger. The degree of conservation is somewhat less for the RXRγ protein, which is in line with what we found for the DNA sequence (see fig. 3). Some 84% of amino acids are identical between birds (zebra finch and chicken) on the one hand and mammals (mouse and human) on the other hand. Differences are again located near the N-terminus, upstream of the zinc finger, where the birds' RXRγ proteins possess five more amino acids than the mammalian ones.

We tested two different RXRα probes for *in situ* hybridization. The smaller one (160bp) spanned the zinc finger, the longer one (658bp, clone obtained from ESTIMA database) spanned the 3' end of the mRNA from the middle of the hormone receptor domain until the poly-A tail (fig. 4). Unsurprisingly, *in situ* patterns obtained with the long 3'-end probe turned out to be more conspicuous and clear-cut in comparison to the zinc-finger spanning probe (see below). Although patterns yielded by this latter probe resembled the other probe's patterns in general, they were blurred by high background. This was most likely because the zinc finger is a relatively conserved domain, suggesting that a probe spanning this region is prone to cross-hybridization with other transcripts' zinc finger domains. Indeed, the probe showed similarity of 86% with the according zebra finch RXRγ sequence, which made cross-hybridization of this probe with RXRγ transcripts conceivable. To assess the possibility of cross-hybridization with other genes' zinc fingers, we performed a post-hoc BLAST of the 160bp-probe sequence on the chicken genome. However, BLASTing only revealed similarities of less than 70% with other genes. Since we cannot exclude that this might still be enough for cross-hybridization to some degree, all RXRα *in situ* hybridizations reported here were done with the long 3'-end-probe.

The RXRγ probe we used for *in situ* hybridizations was 738bp long and spanned large parts of the zinc finger and the hormone receptor domain together with the region between these domains (fig. 4.B). This probe produced distinct and sparse expression patterns on both brain and embryonic sections (see below). The *in situ* hybridization of embryonic sections served as a control for probe specificity: When compared to the known RXRα expression pattern in the chick embryo, our probe produced the same expression pattern (see below). We therefore assume that our probe together with the stringent hybridization conditions used specifically recognized the RXRγ transcript. However, our probe did not distinguish between the two possibly existent different transcriptional variants which only differ in their 5' ends. Therefore, our expression patterns might reflect expression of different transcriptional variants.

### *3.2 In situ hybridization analysis of RXR expression: general comments*

Knowing the expression patterns of RARs and RXRs enables us to determine which brain regions may in general be retinoid susceptible. While the expression patterns of RARα, RARβ, and RARγ are quite widespread with little specificity to the song control system (Jeong et al., 2005), we show here that the expression patterns of the zebra finch RXRs are partly more specific to the song control system (RXRγ), and partly undergo dynamic changes in expression strength within song nuclei (RXRα), suggesting a role for singing or song learning behavior.

We determined RXR expression patterns using *in situ* hybridization with radiolabeled riboprobes followed by phosphorimager autoradiography for an overview, and with digoxigenin-labeled riboprobes for a more detailed regional analysis. Transcript abundance throughout the brain was high for RXRα, and expression was more widespread than expression of the retinoic acid synthesizing enzyme zRalDH (fig. 5). In contrast, overall abundance was extremely low for RXRγ, and expression was restricted to one or very few cell populations (fig. 5 and fig. 6). A detailed description of the RXRα and RXRγ expression patterns with emphasis on the song control system is given below.

### *3.3 RXRγ expression in the zebra finch telencephalon*

Among the retinoid receptors, RXRγ exhibits the most specific expression pattern in the zebra finch brain with respect to the song system. The expression pattern was consistent across all birds studied (n=7). While the overall expression strength was relatively low (fig. 5.B), RXRγ was conspicuously expressed in one or very few cell populations of the learning related song nucleus Area X, a basal ganglia formation (fig. 6). The RXRγ positive cells were large and sparsely distributed (fig. 6.C, D). Due to this sparse distribution as well as the very low expression level in other brain parts, RXRγ expression could not be detected with phosphorimager autoradiography of radiolabeled riboprobe (supplementary fig. 1), but only with the digoxigenin-labeled riboprobe and appropriate microscopic magnification. Distribution and appearance of the RXRγ positive cells reminded of the inhibitory, glutamic acid decarboxylase (GAD)-positive population of Area X cells which project to the thalamic song nucleus DLM. These cells form an essential component of the song learning related

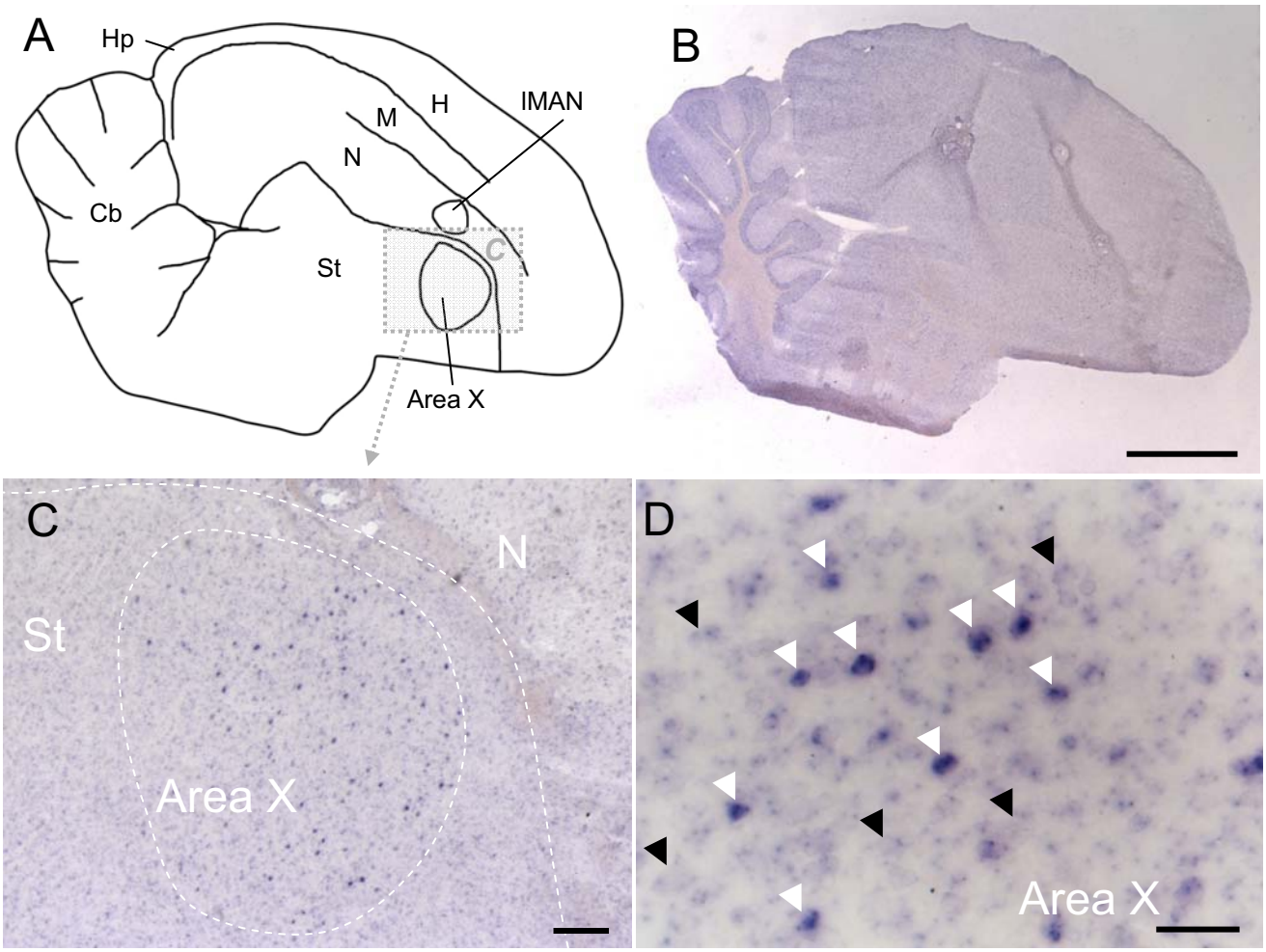


**Fig. 6: RXRγ is conspicuously expressed by a population of sparsely distributed cells in Area X, with otherwise low expression levels in the telencephalon. A:** Drawing of the parasagittal adult zebra finch brain section shown in B and C, depicting specific brain areas and nuclei. Anterior is to the right, dorsal is up. For abbreviations, see introduction. **B:** Digoxigenin *in situ* hybridization of RXRγ probe on sagittal section, followed by NBT/BCIP staining. Expression in Area X is barely visible at this magnification. **C:** Detail of B, showing Area X and the surrounding area as indicated in A. Sparsely distributed RXRγ positive cells in Area X are visible. **D:** High-magnification view of Area X, showing RXRγ expression in large, sparsely distributed cells. Black and white arrowheads indicate examples of unlabeled and labeled cells, respectively. Scale bars: 2mm in B, 200µm in C, 50µm in D.

Anterior Forebrain Pathway (Luo et al., 2004). We attempted to identify the RXRγ positive cells by combining a digoxigenin *in situ* hybridization with backfills of the Area X>DLM projection neurons with a retrograde fluorescent tracer (see *Materials & Methods, 2.6*). However, we were not successful in making this procedure work: The retrograde tracer turned out to be sensitive to high temperatures (starting from roughly 50°C, depending on duration of exposure), while the probe yielded unspecific labeling at or below these temperatures, and no labeling at all when duration of exposure was shortened (i.e., less than about 5h). We were not able to find suitable hybridization conditions for both tracer labeling and (specific) digoxigenin signal to last the procedure. Therefore, the definite identification of the RXRγ positive Area X cells is left for future research.

To see whether RXRγ expression in Area X changes over the course of song learning, we performed *in situ* hybridizations on the brains of two 42 and one 64 day old zebra finch. The expression pattern in all three birds was the same as described above for the adults (data not shown). However, we were not able to quantify RXRγ expression in Area X. This was first due to the sparseness of the pattern which made a quantification based on regional signal intensity as used for RXRα prone to noise and errors. Second, the data originated from different hybridizations involving different room temperature conditions, different amounts of probe etc., which made a post-hoc assessment of transcript abundance based on signal intensity impossible. A more detailed description of RXRγ expression in Area X during song development is therefore left for future research.

Since the overall expression level of RXRγ was low, we also performed an *in situ* hybridization with digoxigenin labeled riboprobes on sagittal zebra finch embryonic sections as a technical control for the probe (supplementary fig. 2). RXRγ transcripts were clearly detected in the eye cup, where RXRγ has been shown to also be expressed in embryos of Xenopus and chicken (Seleiro et al., 1994, Hoover et al., 1998, Cossette and Drysdale, 2004).

### *3.4 RXRα expression in the zebra finch telencephalon*

RXRα exhibited a conspicuous expression pattern in the telencephalon with relatively high and uniform levels in most pallial areas (hyperpallium, mesopallium, and nidopallium) and only low levels in the striatum (fig. 7). Exceptions to the generally high pallial RXRα expression were some lateral pallial areas, i.e. parts of the arcopallium (fig. 7.D-F), the

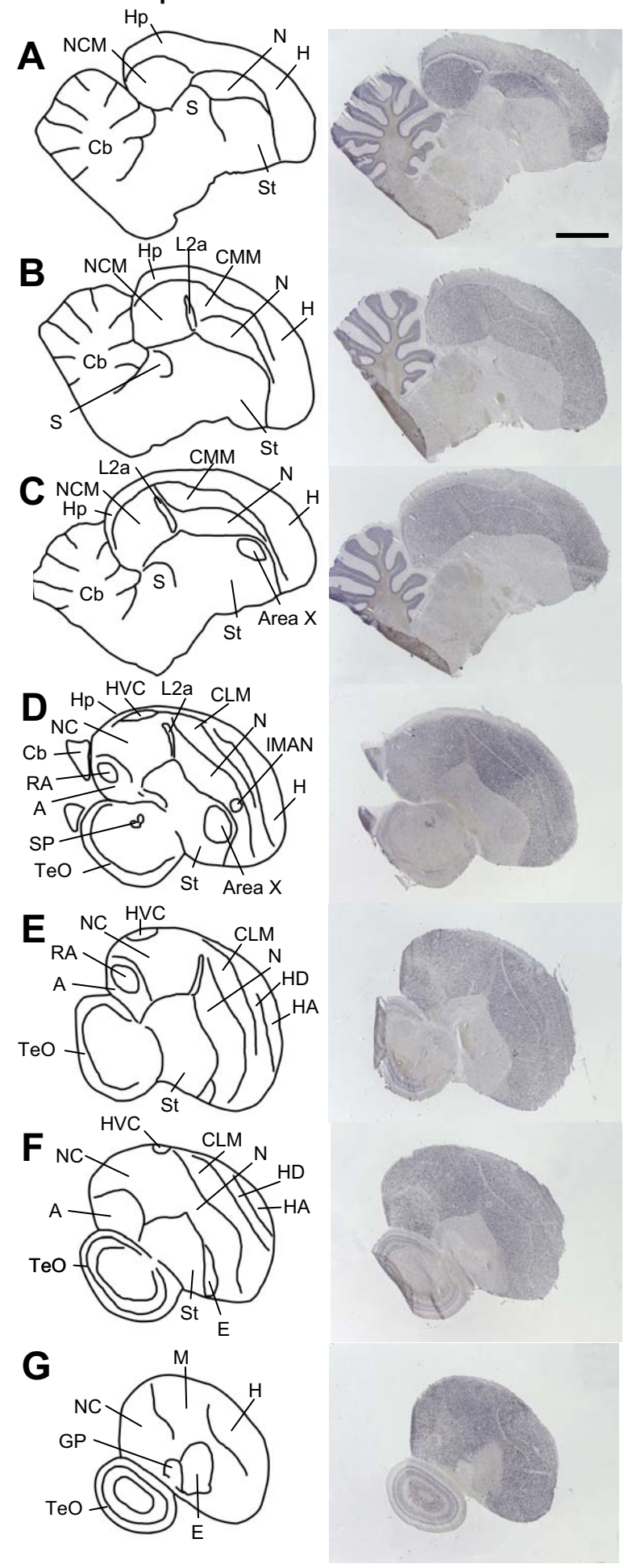


**Figure 7: RXRα expression in adult male zebra finch brain.** The diagrams on the left depict specific brain areas and nuclei of the parasagittal sections shown right. The right column shows sections hybridized with a digoxigenin labeled RXRα antisense probe and stained with NBT/BCIP. For all images, anterior ist to the right and dorsal is up; medial to lateral levels are represented from top to bottom. Scale bar = 2mm (applies to all panels). For abbreviations, see introduction.

entopallium, and the globus pallidus (fig. 7.G). The hippocampus showed only little RXRα expression, either (fig. 7.A-D). Likewise, expression levels in the thalamus, hypothalamus, midbrain, pons, and medulla were generally low to non-detectable, although few isolated thalamic regions did highly express RXRα (see below). In the cerebellum, RXRα was highly expressed in the granule cell layer (fig. 7.A-C) and in the Purkinje cell layer, whereas molecular layer and white matter did not show any labeling.

RXRα expression in the song control nuclei largely reflected their location in the telencephalon: The pallial song nuclei HVC and RA of the premotor pathway as well as lMAN of the AFP showed high expression of RXRα, whereas the striatal song nucleus Area X of the AFP showed only little expression (fig. 8). However, in lMAN, RA, and Area X, RXRα expression differed somewhat from the immediately surrounding tissues, based on a qualitative assessment: Area X exhibited a slightly higher expression than the surrounding striatum (fig. 8.D), and RA showed a considerably higher expression than the surrounding arcopallium (fig. 8.B), whereas RXRα expression in lMAN was slightly lower than in the surrounding nidopallium (fig. 8.D). In contrast, the expression level in song nucleus HVC did not differ from the surrounding nidopallial shelf (fig. 8.G). Within HVC, most strongly labeled cells were large with a neuron-like shape and occurred in clusters, but also single cells with different degrees of labeling were found, as well as non-labeled cells (fig. 8.G, H). In RA, RXRα expression was particularly high in many cells, most of which were located in small clusters of around three to five cells (fig. 8.B, C), and as in HVC, some weakly as well as non-labeled could be found, too. The same applies to lMAN (fig. 8.D, E), where the high number of weakly or non-labeled cells probably contributed to the apparent lower overall expression within lMAN as compared to the surrounding nidopallium. In lMAN, strong labeling was primarily found in large neuronal cells. In contrast, Area X showed no strongly labeled cells at all. Its weakly labeled cells were mostly located in the multicellular clusters typical of this nucleus. Expression levels were low, and many non-labeled cells were also present in Area X (fig. 8.F). A first survey of *in situ* hybridizations of different individuals' brains suggested that RXRα expression levels in some of the song nuclei – especially in Area X and lMAN – might be more variable than expression levels in other brain regions. We therefore investigated more carefully whether expression levels in song control nuclei were correlated with age, singing behavior, or time of the day (see below, section 0).

At a more medial brain level where the higher auditory areas are located (fig. 9.A, B), expression was particularly high in two auditory structures of the caudomedial telencephalon,

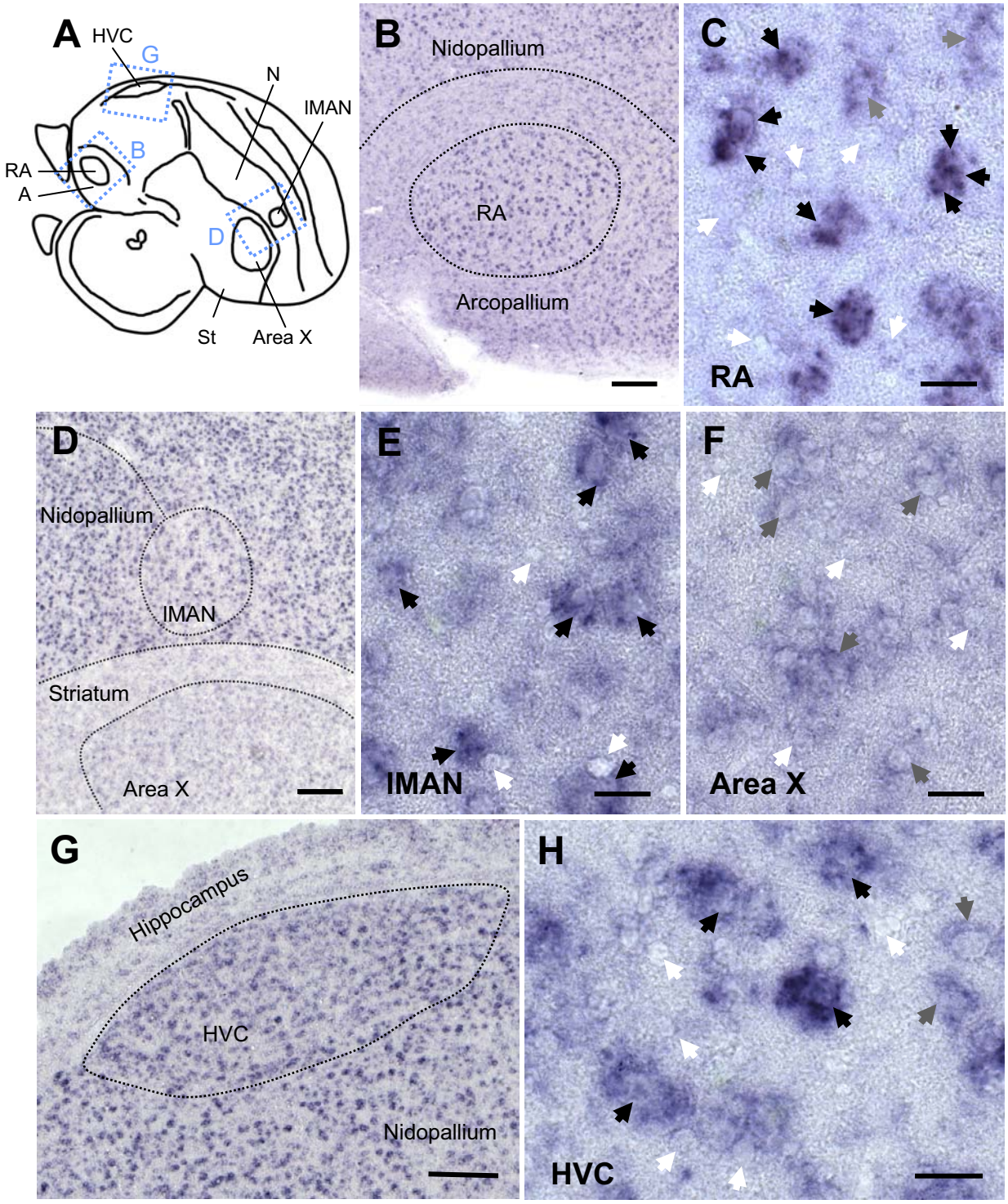


**Figure 8: RXRα expression in song control nuclei of the zebra finch telencephalon. A:** Schematic drawing of a parasagittal section at the level of the song nuclei HVC, RA, Area X, and lMAN. The dashed rectangles indicate the areas shown in B, G, and D. Photos in B-H are bright field views of parasagittal sections hybridized with digoxigenin-labeled RXRα antisense probe and stained with NBT/BCIP. Anterior is right, dorsal is up in all panels. **B, C:** Low and high power views, respectively, of RXRα expression in song nucleus RA. Many cells were strongly labeled; the surrounding arcopallium showed less labeling. **D-F:** Low and high power views of RXRα expression in song nuclei lMAN and Area X of the anterior forebrain pathway. Area X exhibited higher, lMAN lower RXRα expression than the surrounding areas, but overall expression was lower in Area X/striatum than in lMAN/nidopallium. In Area X, only weakly labeled cells were found. **G, H:** Low and high power views of RXRα expression in HVC, showing that the high expression level approximately equaled the surrounding tissue. In **C, E, F, and H,** black arrows indicate examples of strongly labeled cells, gray arrows weakly labeled cells, and white arrows non labeled cells. Scale bars = 200 µm in B, D, G; 20µm in C, E, F, H.

the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM). This was in contrast to the low expression in the adjacent core thalamorecipient auditory zone field L2a (fig. 9.B) and in the overlying hippocampus. Within NCM and CMM, high RXRα expression occurred in various cells throughout the entire regions (fig. 9.C, D), although RXRα-negative cells could also be found (see fig. 9.C, D; white arrows).

### *3.5 RXR expression in the thalamus and midbrain*

Although RXR expression in the thalamus was generally low to undetectable, nucleus spiriformis of the thalamus – a sensorimotor nucleus implicated in the neural integration of visually dependent responses (Gioanni and Sansonetti, 2000, Toledo et al., 2002) – exhibited the highest expression levels of both RXRα and RXRγ in the entire brain (fig. 10.G). Nucleus spiriformis comprises a medial and a lateral subnucleus and projects to the tectum (Reiner et al., 1982); it receives input e.g. from parts of the striatum. Lesions in this nucleus lead to disturbances in movements (Rieke, 1980).

RXRγ expression in the thalamus was restricted to nucleus spiriformis, whereas RXRα also showed expression in other parts of thalamus and midbrain. These included, besides some layers of the optic tectum and the weakly labeled oculomotor nerve, scattered cells in the substantia nigra (SN) and the ventral tegmental area (VTA) (fig. 10.A-F). These two midbrain dopamine centers are part of a brain motivation system and form an anatomical loop with Area X, providing Area X with strong, direct dopaminergic input and in turn receiving indirect input from Area X via the ventral pallidum (Lewis et al., 1981, Gale et al., 2008). Some neurons of the SN/VTA complex show singing-related changes in firing rate, and the complex on its part seems to modify singing-dependent gene expression in the song control nuclei lMAN, RA, and Area X (Hara et al., 2007).

We do not know whether any of the *RARs* is expressed in the RXR thalamic and midbrain expression sites, i.e. whether RXRs at these sites are able to mediate retinoid signaling at all (they might alternatively function as heterodimeric partners for other molecules, see section 4.2). Since the respective regions are not directly implicated in vocal or auditory function, or otherwise related to song, we desist from going into further detail with their analysis here.

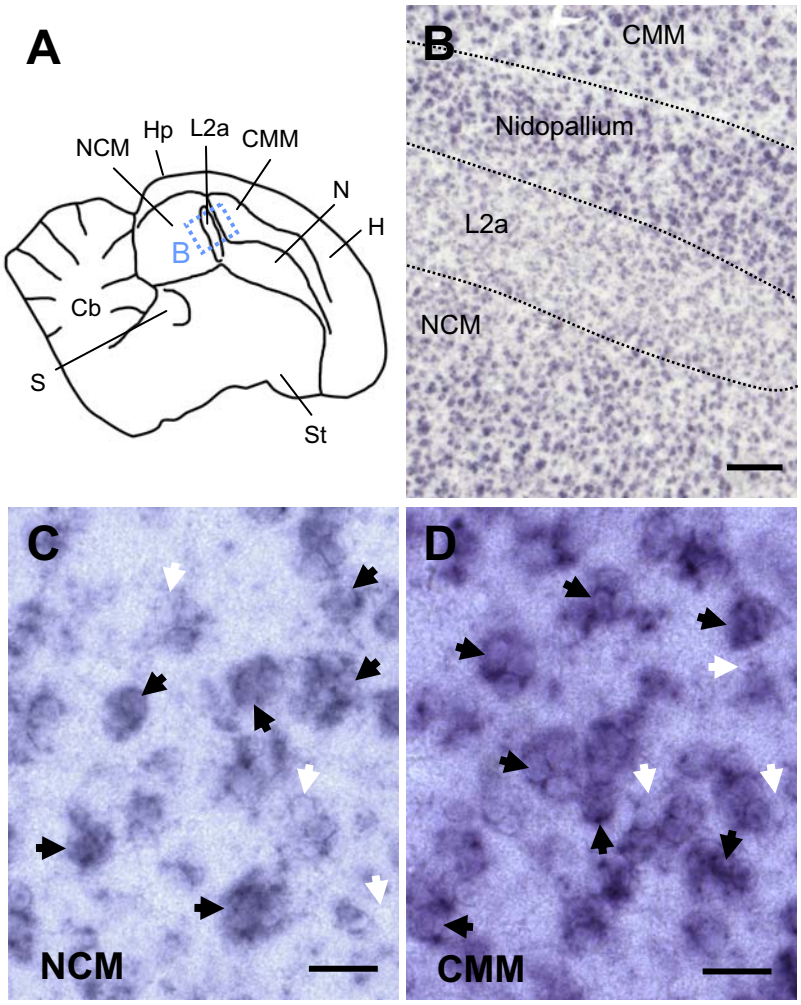


**Figure 9: RXRα expression in higher auditory areas of the zebra finch telencephalon. A:** Schematic drawing of a parasagittal section at the level of the auditory areas NCM, Field L2a, and CMM. The dashed rectangle indicates the area shown in B. Photos in B-H are bright field views of parasagittal sections hybridized with digoxigenin-labeled RXRα antisense probe and stained with NBT/BCIP. Anterior is right, dorsal is up in all panels. **B:** Low power view of RXRα expression in higher auditory areas. Strong labeling occurred in NCM and CMM; L2a showed less labeling. **C, D:** High power views of RXRα expression in NCM and CMM, respectively. In both structures, strongly labeled cells were mostly found in clusters. In **C and D,** black arrows indicate examples of strongly labeled cells, white arrows of non labeled cells. Scale bars = 200 µm in B; 20µm in C, D.

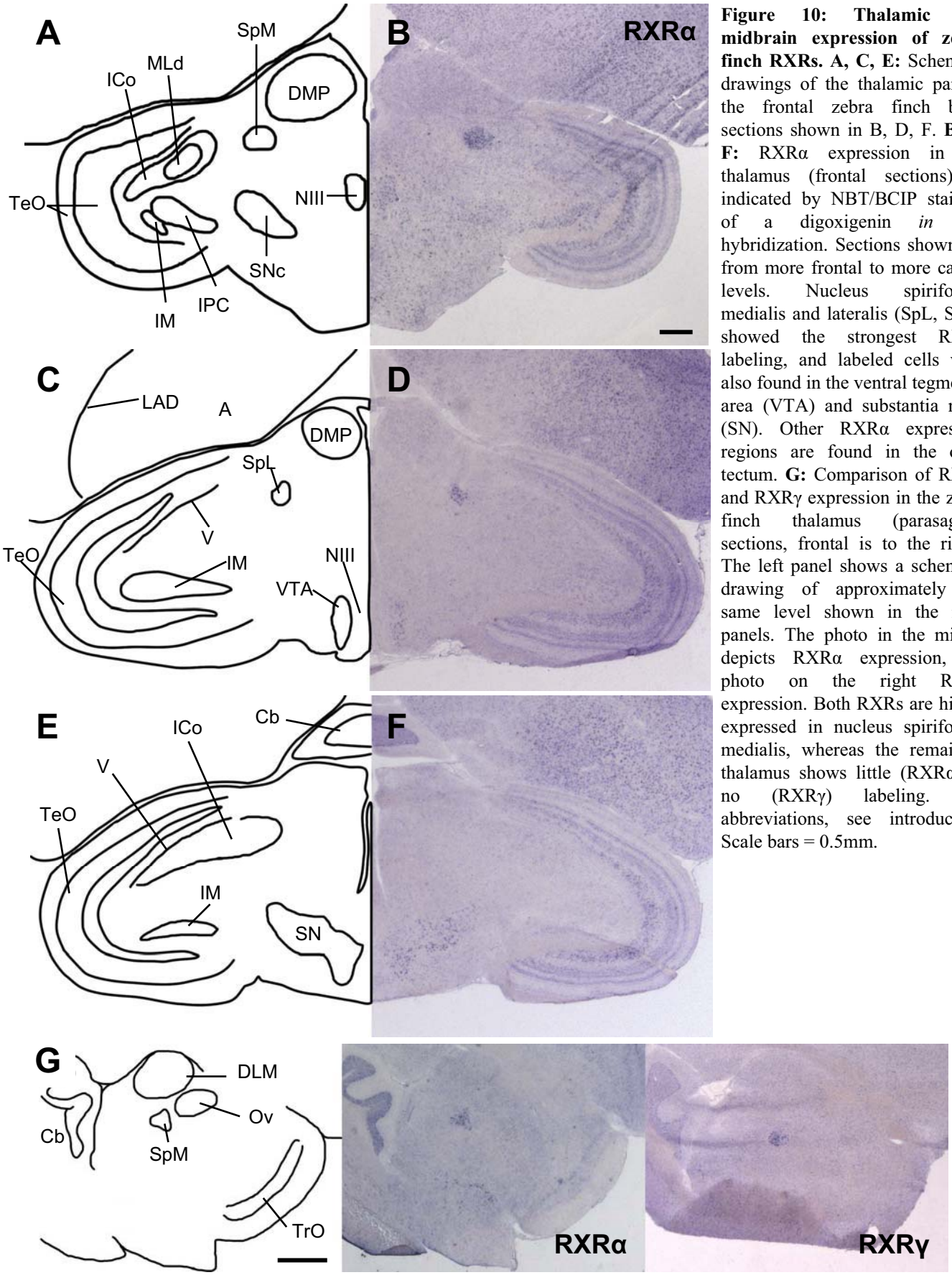


**Figure 10: Thalamic and midbrain expression of zebra finch RXRs. A, C, E:** Schematic drawings of the thalamic part of the frontal zebra finch brain sections shown in B, D, F. **B, D, F:** RXRα expression in the thalamus (frontal sections) as indicated by NBT/BCIP staining of a digoxigenin *in situ* hybridization. Sections shown are from more frontal to more caudal levels. Nucleus spiriformis medialis and lateralis (SpL, SpM) showed the strongest RXRα labeling, and labeled cells were also found in the ventral tegmental area (VTA) and substantia nigra (SN). Other RXRα expressing regions are found in the optic tectum. **G:** Comparison of RXRα and RXRγ expression in the zebra finch thalamus (parasagittal sections, frontal is to the right). The left panel shows a schematic drawing of approximately the same level shown in the right panels. The photo in the middle depicts RXRα expression, the photo on the right RXRγ expression. Both RXRs are highly expressed in nucleus spiriformis medialis, whereas the remaining thalamus shows little (RXRα) or no (RXRγ) labeling. For abbreviations, see introduction. Scale bars = 0.5mm.

## *3.6 Variability of RXRα expression in the song control nuclei*

Based on a number of RXRα *in situ* hybridizations on brains of different individuals, we got the broad impression that RXRα expression in song nuclei lMAN and Area X was relatively variable, in some individuals being more different to the surrounding tissue than in others, where a difference to the surrounding was barely visible. We therefore examined more carefully whether expression levels in song control nuclei might be correlated with age, singing behavior or social context of singing, or time of the day. However, the only nucleus for which we could find significant effects was lMAN. RXRα expression in lMAN was significantly influenced by age (comparison of juveniles, first year adults, and older adults) and by time of the day (comparison of morning, daytime, and evening condition), whereas singing behavior did not significantly influence RXRα expression in any of the song nuclei we investigated. A detailed description of these results is given in the following.

### *3.6.1 Age influences RXRα expression in lMAN, but not in the other song nuclei*

Since we saw a particularly outstanding Area X for the first time in an 68 day old animal, we first compared three animals of 65-68 days of age with adult animals. To this end, we conducted non-radioactive digoxigenin *in situ* hybridizations with NBT/BCIP staining on brain sections of the different animals, and determined labeling strength and its difference between song nuclei and the surrounding tissue as described in *Materials & Methods*, section 2.6. Song nuclei considered were Area X, lMAN, and RA (the RXRα expression level in HVC seemed to never differ from the surrounding nidopallial shelf and was therefore not further analyzed). We could indeed observe a significant effect of age on RXRα expression in lMAN, if three age classes were considered: juveniles of around 65 days, young adult birds from eight months up to one year, and older adults of more than one year (ANOVA, $p$=0.018; fig. 11.A). The older the animals, the stronger the downregulation of RXRα in lMAN as compared to the surrounding nidopallium. However, a regression analysis taking into account the *individual ages of all birds* showed no significant interrelation with RXRα expression in lMAN ($p$>0.05). We have to point out, though, that the three age groups have not been controlled for singing behavior. Actually, the older birds in our samples sang more, which makes it difficult to disentangle the factors *age* and *singing behavior* and to independently assess their effects on RXRα expression. Although an ANOVA testing for correlation of RXRα expression with singing or singing context (see next section for details) did *not* show

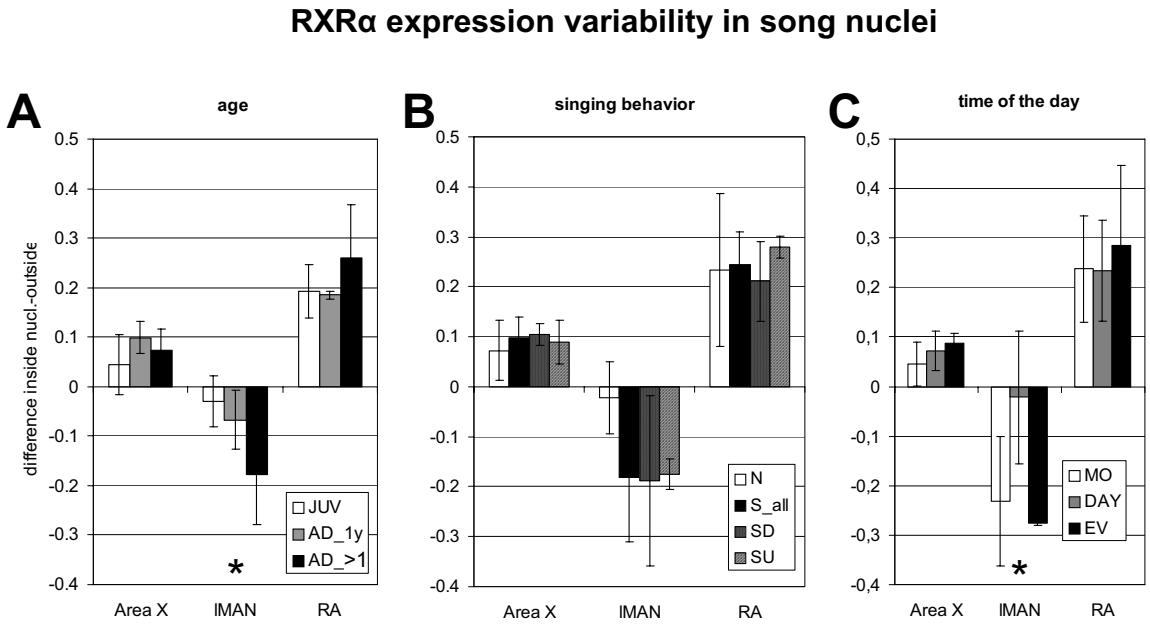


**Figure 11: RXRα expression strength in the song nuclei Area X, lMAN, and RA in relation to age, singing behavior, and time of the day.** In all diagrams, bars represent the difference in normalized RXRα expression strength between a song nucleus and the surrounding area ("Δ RXRα inside nucl.-outside"). For an explanation of how normalized expression strength was determined, see *Materials and Methods*, section 2.7. Error bars represent standard deviation. **A:** In lMAN, a significant effect of age on RXRα expression could be observed, RXRα expression decreasing in older birds (ANOVA, p=0.18). In contrast, RXRα expression in Area X and RA did not differ significantly between age classes. JUV=juveniles (PHD 65-68, n=3); AD_1y=adults from 8 months to 1 year (n=3), AD_>1=adults older than 1 year (n=13). **B:** RXRα expression in Area X, lMAN, and RA did not significantly differ between birds that had been singing (black bars) and birds that had not (white bars), although there is a tendency towards lower expression in lMAN of the singers, irrespectively of whether they sang directed or undirected song. No significant expression differences could be detected between directed (spotted bars) and undirected singers (hatched bars). N=non-singers (n=5); S_all=all singers (n=6, the same ones as in SD and SU); SD=directed singers (n=3); SU=undirected singers (n=3). **C:** At different times of the day, significant RXRα expression differences could be found in lMAN. During day hours, lMAN expression in lMAN was higher (and thus less different from the surrounding nidopallium) than in the morning or evening. MO=adult birds sacrificed in the morning, i.e. within the first 20min after the lights turned on (n=3); DAY=adult birds sacrificed during the day, i.e. 2-9h after the lights turned on (n=12; only birds that did not sing); EV=adult birds sacrificed in the evening, about 1-1.5h before the lights turned off (n=2).

any significant effect, nor did a regression analysis for *singing amount* in relation to RXRα expression yield a correlation significantly different from chance level, we cannot rule out that RXRα expression in lMAN is actually dependent to some extent on both singing behavior *and* age. RXRα expression in the other song nuclei, Area X and RA, did in contrast to lMAN not significantly differ between age groups.

Interestingly, an inspection of RalDH expression which we performed *post-hoc* corroborated an interrelation between RAc signaling in lMAN and age of the birds. We reviewed our data on RalDH mRNA (as determined by *in situ* hybridization) and on RalDH protein (as determined by immunohistochemistry) in song nucleus lMAN (supplementary fig. 3). Indeed, both mRNA and protein appeared to vary across different ages in song nucleus lMAN, RalDH expression being higher in younger and lower in older animals' lMAN. This difference seems to largely depend on the density of RalDH positive cells in lMAN (as judged by inspection): The older the animal, the fewer cells per area are conspicuously expressing RalDH, while the expression strength per RalDH positive cell did not appear to strongly vary with age. This result does of course not directly affect the interpretation of our data on RXRα-lMAN expression in birds of different ages. However, it supports the general idea of age-dependent changes in RAc signaling in song nucleus lMAN, and thereby lends further plausibility to a scenario where also other molecules involved with RAc signaling may be mediating, or be subject to, age-dependent changes in song nucleus lMAN.

### *3.6.2 Singing behavior does not significantly influence RXRα expression in song nuclei*

The second variable we investigated as a potential regulator for RXRα expression variability in song nuclei was singing behavior (fig. 11.B). To this end, we separated adult males in recording boxes and recorded their singing activity. Birds that sang during a time span of at least 30 minutes were sacrificed within 30 minutes after singing and represented the group of undirected singers. Birds that did not sing for more than one day were sacrificed and represented the non-singers group. A different group of adult males was presented with females to produce directed singing. After they had been singing during at least 30 minutes, these birds were sacrificed within another 30 minutes, and represented the group of directed singers. RXRα expression in Area X, lMAN, and RA did not significantly differ between any of the groups. The different singer groups showed RXRα expression values highly similar to each other, whereas the non-singers tended to have a higher RXRα expression in lMAN (which was therefore *less* different to the surrounding nidopallium) as compared to all singers

irrespective of social context of singing, but this difference was not significant (ANOVA, $p>0.05$). Note also that on average, the non-singers were younger than the singers, again making a clear distinction between the factors *singing behavior* and *age* difficult (see previous section): The non-singers' tendency to a less suppressed RXRα expression in lMAN might be mainly due to age effects.

### *3.6.3 Time of the day influences RXRα expression in lMAN*

The third variable we considered as a potential regulator of RXRα expression in song nuclei was time of the day (fig. 11.C). Song structuredness has been shown to undergo circadian oscillations, which are in juveniles relevant for song learning success (Deregnaucourt et al., 2005). Retinoid signaling may potentially be involved with this circadian rhythmicity of song features, given that previous observations have hinted at a circadian role for retinoid nuclear receptors and vitamin A: For instance, targeted gene disruption of the retinoid-related orphan receptor, RORβ, mildly affects circadian rhythmicity (Andre et al., 1998), and several binding proteins which transport the retinoic acid precursor molecule retinol through plasma (retinol binding proteins, or RBPs) and within cells (cellular retinol binding proteins, or CRBPs) have been shown to undergo circadian variation (Hongo et al., 1993, Rajendran et al., 1996). These findings suggest circadian oscillations in retinoid signaling itself, and therefore make time of the day a conceivable regulator of retinoid related gene expression. Indeed, comparing RXRα expression in lMAN, Area X, and RA of birds in morning condition (i.e. sacrificed within the first 20 minutes after the lights turned on), evening condition (birds sacrificed within the last 1-1.5h before the lights turned off), and daytime condition (birds sacrificed at least 2h after the lights turned on in the morning to at least 2h before they turned off in the evening), we saw significant RXRα expression differences in lMAN (fig. 11.C; ANOVA, $p=0.003$). During day hours, RXRα expression in lMAN was higher (and thus less different from the surrounding nidopallium) than in the morning or evening. The three groups were relatively comparable as to their average age (446 days for the morning condition, 482 days for the daytime condition, days 396 for the evening condition) and their singing behavior (all birds were non-singers). We therefore assume that the significant difference in RXRα expression in lMAN was indeed dependent on time of the day.

The other song nuclei did not show a significant RXRα expression difference depending on time of the day; however, we could observe a tendency to slightly lower RXRα expression in Area X in the morning.

### *3.7 Sites of overlapping RXR and RAR expression that define regions of potential RAc signaling include many song control regions*

Since RARs are thought to mediate RAc induced target gene transcription only upon heterodimerization with RXRs (Hermann et al., 1992, Heery et al., 1993, Chambon, 1994, Xiao et al., 1995, Kastner et al., 1997), sites of potential RAc signaling are defined by the co-occurrence of at least one RXR with one RAR. As already mentioned, RAR expression in the zebra finch brain has been shown to be widespread and much less confined and conspicuous than RalDH expression (Jeong et al., 2005). We aimed at investigating whether RXR expression in the bird brain is more confined than RAR expression and thus able to narrow down putative sites of potential RAc signaling.

The results concerning this matter were mixed (fig. 12): RXRα was expressed as broadly as the RARs, thus allowing for potential RXRα-mediated RAc signaling in most brain regions. However, RXRγ exhibited a very sparse expression largely restricted to few cells in song nucleus Area X. RAc effects mediated by RXRγ containing receptor dimers must thus be spatially very restricted. Assuming that each receptor combination regulates its own subset of target genes, the RXRγ expression pattern might allow for specific RAc effects in a spatially restricted part of the song control system.

Focusing on the telencephalic song nuclei, we observed that (1) all song nuclei but HVC exhibited receptor expression profiles that differed from the surrounding tissue (fig. 12.A). (2) When compared to each other, each song nucleus had its own receptor expression profile differing from that of the other nuclei (fig. 12.B) – if both receptor identity and expression strength were taken into account. Expression profiles of the three pallial song nuclei resemble each other more than the expression profile of striatal nucleus Area X: HVC, lMAN, and RA all predominantly expressed RARα and RXRα. However, expression strength was generally lower in lMAN than in the other two pallial song nuclei. Furthermore, HVC also expressed a significant amount of RARγ whose expression was very low in both RA and lMAN. RA was characterized by a particularly high RXRα expression in comparison to HVC and lMAN. The striatal nucleus Area X differed from the pallial nuclei most notably in that it expressed RXRγ – at least in some cells – but only very little RXRα. RAR expression in Area X resembles HVC, although both RARα and RARγ, of which we find a considerable amount in HVC, are expressed even more strongly in Area X (Jeong et al., 2005).

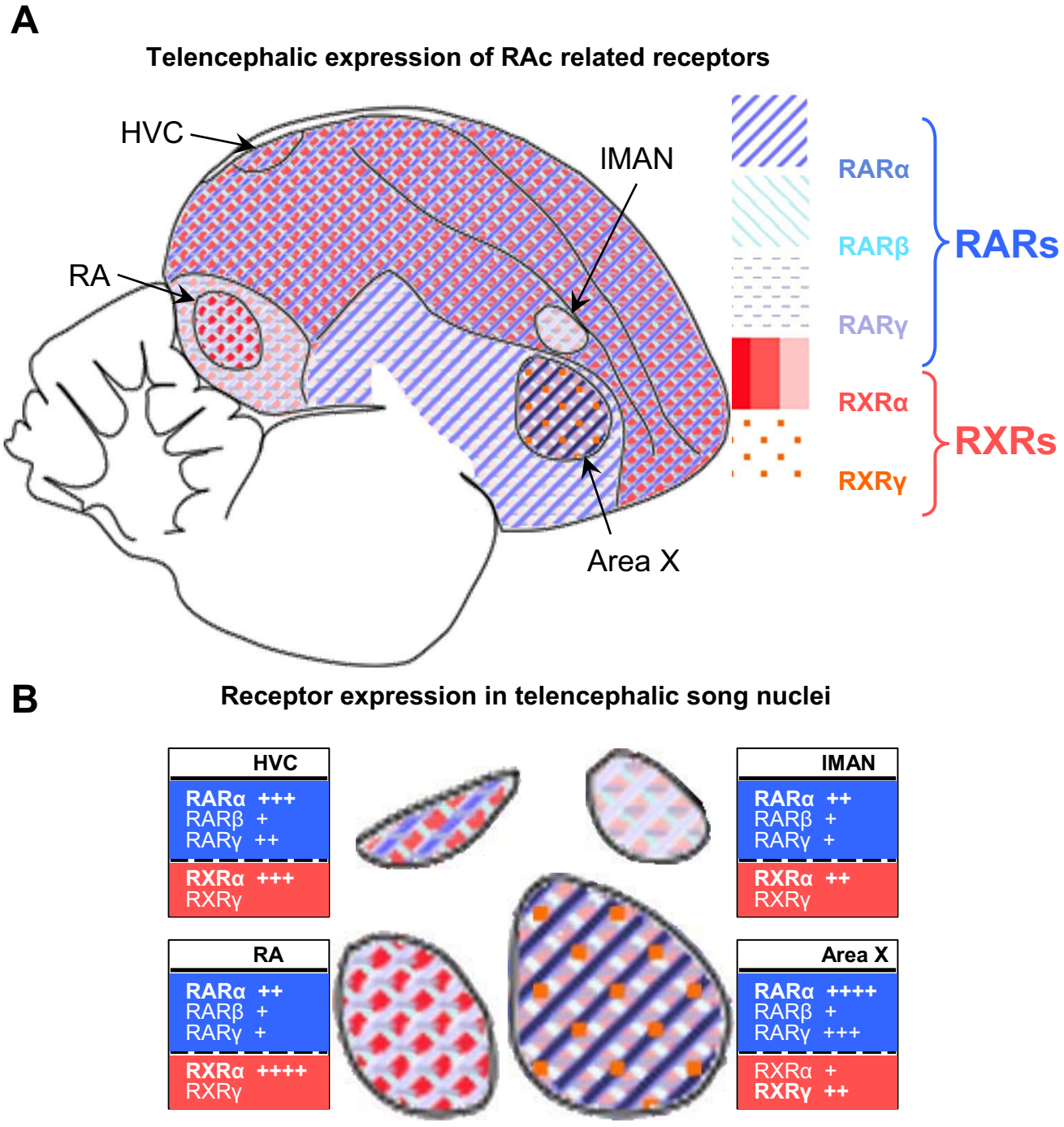


**Figure 12: Sites of differentially overlapping RAR and RXR expression include telencephalic song nuclei. A:** Schematic drawing of RAc receptor expression in the zebra finch telencephalon. The parasagittal section depicted contains song nuclei HVC, RA, lMAN, and Area X. Expression of the five RAc related nuclear receptors is represented by blue (RARs) and red (RXRs) patterning of areas. Each receptor is represented by its own pattern and tone; expression strength is symbolized by color intensity. Expression outside the telencephalon is neglected in this picture. Note that all song nuclei but HVC show a receptor expression profile different to their surrounding. **B:** Receptor expression in telencephalic song nuclei, separated for easier comparison. The tables indicate expression strength of the five RAc related receptors for each song nucleus. The number of crosses per receptor reflects visual estimates of the relative signal intensities from non-radioactive *in situ* hybridizations for RXRs; for RARs, data are adopted from Jeong et al. (2005). Prevailing receptors per song nucleus are represented by bold text, receptors with little or no expression by pastel fonts. Side-by-side comparison of the song nuclei reveals that receptor expression profiles are not only largely different to the surrounding tissue, but also between the different song nuclei. Taking into account both receptor identity and expression strength, each song nucleus has its own combination of RAR(s)/RXR(s) which might allow for a song-nucleus specific set of RAc effects.

Taken together, the expression patterns of the two RXRs did not in general narrow down potential sites of RAc signaling in comparison with RAR expression. This was due to the widespread expression of RXRα, which may act as an RAR heterodimerization partner in almost all telencephalic regions. However, *what* effects are provoked by RAc may be region specific, mediated by the particular receptor combination present at a given site. While some effects might be widespread – for instance gene regulations through RARα/RXRα heterodimers – others could be spatially very restricted, as those mediated by RXRγ-containing receptor dimers which only occur in some Area X cells. Spatial specificity of potential RAc effects also characterized the telencephalic song nuclei: Each nucleus having its own receptor expression profile, RAc may there trigger a specific bunch of effects different to the other song nuclei.

# 4 Discussion

We cloned the zebra finch homologues of RXRs and examined their expression patterns in the brain of adult and juvenile zebra finches as a step towards determining sites of RAc action in the songbird brain. Our data provide a characterization of the brain distribution of RXR transcripts in zebra finches and provide some new insights into the involvement of RAc in regulating the song control system during and after the period of song learning.

## *4.1 Zebra finch RXR properties and probes*

Based on homology searches in GenBank and different genomic sequence databases, we could state that chicken and zebra finches (and possibly all birds) have only two RXR genes instead of the mammals' three RXRs. Chicken and zebra finches possess homologues to the mammals' RXRα and RXRγ gene, but no RXRβ homologue. The number of possible RAR-RXR heterodimers is therefore reduced in zebra finches as compared to mammals. However, this does not necessarily mean that the number of RAc effects is smaller in the birds: First, mammals' retinoid receptors show functional redundancy to some degree, as revealed by single and double knockouts (Kastner et al., 1995, Samad et al., 1997, Krezel et al., 1998, Mark et al., 2006), suggesting that not all RAR-RXR combination options are actually functionally exhausted. Second, a possibly still unknown number of retinoid receptor variants resulting from differential splicing or alternative promoter usage might add to the receptor combinatorics, and thus increase the number of possible effects in birds (although for RXRs, so far very few incidences of transcriptional variants are known in general – see below).

We cloned the ORFs of the two zebra finch RXRs from adult brain cDNA, and identified a single transcriptional variant for either gene. It is possible, though, that like for RARs, more transcriptional variants exist (although this is known only for chicken embryos which have two different RXRγ transcripts; Seleiro et al. (1994)). Protein sequences that correspond to our cloned RXRα and RXRγ ORFs are relatively conserved (similarity with human and mouse proteins ~95% for RXRα and ~85% for RXRγ, where differences accumulated at the N-terminus upstream to the zinc finger domain). The probes we designed for *in situ* hybridization would not be able to discriminate between variants differing at the 5' end.

Based on sequence analysis, homology searches in genomic and other sequence databases, and their distinct *in situ* hybridization patterns, we reason that our probes specifically

recognized RXRα and RXRγ. For our RXRγ probe which spanned regions with a higher degree of conservation, we rechecked probe specificity on embryonic sections which could be compared to known chick embryo expression data, and we found that our probe detected RXRγ transcripts at the expected sites. The specificity of our probes allowed us to separately determine their expression patterns in the brain.

### *4.2 RAR-RXR regional overlap in relation to RalDH distribution*

We found that both zebra finch RXRs were expressed in the adult brain. RXRα was more abundant than RXRγ and showed a broad distribution. In this respect, it resembled the RARs whose expression patterns are equally (or even more) widespread (Jeong et al., 2005). The RXRα distribution did therefore not resolve the problem of widespread receptor versus narrow RalDH distribution (Jeong et al., 2005): Had all RXRs turned out to be restricted to regions where RalDH is expressed, or regions close to them, we could reason that RXRs in general constitute a limiting factor for RAc controlled transcriptional regulation, and that their restricted distribution would delineate regions of potential RAc action (while RARs would not be a spatially limiting factor for RAc action). However, what we found was that RXRα expression overlapped with the broad RAR distribution in most brain areas, so that functional RAR-RXR heterodimers might be available across large regions.

This is in particular true for the pallium where RXRα was abundantly expressed (almost) ubiquitously. However, the situation was somewhat different in the striatum where song nucleus Area X is located: RXRα expression was very low in the striatum, and just a little enhanced in Area X. In the striatum, RXRα expression might therefore very well constitute a limiting factor for RAc action, providing restricted opportunity for the abundantly expressed RARα/γ receptors (Jeong et al., 2005) to build functional RAc activatable dimers. Limiting factors are in general interesting targets for investigation: They constitute bottlenecks within the biological processes we try to understand; fluctuations of their availability are likely to result in significant effects and thus to provide insights into the functioning of the process. RXR expression in the striatum is therefore an interesting target for future functional studies on retinoid signaling in the songbird brain.

For those RAc effects in the zebra finch brain that are mediated by receptor dimers containing RXRγ, the “limiting factor” notion is all applicable: Being restricted to some cells in Area X,

RXRγ distribution does indeed narrow down potential sites of *certain* RAc effects to a small region.

Yet, it is puzzling that the *striatum* constitutes a region where RXRs might function as limiting factors. There is no RalDH transcript found throughout the striatum, and it is therefore unclear whether RAc reaches it at all. We meet the same problem here as when comparing pallial RAR-RXRα overlap to RalDH expression: What are retinoid receptors for in pallial regions like the mesopallium, arcopallium, and caudal nidopallium, as well as in the entire striatum, if RalDH is not being expressed there? In adult birds, RalDH mRNA is only present in HVC, parts of the hyperpallium, and the rostral nidopallium including lMAN (Denisenko-Nehrbass et al. (2000), and see fig. 5. In juveniles, additional RalDH expression is found in song nucleus RA; ibid.). There are several possibilities why receptors might occur far from these sites:

(1) RAc could diffuse from its production sites to distant target sites. Diffusion of RAc is thought to play an important role in embryonic development, leading to morphogenetically relevant RAc concentration gradients (Boncinelli et al., 1991, Ross et al., 2000, Gavalas, 2002, Luo et al., 2004). However, it is unknown how far RAc can maximally diffuse through brain tissue; diffusion distances described so far are rather in the range of hundreds of microns than of several millimeters (Smith et al., 2001, Molotkov et al., 2005), which is considerably smaller than the long distance from HVC or rostral nidopallium to song nucleus RA. But even if diffusing RAc seems unlikely to reach sites like nucleus RA, diffusion could be a relevant mechanism to overcome smaller distances from production to target sites. Area X, for instance, is close to the strongly RalDH expressing rostral nidopallium and therefore perhaps within reach of diffusing RAc. A possibility related to RAc diffusion is the *transport* of RAc molecules bound to cellular retinoic acid binding proteins (CRABPs). While the exact functions of CRABPs are not known, a role in intracellular transport of RAc within neurons is one of the possibilities that have been discussed (reviewed by Ross et al., 2000). Target regions would in this case be restricted to projection sites of RalDH expressing neurons.

(2) An alternative to diffusion or transport of RAc itself is the anterograde transport of the RalDH *enzyme*, which has been reported for RalDH 1 in the mouse brain: It is being transported from the ventral tegmentum to ventral parts of the telencephalon (Wagner et al., 2002). So far, sites of RAc production in the zebra finch brain have only been inferred from mRNA localization by *in situ* hybridization (Denisenko-Nehrbass et al., 2000), while the distribution of RalDH protein was actually unknown. RalDH protein transport could permit

the production of RAc in more widespread regions than RalDH mRNA transcription; i.e., in all projection sites of RalDH expressing neurons. Being energy dependent, intracellular transport is not distance restricted in contrast to passive diffusion. The enzyme could be transported along both axonal projections and dendritic processes. Below, we present data indicating that anterograde RalDH transport does actually occur: RalDH protein indeed reached nucleus RA and Area X (see section 3.9 of Chapter III). Consistent with this, we could also detect RAc in these regions (see section 3.7 of Chapter III). RalDH transport seems to be an effective mechanism to selectively provide brain regions with RAc over distances. Still, this doesn't exclude that diffusion of RAc over some distances might also take place and be relevant for RAc signaling in the postembryonic brain.

(3) Another possible explanation for the receptors to be expressed in RalDH-free brain regions is that other aldehyde dehydrogenases with a different brain distribution than RalDH might also be involved in RAc production, and provide brain regions with RAc that RalDH does not reach. However, we consider this scenario unlikely, given that there is no evidence for other aldehyde dehydrogenases in songbird brain tissue, when tested in Western blots and enzymatic assays with a general aldehyde dehydrogenase inhibitor (Denisenko-Nehrbass and Mello, 2001). Furthermore, RalDH shows a much higher affinity for retinaldehyde, the precursor molecule for RAc, than other aldehyde dehydrogenases, and in both mammals and birds, RalDH is the only aldehyde dehydrogenase known to produce RAc (Wang et al., 1996, Zhao et al., 1996).

(4) Another important point is that we examined here *mRNA* expression of RXRs, and their *regional* overlap with RARs. Strictly speaking, we cannot say whether functional RAR-RXR dimers really occur in the regions of overlapping expression. It is conceivable that the functional dimers have a sparser distribution than the single molecules' mRNA. This might be the case, for instance, if differential translational control takes place, leading to more restricted protein than mRNA distribution. Indeed, two studies on retinoid receptors in mammals detected mRNA, but not proteins in some, albeit rare, instances (Krezel et al., 1999, Zetterstrom et al., 1999). Another possibility is that, despite *regional* overlap, RXR and RAR expression might not or only partly overlap in individual *cells*. This would also result in a more constricted availability of functional receptor heterodimers than suggested by their mRNA distribution. This possibility seems unlikely, though, for most receptors and regions of interest, because expression generally covered *most cells* in areas of marked expression (our data presented here and Jeong et al., 2005): This concerns RXRα and RARβ in most pallial and RARα and RARβ in pallial as well as striatal areas, including Area X.

We would like to point out that the scenarios summarized under (4) – translational control of receptor genes, or lack of cellular overlap – would not be suited to *solve* the problem of widespread receptor presence versus constricted RalDH expression. Even if the availability of functional RAR-RXR dimers in contrast to their mRNA was indeed neatly restricted to RAc producing areas, the question would just be shifted to a different level: Why would then *receptor mRNA* be abundantly transcribed in widespread patterns, including large areas possibly never reached by RAc, and never be translated there? Or, what should an abundance or receptor protein be good for in cells where they never meet a heterodimerization partner to become functional? However we phrase the question, the basic problem of locally constricted RAc production versus widespread receptor transcription remains untouched.

(5) Finally, functional receptors that may be located at sites far from RAc could potentially serve other purposes than RAc induced target gene transcription. For the RXRs, other dimerization partners than RARs are known. They include the thyroid hormone receptor and the vitamin D receptor, the nuclear receptors PPARg, LXR, FXR, and the orphan receptor nur77/NGFB-1 (Bugge et al., 1992, Kliewer et al., 1992, Forman et al., 1995, Perlmann and Jansson, 1995, Janowski et al., 1996). There is also some evidence that RXR homodimers can function as transcriptional activators (Ijpenberg et al., 2004). In regions where RAc is not available, RXRs might therefore be able to control RAc independent processes (however, their ligand is not all-trans-RAc but 9-cis-RAc, the physiological role of which has been questioned as it has never been identified as an endogenous compound). For the *RARs*, in contrast, no ligands or binding partners other than RAc and RXRs are known. Since the RAR distribution tends to be even broader than RXR expression, RARs are – according to their mRNA distribution – available in all regions where RXRs are present. This suggests that, despite the ability of RXRs to dimerize with numerous other nuclear receptors, RARs probably represent a major dimerization partner for RXRs in all regions, even those far from RAc production.

### *4.3 Comparison with mammals*

In general terms, the RXR distribution we found in the avian brain was consistent with data about RXR expression in the rodent brain (Krezel et al., 1999, Zetterstrom et al., 1999). RXRγ shows an equally sparse expression pattern in the mouse as in the avian telencephalon: Only the basal ganglia express RXRγ. RXRα distribution in the avian telencephalon was also comparable to what is known from mice, RXRα expression being detectable in the

cortex/pallium but low to undetectable in striatum and hippocampus. In both mice and zebra finches, RXRα shows a considerably more widespread distribution than RXRγ. Cerebellar expression of RXRα and lack of cerebellar expression for RXRγ are also similar between rodents and zebra finches. Two minor differences between zebra finch and rodents' RXR expression concern the olfactory bulb, which does express RXRα in rodents but not in the zebra finch, and the overall expression level of RXRα, which was relatively high in the zebra finch brain, and only moderate in the mouse brain.

Avian thalamic, diencephalic, and midbrain RXR expression seem to differ from mice. Within these structures of the mouse brain, RXRα is restricted to the medial habenula. The zebra finch habenula does not express RXRα; instead, RXRα transcript was detected in the tectum and the pretectal nucleus spiriformis, which also showed RXRγ expression. Nucleus spiriformis has a motor related function, probably being involved with facilitating desired movements (Reiner et al., 2005), and does not have an obvious equivalent in the rodent brain: It is a target region for a direct striatal-pretectal connection which has been shown to exist in birds, amphibians, and reptilians, but not mammals (Reiner et al., 1998), and RXR expression in this nucleus thus cannot be matched in the mouse brain. Finally, marked rodent RXRγ expression in hypothalamic regions has no correspondence in the zebra finch hypothalamus.

Despite some differences particularly in non-telencephalic regions, the generally comparable RXR distribution patterns between rodents and the zebra finch also corroborate our identification of the two RXRs as RXRα and RXRγ. The mouse RXRβ, which probably doesn't exist in birds, exhibits a quite different distribution, including hippocampal expression, which neither of our RXR genes showed.

### *4.4 Retinoid receptors in the avian song control system*

By completing the available data about RAR distribution (Jeong et al., 2005) with the RXR expression patterns, our data rounds the picture about the potential for specific song control nuclei to respond to RAc. We observed that the four telencephalic song nuclei HVC, RA, lMAN, and Area X may all potentially respond to RAc, according to their expression of at least one RAR and one RXR. Moreover, retinoid receptor expression was, to a certain extent, specific to the song control system – one receptor being expressed exclusively in a song control nucleus (RXRγ in Area X), receptor expression being different between most song nuclei and their surrounding tissue (e.g. RXRα in RA and lMAN), and receptor expression

profiles being different between individual song nuclei (see fig. 12). The RXR distribution thereby provides further experimental evidence for an involvement of RAc in regulating the song control system and possibly song behavior.

### *4.4.1 Area X*

The striatal song nucleus Area X showed the most specific retinoid receptor expression profile. Area X is the only telencephalic expression site for RXRγ and exhibited little RXRα expression (but still more than the surrounding striatum), against a background of particularly high RARα/g expression (Jeong et al., 2005). Area X is therefore the only song nucleus potentially capable of RAc signaling via both the RXRγ and RXRα pathway, and levels of both RXRs may be crucial limiting factors for RAc signaling in this nucleus. We therefore examined RXRα expression variability in Area X, assuming that expression fluctuations may have a relatively strong influence on RAc signaling if RXRα constitutes a limiting factor. However, at least for RXRα, we could not find any correlation of RXRα expression strength with age of the animals, singing, social context of singing, or time of the day. Nevertheless, the RXR expression patterns suggested that complex RAc signaling processes might take place within Area X. Different cell types may respond differently to RAc, depending on their receptor profile: Many cells might show RARα-RXRα and/or RARγ-RXRα mediated RAc effects, given the dense expression of these three receptors across Area X cells. RXRγ mediated RAc effects, in contrast, could be shown by only few cells.

Area X is part of the AFP essential for vocal learning (for reviews, see Bottjer, 2004, Farries, 2004, Perkel, 2004), and belongs to the avian basal ganglia. Interestingly, RAc is thought to play a role for the maintenance of normal basal ganglia motor function in the adult mammalian brain: Disturbed RAc signaling (for instance by Vitamin A deficiency or in transgenic retinoid receptor knockouts) leads to locomotor and spatial learning impairment, along with a number of molecular signaling abnormalities in the striatum (Krezel et al., 1998, Husson et al., 2004, Carta et al., 2006). The particularly high RAR and specific RXR expression in the zebra finch striatum may point to an analogous link between RAc and striatal motor function. In this context, Area X could constitute a key region: It has a unique retinoid receptor expression profile on the one hand, and, with its well-described role for song learning and context-depending song variability, it serves a specialized kind of motor function on the other hand. Whether there is any causality between the two, and which pathways and molecules may be involved, seem to be interesting questions for future research.

Some studies suggest that altered dopamine signaling in particular is responsible for the RAc effects on striatal motor control (McCaffery and Drager, 1994a, Krezel et al., 1998): Dopamine reaches the striatum from the midbrain, more precisely the ventral tegmental area / substantia nigra complex (VTA/SN), and is known to mediate contextual modulation of motor behaviors, in mammals as well as songbirds. This pathway is linked to RAc signaling not only by expressing RalDH and several retinoid receptors in mammals (McCaffery and Drager, 1994a, Krezel et al., 1999), but also by the ability of RAc to regulate the D2 dopamine receptor (Samad et al., 1997). In songbirds, an attractive aspect of a possible RAc influence on dopamine signaling is that thereby, RAc might indirectly affect context-dependent modification of song, which has been linked to dopamine signaling (Sasaki et al., 2006, Hara et al., 2007). However, in contrast to the mammalian nigrostriatal dopamine pathway, the avian one is not thought to express RalDH (see Chapter III, section 4.5). If RAc indeed affects dopamine signaling in the avian striatum (for instance by D2 receptor regulation), this should probably rather concern RAc of pallial origin, either from lMAN and/or the surrounding nidopallium, or from HVC, which both project to Area X.

Although the link between RAc and dopamine signaling has been focused in many studies, there are alternative explanations for the RAc influence on basal ganglia motor function: Carta et al. (2006) identify striatal cholinergic, rather than dopaminergic, dysfunction as a consequence of vitamin A deficiency, and Husson et al. (2004) report an RAc influence on striatal neuromodulin (GAP 43) and neurogranin (RC3), two genes that are supposedly involved with synaptic plasticity by regulating calcium availability and calcium/calmodulin-dependent protein kinases (Gerendasy and Sutcliffe, 1997, Krucker et al., 2002).

We would like to point out that whichever pathway RAc might control in the avian striatum, some kind of RAc transfer is always required: Since RalDH mRNA is not expressed in the striatum, RalDH protein or RAc must be transferred to the striatum in order to allow RAc signaling to take place there (see section 4.2). In Chapter III, we show that RalDH protein from extrastriatal areas indeed reaches Area X, and we show RAc to be present there.

### *4.4.2 lMAN*

The nidopallial song nucleus lMAN strongly expresses RalDH (Denisenko-Nehrbass et al., 2000) as well as the three RARs, although expression is for all four molecules lower than in the surrounding nidopallium in adult birds (Jeong et al., 2005, Kim and Arnold, 2005). We show here that the same applies to RXRα, the only RXR expressed in lMAN. Altogether, this

suggests that song nucleus lMAN constitutes a target region for RAc signaling. We did not investigate cellular colocalization of RXRα with the RARs and RalDH, but given that a large fraction of lMAN cells expresses RXRα, which also applies to the RARs and RalDH (Jeong et al., 2005), RXR, RAR, and RalDH expression is likely to overlap. Autocrine as well as paracrine actions of RAc may exist in lMAN, as already suggested by RAR and RalDH expression data (Jeong et al., 2005), but is must be taken into account that receptor mRNA localization only gives and indirect assessment for receptor protein localization.

Interestingly, we observed that RXRα mRNA expression strength in lMAN is relatively variable, which also points to the existence of active RAc signaling in this nucleus. We could identify two factors that are significantly correlated with RXRα expression changes in lMAN: age of the birds, and time of the day. In contrast, no correlation of RXRα expression with acute singing behavior could be detected. This is in line with the results of a very recent study which investigated expression of a number of RAc related genes, among which RXRα, in juvenile birds fed with a RAc rich diet versus control birds who had received a regular diet (Wood et al., 2008). The effect of acute singing behavior was not tested in this study, but dietary treatment was observed to influence the development of song variability. The expression of several genes in song nuclei was shown to covary with diet-induced modifications in song variability. RXRα, however, was not among those: Its expression in song nuclei did *not* change with diet-induced song variability (although its overall telencephalic expression strength did). Thus, RXRα *expression levels* in lMAN, Area X, and RA seem to be rather independent of singing activity: They are neither significantly correlated with acute singing behavior, nor with variability of acquired song following a retinoic acid-enriched diet. However, the (non-significant) tendency towards higher RXRα expression in singers' lMANs, and the observation of covarying *overall* RXRα levels with diet-affected song variability, still hint to an indirect role of RXRα for singing or song learning.

Such an indirect role for song might be based on the more direct dependency of RXRα expression on age which we report here: Juvenile birds around PHD 65 showed higher RXRα expression in lMAN than finches in their first year, and finches from their second year on expressed even less RXRα. Although the three age groups were not controlled for singing behavior, and we therefore cannot rule out that it is a *combination* of singing behavior and age which drives RXRα expression in lMAN, age seems to be the more important factor, since singing behavior alone did not significantly correlate with RXRα expression. Furthermore, a *post-hoc* analysis of RalDH expression data (including RalDH mRNA as well as protein) also suggested that RAc signaling in lMAN varies with age: The older the birds, the less lMAN

cells were strongly expressing RalDH. Against this background, an age-dependent decrease of RXRα expression in lMAN seems plausible. It might seem surprising that in our three age groups, RXRα expression in lMAN decreased continuously over two years, since song learning is thought to be terminated around 100-120 days after hatching. Why a song nucleus should, after that, continue to change its expression profile is not obvious. However, a recent study showed that adult finches continue to modify their song in a more subtle way after the imitation process is accomplished, resulting in increasingly stereotyped syllable structure until at least 15 months of age (Pytte et al., 2007). In fact, lMAN is known to be involved in stereotypy in juvenile as well as adult birds (albeit in an inverse way, *reducing* stereotypy, or enhancing variability): Juveniles with lMAN lesions develop a defective, abnormally stereotyped song (Scharff and Nottebohm, 1991). This is consistent with the finding that the variable, exploratory song patterns of juvenile sub- and plastic song seem to be driven by highly variable activity patterns that lMAN "injects" into RA (Olveczky et al., 2005, Aronov et al., 2008). In the crystallized song of adult birds, lMAN seems to play a similar role of providing variable motor patterns (Brainard and Doupe, 1997, Doupe, 1998, Kao and Brainard, 2006, Thompson and Johnson, 2007, Thompson et al., 2007), which, for instance, distinguish more exploratory undirected song from the more stereotyped directed song (Sossinka and Böhner, 1980). The decrease of RXRα expression in lMAN with age would be consistent with a variability enhancing role of RXRα mediated RAc signaling in lMAN: The more variable and plastic the song (i.e., the younger the bird), the higher the RXRα expression in lMAN.

While our findings are consistent with a variability-enhancing role for RXRα in juvenile vs. older birds, this does not directly translate to adult birds: When comparing directed (more stereotyped) with undirected (more variably) singing adult birds, we could not detect any correlation of plasticity with RXRα expression. We cannot exclude that we missed a potentially significant expression difference due to our small sample size of three birds per group. Given the large variability within the groups (see fig. 11), this possibility doesn't seem too unlikely. Alternatively, it is of course conceivable that RXRα expression is correlated with song variability only on a long-term scale, and insensitive to acute short-term behavior as directed vs. undirected singing, even if this involves differences in syllable variability.

The second factor we found to be correlated with RXRα expression in lMAN was time of the day (comparison of morning, daytime, and evening condition). During day hours, RXRα expression is significantly higher than right after waking up and at the end of the day. Why should gene expression in a song nucleus vary over the course of a day? Zebra finches are

non-seasonal breeders and singers, and day length, for instance, should not be of much significance for the song system. Interestingly, song structure has been found to show circadian oscillations in young zebra finches during song learning (Deregnaucourt et al., 2005): After waking up, syllable structure and similarity to tutor song are poorer than in the preceding evening, but recover during the first 2-3 hours of morning song. Overnight deterioration might result from neural song-replay during sleep (Deregnaucourt et al., 2005, Crandall et al., 2007). Comparing individuals, larger circadian oscillations (which also means stronger post-sleep deterioration) are associated with better overall learning success. This can be interpreted as a tradeoff between plasticity and consolidation: Periodic increases in plasticity might help to correct inappropriately consolidated structure and thereby enhance overall improvement (Deregnaucourt et al., 2005). Although the circadian oscillations decrease over the course of song learning, it is possible that they persist to a low extent in adults. An obvious molecular candidate for their control is the hormone melatonin which regulates circadian and circannual rhythms: There is indeed evidence for melatonin signaling in song nuclei HVC and RA (Gahr and Kosar, 1996, Jansen et al., 2005). However, other molecules with circadian expression changes in any song nucleus may play a role, too. An *in vitro* study has actually implicated RAc in the regulation of circadian genes which are found in the suprachiasmatical nucleus, the mammalian master circadian pacemaker (Shirai et al., 2006). This link between RAc and circadian gene regulation is bi-directional, and it is conceivable that RXRα is a part of this pathway.

How could the reported oscillations in syllable structure possibly link up with circadian RXRα expression changes in lMAN? Our morning group with low RXRα expression in lMAN would correspond to birds with maximally deteriorated post-sleep song. The daytime group with higher RXRα expression would comprise birds in a state after their structure-restoring morning song up to a state of stable daytime song in the afternoon. The evening group which shows again low RXRα expression in lMAN would only represent birds whose last structure-restoring morning song was several hours ago.[2] Our data would thus be

[2] Birds used in this time-of-the-day comparison have been controlled for singing to avoid confounding circadian effects with effects of acute singing. None of the birds had been singing during at least the last 2h before his death. Therefore, all groups are likely to also contain general non-singers that have not sung for the entire day or even longer. This does *not* pose a problem for the interpretation of RXRa expression related to circadian song oscillations: The latter ones have been shown to *not* result from acute practice during the day, versus overnight lack of practice. Instead, they seem to be due to neural processes that happen during night-sleep, so that actual singing activity is rather irrelevant for the existence of the oscillations (Deregnaucourt et al., 2005).

consistent with an increased RXRα expression around the morning phase of the structure-restoring song. This morning song is thought to have the strongest effect on overall learning success (Deregnaucourt et al., 2005), and RXRα in lMAN might be an interesting molecular candidate to contribute to its particular effectiveness. Enhanced RXRα around the morning hours might, for instance, induce genes for activity dependent changes at lMAN synapses, whereas activity of the same cells in the evening (i.e. in the presence of less RXRα) might lead to less synaptic reorganization. In such a scenario, RXRα would probably not be responsible for the song structure recovery itself: This happens in too short a time frame to be transcription dependent, starting immediately with the onset of morning singing (Deregnaucourt et al., 2005). A (speculative) RXRα influence on synapses would rather somehow contribute to more long-ranging changes ultimately leading to good imitation of the tutor song. As to a role of RAc signaling for synaptic plasticity in the postembryonic brain, a growing body of evidence is indeed available from rodents: Both adult long-term potentiation (LTP) and long-term depression (LTD) of synaptic function in the hippocampus seem to be RAc dependent (Chiang et al., 1998, Etchamendy et al., 2001, Misner et al., 2001), and known target genes of RAc controlled transcription include genes related to synaptic plasticity, such as neurogranin (RC3) and neuromodulin (GAP43) (Iniguez et al., 1994, Etchamendy et al., 2003, Husson et al., 2004), although it is not known whether their regulation depends on RXRα or other retinoid receptors. A recent study shows that RAc rapidly enhances dendritic growth and spine formation on cultured mouse hippocampal neurons (Chen and Napoli, 2008). RAc signaling is also essential for another form of adult neuronal reorganization, albeit in the peripheral nervous system: Neurite outgrowth following nerve injury is RAc dependent (see Maden, 2007 for a recent review).

Would such a function of RXRα in lMAN be compatible with the variability-enhancing role which we considered after finding an RXRα decrease with age? At first sight, enhancing variability and contributing to long-lasting changes in synapses and song seem like two quite different hypotheses. But the two roles are not necessarily contradictory, if they occur in different time frames, or on different levels. The “injection” of variable motor patterns by lMAN into the posterior premotor pathway should not be thought of as a random process: Firing of the lMAN>RA neurons is variable, but not completely unlinked to moments in the song (Aronov et al., 2008). LMAN thus seems to “inject” variability in a somewhat *directed* manner, possibly allowing for exploratory variations of *certain moments* in the song, or notes. Variability could thereby be generally confined, or redirected, to moments in the song which are not or inappropriately consolidated, and thus provide the basis for a new, better

consolidation. The certain timing of lMAN's variable output firing patterns, and of the resulting variability in the song output, must somehow be coded in the synapse strength and connectivity of lMAN neurons. If high RXRα associated with morning song influenced these very connections, it could, by contributing to confining or directing the variability, indirectly lead to a long-term improvement of song. On a broader time scale, high RXRα expression in the juvenile lMAN could at the same time very well be associated with high overall variability: The need for *directing* the variability might be highest at periods when variability is generally high to provide exploratory song patterns as "raw material" for song imitation, i.e. in the juvenile bird. In adult birds with crystallized song, RXRα could still fulfill the role of redirecting variability "injections", but this might be required to a much lesser extent than in the learning juvenile. However, this scenario for RXRα function in lMAN is pure speculation so far, and needs further experimental research to be verified. In particular, it would be interesting to know whether juvenile birds show more pronounced circadian oscillations in lMAN-RXRα expression than adult birds, which should be the case if the above scenario applies. A more fine-grained picture of circadian lMAN-RXRα oscillation (e.g. at 4-5 time points during the day) would be helpful to gain more certainty about the association with morning vs. later singing. Moreover, identifying which lMAN cells express RXRα – projection neurons to RA and Area X, or local interneurons? – is necessary to build hypotheses about which synapses might be affected by RXRα mediated RA signaling. Finally, only functional investigations (e.g. with the local use of RXRα blockers, or virally mediated RXRα overexpression or knock-down) can provide definite information about RXRα's influence on song variability, or on the variability of lMAN>RA firing patterns.

### *4.4.3 RA and HVC*

Song nucleus RA shows a specific receptor expression profile which contrasts with the surrounding arcopallium. RARα shows a moderately high, RARβ and γ low expression in RA (Jeong et al., 2005). We report here that RXRα was strongly expressed in many cells, so that RA stood out as one of the areas of highest RXRα expression in the telencephalon. Abundance of RXRα in RA was in sharp contrast to the surrounding arcopallium which showed only weak labeling. RARs and an RXR being present, RA is a potential target for RAc signaling. However, RalDH is not expressed in the adult RA, again raising the question of whether RAc can reach this region at all. Below, we present data showing that RalDH protein and RAc are in fact present in the adult RA, corroborating the idea that RAc signaling takes place in this nucleus in juvenile as well as adult zebra finches. RA is the site where the

learning-related AFP and the posterior premotor pathway converge. In the young male bird which produces highly variable subsong, activity in RA and its downstream motor nuclei is dominated by variable input from the AFP. Around PHD 30-45, HVC starts to take over input dominance over RA (Aronov et al., 2008). Highly stereotyped HVC input to RA dominates over variable AFP input in the adult bird with crystallized song, but input from the AFP still plays a role for the differentiation between more variable undirected vs. more stereotyped directed song (Kao and Brainard, 2006). AFP input into the adult RA becomes visible upon experimental disturbance of the ratio of HVC and lMAN input, showing that the adult RA still receives a balanced mixture of more variable input from the AFP and more stereotyped input from HVC (Kao and Brainard, 2006, Thompson and Johnson, 2007, Thompson et al., 2007). Song nucleus RA is one of the sites where weighting of either input pathway might be controlled, e.g. by synaptic strength and connectivity. It is conceivable that RAc signaling in RA plays a role in this context: Both input nuclei to RA express RalDH; functional receptor dimers may be available (judging from their mRNA distribution); and above all, there is additional RalDH expression in the juvenile RA itself around the very time when the dominance shift from the AFP to HVC takes place (around PHD 38; Denisenko-Nehrbass et al., 2000). Evidence for the involvement of RAc in synaptic plasticity has already been mentioned (see above, section 4.4.2).

Song nucleus HVC might be a target for RAc signaling as well: Besides RalDH, all RARs and RXRα are expressed there (Denisenko-Nehrbass et al., 2000, Jeong et al., 2005, and data presented here). As in lMAN, a high fraction of cells expressed RXRα, suggesting that RXR-RAR-RalDH overlap may occur, so that paracrine as well as autocrine RAc effects are possible. HVC belongs to the posterior premotor pathway of the song control system which is necessary for song production from the juvenile singing plastic song to the adult bird. HVC projects to RA as well as to Area X of the AFP, and interestingly, HVC>RA projection neurons are subject to constant replacement throughout life (Alvarez-Buylla et al., 1990, Kirn et al., 1991, Alvarez-Buylla, 1992, Alvarez-Buylla and Kirn, 1997). In adult rodents, RAc has been shown to be involved in several aspects of neurogenesis, such as differentiation and survival of the new cells (Crandall et al., 2004, Jacobs et al., 2006). It is possible that RAc produced in HVC also affects incorporation or survival of newly arriving neurons that will become HVC>RA projection neurons. If new neurons arriving in HVC express retinoid receptors, RAc might influence their differentiation, incorporation, or survival directly by transcriptionally regulating genes that are essential for the new cells functional incorporation, such as growth factors or factors that support differentiation or survival. But also indirect

effects are possible, if RAc acting on already present HVC cells contributes to a molecular niche for the newly arriving cells which might be either favorable or disadvantageous for their functional incorporation. Below, we investigate a possible effect of RAc signaling on HVC neurogenesis rates (see Chapter IV).

In sum, we demonstrate here the existence of RXR transcripts in the brain and song control areas of the zebra finch, completing the available data about transcript distribution of the RAc producing enzyme RalDH and the three retinoic acid receptors, RARα, RARβ, and RARγ. Most telencephalic areas qualify as potential targets of RAc signaling, according to transcription of RARs and RXRs, while RalDH expression is considerably more confined. This raises the question of whether RAc actually reaches all sites of co-occurring RXR/RAR expression, a question we approach in the following chapter.

# III Localizing sites of retinoic acid action in the songbird brain

## 1 Introduction

Identifying sites of RAc signaling in the brain ideally requires knowledge about all sites at which RAc is available. The expression pattern of the RalDH gene, together with expression patterns of the different retinoid related receptors, could in principle be suited to predict sites of retinoid signaling fairly well. In the case of the zebra finch brain, however, there is a large discrepancy between the two, as we have described in the previous chapter. Most of the receptors turned out to be so widely expressed relative to the RalDH gene that vast regions very far from RalDH expression away seemed, by their receptor expression, qualified for RAc signaling, but whether and how RAc could reach them is unclear. Only one receptor, RXRγ, was sparsely expressed, suggesting that the striatal song nucleus Area X is a site of specific locally constricted RAc effects. However, the question of whether RAc signaling does actually take place at RalDH transcript-negative regions remained; the case of Area X even seemed to exacerbate this issue since RalDH is not expressed in this nucleus either (see fig. 5, right panel, or fig. 23, right panel).

We chose a combination of other strategies to identify sites of RAc signaling in the zebra finch brain, which we present in this chapter. They consist in a) determining expression patterns of the RAc degrading cytochromes CYP26A1, CYP26B1, and CYP26C1; b) localizing the signaling molecule RAc itself in brain sections; and c) localizing RalDH enzyme in brain sections.

In vertebrate embryos, enzymes that are able to degrade RAc build up local RAc sinks or gradients, resulting in complex patterns of RAc availability which are crucial for developmental pattern formation (see Reijntjes et al., 2003, Reijntjes et al., 2004, Reijntjes et al., 2005 for chick brain development, Glover et al., 2006 for a review on mammalian hindbrain patterning, Duester, 2007 for a review on somitogenesis). These enzymes, cytochromes of the P450 family called CYP26s, are mono-oxygenases which use haem to oxidize their substrate. They catabolize RAc to inactive, polar water-soluble metabolites, such as 4-oxo retinoic acid, 4-OH retinoic acid, 18-OH retinoic acid, and 5,8-epoxy retinoic acid (White et al., 1996, Fujii et al., 1997, White et al., 2000, Reijntjes et al., 2005).

In the embryo, they are expressed in a non-overlapping manner, suggesting individual roles for each of the CYP26 enzymes in the catabolism of RAc in the embryonic context (Reijntjes et al., 2004). To see whether this also applies to the posthatch songbird brain, we cloned the zebra finch CYP26A1, CYP26B1, and CYP26C1, and determined their distribution in the brain by *in situ* hybridization.

We found that the expression pattern of CYP26B1 revealed higher auditory areas as a site of complex, gradient-like RAc signaling. However, as the other CYP26s showed either ubiquitous or no expression, the CYP26 expression patterns did, in sum, not turn out to significantly constrict candidate sites of retinoid signaling, especially not in those parts of the song control system where receptors were highly expressed, while RalDH transcript was lacking, such as RA and Area X.

Given that the attempts to indirectly determine sites of RAc signaling through RalDH, receptor, and CYP26 expression had led to inconclusive results, especially with regard to parts of the song control system, we next sought to directly localize the signaling molecule RAc itself. Localizing RAc in tissues has always been difficult due to its extremely low physiological concentrations and its labile, lipophilic nature, which even makes detection with highly sensitive methods such as HPLC error-prone, especially for the lipid-rich brain tissue (Maden et al., 1998, Wagner et al., 2002, Blomhoff and Blomhoff, 2006, Gundersen, 2006). While the most *sensitive* method available to date for RAc quantification in tissues seem to be combined liquid chromatography tandem mass spectrometric assays (Kane et al., 2008), such assays determine the retinoid amount of an entire tissue sample and are therefore limited in spatial resolution. Since we were more interested in visualizing RAc distribution across the entire brain – even if at the expense of quantitative exactness –, we chose a retinoid sensitive reporter cell assay to visualize RAc presence, and adapted it for application on entire brain sections. The reporter cells use a LacZ gene under the control of a RARE (retinoic acid response element) and are co-cultured with tissue sections (Wagner et al., 1992). We show here that RAc presence in the sections is indicated by the reporter cells expressing LacZ with high local specificity. The RAc distribution we found could largely account for the discrepancy between receptor and RalDH transcription sites: RAc was present in considerably larger regions than RalDH expression suggested, including song nuclei Area X and RA, which are RalDH transcript-negative.

Moreover, we show, using immunohistochemistry, that these regions obtain their RAc by transport, not of the signaling molecule itself but rather its synthesizing enzyme RalDH. We

have started to explore possible input sources for RalDH enzyme in Area X and RA using immunohistochemistry in combination with retrograde tracers to mark projection neurons of different origin, and lesioning the candidate input regions. Prelimiary data indicate that both RA and Area X most likely receive RalDH enzyme from both HVC and lMAN via axonal fibers of the connecting projection neurons. Across the entire brain, RalDH enzyme and RAc distribution are largely overlapping, RAc covering just slightly more extended areas; but its distribution seems to be fully accounted for by RalDH enzyme presence plus diffusion over short distances. Our data therefore suggest that no other RalDH than the RalDH2-like type described by Denisenko-Nehrbass et al. (2000) is active in the zebra finch brain.

RAc in the postembryonic brain has been primarily considered in the context of learning and plasticity (see, for instance, Misner et al., 2001, Thompson Haskell et al., 2002, Wagner et al., 2002, Mey and McCaffery, 2004, Lane and Bailey, 2005, and McCaffery et al., 2006 for reviews, Wagner et al., 2006, Chen and Napoli, 2008). While the crucial role of postdevelopmental plasticity in the zebra finch song control system is obvious in the context of song learning, it is less so for the adult zebra finch brain, since adults do not seem to alter their once-crystallized song significantly. However, more and more studies indicate that there is still residual capacity for song adjustment after crystallization (Woolley and Rubel, 2002, Helekar et al., 2003, Tumer and Brainard, 2007, Funabiki and Funabiki, 2008), or that the maintenance of stable, crystallized song is a dynamic process (that might rely on some neuronal plasticity), rather than an inert state (Nordeen and Nordeen, 1992, Williams and McKibben, 1992, Leonardo and Konishi, 1999, Pytte and Suthers, 2000, Woolley, 2004 for a review on auditory feedback for song maintenance). We consider RAc a candidate molecule to allow for and mediate the dynamic adjustment of adult song: Adult song plasticity or dynamic song maintenance likely relate to the brain regions which we find here to be strongly concerned with RAc signaling. The telencephalic song control system shows large-scale (and supposedly energy-costly) RalDH trafficking, and higher auditory areas are equipped for fine-tuned RAc signaling. These findings are in line with RAc controlling neuronal plasticity for dynamic song maintenance or adjustment in the adult bird brain. Or, to put it the other way round, the extensive role a signaling pathway implicated with neuronal plasticity seems to play in the adult songbird brain strongly supports the assumption that a great amount of plasticity is still going on there, and characterizes the adult maintenance of song as a complex learned behavior.

# 2 Materials & Methods

## *2.1 Animals*

All male zebra finches (*Taeniopygia guttata*) were obtained from breeding colonies at the Free University and the Max Planck Institute for Molecular Genetics, Berlin, and kept as described in *Materials & Methods* of Chapter II.

In total, brains of 13 male and two female zebra finches and one whole embryo were used for RAc degrading cytochrome (CYP26s) *in situ* hybridization. Seven of the males were adults between one and two years, two of them were 20 days old, two 41-49 days, and two 64-68 days (except for two birds, these were the same as used for RXR *in situ* hybridization; see Chapter II). Both females were adult. The embryo was the same as used for RXR *in situ* hybridization (see section 2.1).

For RAc detection with a RARE-LacZ F9 reporter cell line, we used brains of 17 male and two female zebra finches. All animals were adults (age between 241 and 1110 days, average 439 days) except for three juvenile males which were 38-42 days old. In two of the juveniles and three of the adult males, HVC was lesioned unilaterally, while the HVC>RA fiber tract was cut with a scalpel in two more adult males (see section 2.8).

Twelve male zebra finches were subjected to RalDH immunohistochemistry. Two of them were juveniles of 51-52 days of age, five were adults in their first year (6-8.5 months old), three adults in their second year, and two were old males of more than three years of age. Two of the adult males received RA injections with a retrograde tracer, resulting in labeling of HVC>RA and lMAN>RA projection neurons, without any further treatment. The two juveniles and one more adult male had HVC lesioned unilaterally, and unilateral lMAN lesions using the excitotoxin ibotenic acid had been performed on four more adult males but were unsuccessful (for details on lesion surgeries, see sections 2.8 and 3.10).

## *2.2 Cloning of fragments for the retinoic acid degrading cytochromes (CYP26s) for subsequent usage as in situ hybridization probes*

Fragments of the zebra finch homologues of CYP26A1, CYP26B1, and CYP26C1 used as probes for *in situ* hybridization were obtained by cloning from zebra finch total brain cDNA; for details see *Materials & Methods* part of Chapter II, section 2.2. Primer design for cloned

fragments was based on conserved regions identified with the aid of the UCSC genome browser and GenBank data of other species' mRNA sequences (CYP26A1: forward 5'-CTGAATGAGTCTGCCACAG-3', reverse 5'-CTTCATGTCTCCATCTCCAG-3'; resulting fragment length 406bp), or was adopted from the literature (CYP26B1, CYP26C1, fragment lengths 386 and 495bp resp.; see Reijntjes et al., 2003, Reijntjes et al., 2004), as the zebra finch genome sequence was not available yet at the time of primer design. Hybridization temperatures for the CYP26B1 probe was 56°C; CYP26A1 and CYP26C1 probes were hybridized at 53°C, followed by an RNAse treatment (see Chapter II, section 2.5).

### *2.3 Generation of full ORF clones of zebra finch CYP26s*

Starting from cDNA fragments, we cloned the full-length sequences of zebra finch CYP26A1 and CYP26B1 as described for the RXRs in the *Material and Methods* part of Chapter II. Of CYP26C1, however, we were not able to generate a full-length clone; for this gene, we have cloned two different fragments, covering a sequence from the end of exon 2 to exon 7 (out of 10 exons in total, according to the zebra finch genome database now available on NCBI).

Primer design for the 3' and 5' ends of the CYP26A1 and CYP26B1 ORFs was based on conserved regions at the 3' end 5' ends of the CYP26s of chicken, human, and mouse (sequences were again obtained from the UCSC genome browser), since the according sequences zebra finch could not be found by BLASTing the WGS archive (analysis and cloning were done before the zebra finch genome became available). 5' and 3' end primers were as follows:

- for CYP26A1: forward 5'-GAGTCGCTCCCCAGTCAT-3',
  reverse 5'-CCATTTGGAGGTGAGTGTTG-3'
- for CYP26B1: forward 5'-TTCTTTCCAAATATGCTT-3',
  reverse 5'-ACTGGTGCGGGCTCTACA-3'
- for CYP26C1: forward 5'-CTCCCATACTCTGCTCAGTT-3',
  reverse 5'-TGCCAGGAGCAGGTTTCTGCTG-3'.

PCR, gel purification, cloning and sequencing was done as described for the initially cloned fragments (see *Materials & Methods* of Chapter II, section 2.2).

### *2.4 Labeling of riboprobes, radioactive and non-radioactive in situ hybridization*

Labeling of riboprobes and *in situ* hybridization were done as described for RXR riboprobes in *Materials & Methods* of Chapter II, sections 2.4 and 2.5.

### *2.5 RAc detection in brain sections with a RARE-LacZ reporter cell assay*

The F9-reporter cells which carry a β-gal reporter gene under control of a retinoic acid-sensitive promoter element were a gift from Prof. Michael Wagner, State University of New York Downstate Medical Center, Brooklyn. F9-cell assays were essentially performed as described elswhere (Wagner et al., 1992) with some modifications. In brief, F9-reporter cells were grown to subconfluence in 10cm Petri dishes for subsequent coculture with entire brain slices of 110µm thickness. Growth medium for the cells was Dulbecco's MEM (DMEM; Biochrom) with high glucose content (4.5mg/ml) and L-glutamine, 20% fetal calf serum (FCS; Biochrom), 1% (v/v) Penicillin/Streptomycin (Roth), and 0.8mg/ml Geneticin (Gibco). Brains for coculture were obtained from birds which had been anesthetized with an intramuscular injection of a solution containing ketamine (50mg/kg) and xylazine (10mg/kg) in sterile saline solution, intracardially perfused with approximately 20ml PBS to remove blood, and quickly dissected. After dissection, brains were placed immediately into an ice cold mixture of half PBS and half DMEM medium (4.5 mg/ml glucose, Biochrom), prepared from half frozen and half 4°C cold solutions with a kitchen blender. Sagittal brain slices of 110µm thickness were cut freshly with a vibrating tissue slicer (Leica) in the same mixture. Until transfer to the cell cultures, sections were kept in ice cold medium. Immediately prior to coculture, growth medium in the cell dishes was replaced with a thin layer of assay medium (serum-free, antibiotics-free DMEM medium containing 4.5mg/ml glucose, Biochrom). Using a brush, brain sections were placed very carefully on top of cell monolayers (typically three to six sections per 10cm Petri dish) to not destroy the cell layer, and dishes were transferred to a $CO_2$ incubator (Binder) for 2.5-3h for brain slice attachment. Before moving the dishes, medium had to be removed almost completely to prevent sections from dislodging. In the incubator, some drops of medium were added to each dish every 30min to keep sections covered, until at the end of 2.5-3h attaching time additional medium up to 10ml was carefully added to each dish without moving it. The cocultures were incubated for 24h, then carefully washed with PBS (again, liquid had to be removed before moving the dishes to the hood, to prevent sections from detaching), fixed with 1% glutaraldehyde (Sigma) in PBS for 15min at room temperature, washed again with PBS and incubated with standard X-gal solution (0.2%

X-gal (Roth), 3.3mM $K_3Fe(CN)_6$ (Roth), 3.3mM $K_4Fe(CN)_6$ (Roth), 150mM NaCl, 1mM $MgCl_2$ in phosphate buffer pH 7.0) in an oven (Binder) at 37°C over one to three nights (duration depended on staining intensity as judged by inspection). After X-gal staining, sections were washed with PBS, coverslipped within the dishes with PBS/50% glycerol (Roth) and stored at 4°C. Photographs of the sections were taken with a Leica Macroscope (MacroFluo Z16APO) from below to capture the cell layer as well as the sections.

Three controls were run in parallel and behaved as expected: (1) F9-reporter cell monolayers not exposed to brain sections but incubated with X-gal (negative control 1), (2) F9-cells without reporter construct cocultured with brain sections and incubated with X-gal (negative control 2), and (3) F9-reporter cells exposed to 5 X $10^{-8}$ M RAc and incubated with X-gal (Sigma; positive control).

### *2.6 Immunohistochemistry*

RalDH protein was detected by diaminobenzidine staining with a goat anti-Human ALDH1A2 antibody (1:50, Santa Cruz Biotechnology) raised against a region near the N-terminus of the human protein (human ALDH1A2 and mouse or zebra finch RalDH2 are homologous). Male zebra finches were perfused intracardially with about 60ml of PBS, followed by about 60ml of 4% paraformaldehyde (PFA, Sigma) in PB (phosphate buffer), post-fixed in 4% PFA/PB over night and stored in PBS at 4°C until processed. Brains were cut sagitally into 40 to 60µm sections with a vibratome (Leica). Floating sections were permeabilized with 0.2% triton/PBS (PBST). The antibody required a heat treatment to expose masked antigen, which was performed in a 10mM sodium citrate buffer, pH 6.0 (Fluka). Sections were heated in 2ml Eppendorf tubes in a heating block (Eppendorf) to 95°C for 30min (with some fresh buffer added after 15min). The tubes were taken out of the heating block and sections were allowed to cool down in the buffer at room temperature. After heat treatment, the endogenous peroxidase was quenched with 3% $H_2O_2$ (Roth) in PBS, followed by three washes in PBS (10min each), saturation in 10% fetal calf serum (FCS; Biochrom) for 30min, and application of primary antibody (1:50 in PBST/2% FCS). Antibody was allowed to incubate over one to two nights at 4°C. Excess primary antibody was washed off with PBS (three washes) before the secondary antibody was applied (anti-goat biotinylated antibody (Vector Laboratories), applied 1:200 in PBST/2% FCS) for 2h at room temperature. Excess antibody was washed off with three PBS washes. Reactions were developed with the help of the avidin-biotin peroxidase method using diaminobenzidine (DAB, Sigma-Aldrich) as a

chromogen substrate. Briefly, sections were incubated in avidin-biotin-complex solution (Vector Laboratories; prepared 30min in advance to allow for avidin and biotin to aggregate) for 30min at room temperature, followed by three washes in PBS. Sections were incubated in DAB staining solution (1 DAB tablet per 15ml Tris-HCl, activated with 12µl $H_2O_2$) for about 5min until brown staining was clearly visible. The reaction was stopped with PBS, followed by three more PBS washes. Sections were counterstained with DAPI (4'-6-Diamidino-2-phenylindole; Serva) before mounting with Roti-Histokitt (Roth).

As a control for the specificity of the antibody, a regular αRalDH immunostaining as described above was run in parallel to an immunostaining where the antibody had been pre-incubated with an ALDH1A2 immunizing peptide (also Santa Cruz Biotechnology). The immunizing peptide was used in a concentration twice as high as that of the antibody. I.e., antibody was diluted 1:50 in PBST/2% FCS, immunizing peptide was added to a final concentration of 1:25, and the mixture was incubated for 30min at room temperature to allow for the antibody to bind the peptide. The protocol was completed as described above and the control behaved as expected: no staining occurred when antibody was already bound by immunizing peptide.

### *2.7 Retrograde labeling of song nuclei*

Stereotaxic injections with Alexa-488 conjugated latex beads (Lumafluor) or cholera toxin subunit B conjugates (Molecular Probes) as retrograde neuronal tracers were performed under complete anesthesia resulting from an intramuscular injection of a solution containing ketamine (50mg/kg) and xylazine (10mg/kg) in sterile saline solution. The birds' heads were fixed with the help of a stereotaxic apparatus (MyNeurolab) and the retrograde tracer was injected into song nucleus RA (stereotaxic coordinates, relative to the 0-point at the bifurcation of the midsagittal sinus: medial/lateral 2.4mm, anterior/posterior -1.8 and -1.5mm, dorsal/ventral -2.0 and -1.8mm; injection needle tilted in anterior/posterior plane by -0.9mm) with a hydraulic micromanipulator (Narishige). Per injection site, approximately 200nl of tracer were injected. Birds received painkiller (Meloxidyl; active is meloxicam, dose 0.1 mg/kg) half an hour before anesthesia and and once per day for three days post surgery. After receiving injections, birds were allowed to survive for at least four more days and then killed by decapitation. Brains were quickly dissected, hemispheres were separated and frozen immediately over liquid nitrogen and stored at -75°C until processed. Cutting was performed with a cryostat (Leica) in the sagittal plane at 14µm thickness. If brains had to be used for

immunohistochemistry, birds were perfused intracardially with PBS followed by 4% paraformaldehyde (PFA; Sigma), their brains were dissected, post-fixed in 4% PFA over night and stored in PBS at 4°C until cut with a vibratome (VT 1000S, Leica).

### *2.8 Lesion surgeries*

Birds were analgized, anesthetized and fixed in a stereotaxic apparatus as described in section 2.7. For HVC lesions, the skull and the hippocampus overlying HVC were opened using an injection needle to expose HVC. HVC was then removed with the help of a Delicate Bone Scraper (Fine Science Tools). The removed area was shaped like an egg with its tip towards frontal-anterior, its axis forming a 45-degree angle with the midline. Coordinates for the tip of the egg-shaped removal site were medial/lateral 3.0mm and anterior/posterior 1.8mm; the opposite side (towards medial-posterior) reached medial/lateral 0.9 and anterior/posterior -0.2mm. Dorsoventral depth of the lesion was about 1mm. For lesions of the fiber tract between HVC and RA, a knife cut was set about 1.5mm ventrally to the posterior end of HVC as estimated with the above coordinates and extended about 2mm into the tissue. For excitotoxic lMAN lesions, ibotenic acid solution (10mg/ml in PBS; Biotrend) was mixed 5:1 with rhodamine beads (Lumafluor) to visualize the injection site. Twelve injections were applied with a hydraulic micromanipulator (Narishige) at the following coordinates (relative to the 0-point at the bifurcation of the midsagittal sinus): medial/lateral (m/l) 1.3mm and anterior/posterior (a/p) 3.4 / 3.6mm; m/l 1.6mm and a/p 3.7 / 3.9mm; m/l 1.8mm and a/p 4.0 / 4.2mm. Dorsal/ventral coordinates were 2.5 / 2.6mm for all positions. Injection volume was about 200nl per injection site. Before performing lesions, a retrograde tracer was injected into nucleus RA as described in section 2.7, to retrogradely label HVC or lMAN, respectively, which allowed us to determine completeness of lesions. After surgery, birds were allowed to survive 14 more days before being sacrificed (with PBS perfusion if brain was to be used for reporter assay, or PBS/PFA perfusion for immunohistochemistry, as described in sections 2.5 and 2.7).

# 3 Results

## *3.1 Cloning of RA hydroxylating cytochromes CYP26A1, CYP26B1, and CYP26C1*

We cloned full ORFs of the zebra finch genes for the RAc hydroxylating cytochromes CYP26A1 and CYP26B1, and two fragments of CYP26C1. The degree of conservation of these proteins between species is moderately high: Mouse, human, and zebra finch CYP26A1 share 78.3% of their amino acids; for CYP26B1, similarity is 87.3%. As we are lacking the full zebra finch ORF sequence for CYP26C1, we have to estimate interspecies similarity based on the predicted zebra finch gene in the recently available NCBI genome database: It shares 62.6% of its amino acids with its human and mouse homologs. In contrast, similarity *between the different members* of this protein family is considerably less – the human CYP26A1, B1, and C1 share only 32.7% of their amino acids; for the three mouse proteins, similarity is 32.1%.

The probes we used for CYP26A1 *in situ* hybridization (see fig. 13.A) had a length of 590bp (probe named "probeCYPA_antiORF2") and 406bp (probe named "probeCYPA_3'_end"). ProbeCYPA_antiORF2 was located towards the 5' end of the gene, and covered 70% of a putative additional antisense open reading frame in the first half of the gene (see below and fig. 13.A). ProbeCYPA_3'_end was positioned towards the 3' end of the gene, downstream of the putative antisense open reading frame. For CYP26B1, we used a 386bp probe which was located towards 5' end of the gene (40bp from the beginning of the open reading frame; fig. 13.B). The probe we used for CYP26C1 (fig. 13.C) was 495bp long and was located directly in the middle of the open reading frame.

Similarities between the different zebra finch CYP26 family members in the probe spanning regions were all lower than 49%, making cross-hybridization of the probes rather unlikely. The CYP26B1 probe produced distinct and sparse expression patterns on zebra finch brain sections (see below and fig. 14 to fig. 16). The CYP26C1 probe produced hybridization patterns on brain sections that were rather uniform and little conspicuous (see below and fig. 17). To ensure that this was not due to lacking probe specificity, we also performed *in situ* hybridizations on embryonic sections as a test (see below, supplementary fig. 6). The replication of the same strong and conspicuous expression patterns as found in chicken embryos (Reijntjes et al., 2004) lead us to the conclusion that our probe was suited to specifically detect CYP26C1 transcript.

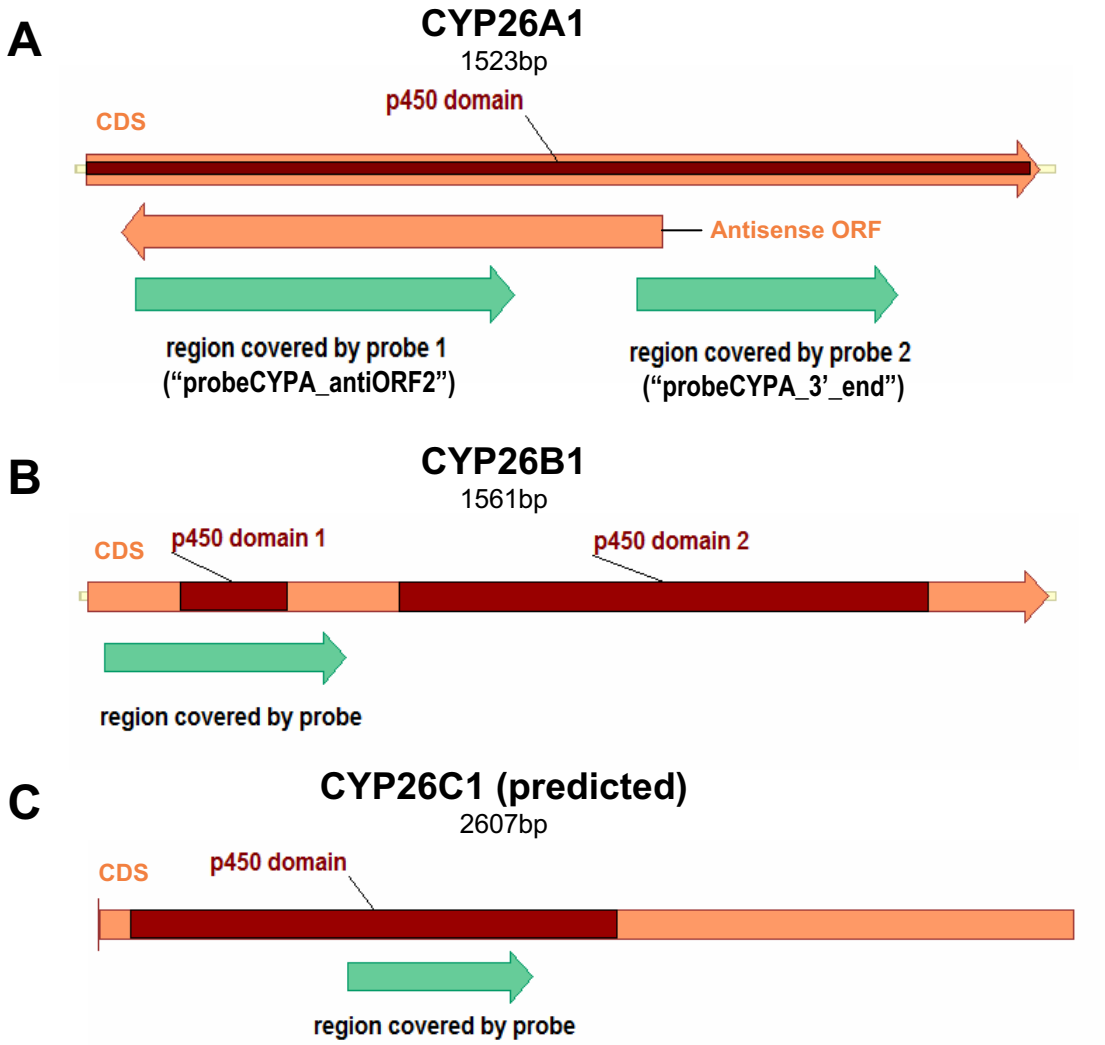


**Figure 13: Positioning of ISH probes for the RAc degrading cytochrome genes.** Pink bars represent coding sequences (CDS) of the three genes (and in case of CYP26A1, an additional antisense open reading frame [ORF]); regions coding for p450 domains which are specific for this class of cytochromes are marked in dark red. Regions covered by probes are represented as aligned green arrows. **A:** For CYP26A1, we used two different probes, one covering a region close to the 5' end of the CDS which falls into a potential additional antisense ORF, and one covering a region further downstream. **B:** Our CYP26B1 probe covered a region close to the 5' end of the CDS. **C:** As the zebra finch CYP26C1 sequence is unknown, a putative CYP26C1 sequence predicted by automated computational analysis of the zebra finch genome is shown (NCBI Reference Sequence: XM_002189751.1). We used a probe near the middle of this predicted gene.

For CYP26A1, we used two different probes for the following reason: Other than the human, mouse, and chicken CYP26A1 genes, the zebra finch gene contains an additional possible open reading frame of 837 bp on the complementary strand (see fig. 13). The according protein sequence is not similar to any known protein sequences as confirmed by BLAST on the NCBI database of non-redundant protein sequences (very low BLAST alignment scores - below 40). We therefore doubt that an actual protein is translated based on this sequence. To anyway verify whether the putative antisense-ORF is translated, we used six different strand specific primers that would bind to the antisense transcript only, to generate strand-specific cDNA from whole brain RNA. The cDNA was obtained with the the protocol specified in Chapter II, section 2.2, except that we used the strand-specific CYP26A1 primers instead of Oligo(dT)s. We were able to amplify cDNA stretches of the predicted lengths (data not shown), indicating that a CYP26A1-antisense-ORF transcript was present, although we did not clone and sequence the resulting PCR products. Furthermore, radioactive *in situ* hybridization with a sense probe covering most parts of the antisense-ORF (see fig. 13) suggested ubiquitous distribution of this transcript in the adult brain, whereas a different probe covering a region beyond the putative antisense ORF (see below and fig. 13) detected neither sense nor antisense transcript in adult brain. In contrast, applying this latter probe located outside the putative antisense-ORF on embryonic sections revealed a strong, conspicuous expression pattern resembling the one known from chick embryos (also see below, and fig. 18.E and F), which shows that the probe was functional.

### *3.2 In situ hybridization analysis of RAc catabolizing enzyme expression: general comments*

To assess availability of RAc in different regions of the bird brain, knowing the expression patterns of the RAc degrading enzymes is as important as knowing where RalDH is expressed, to identify potential RAc sinks. We cloned the zebra finch RAc degrading cytochromes CYP26A1, CYP26B1, and CYP26C1 and determined their expression patterns using *in situ* hybridization. Like for the RXRs, *in situ* hybridization was done with radiolabeled riboprobes followed by phosphorimager autoradiography for an overview, and with digoxigenin-labeled riboprobes for a more detailed regional analysis. The only RAc degrading cytochrome with a conspicuous expression pattern in the brain was CYP26B1, whereas CYP26C1 showed low ubiquitous expression, and CYP26A1 was not expressed at all in the adult brain except for some cells in the brain stem. A detailed description of the P450 cytochrome expression patterns is given in the following.

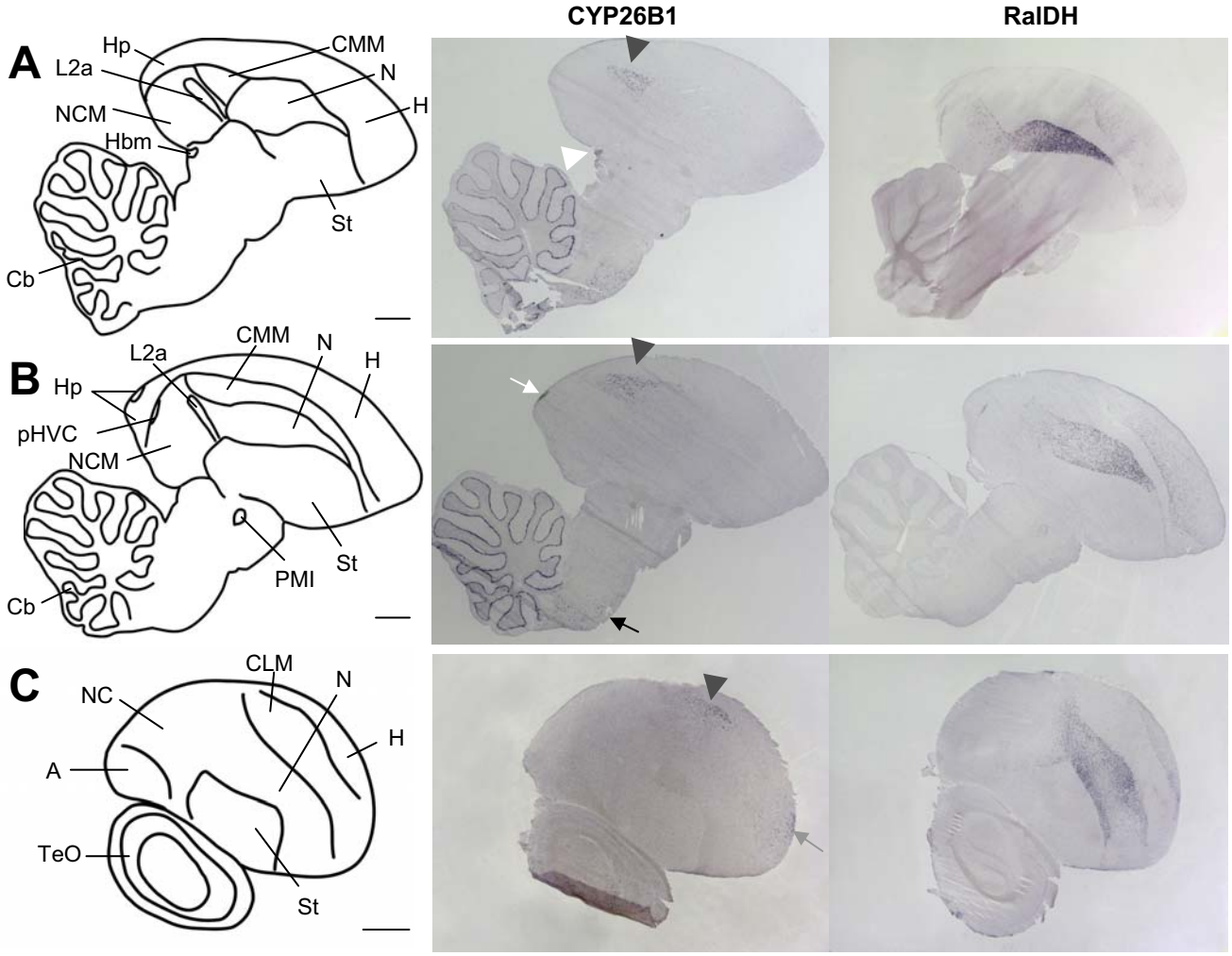


**Figure 14: CYP26B1 expression in the zebra finch brain.** The diagrams on the left depict specific brain areas of the parasagittal sections shown right. The middle and right panels show sections hybridized with a digoxigenin labeled CYP26B1 and RalDH antisense probe, respectively, and are stained with NBT/BCIP. CYP26B1 photos are slightly contrast enhanced. RalDH in situ hybridizations in A and C are from a different animal. For all images, anterior is to the right and dorsal is up. Medial to lateral levels are represented from top to bottom (in irregular intervals: A and B represent medial sections, C a lateral one. Levels were chosen as to illustrate all brain regions exhibiting CYP26B1 expression). CYP26B1 is expressed in a sparse pattern which does not overlap with RalDH expression. CYP26B1 expression forms a dorsoventral gradient in the caudal mesopallium (including both its medial and lateral parts, CMM and CLM; gray arrowheads), and is furthermore expressed in the fronto-lateral mesopallium (gray arrow), in the medial habenula (white arrowhead), in a small distal region of the caudal hippocampus (white arrow), the Purkinje cell layer of the cerebellum, and some scattered cells in the brain stem (black arrow). For abbreviations, see introduction. Scale bars = 1mm.

### *3.3 CYP26B1 expression in the zebra finch brain*

The RAc degrading cytochrome CYP26B1 exhibited a sparse and conspicuous expression pattern in the zebra finch brain which was partly adjacent to, but did not overlap with RalDH expression (fig. 14). While the CYP26B1 expression pattern was not related to song control areas, the higher auditory areas CM and NCM exhibited an interesting combination of RalDH and CYP26B1 expression, suggesting that a RAc *gradient* plays a role in these areas. In vertebrate embryos, RAc gradients are crucial for pattern formation, by providing positional cues through concentration-dependent Hox-gene activation (Boncinelli et al., 1991). In the following, the expression of CYP26B1 is described and related to RalDH expression in more detail, with a focus on the higher auditory areas.

Within the telencephalon, CYP26B1 expression was restricted to the mesopallium and the hippocampus: CYP26B1 was found throughout the caudal mesopallium (CM), which comprises two subregions, a more medial (CMM) and a more lateral one (CLM) which both function as higher auditory areas. In the CM, CYP26B1 was expressed in a dorsoventral gradient, with highest expression in the most dorsal part of CM (fig. 14 and fig. 15). The most fronto-lateral part of the mesopallium also showed some CYP26B1 expression. In the hippocampus, CYP26B1 was only expressed in a small, very distal caudomedial region. Cells expressing CYP26B1 were neurons, as shown by cellular colocalization of CYP26B1 mRNA and the neuronal marker Hu, against which we stained immunohistochemically (fig. 16).

Outside the telencephalon, CYP26B1 expression was found in the medial habenula, the Purkinje cell layer of the cerebellum, and in scattered cells in the optic tectum and the brainstem (fig. 14). Among the regions where CYP26B1 expression was found, the density of CYP26B1 positive cells was high only in the medial habenula, where almost all cells expressed CYP26B1 (fig. 16.F-H). In the mesopallium, only a minority of neurons was CYP26B1 positive (fig. 16.B-E).

The observation that CYP26B1 and RalDH expression were non-overlapping (fig. 14 and fig. 15) suggests that the role of CYP26B1 in the zebra finch brain rather consists in establishing regional sinks or gradients of RAc than in generally limiting or regulating the levels of RAc in its production sites. This concerns in particular the higher auditory areas NCM and CM (including its medial part, CMM, and the lateral part, CLM) (fig. 15): In both NCM and CM,

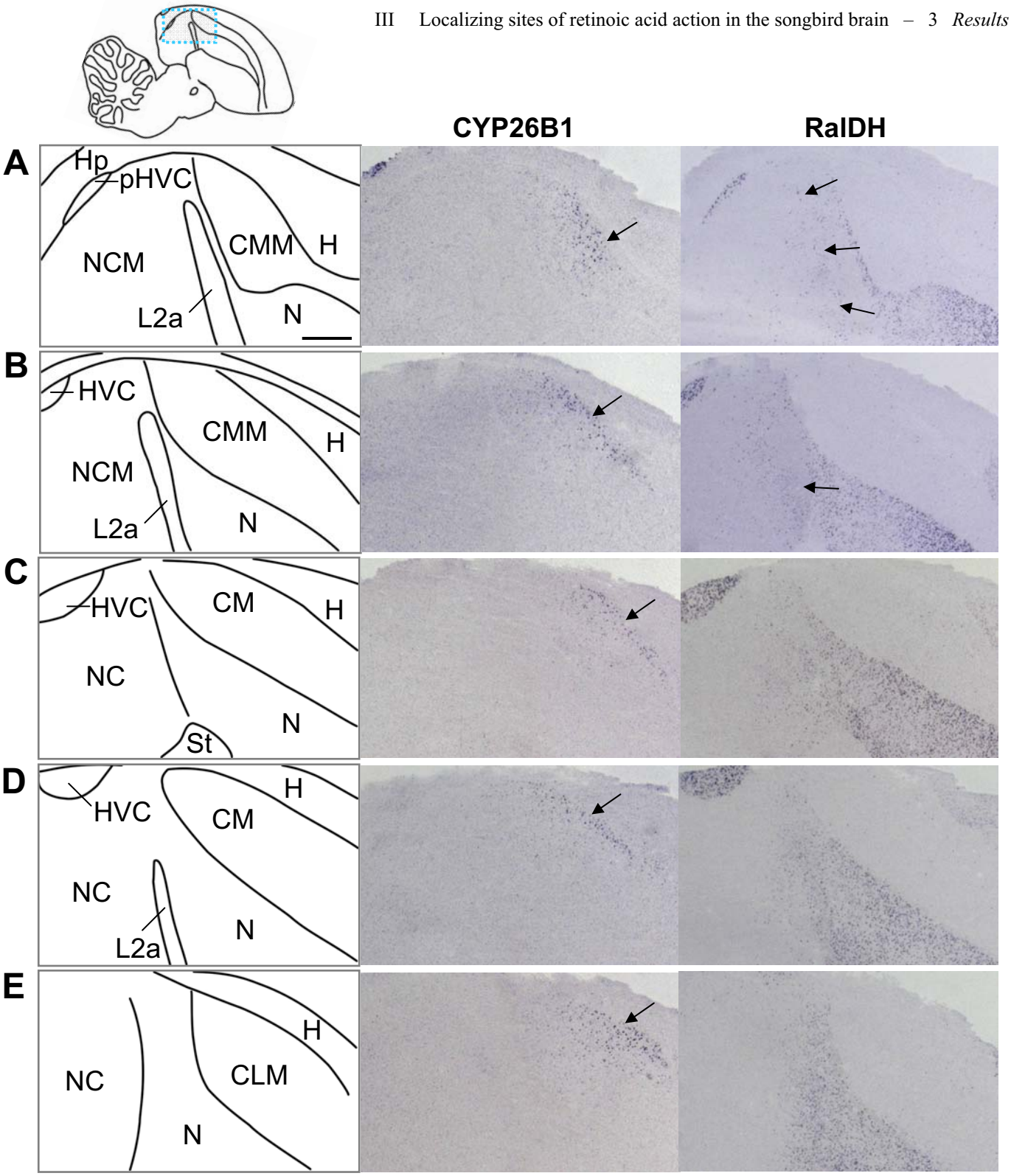


**Figure 15: CYP26B1 and RalDH exhibit expression gradients in higher auditory areas CM and NCM.** The small drawing in the top left corner approximately outlines the region shown in A-E (irrespective of mediolateral plane). The diagrams on the left depict specific brain areas of the parasagittal sections shown right. The middle and right columns show sections hybridized with a digoxigenin labeled CYP26B1 and RalDH antisense probe, respectively, and are stained with NBT/BCIP. CYP26B1 photos are slightly contrast enhanced. For all images, anterior is to the right and dorsal is up. Medial to lateral levels are represented from top to bottom. CYP26B1 is expressed in a dorsoventral gradient throughout the caudal mesopallium (CM), a higher auditory area (arrows point to region of high expression within the CYP26B1-gradient in CM), while RalDH expression is largely complementary: No RalDH is found in the caudal mesopallium, but both hyperpallium and nidopallium express RalDH. In the caudal nidopallium comprising the auditory region NCM, RalDH expression forms an antero-posterior gradient (arrows point to regions of high RalDH-expression in NCM). For abbreviations, see introduction. Scale bar = 0.5mm.

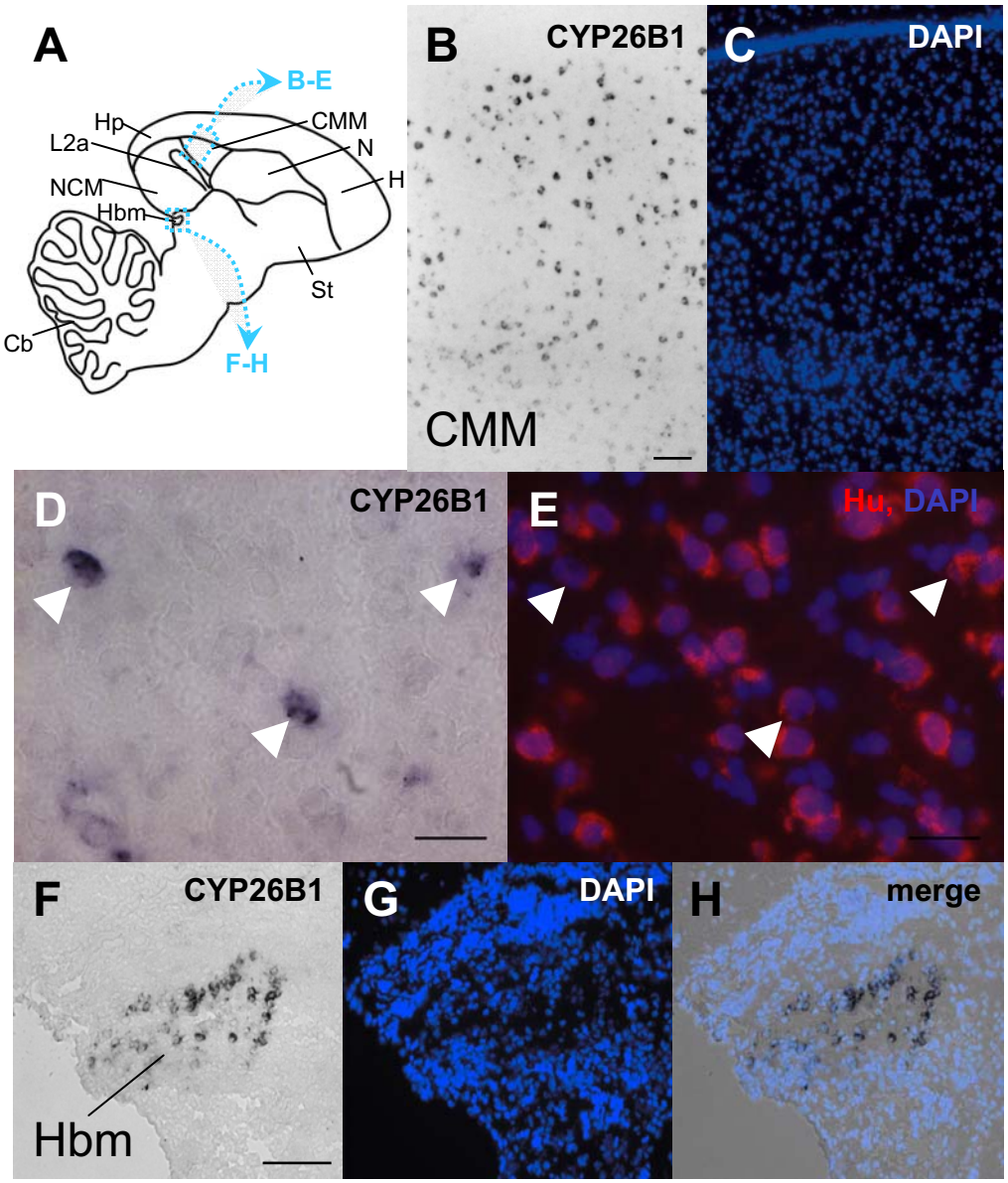


**Figure 16: CYP26B1 is expressed in neuronal population(s) with medium to high density.** **A:** Schematic diagram depicting the CMM region shown in photos B-E to illustrate distribution of CYP26B1 positive cells. **B and C:** Medium power views of a brain section hybridized with a CYP26B1 antisense riboprobe, stained with NBT/BCIP, and counterstained with DAPI to visualize cell nuclei. B is a bright field view illustrating the dorsoventral CYP26B1 expression gradient in CMM, C shows the according DAPI counterstain, illustrating that the density of the CYP26B1 positive cell population is medium high. **D and E:** High power bright field and fluorescence views of a non-radioactive CYP26B1 *in situ* hybridization immunostained for the neuronal marker Hu (red), and counterstained with DAPI (blue). CYP26B1 positive cells are also Hu positive (white arrowheads). **F-H:** Medium power view of CYP26B1 expression in the medial habenula. F is a bright field image of the non-radioactive CYP26B1-*in situ* hybridization, G is the according DAPI stain, and F the merged image. Most cells particularly at the margins of the medial habenula express CYP26B1. Scale bars for B and C = 50µm, for D and E = 20µm, for F-H = 100µm.

expression gradients were found; an antero-posterior RalDH gradient in NCM, and a dorso-ventral CYP26B1 gradient in CM. CMM and NCM are separated by a thin stretch of caudal nidopallium with high RalDH expression, and field L2a which did not express either of the genes. The distribution of CYP26B1 and RalDH mRNA suggests that RAc gradients may be established in both CM and NCM: In NCM, RalDH may directly produce such a RAc gradient by means of its own expression gradient. In CM, a RAc gradient could be established indirectly by CYP26B1 which may gradually catabolize RAc reaching CM by diffusion. RAc might trickle into CM from the caudal nidopallium with its high RalDH expression, and its degradation could depend on CYP26B1 expression strength, leaving an antidromic ventro-dorsal RAc gradient in CM.

Since song control regions did not express CYP26B1, the expression pattern did not differ between male and female brains (supplementary fig. 4 shows a medial section of a female brain as an example). Also, CYP26B1 expression did not show any major changes during song development, as illustrated by radioactive CYP26B1 *in situ* hybridizations of juvenile brains at PHD 45 and 68 as examples (supplementary fig. 5).

### *3.4 CYP26C1 expression in the zebra finch brain*

The RAc degrading cytochrome CYP26C1 was expressed ubiquitously at low levels throughout the zebra finch brain, with highest expression levels in the cerebellum (fig. 17). To demonstrate that lacking clearness of pattern was not due to unsuitable probe or hybridization conditions, we performed an *in situ* hybridization on zebra finch embryonic sections with the same sense and antisense probes and hybridization conditions (supplementary fig. 6). Our probe clearly detected the CYP26C1 transcript in the same regions where the gene is known to be expressed in the stage 20 chick embryo, which approximately corresponds to our zebra finch embryo. Sites of expression were the hindbrain roof plate and the mesenchyme adjacent to the otic vesicle (Reijntjes et al., 2004). In the adult zebra finch brain, however, the same probe detected no conspicuous expression pattern. The overall very low expression level seemed minimally increased in some layers of the optic tectum (fig. 17.E), but the difference to the surrounding tissue was quite low.

No changes in CYP26C1 brain expression could be detected at different points in song development (PHDs 20 and 68; supplementary fig. 7). At best, a minimal CYP26C1

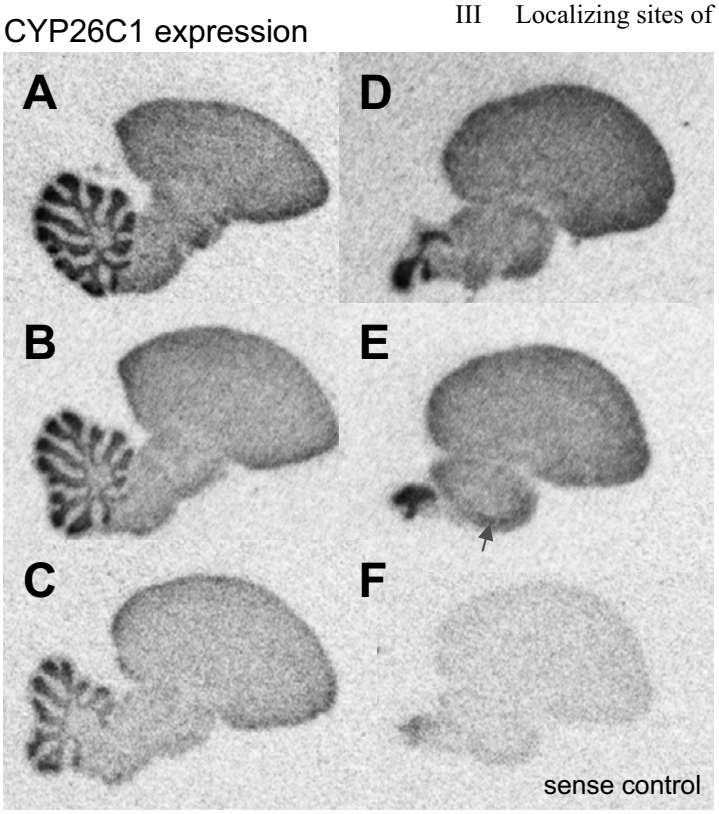


**Fig. 17: Expression of CYP26C1 is low and ubiquitous in the adult zebra finch brain, with highest expression levels in the cerebellum. A-E:** Radioactive *in situ* hybridization of CYP26C1 antisense probe on sagittal sections, from medial to lateral. Pictures are strongly contrast enhanced, revealing a minimally increased expression in layers of the optic tectum (gray arrow). **F:** Sense control probe did not yield any signal.

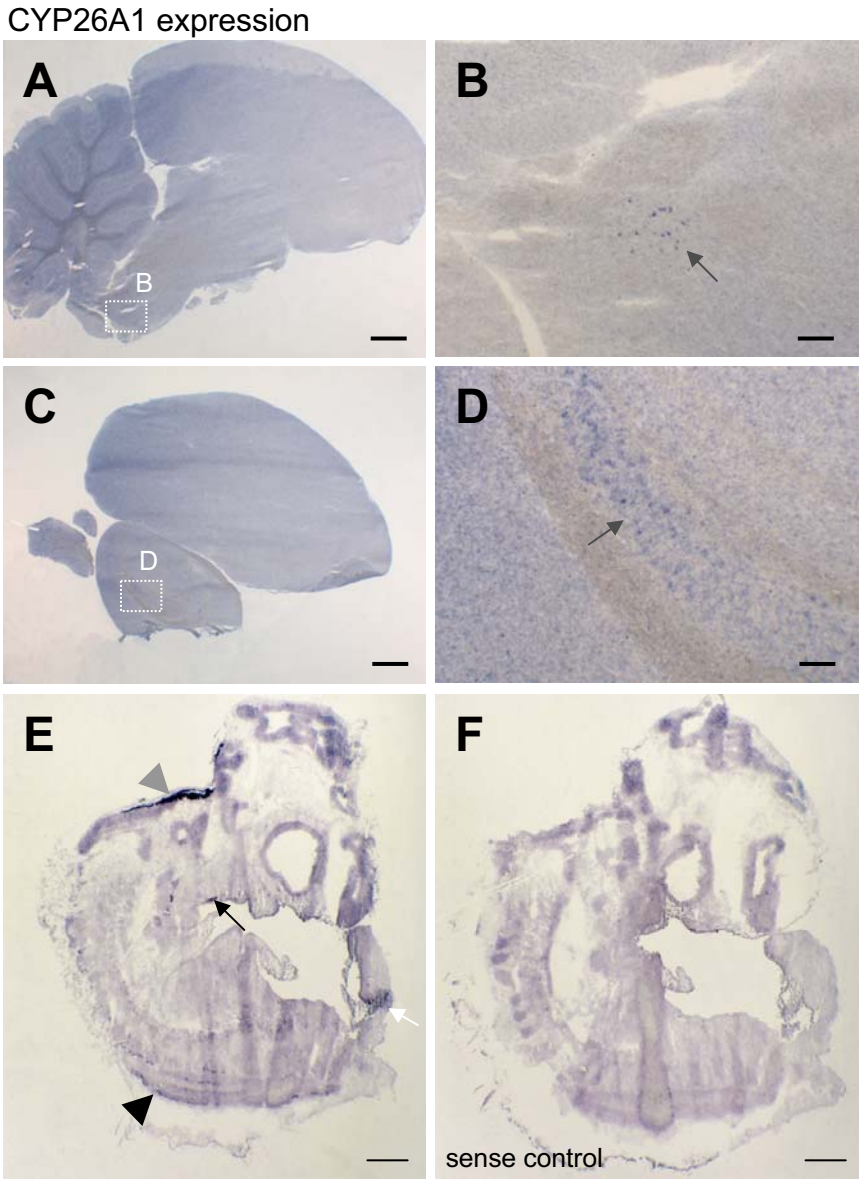


**Figure 18: CYP26A1 is practically not expressed in the postnatal brain, but we can detect its transcript in the zebra finch embryo.** All photos show non-radioactive *in situ* hybridizations (ISHs) followed by NBT/BCIP staining. **A-D** show CYP26A1 ISHs on para-sagittal brain sections (A and B show a more medial, C and D a more lateral section of the same brain). Frontal is to the right, dorsal is up. **B and D** are detail views of the only CYP26A1 expression sites in A and C, respectively. In the brain, no CYP26A1 transcript is detected outside expression in few cells in the brainstem (B) and some cells in a layer of the optic tectum (D; gray arrows). **E and F** show ISHs with CYP26A1 antisense (E) and sense probe (F) on sagittal embryonic sections, demonstrating that the probe we used detected a CYP26A1 transcript at sites where the gene is known to be expressed from the chick embryo. Dorsal is to the left, rostral is up. CYP26A1 is expressed in the embryonic hindbrain (gray arrowhead), in the neural tube of the trunk (black arrowhead), the tail bud (white arrow), and a ventral site probably corresponding to a part of the first pharyngeal groove (black arrow). No expression is detected with a CYP26A1 sense probe (F). Scale bars: 1mm for A/C and E/F, 100µm for B and D.

upregulation might be visible in song nucleus HVC at PHD 68. Furthermore, not being in any way specific to the sexually dimorphic song control structures of the brain, CYP26C1 expression did not show any major difference between male and female brains (supplementary fig. 8).

In contrast to CYP26B1, the expression of CYP26C1 in the postembryonic zebra finch brain does therefore not suggest a role of CYP26C1 in building local RAc sinks. At best, it might be suited to set a general limit to excess RAc availability throughout the brain.

### *3.5 CYP26A1 expression in the zebra finch brain*

An *in situ* hybridization with a CYP26A1 antisense probe on the postembryonic zebra finch brain showed that no CYP26A1 transcript could be detected in the telencephalon (fig. 18.A-D), suggesting that this RAc catabolizing cytochrome is not expressed there. The lack of signal was not due to unsuitable probe design or hybridization conditions, since the same probe and conditions used on a zebra finch embryo clearly detected the CYP26A1 transcript at sites where it is known to be located in the chick embryo (fig. 18.E, F). Expression sites in the finch embryo roughly corresponding to a stage 20 chick embryo were the hindbrain, the neural tube of the trunk, parts of the tail bud, and a ventral site opposite the hindbrain which probably corresponds to a part of the first pharyngeal groove. These sites were identical to CYP26A1 expression sites in the chick embryo (Reijntjes et al., 2004). In the adult brain, in contrast, CYP26A1 expression was restricted to few cell populations in the brainstem and a layer of the optic tectum (fig. 18.B, D). CYP26A1 was not expressed in any brain structures relevant to singing, song learning, or hearing.

In sum, the expression patterns of the RAc degrading CYP26 cytochromes did suggest the existence of regional RAc sinks generated by CYP26B1 in the caudal mesopallium and in some smaller regions like the medial habenula and a caudo-distal hippocampal spot. However, the question of whether other RAc sinks may exist remains unsolved, due to ubiquitous (albeit low) expression of CYP26C1: We cannot exclude that it might be able to either generate further local sinks (e.g. depending on locally restricted translation, or on a differential subcellular localization), or set a global limit to RAc levels, though the mere existent of transcript is not enough to prove either of these possibilities. This reminds of the conclusions drawn from the receptor expression patterns: They did indicate that RAc may

play an important and specific role for certain brain regions, but did not allow to undoubtedly identify all sites of actual RAc signalling. The indirect determination of RAc signalling sites via retinoid receptor and degrading enzyme mRNA distribution is likely to remain ultimately inconclusive, because information about local RAc availability is lacking – even though distribution of the pathway molecules is necessary to assess which regions may be RAc responsive. To address this problem, we aimed at finding a way to determine the distribution of RAc itself in the zebra finch brain, presented in the next section of this study.

### *3.6 Localization of RAc with the help of a retinoic acid sensitive reporter cell assay*

Measuring RAc in brain tissue to determine sites of its presence has always been a challenging task, due to its minute concentrations in low nanomolar ranges and the labile, lipophilic nature of the molecule, which makes it difficult to detect RAc in the lipid-rich brain, even with ultra sensitive HPLC (see Gundersen, 2006 for a review). At present, the most sensitive and quantitative method to detect RAc is with combined mass spectrometric and chromatographic assays (Kane et al., 2008). However, such assays are impractical if one is to investigate the distribution of RAc over widespread brain areas. Reporter assays are a different way to analyze RAc in tissues. Cells transformed with a reporter construct are used to indicate the presence of RAc by expression of a reporter gene which can be visualized, detected, or developed in some way – examples are LacZ, GFP, or luciferase (Wagner et al., 1992). The reporter gene is specifically activated by RAc because it is located downstream of a retinoic acid response element (RARE). If such reporter cells are co-cultured with tissue explants, they will indicate RAc release from the tissue by reporter gene expression. Although they are not as quantitative as spectrometric/chromatographic assays, reporter cell assays are relatively sensitive (Gundersen, 2006). Up to now, reporter cell assays have been used for small pieces of tissue, such that spatial resolution posed no challenge. Since we were interested in determining RAc distribution over broad brain areas, we modified an existing RAc reporter assay (Wagner et al., 1992) and applied it to entire brain sections.[3] The reporter cells we used were mouse F9 cells (derived from an embryonic teratocarcimona) transformed with a RARE-LacZ construct (see fig. 19.A and B), kindly provided by Michael Wagner,

[3] Our modifications to the assay mainly consisted in using large Petri dishes, prolonged times for tissue attachment to the cell layer and repeated wetting of explants during that time, reducing the number of PBS washes, as well as carefully draining the dishes every time before moving them (see *Materials & Methods*).

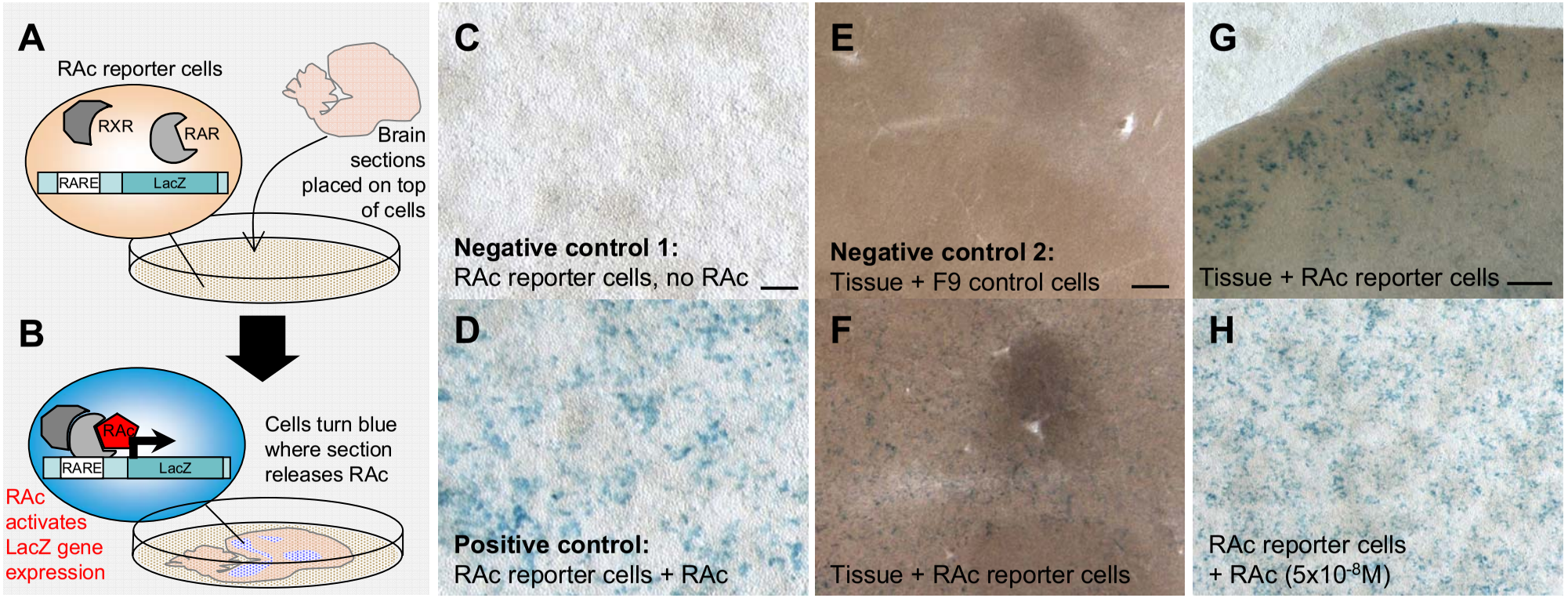


**Figure 19: RAc reporter cells indicate regional presence of RAc in co-cultured brain sections by specific expression of LacZ. A and B:** Schematic overview over RAc reporter cell assay. RAc reporter cells contain a LacZ gene under the control of a retinoic acid response element (RARE), as well as retinoic acid receptors (RARs) and retinoid X receptors (RXRs) necessary for RAc induced gene expression (**A**). If RAc enters the cell, it binds to the RAR/RXR complexes and causes them to induce LacZ expression (**B**). LacZ positive cells are stained blue with the help of a standard LacZ/X-gal staining. Reporter cells are co-cultured in Petri dishes with freshly cut brain sections. The sections attach to the cell layer, and regions containing or producing RAc generate blue LacZ labeling in the underlying cell layer (**B**). Photos shown in C-H are taken from the bottom side of the Petri dish; tissue sections are thus photographed through the cell layer. **C and D:** LacZ expression in reporter cells is specifically induced by RAc. Blue LacZ labeling is only generated in the presence of RAc ($5x10^{-8}$M, added to culture medium as a positive control) (**D**); in the absence of RAc, reporter cells are LacZ negative and do not turn blue upon standard LacZ/X-gal staining procedure (**C**; negative control 1). **E and F:** Blue labeling specifically indicates LacZ expression, instead of being a non-specific result of the LacZ/X-gal staining procedure, or of tissue co-culture with underlying cell layer. Blue labeling occurs in F9 reporter cells containing the LacZ gene under the control of a RARE, if RAc is present (**F**). Tissue co-cultured with an F9 control cell line *without* RARE and LacZ does not generate blue signal upon standard LacZ staining procedure, since no LacZ is present (**E**; negative control 2). The part of the brain sections shown here is a rostral region containing song nucleus lMAN (dark region in upper half of photos E and F) and the surrounding nidopallium, which both highly express RalDH. **G and H:** Blue LacZ labeling induced by RAc released from tissue is of comparable strength as labeling generated by physiological concentrations of RAc added to the assay medium. G shows LacZ labeling in reporter cells underlying song nucleus HVC, a region which highly expresses RalDH. Labeling is comparable, if not stronger, than labeling induced by $5x10^{-8}$M RAc added to the medium, a concentration in the range of physiological RAc (**H**). Photos G and H have the same scale. Note that G illustrates spatial specificity of the assay: Cells that are not touched by co-cultured tissue, or cells that underlie tissue which does not produce RAc, are devoid of blue signal. Scale bars: for C, D=200µm; for E, F=100µm, For G, H=100µm.

Ph.D., SUNY Downstate Medical Center, Brooklyn, NY. Besides the reporter construct, the cells contain RARs and RXRs that bind to the RARE and induce expression of the gene located downstream, provided that RAc is present to bind the RAR/RXR complex (without RAc being present, the LacZ gene is not being expressed, see fig. 19.A and B). LacZ protein is subsequently visualized using a standard staining with the substrate X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside): LacZ catalyzes the conversion of X-gal to the deep blue 5,5'-dibromo-4,4'-dichloroindigo in the presence of $O_2$. Blue color is therefore indicative of LacZ expression and thus of presence of RAc. Due to silencing elements in the reporter construct, there should be no unspecific, "leaky" LacZ expression. We verified that LacZ expression was specifically dependent on the presence of RAc by adding physiological concentrations of RAc ($5x10^{-8}$M) directly to the culture medium for positive control (fig. 19.C and D): Blue labeling was only generated in the presence of RAc. It did not occur when no RAc was added (negative control), showing that LacZ is specifically induced by RAc. To also make sure that in the context of tissue-reporter cell co-culture, blue label was not a non-specific result of the X-gal staining procedure but indeed indicative for LacZ expression, we co-cultured brain sections with F9 cells that did not contain a RARE-LacZ reporter construct as a second negative control (fig. 19.E and F). The brain sections used contained song nucleus lMAN and frontal nidopallial regions which both highly express RalDH. RAc was therefore available, but blue labeling only occurred in the RARE-LacZ reporter cells, not in the empty F9 control cells, showing that it was specifically dependent on RAc-induced LacZ expression. Note that photos of the co-cultures are taken from the bottom side of the Petri dishes; the brain sections are therefore viewed *through* the reporter cell layer.

Although the reporter cell assay is not designed for exact quantification of RAc in tissues, the density of blue cells and the intensity of staining are approximately linearly correlated with amount of RAc (Wagner et al., 1992). We verified whether LacZ expression induced by tissue-released RAc is comparable to physiological concentrations of RAc ($5x10^{-8}$M) added to the culture medium (fig. 19.G and H). Blue labeling of cells lying beneath a strongly RalDH expressing region like HVC turned out to be a little stronger than, but in a comparable range of a concentration of $5x10^{-8}$M RAc directly added to the culture medium.

Using the reporter assay to determine the distribution of RAc over widespread brain areas requires that the blue signal is spatially specific: RAc released by a brain region should not spill over to adjacent regions, inducing LacZ expression in cells lying beneath regions which actually do not produce RAc. fig. 19.G shows that this is not the case: Cells that are not

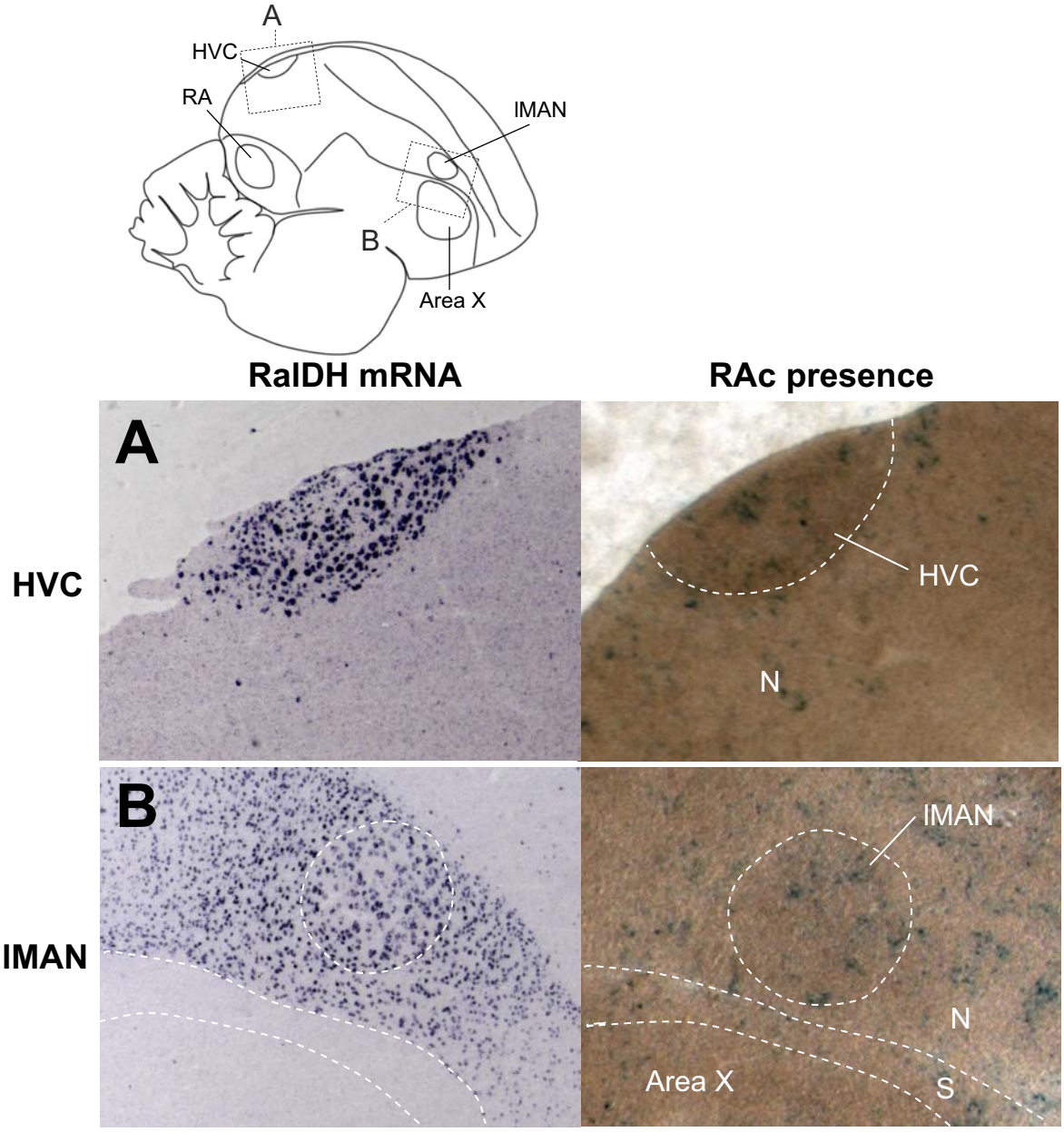


**Figure 20: RA presence in song nuclei HVC and lMAN is consistent with RalDH expression.** The line drawing illustrates where regions shown in A and B are located within a brain section. Left panels in A and B show non-radioactive *in situ* hybridization with RalDH antisense probe. Right panels show presence of RAc as determined by RAc sensitive RARE-LacZ F9 reporter cells on top of which the brain sections were cultivated (photographed from below; blue label indicates LacZ expression by reporter cells in regions where brain section produced RAc). **A:** Song nucleus HVC strongly expresses RalDH mRNA, and blue LacZ label in reporter cell layer indicates presence of RAc, showing that RalDH protein is functional in this region. **B:** The same applies to song nucleus lMAN of the AFP and the surrounding nidopallium, where both RalDH mRNA and RAc are detected. In all panels, frontal is to the right and dorsal is up.

touched by co-cultured tissue do not show any blue staining, even right at the edge of an adjacent piece of tissue which heavily produces RAc and generates strong blue staining in the cells located beneath. In addition, boundaries between tissue regions that do produce RAc (and thus blue LacZ signal) and those which do not can be relatively sharp, as is the case for some regions proximal to HVC (fig. 19.G).

### *3.7 RAc distribution in the zebra finch brain as determined with the RAc reporter assay*

Using the RAc reporter cell assay, we detected RAc in all parts of the brain which express RalDH (see supplementary fig. 9 for an overview), suggesting that RalDH gene transcription in the adult brain generally results in local translation into functional protein which synthesizes RAc. With regard to the song system, we found RAc, as expected from RalDH expression, in song nuclei HVC and lMAN (fig. 20). RAc detection is thus fully consistent with RalDH expression.

We also detected RAc in some regions which do not express RalDH themselves, but are adjacent to RalDH expressing brain regions. A particularly interesting example is the mesopallium (fig. 21): There, RAc formed an anteroposterior concentration gradient, with high concentrations in anterior regions and concentrations below detectability in its most caudal and dorsal part. The mesopallium does not express RalDH (Denisenko-Nehrbass et al. (2000); see also fig. 5 and fig. 14). Therefore, mesopallial presence of RAc required diffusion from the adjacent RalDH expressing hyper- or nidopallium (or, alternatively, transport of RalDH protein from regions with neuronal projections to the mesopallium, but we show in section 3.9 that this is not the case). The lack of RAc in the dorso-caudal mesopallium was consistent with expression of CYP26B1 (see above, section 3.3): As predicted from its expression in this region, CYP26B1 did generate a RAc sink here. As CYP26B1 expression decreased towards the more rostro-ventral mesopallium, RAc increased, leading to a RAc gradient in the caudal mesopallium (CM; see fig. 21). The CM is of significance in the context of song, since both subregions it comprises, the caudomedial (CMM) and the caudolateral mesopallium (CLM), serve higher auditory function, analogous to the mammalian secondary auditory cortex (Vates et al., 1996).

Interestingly, we found RAc not only to be present in brain areas of RalDH expression, such as HVC and lMAN, and regions close to RalDH expressing areas, such as the mesopallium.

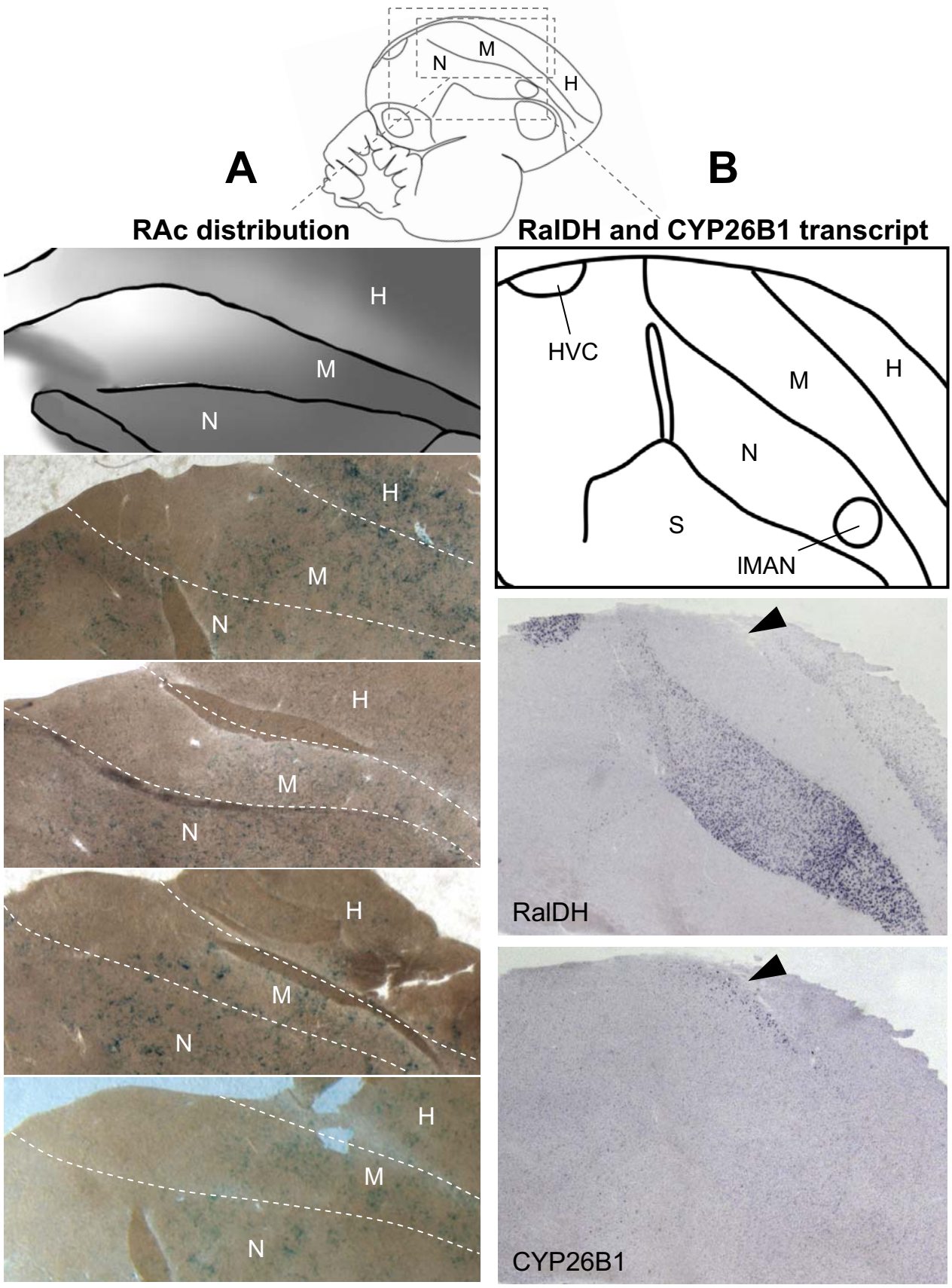


**Figure 21: In the mesopallium, RAc forms an anteroposterior concentration gradient that is consistent with CYP26B1 expression but requires RAc diffusion from hyper- or nidopallium.** The schematic drawing on top depicts regions shown in panels A and B. **A:** RAc distribution as detected with RAc reporter cell culture assay. Blue label on tissue indicates presence of RAc. The upper picture is a summing-up overview of RAc distribution in the dorso-caudal area (gray indicates RAc presence), below are corresponding examples of 5 different animals. **B:** Non-radioactive *in situ* hybridizations with RalDH and CYP26B1 antisense probe. Note that RalDH and CYP26B1 expression are non-overlapping. The caudo-dorsal mesopallium is devoid of RAc, consistent with CYP26B1 expression and lack of RalDH expression (see arrowheads in right panels). As CYP26B1 expression decreases towards the more rostro-ventral mesopallium, RAc increases. Its presence in mesopallium requires transport or diffusion from adjacent hyper- or nidopallium, where RalDH is expressed.

We could also detect RAc in some brain regions more or less distant from RalDH expression sites: Overall RAc distribution in the brain was considerably more widespread than RalDH gene transcription (supplementary fig. 9). Most importantly in the context of song, we found RAc to be present in song nuclei Area X and RA (fig. 22). Neither of these nuclei expresses RalDH. The dorsal part of Area X is relatively close to the rostral nidopallium which highly expresses RalDH, and might thus be in reach for diffusing RAc from lMAN and the surrounding nidopallial area. However, RAc was also present to the same degree in ventral Area X and the surrounding striatum (fig. 22.A and B), which are more distant from the RalDH expressing nidopallium. It seems more unlikely that RAc originating from the nidopallium reaches these areas by mere diffusion. Song nucleus RA seems even highly improbable to be in the reach of RAc diffusing from RalDH positive areas (fig. 22.A and C): RA is located at several millimeters distance from the nearest RalDH expressing regions, HVC and the rostral nidopallium. Furthermore, there was less RAc in the immediate arcopallial vicinity of RA than inside the nucleus (fig. 22.C and supplementary fig. 9), which could not be easily accounted for by RAc diffusion.

If diffusion seems unlikely, how does RAc then reach regions far from RalDH expression sites? One possibility is the existence of a second RalDH in the zebra finch brain which produces RAc in those regions that are not covered by transcription sites of the known RalDH, which is homologous to the mammalian RalDH2 (Denisenko-Nehrbass et al., 2000). However, no such other RalDH has been described to date for zebra finches, and Western blots with antibodies against all retinaldehyde dehydrogenases show only one target protein (corresponding to the described zebra finch RalDH2) in homogenates of different brain regions (Denisenko-Nehrbass et al., 2000, Denisenko-Nehrbass and Mello, 2001), suggesting that the described zebra finch RalDH is the only retinaldehyde dehydrogenase in the zebra finch brain. An alternative explanation for the presence of RAc far from RalDH expressing regions may be transport of RalDH enzyme. Indeed, all RAc positive regions that were far from RalDH expression sites do receive neuronal projections from those latter ones: RA and Area X are target sites for projections from both lMAN and HVC, which express RalDH. Both the RAc positive striatum surrounding Area X and the rostroventral arcopallium (see supplementary fig. 9) receive projections from the RalDH positive hyperpallium (Veenman et al., 1995, Wild and Williams, 1999, Reiner et al., 2001). Via these afferent projections, RalDH enzyme could thus reach the areas in question. To investigate this possibility, we performed immunohistochemical stainings of zebra finch brain sections with an antibody against human ALDH1A2, which is homologous to zebra finch RalDH.

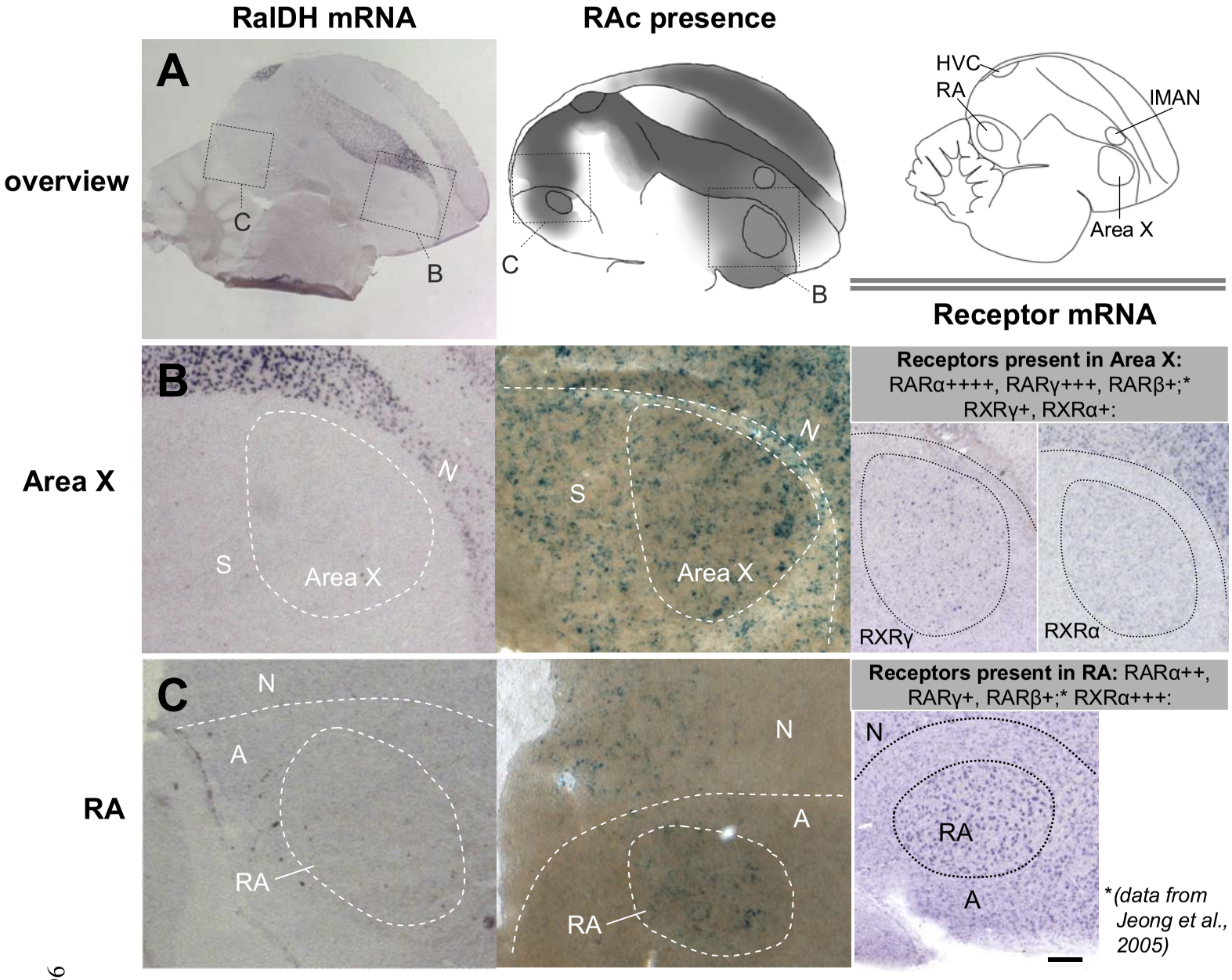


**Figure 22: RAc is present in song nuclei Area X and RA without RalDH being transcribed there.** Left panels show non-radioactive *in situ* hybridization with RalDH antisense probe. Middle panels show RAc distribution as determined with RARE-LacZ F9 reporter cells. Right panels list transcript distribution for the five retinoid receptors in the respective region, and show examples (non-radioactive *in situ* hybridization with RXR antisense probes). The line drawing in the top right corner corresponding to the overviews shown in A indicates song control regions. In all panels, frontal is to the right and dorsal is up. **A:** Overview over RalDH transcription and RAc distribution (the latter one as a schematic; gray indicates presence of RAc) in an adult zebra finch brain section containing song nuclei HVC, RA, lMAN, and Area X. Distribution of RAc is more widespread than distribution of RalDH transcript. **B:** RalDH mRNA, RAc distribution, and receptor mRNA in Area X. Although RalDH is not transcribed in Area X, RAc is detected, and receptors are transcribed that may mediate its effects. **C:** The same applies to RA, where receptor mRNA is present and RAc is detected, while RalDH transcript is not.

### *3.8 An antibody against human ALDH1A2 specifically binds zebra finch RalDH*

With the antibody against human ALDH1A2 (in the following termed "αRalDH"), we obtained a specific staining of RalDH protein in zebra finch brain sections, if using peroxidase-diaminobenzidine (DAB) staining and a heat treatment to expose antigen which appeared to be masked by paraformaldehyde fixation (see *Materials & Methods*, and supplementary fig. 10). The antibody generated a distinct staining pattern in which all regions known to be positive for RalDH transcript (e.g., rostral nidopallium, hyperpallium, HVC) were positively labeled for RalDH protein; no staining pattern whatsoever occurred by the staining procedure when αRalDH antibody was omitted for negative control (supplementary fig. 10.A and B). This suggests that the observed staining pattern was due to antibody binding, instead of, for instance, any non-specific DAB staining of brain structures. To verify whether the antibody specifically binds to RalDH and not to additional other peptides, we performed a second control where we pre-incubated the αRalDH antibody with human RalDH immunizing peptide for one hour (supplementary fig. 10.C and D). During this time, the RalDH immunizing peptide blocks the antibody's specific binding sites for RalDH protein. Treated this way, the antibody can no longer bind to tissue RalDH protein when applied to brain sections. However, if the antibody is non-specific and additionally binds to other peptides than its RalDH target, such binding will still occur, as the RalDH immunizing peptide only blocks the RalDH specific binding sites of the antibody. However, this was not the case when the blocking peptide-treated antibody was applied to zebra finch brain sections: They remained clean, indicating that the αRalDH antibody specifically bound to the zebra finch RalDH (supplementary fig. 10.C). This was not due to a lack of RalDH positive regions in the brain section shown: The dark field view of the section shown made clear that it contained structures like HVC which were actually RalDH positive (supplementary fig. 10.D vs. A).

### *3.9 Distribution of RalDH protein in the zebra finch brain*

The αRalDH antibody revealed that RalDH protein was not only present in all those brain areas that are positive for RalDH mRNA, i.e. the rostral nidopallium including lMAN, the hyperpallium, and HVC, but additionally in song nuclei Area X and RA (fig. 23). In these latter nuclei, RalDH transcript is not present, but RAc itself could be detected using the RAc reporter cell assay (see section 3.7). The immunohistochemical RalDH staining overcame the discrepancy between the distribution of RalDH mRNA on the one hand and RAc on the other

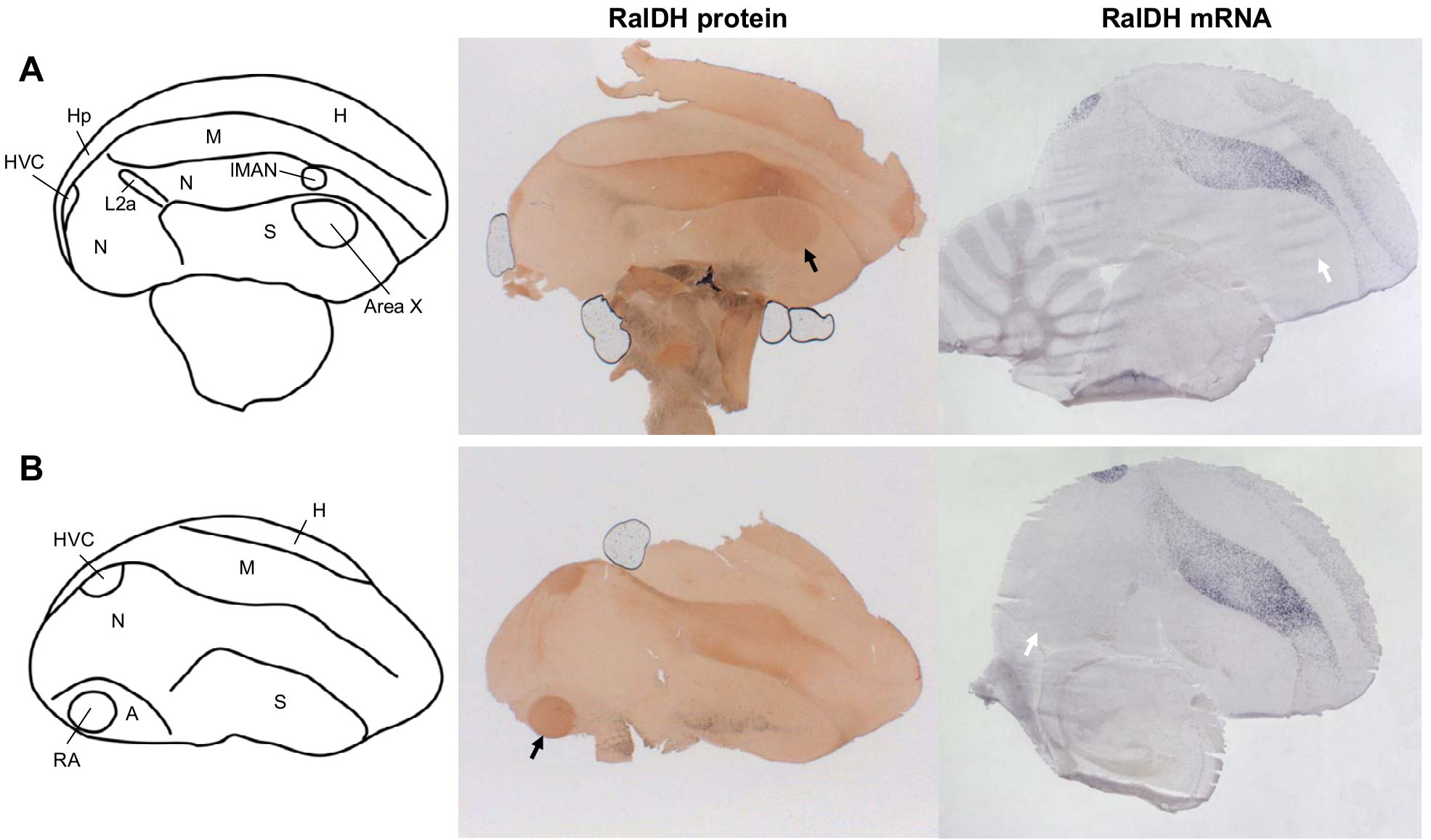


**Figure 23: RalDH protein is present in brain areas which are known to express RalDH mRNA, and additionally in song nuclei Area X and RA where no RalDH transcript is detectable.** Schematic diagrams on the left depict the parasagittal brain sections shown in photos to the right. Panels in **A** show a more medial, in **B** a more lateral brain level. Frontal is to the right, dorsal is up. Middle panels show sections of adult zebra finch brain immunohistochemically labeled for RalDH protein, and stained with DAB. Right panels show non-radioactive *in situ* hybridizations with RalDH antisense probe on sections of a different animal (mediolateral levels approximately corresponding to sections C and D), to illustrate RalDH mRNA distribution. RalDH protein is detected in all areas where the transcript is present (HVC, rostral nidopallium including lMAN, hyperpallium), but additionally in song nuclei Area X (in **A**) and RA (in **B**) (see black arrows in middle panels). RalDH transcript is not found in either of these nuclei (see white arrows in right panels).

hand, by showing that in both RA and Area X RalDH protein was present. This corroborates a scenario where Area X and RA obtain their RAc from RalDH enzyme that is transported from RalDH expressing regions to Area X and RA (rather than exclusive RAc diffusion and/or transport to these areas, although this may play an additional role, especially in the case of Area X; see section 3.7).

A closer look at the RalDH immunolabeling in the song nuclei supports this view (fig. 24). HVC and lMAN on the one hand, which were positive for RalDH mRNA, and RA and Area X on the other hand, which were not, were all labeled for RalDH protein (fig. 24). While RalDH immunolabeling in HVC and lMAN was concentrated in cell bodies, it was diffuse in RA and Area X. This suggests that RalDH expressing HVC and lMAN neurons had RalDH protein in the cytoplasm of their somata, where the protein is synthesized, while RalDH in RA and Area X was not found in cell somata, consistent with the absence of RalDH transcript in these nuclei. The diffuse RalDH distribution in these two nuclei rather suggests protein presence in the synaptic endings of afferent projection neurons.

Where could RA obtain RalDH protein from? The strongly labeled fiber tracts visible between HVC and RA (fig. 24.A, B, D) seem to be particularly suggestive of RalDH protein transport from HVC to RA. But they are also consistent with transport from lMAN to RA: lMAN axon fibers to RA traverse very close to HVC (Mooney, 1992) and may then partly run in parallel with fibers coming from HVC. Therefore, the RalDH positive fiber tracts may belong to both lMAN>RA and HVC>RA projection neurons (for a schematic overview, see fig. 2). Moreover, we show here by RalDH immunolabeling on a brain where we injected the retrograde tracer cholera toxin subunit B into song nucleus RA, that RalDH protein was present in projection neurons to RA from both HVC and lMAN (fig. 25): In both song nuclei, we found RalDH immunolabel to coincide with the retrograde tracer in the same cell bodies. Conditions are thus in principle met for both HVC and lMAN to transport RalDH protein via axonal projections to RA. Moreover, RAc distribution as localized with the reporter assay is consistent with putative RalDH protein transport from lMAN to RA (supplementary fig. 13): RAc was detected along the fiber tracts originating in MAN and extending towards posterior. On brain sections lateral to song nucleus HVC, these fiber tracts extending dorsally from MAN were clearly visible and unlikely to be confounded with HVC>RA projection neurons, and RAc was detected there (supplementary fig. 13.B). The same applies to transverse sections, where the course of these lMAN efferent fiber tracts in posterior-lateral direction can be followed (supplementary fig. 13.C). Finally, RAc distribution in the female brain was also

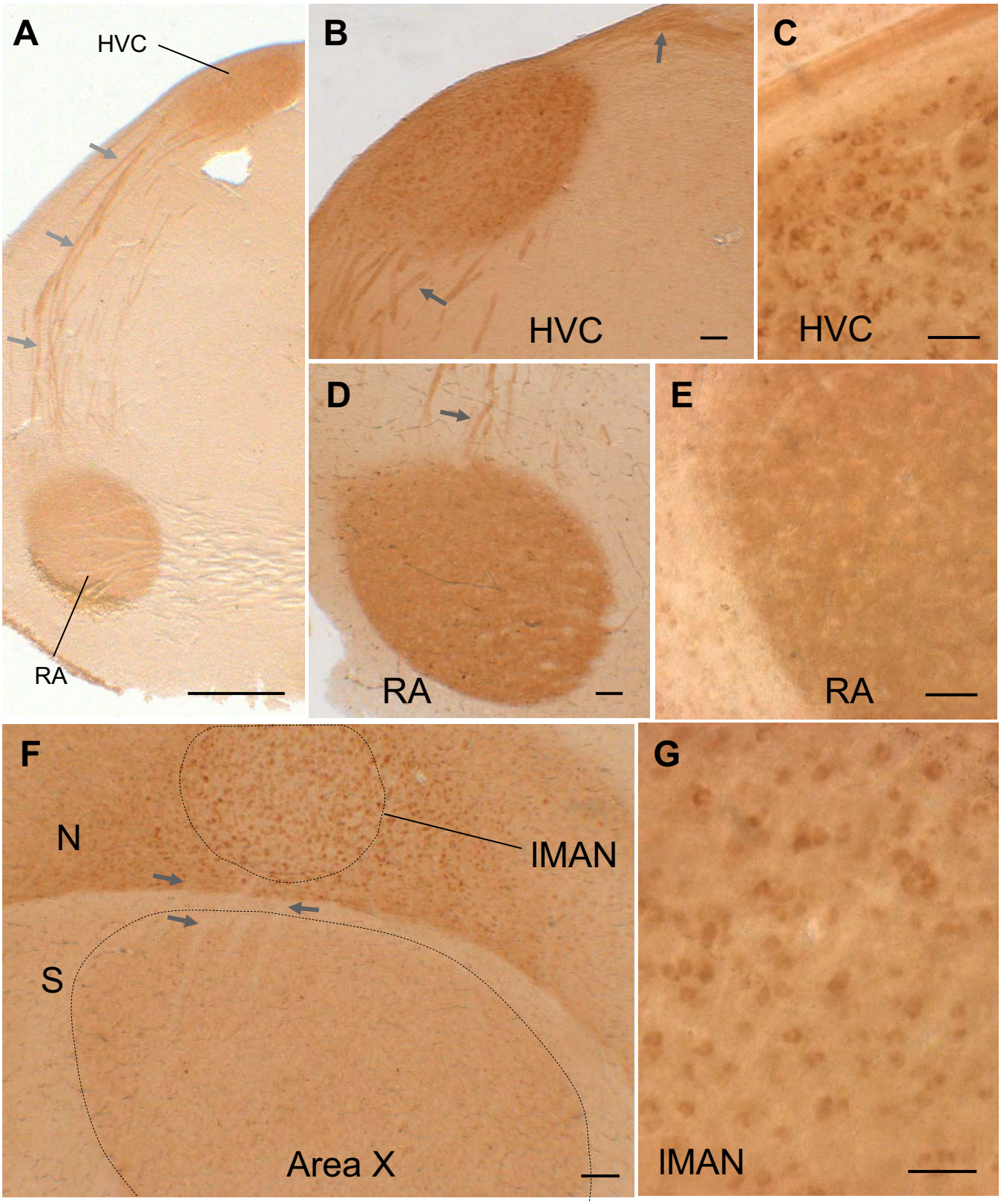


**Figure 24: RalDH protein distribution in telencephalic song control nuclei of the adult zebra finch brain.** All views show adult zebra finch brain sections immunolabeled for RalDH protein, stained with DAB. In all photos, frontal is to the right, dorsal is up. **A:** RalDH protein in caudal part of a zebra finch brain section. Song nuclei HVC and RA are positively labeled for RalDH protein, as well as the fiber tracts extending from HVC to RA (see arrows). **B and C:** Detail views of RalDH protein in song nucleus HVC. Note that fiber tracts extending caudally as well as rostrally from HVC are labeled for RalDH (B, see arrows), and that cell bodies are enriched for RalDH (C), consistent with its transcription in HVC cells. **D and E:** Different medium power views of RalDH protein in song nucleus RA. RA itself as well as fibers entering RA are positively labeled (D, see arrow), and labeling within RA is diffuse and not concentrated in somata (C and D). **F:** Medium power view of RalDH protein in a rostral region comprising song nuclei lMAN and Area X. Note that labeling in Area X is diffuse and not concentrated in somata, and that there are RalDH negative fibers entering Area X (see arrows). **G:** High power view of RalDH in lMAN. Cell bodies are enriched for RalDH, consistent with RalDH mRNA presence there. Scale bars for A=0.5mm; B, C, D, E, F=100µm, G=50µm.

in line with RalDH transport from lMAN to RA (supplementary fig. 13.D and E): As in the male RA, RAc was detectable to considerable amounts in the female RA. HVC is rather unlikely to be the only source of this RAc, since the female HVC is tiny, and RAc was instead detected along the caudally extending fiber tracts from lMAN. This is in line with female RA obtaining RalDH from lMAN (and possibly additionally from the small HVC). Of course, the situation in the female brain does not allow direct conclusions for the male brain. However, molecular properties of cells which exist in both sexes, as lMAN>RA projection neurons, are not unlikely to have similar molecular properties between males and females. Taken together, RAc reporter assays and immunohistochemical data are consistent with HVC and lMAN providing RA with RalDH protein, although a definite proof of this scenario would require HVC and lMAN lesion experiments, which we just started to perform (see section 3.10).

How is the situation in the AFP where Area X resides? It seems largely similar to the posterior premotor pathway: Area X exhibited a diffuse RalDH immunostaining along with lack of RalDH gene expression (fig. 24.F), while the RalDH expressing lMAN, as already mentioned, showed RalDH protein enrichment in somata (fig. 24.G). Again, the sources of RalDH in Area X could be both lMAN and HVC, as both nuclei send axonal projections to Area X and thus may provide it with RalDH enzyme. In the case of Area X, however, RalDH positive fiber tracts could not be detected by eye (fig. 24.F), while some clearly RalDH negative fiber tracts entering Area X from dorsally seemed to originate in lMAN (fig. 24.F), but we cannot be certain about their origin, and how representative they are for other fiber tracts running in parallel. But more importantly, it has been reported that projection neurons from HVC to Area X are positive for RalDH mRNA (Denisenko-Nehrbass et al., 2000) and could therefore provide Area X with the enzyme. While it is not known whether the LMAN>Area X projection neurons are positive for RalDH *transcript*, we showed them here to contain RalDH *enzyme*: They are the very same neurons which send projections to nucleus RA (Vates and Nottebohm, 1995) and were positively RalDH immunolabeled in our experiments (see fig. 25). This opens up the possibility for lMAN as well to provide Area X with RalDH protein.

We would like to point out that RalDH protein distribution over the entire brain roughly matched RAc distribution. Exceptions were few RAc positive and RalDH negative regions like the anterior mesopallium close to the lMAN area. However, all such regions were close to RalDH expressing regions, so that diffusion can account for the RAc detected there. In the rest of the brain, RAc presence closely matched RalDH enzyme distribution, leading us to the

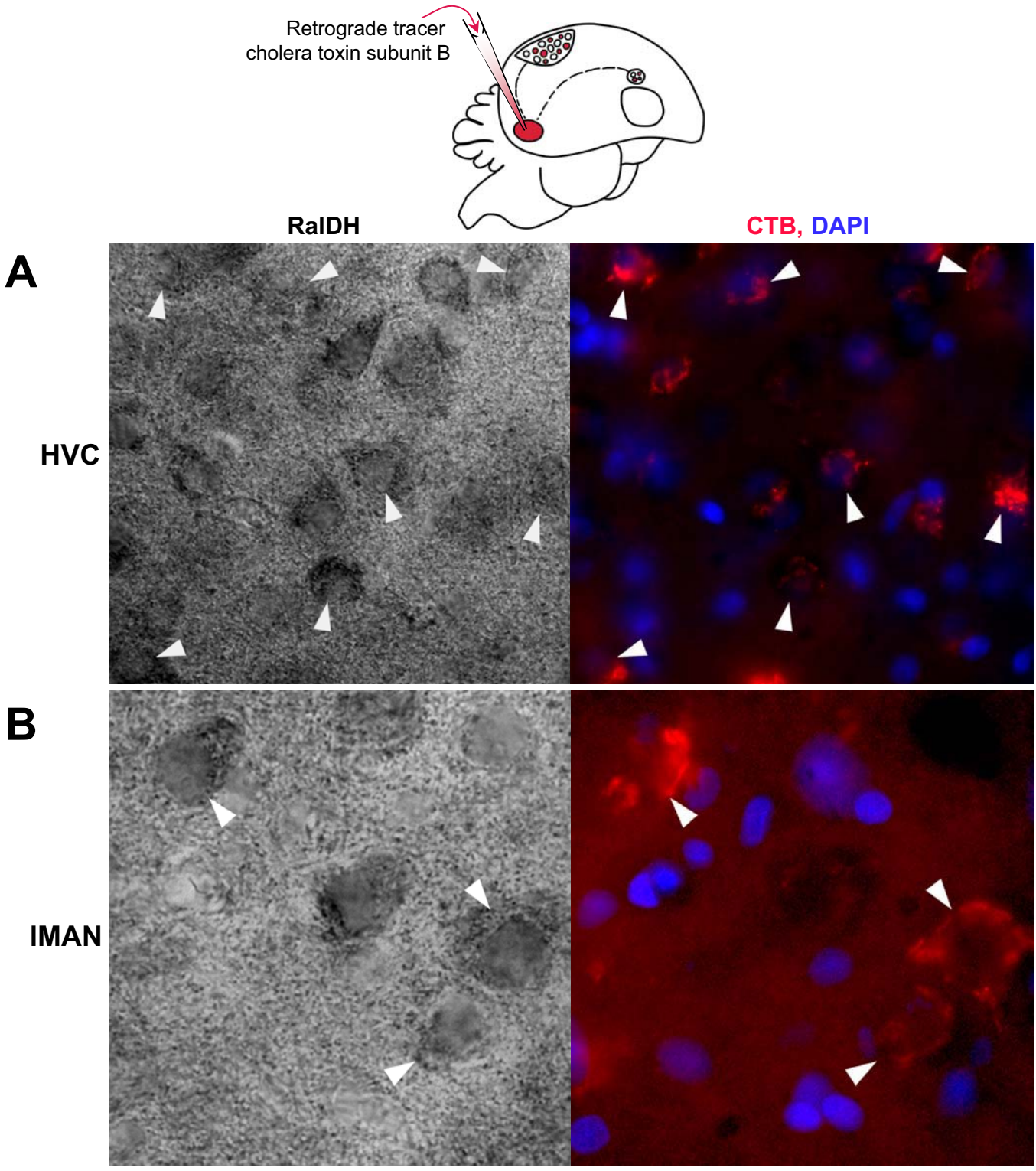


**Figure 25: RalDH protein is found in RA projecting neurons of song nuclei HVC and lMAN.** Schematic drawing on top illustrates how HVC>RA projecting neurons were labeled with the retrograde tracer cholera toxin subunit B (CTB). **A and B:** High power views of HVC (**A**) and lMAN (**B**) of RalDH-immunolabeled brain sections from a bird that received an RA backfill with CTB. RA projecting neurons from HVC and lMAN are CTB labeled. Left photos show bright field views of immunolabeling for RalDH protein (stained with DAB and contrast enhanced). Right photos show the according CTB signal (red) and cell nuclei stained with DAPI (blue) of the area shown left. In both song nuclei, HVC and lMAN, RalDH protein is found in RA projection neurons with CTB labeling (see arrows).

assumptions that 1) RAc distribution is fully accounted for by RalDH enzyme transport over long distances plus RAc diffusion over shorter distances. 2) No additional retinaldehyde dehydrogenase is required to produce this RAc distribution in the zebra finch brain. For matching of RAc and RalDH enzyme presence in the song control regions, compare fig. 20 and fig. 22; for a region outside the song system, see supplementary fig. 11 as an example, showing RalDH enzyme in the striatum surrounding Area X (for further RAc positive regions outside the song system, data is not shown).

### *3.10 Preliminary data from lesion experiments to identify RalDH input sites to Area X and RA*

A way to determine which one of the two candidate nuclei, HVC or lMAN, is responsible for RalDH supply to Area X and RA, is to lesion these nuclei and then determine resulting RalDH protein or RAc amount in Area X and RA. We started such lesion experiments and removed HVC unilaterally in different animals. We assumed that it might take some time after lesioning to be sure that RalDH protein in synaptic endings of the damaged cells would be depleted. We therefore waited 14 days before killing the animals. Subsequently, brains were cut and immunolabeled for RalDH, or used for RAc reporter cell assay, to determine the amounts of RalDH protein and RAc in Area X and lMAN. In the following section, we present preliminary data from HVC lesion experiments. Complementing HVC lesions, lMAN lesions are necessary to determine to what extent either source nucleus influences RalDH/RAc in target nuclei RA and Area X. Excitotoxic lesions with ibotenic acid have been used to lesion lMAN (see e.g. Akutagawa and Konishi, 1994, Kubikova et al., 2007) and are supposed to strongly and specifically affect glutamatergic neurons while preserving other cells or fibers of passage. We are currently working on lMAN lesion experiments using ibotenic acid (see below).

First HVC lesion experiments performed in adult zebra finches showed that in the absence of axonal input from HVC, RAc was strongly reduced in song nucleus RA (fig. 26. B and C). The result was comparable in a bird where, instead of removing HVC, the fiber tracts from HVC to RA were cut with a knife (fig. 26.D and E). Even though the reporter assay provides no strictly quantitative information about regional amounts of RAc, it can be inferred from the very weak LacZ labeling in reporter cells beneath RA that the remaining amount of RAc after HVC removal was very low to non-detectable (fig. 26.C and E, see arrowheads). This seems

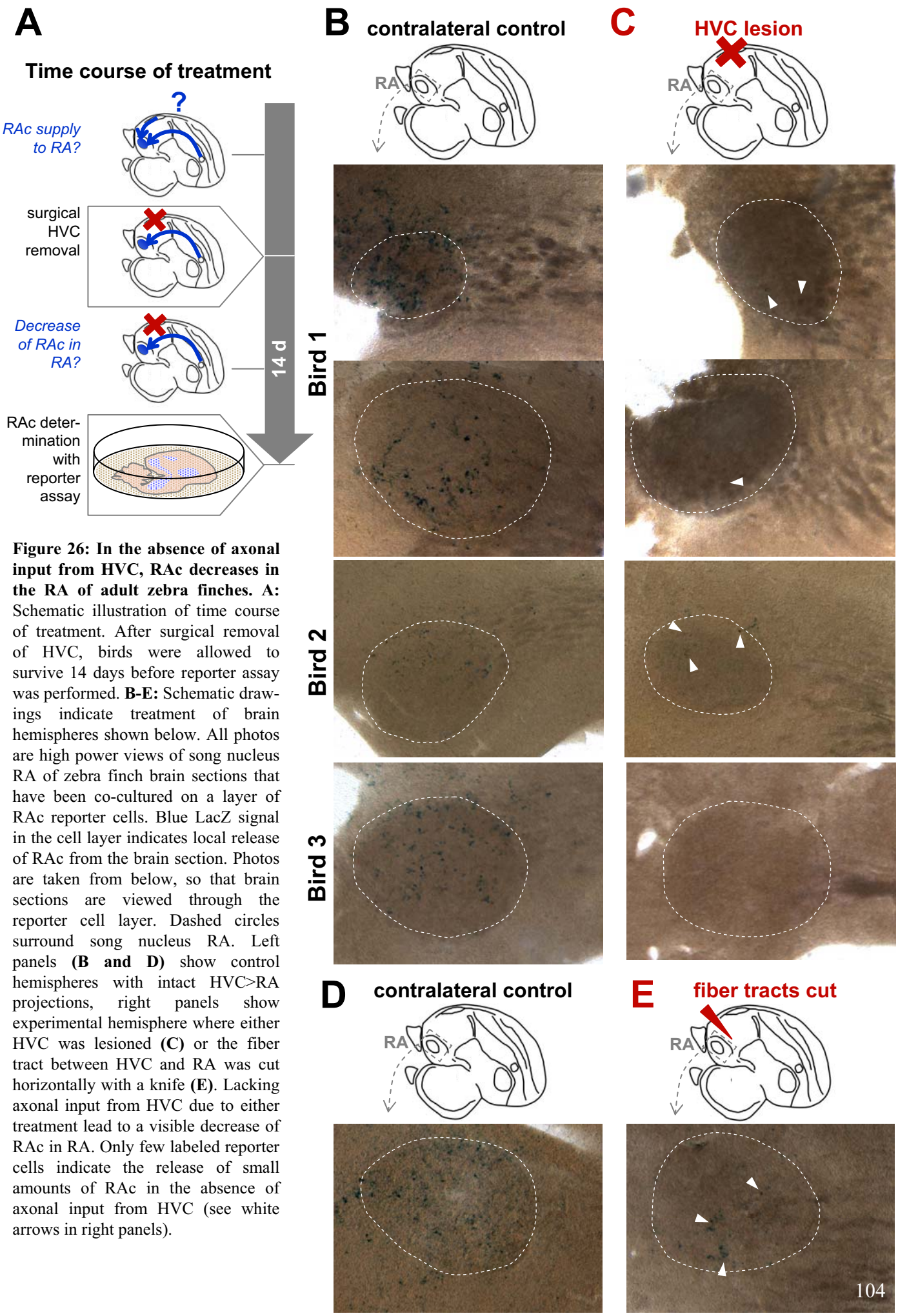


**Figure 26: In the absence of axonal input from HVC, RAc decreases in the RA of adult zebra finches. A:** Schematic illustration of time course of treatment. After surgical removal of HVC, birds were allowed to survive 14 days before reporter assay was performed. **B-E:** Schematic drawings indicate treatment of brain hemispheres shown below. All photos are high power views of song nucleus RA of zebra finch brain sections that have been co-cultured on a layer of RAc reporter cells. Blue LacZ signal in the cell layer indicates local release of RAc from the brain section. Photos are taken from below, so that brain sections are viewed through the reporter cell layer. Dashed circles surround song nucleus RA. Left panels **(B and D)** show control hemispheres with intact HVC>RA projections, right panels show experimental hemisphere where either HVC was lesioned **(C)** or the fiber tract between HVC and RA was cut horizontally with a knife **(E)**. Lacking axonal input from HVC due to either treatment lead to a visible decrease of RAc in RA. Only few labeled reporter cells indicate the release of small amounts of RAc in the absence of axonal input from HVC (see white arrows in right panels).

to suggest that HVC was responsible for most, if not all, RalDH and RAc in RA. However, we cannot exclude with absolute certainty that we underestimate here the amount of RalDH that comes from lMAN: Both surgeries, HVC removal and knife-cutting of fiber tracts, might possibly affect axon bundles from lMAN to RA, too (Akutagawa and Konishi, 1994). These fibers traverse very close to HVC on their pathway to RA and may have been partly damaged while HVC was removed (Mooney, 1992). It is therefore necessary to complete these data with RalDH immunohistochemical experiments on HVC lesioned birds, because immunohistochemistry can (in contrast to the reporter assay) be combined with RA backfills with a retrograde tracer. If done at the same time as HVC removal, the tracer should be retrogradely transported to lMAN if and only if its axonal fibers are still intact. Alternatively, HVC might be lesioned with ibotenic acid which is thought to leave fibers of passage intact (Schwarcz et al., 1979).

HVC lesions in two juvenile birds (PHD 39) with subsequent immunolabeling for RalDH protein revealed that the juvenile Area X receives differential RalDH input (fig. 27): While RalDH in medial Area X was not reduced in the HVC lesioned hemisphere as compared to the contralateral control hemisphere, the lateral part of Area X received less RalDH protein. We conclude that lateral Area X receives its RalDH protein input from HVC, while the medial Area X must have other sources, perhaps song nucleus lMAN.

These results should be completed with more HVC lesion experiments followed by RalDH immunohistochemistry in adult birds, to see whether they show the same pattern of differential RalDH input to Area X as juveniles; as well as lMAN lesion experiments, to determine lMAN's share in RalDH protein supply to RA or Area X. We have started to lesion lMAN with ibotenic acid as has been done by several investigators (Herrmann and Arnold, 1991, Akutagawa and Konishi, 1994, Foster and Bottjer, 2001, Kubikova et al., 2007), but met a surprising problem: Although lesions were on target (as visualized by the rhodamine beads of which we added a small amount (1:5) to the ibotenic acid), most if not all neurons in lMAN remained intact, as we could see by immunolabeling for the neuronal marker Hu and by intact backfill of the lMAN>RA projection neurons with green fluorescent beads (supplementary fig. 12). It is unlikely that this was due to non-functional ibotenic acid, because large areas *around* lMAN were readily damaged. We have no explanation so far why ibotenic acid failed to lesion lMAN neurons. A different excitotoxin, such as glycine or glutamate, could be tested in future experiments.

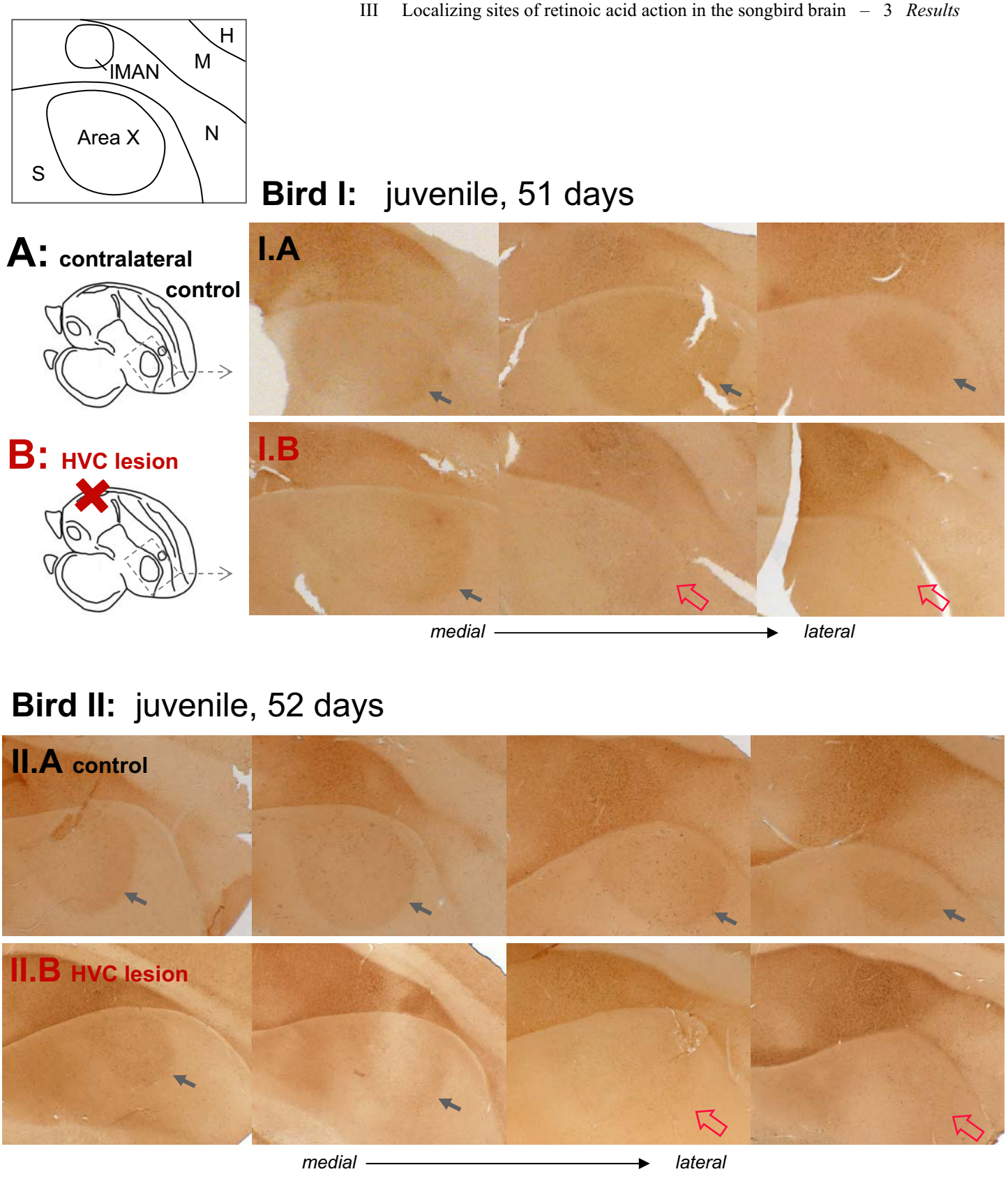


**Figure 27: RalDH protein in lateral Area X is provided by song nucleus HVC In juvenile male zebra finches.** Schematic drawing on top depicts the rostral brain region comprising song nuclei Area X and lMAN, approximately corresponding to the photos shown below. Photos are detail views of brain sections from two different juvenile birds, I and II, immunolabeled for RalDH protein and stained with DAB. HVC was removed in hemisphere B of either bird; hemisphere A served as a control, as indicated by schematic drawings. Photos are arranged according to their mediolateral level (left to right = medial to lateral). In both birds, HVC removal resulted in reduced RalDH protein level in lateral Area X (red arrow contours), in comparison to Area X of the control hemisphere (gray arrows).

# 4 Discussion

In the previous chapter, we described the considerable discrepancy between the spatially restricted sites of RalDH expression on one hand and the widespread sites of potential RAc action as determined by expression of the retinoid related receptors on the other hand. This discrepancy raised the question of whether RAc signaling via retinoid related receptors may actually take place in regions far away from the RalDH expression sites. To address this question, we first cloned the zebra finch homologues of the RAc hydroxylating cytochromes CYP26A1, B1, and C1, and determined their expression patterns in the brain, considering the possibility that these RAc degrading enzymes might – dependent on their brain distribution – narrow down the broad areas of potential RAc signaling as suggested by receptor distribution. This is the case in the vertebrate embryo, where the cytochromes create local RAc sinks or gradients that contribute to the normal course of pattern formation (review by Boncinelli et al., 1991, Reijntjes et al., 2004, Hernandez et al., 2007, Maden, 2007, review by White et al., 2007). However, our results in this respect were mixed: The conspicuous expression pattern of CYP26B1 did indeed suggest for some (and in the case of CM, potentially song relevant) regions to be sinks of RAc, but overall CYP26B1 expression was very sparse so that most receptor expressing regions in the brain remained unconcerned and thus still qualified as potential targets of RAc signaling, even though being far from RalDH expression. The distribution of CYP26C1 transcript was entirely inconclusive with respect to the identification of local potential RAc sinks: Its ubiquitous, albeit low, expression suggests at best a role in general limitation of RAc availability. While in principle there are also the possibilities that CYP26C1 serves to completely break down RAc in the brain, or to create local RAc sinks – e.g. through differential translation of the CYP26C1 mRNA into protein, or differential subcellular localization – we do not see strong evidence for either of these scenarios: The distribution of RAc itself eventually determined through the reporter cell assay could be accounted for by RalDH enzyme distribution; it did not imply the presence of a further RAc sink creating agent (and was not indicative of a general RAc breakdown, either). CYP26A1 is no candidate to create RAc sinks in the posthatch bird telencephalon either since it turned out to be basically not expressed in the brain at all. In sum, determining expression patterns of RAc hydroxylating cytochromes did not resolve the discrepancy between spatially restricted RalDH and widespread RAc receptor expression, although it unexpectedly identified the higher auditory region CM as an interesting target of RAc signaling (see below).

Since the identification of RAc responsive regions was still uncertain after the identification of the expression patterns of the different RAc pathway molecules (i.e., RalDH, receptors and degrading enzymes), we aimed at localizing RAc itself in the brain. We show here that a RAc sensitive reporter cell assay, a method which has up to now only been used to detect RAc in small tissue samples (Wagner et al., 1992), was suited to detect RAc in entire zebra finch brain sections with high local specificity. It allowed for an at least coarse quantitative estimation of local RAc content. To our knowledge, this is the first time that RAc distribution has been visualized in entire brain sections of a non-transgenic animal. Using this assay, we not only detected RAc in all regions of RalDH gene transcription, as expected, but also to considerable amounts in other brain areas that were in part surprisingly far from RalDH expression sites. Interestingly, two areas of particularly high RAc presence were the RalDH transcript-negative song nuclei Area X and RA. Both nuclei have previously been shown to express different combinations of retinoid receptor genes (Jeong et al., 2005, and data presented in Chapter II), so that conditions are met for RAc signaling to take place in these regions. Using the RAc reporter assay, we thus found that RAc distribution in the zebra finch brain mediates between the narrow RalDH expression sites and the widespread receptor distribution.

Moreover, we detected that RAc presence in the song nuclei Area X and RA can be accounted for by transport of RalDH enzyme to these areas via afferent axonal projections: Immunohistochemical localization of RalDH enzyme was consistent with nuclei RA and Area X being provided with RalDH enzyme via axonal projections from RalDH expressing nuclei HVC and/or lMAN. We show here that lMAN>RA and HVC>RA projection neurons contained RalDH enzyme which they may have transported to RA. However, we have not investigated directly whether lMAN>Area X and HVC>Area X projection neurons are positive for RalDH protein, and therefore have to speculate about how Area X obtains its RalDH enzyme. We consider the most likely scenario that Area X, like RA, receives RalDH enzyme from both HVC and lMAN, since HVC>Area X projection neurons are known to be positive for RalDH *transcript* (Denisenko-Nehrbass et al., 2000). lMAN>*RA* projection neurons which we found to be positive for RalDH *protein* are the same neurons that project to Area X (Vates & Nottebohm, 1995). Therefore, conditions are met for Area X, too, to receive RalDH protein from both HVC and lMAN. Moreover, preliminary results from lesion experiments in juvenile birds suggested that *medial* Area X received RalDH enzyme mainly from HVC, while *lateral* Area X received it mainly from a different source, most likely lMAN.

We conclude that the RAc we found in Area X and RA was not (or, at best, only partly) being transported to these areas, but rather produced there directly by RalDH enzyme that has been transported there from HVC and/or lMAN.[4] Axonal transport of RalDH enzyme has also been reported to take place in the postnatal mouse nervous system, where RalDH 1 is transported from the ventral tegmentum to ventral telencephalic sites, and RalDH 3 undergoes axonal transport along the auditory nerve during early postnatal development (Wagner et al., 2002). Our data indicate that RAc distribution in the song control and auditory system can be fully explained by anterograde transport of the known zebra finch RalDH enzyme (which is homologous to the mammalian RALDH2; Denisenko-Nehrbass et al., (2000)). It is therefore not necessary to assume the existence of another RalDH in the song system, confirming the findings of Denisenko-Nehrbass & Mello (2001). Moreover, this seems to apply to the remaining telencephalic regions as well: No significant amounts of RAc were detected in regions that were devoid of RalDH enzyme and *not* at the same time in the likely reach of diffusing RAc from RalDH enzyme positive adjacent regions.[5]

Anterograde enzyme transport within projection neurons has been observed for different neurotrophins (see also review by von Bartheld et al., 2001): In the songbird brain, BDNF and NT-3 reach RA from lMAN's RA-projecting neurons (Johnson et al., 1997); the rat olivary-cerebellar system shows a similar kind of afferent transport of IGF-I (Nieto-Bona et al., 1993); chicken retinal ganglion cells anterogradely transport BDNF and NT-3 to the midbrain tectum/superior colliculus (von Bartheld and Butowt, 2000, Butowt and von Bartheld, 2005, Butowt and von Bartheld, 2007), and especially BDNF has also been described to undergo such transport in different other rodent neurons, such as hippocampal, noradrenergic, or cultured cortical neurons (Altar et al., 1997, review by Altar and DiStefano, 1998, Fawcett et al., 1998, Mowla et al., 1999, Kohara et al., 2001). Thus, anterograde transport over distances seems to be a common mechanism in the vertebrate brain to coordinate growth, plasticity, or cell survival between different regions. Although not being implicated in such a context so

[4] Besides, our and others' (e.g., Wagner et al., 2002) findings of long range *RalDH enzyme transport* along axonal projections (in contrast to RAc being transported itself) put into perspective the hypothesis that cellular retinoic acid binding proteins (CRABPs) play a main role in long range retinoid signaling in the nervous system (see for instance McCaffery et al., 2006). Both mechanisms, RAc transport by CRABPs as well as RalDH enzyme transport, may of course exist at the same time. However, our data indicate that in the bird brain, CRABPs are not necessary to explain the actual RAc distribution, which is largely accounted for by RalDH enzyme transport.

[5] An example is the RAc-rich anterior mesopallium which is negative for RalDH enzyme, but very close to RalDH positive anterior nido- and hyperpallium and therefore probably in the range of diffusing RAc.

far, the RAc/RalDH distribution in the bird brain suggests that this could apply to retinoid signaling, too.

We would like to point out that detecting RalDH enzyme in the HVC>RA projection neurons was unexpected, as these neurons have been shown to not transcribe RalDH mRNA (Denisenko-Nehrbass et al., 2000). RalDH transcript within HVC is only reported for the Area X-projecting neurons (ibid.), which raises the question of how RalDH enzyme came into the RA-projecting cells. One possibility is that Area X-projecting neurons provide the RA-projecting neurons with the enzyme, perhaps via the gap junctions that connect the clustered somata of HVC neurons (Burd and Nottebohm, 1985, Gahr and Garcia-Segura, 1996). A particularly interesting finding in this context is that RAc regulates the expression of connexins, the transmembrane proteins that make up gap junctions, in endometrial stromal cells and liver carcinoma cell lines (Liu et al., 2005, Tanmahasamut and Sidell, 2005). If RAc activates connexin expression in the songbird HVC, it could thereby regulate the transfer of other neurotrophic substances between HVC cells, besides the transfer of its own synthesizing enzyme RalDH. Another possible, albeit more remote source of RalDH enzyme in RA-projecting neurons might be the mMAN neurons that send projections to HVC, as RalDH transcript seems to be present in the entire MAN (Denisenko-Nehrbass et al., 2000, Kim and Arnold, 2005). The very same distribution of mRNA and peptide in the songbird HVC has been described for IGF-II (Holzenberger et al., 1997).

We have started to investigate the origin of RAc in nuclei RA and Area X in more detail with lesion experiments: HVC or lMAN were lesioned to evaluate whether RalDH protein or RAc presence are reduced or depleted in Area X or RA by this treatment. Our preliminary data suggested that 1. HVC provided medial Area X of juvenile birds with RalDH protein, while lateral Area X got its RalDH protein from a different source; and 2. HVC was the major provider of RAc for song nucleus RA in adult birds.

In sum, we showed here that the distributions of RalDH transcript, RalDH protein, and the signaling molecule RAc itself differed to different degrees: The transcript distribution was sparsest, while the enzyme reached a number of large additional regions, of which the most prominent were song nuclei Area X and RA. RAc was found in even slightly broader areas than the protein, including the ventral striatum and the rostral mesopallium. Axonal transport of RalDH enzyme from RalDH expressing regions could account for the broader distribution of RalDH enzyme as compared to its mRNA, and RAc diffusion is the most straightforward explanation for RAc found in regions without RalDH enzyme.

## *4.1 Does retinoid signaling in the song control system and the higher auditory system point to enhanced plasticity?*

RAc being a signaling molecule which induces complex plastic processes in the embryo, it has been claimed to mark sites of enhanced plasticity in the adult nervous system as well. Indeed, this is the case for the mouse olfactory bulb, spinal cord, or hippocampus (Thompson Haskell et al., 2002, Wagner et al., 2006), and for cortical areas with high local expression of plasticity-related genes (Wagner et al., 2006). RAc has been implicated with a number of plasticity-related functions in the adult brain, such as hippocampal long term potentiation and depression (Chiang et al., 1998, Etchamendy et al., 2001, Misner et al., 2001), dendritic spine formation (Chen and Napoli, 2008), neurogenesis (Crandall et al., 2004, Jacobs et al., 2006), as well as with hippocampal memory formation (Etchamendy et al., 2001, Cocco et al., 2002, Etchamendy et al., 2003, Dopheide and Morgan, 2008, Mingaud et al., 2008).

Retinoid signaling seems to be even more prevalent in the adult songbird brain than in the rodent brain: In rodents, it is restricted to the hippocampus, midbrain dopamine centers and their striatal targets, and the olfactory bulb, while the songbird brain additionally shows persistent retinoid signaling in all parts of its large telencephalic song system. We show here that the song control system is actually the most prominent site of adult RalDH and RAc presence, and that extensive RalDH enzyme trafficking takes place within this system. In addition, parts of the higher auditory system seem to be qualified for spatially fine-tuned retinoid signaling. As claimed for the rodent brain, RAc may regulate plasticity related processes in the adult songbird brain as well. Zebra finches learn their song as juveniles and do not alter it significantly as adults, but nevertheless, many observations suggest that neuronal plasticity continues to play a role after song learning is completed. On one hand, adult zebra finches are under normal conditions still able to modify their song in subtle ways (Helekar et al., 2003, Funabiki and Funabiki, 2008), and on the other hand, experimental evidence suggests that the *maintenance* of the adult song is not a static state, but rather depends on complex dynamic processes (Nordeen and Nordeen, 1992, Williams and McKibben, 1992, Leonardo and Konishi, 1999, Pytte and Suthers, 2000, Woolley, 2004 for a review on auditory feedback for song maintenance). It may include the continuous ability to use trial-to-trial variability in song output to dynamically optimize performance (Tumer and Brainard, 2007). RAc signaling seems to take place in all those telencephalic brain areas that

are potentially crucial for the dynamic maintenance of song, i.e. HVC and RA of the premotor pathway, Area X and lMAN of the AFP, as well as the higher auditory areas.

The striking occurrence of long-range RalDH trafficking to synaptic terminals within the song system suggests that RAc is suited to induce plasticity with high local specificity: It could regulate growth processes directly in its target synaptic terminals, as well as in adjacent dendrites of postsynaptic cells, if released, or diffusing, from the presynaptic sites. This would require non-transcriptional mechanisms of RAc action. They might include stabilization of mRNAs locally stored at synapses, or translational control of stored mRNAs. RAc has actually been implicated in both mechanisms (Busam et al., 1993, Zhou et al., 1994, Chen and Napoli, 2008). These and other mechanisms of RAc actions will be discussed in more detail in the in the following, with respect to the different regions of the bird brain where we found RAc. Possible roles for RAc in these regions will be focused, particularly with regard to adult plasticity in the context of dynamic song maintenance.

## *4.2 HVC and lMAN could use RAc to "telecommand" connectivity within RA, to equilibrate variability and stereotypy of song*

In the juvenile zebra finch, the AFP seems to "inject" highly variable, exploratory activity patterns via lMAN into the premotor pathway, leading to the high variability that characterizes the exploratory, babbling-like song output during early learning phases (Olveczky et al., 2005, Aronov et al., 2008). Although the adult birds' crystallized song is stable and highly stereotyped, the variability-injecting mechanism apparently persists into adulthood, where it is responsible for the subtle variability differences between directed and undirected song output, undirected song being slightly more variable (Kao et al., 2005, Kao and Brainard, 2006). The influence of this mechanism seems to be lower on adult as compared to juvenile song because variable input from lMAN to RA gets largely outweighed by the stereotyped input patterns from HVC to RA which slowly take over during song development (Thompson and Johnson, 2007, Thompson et al., 2007, Aronov et al., 2008). Successful imitation of the tutor's song patterns by the juvenile bird is crucially dependent on the exploratory lMAN-driven pattern production: Preventing birds from exploratory singing by early lMAN lesions leads to stereotyped, prematurely crystallized song which is much simpler than the target song (Scharff and Nottebohm, 1991). Maintenance of the adult song might in a similar way still require lMAN-dependent exploration to some extent, for constant dynamical re-adjustment of the motor output, albeit probably less than in the juvenile.

Interestingly, the RAc distribution we found in the song system would be perfectly consistent with a role for balancing pathway dominance: Which pathway is dominant on song output – the "exploratory" AFP or the "stereotyped" premotor pathway – should depend, at least in part, on synaptic connectivity of HVC/lMAN>RA projections neurons within RA (Thompson and Johnson, 2007, Thompson et al., 2007, Aronov et al., 2008). We found that both lMAN and HVC may send RalDH enzyme to their presynaptic endings in nucleus RA, where RAc could locally regulate connectivity (for possible mechanisms of synapse modification by RAc, see next section). This way, the nuclei lMAN and HVC would, by controlling trophic input they provide to nucleus RA, regulate their own influence on the target nucleus. Preliminary data we present here suggests that RalDH enzyme in lMAN decreases with age, which would be in line with the observation that song becomes increasingly stereotyped in older animals, even after song acquisition is completed (Pytte et al., 2007): Trophic input from lMAN to RA being taken away, the exploratory influence of lMAN on song might decrease, while the share of stereotyped input from HVC increases. Whether this scenario is actually true for RAc in the lMAN/HVC>RA system could be tested with a blocking experiment: Blocking of RalDH (e.g. using disulfiram, as Denisenko-Nehrbass et al., 2000) in lMAN of a juvenile bird should then result in premature crystallization of the song.

### *4.3 How RAc might mechanistically influence synaptic connectivity within nucleus RA*

Possible mechanisms by which RAc, brought to RA via HVC/lMAN>RA axonal projections, might influence synaptic connectivity can be numerous, due to RAc's ability to cross membranes.

They may first include autocrine non-genomic processes within the presynapse of the respective RAc producing cell. RAc can locally induce neurotransmitter release by opening intracellular $Ca^{2+}$ stores (which might ultimately lead to strengthening of the synapse), which has been shown to be the case at the developing neuromuscular synapse in Xenopus (Liao et al., 2004, Liou et al., 2005).

Such a non-genomic, local mechanism of RAc action might also be used in a paracrine way to alter synaptic connectivity of the *other* pathway's projections. Indeed, lMAN projections onto RA have been shown to influence synaptic connectivity of the descending HVC>RA inputs in juvenile birds: lMAN lesions lead to a sudden numerical *decrease* of HVC>RA synapses, along with a *strengthening* of their excitatory transmission (Kittelberger and Mooney, 1999). Interestingly, synaptic terminals of the HVC>RA neurons contain protein kinase C (PKC;

Watanabe et al., 2002, Watanabe et al., 2006), a molecule involved in synaptic plasticity and a direct target for RAc (Radominska-Pandya et al., 2000, Ochoa et al., 2003). Taken together, this could allow for a local, paracrine effect of RAc released from lMAN>RA presynapses on HVC>RA synapses via their PKC. Strikingly, PKC content in the HVC>RA nerve terminals increases in parallel to song alterations that take place after deafening (Watanabe et al., 2006). In addition, an intact LMAN is required for the deafening-induced song changes to take place (Williams and Mehta, 1999, Scott et al., 2007)! Taken together, RAc released from lMAN>RA terminals is a conceivable molecular candidate to mediate the deafening-induced song change, by entering HVC>RA cells and locally altering their synapses via their PKC. It would be interesting to test this hypothesis experimentally: It must be true if blocking of RalDH in the HVC>lMAN cells is enough to abolish the deafening-induced song destabilization – as does lesioning of lMAN. Another possible effect of RAc could be tested with the same experiment (blocking RalDH activity in lMAN): RAc might be responsible for the above described decrease of HVC>RA synapses in lMAN-lesioned brains (Kittelberger and Mooney, 1999). If so, blocking RalDH activity within lMAN should have the same effect as lesioning lMAN.

RAc brought to RA from HVC or lMAN might alternatively influence connectivity by targeting the postsynaptic RA cells in a transcription-dependent way. In a paracrine manner, RAc would diffuse out of its synaptic terminals of origin and enter RA cells, where it could regulate plasticity-related gene transcription via retinoid receptors, which are continuously expressed within RA (Jeong et al., 2005, and data presented here, Chapter II). Alternatively, RAc could induce the formation of postsynaptic dendritic spines in RA cells through another unconventional, extremely rapid, non-genomic mechanism which has been reported recently to take place in mouse hippocampal cells (Chen and Napoli, 2008, Chen et al., 2008): Via a membrane-associated (!) RARα, RAc enhances dendritic translation (but not transcription) of locally stored mRNA, e.g. for GluR1, an AMPA receptor subunit. AMPA receptors are incorporated into the synaptic compartment and contribute to the formation and maintenance of stable spines, a process which is detectable within minutes. This mechanism allows for rapid and local *translational* control through retinoid receptors. Whether there is RARα protein associated with synaptic terminals of HVC>RA projections is a question left for future research. Yet another non-transcriptional effect of RAc that may affect structure and stability of presynaptic terminals of the HVC/lMAN>RA projections might be the stabilization of mRNAs (Busam et al., 1993, Zhou et al., 1994) of possibly plasticity related molecules.

Finally, RAc could of course affect connectivity within RA in the "classical" transcriptional way by regulating plasticity-related target gene expression[6] within its cells of origin, i.e. in the somata of RA projecting neurons in HVC and lMAN. This would not require the RalDH transport to the synaptic terminals that we assumed here, but if it was to affect their connectivity, the products of gene transcription and/or translation would need to be transported to the synapses. As shown previously (Jeong et al., 2005) and by our data (see Chapter II), retinoid receptors to mediate RAc controlled transcription are present in both HVC and lMAN, so that transcriptional effects of RAc can be expected to take place here. HVC is a site of particular interest for such local RAc signaling in the context of plasticity, because its HVC>RA projection neurons undergo permanent replacement, even in adulthood. A growing body of evidence suggests that RAc might be implicated in the regulation of ongoing neurogenesis, in axonal outgrowth (which is crucial for newly incorporated HVC>RA neurons to become functional), and even in electric coupling of cells (which has been suspected to help new HVC>RA neurons to "learn" from neighboring cells when to fire). These possibilities related to HVC neurogenesis will be discussed more thoroughly in Chapter IV.

The variety of mechanisms through which RAc could act on the (re)construction of synapses – autocrine as well as paracrine; local non-genomic as opposed to transcriptional, PKC-dependent versus independent mechanisms – might allow for highly specific effects for RA's different subcompartments, the synaptic terminals of lMAN>RA and HVC>RA cells and RA's own neurons, respectively. Thereby, differential control of relative HVC and lMAN input strength to RA by RAc is conceivable.

### *4.4 Complex RAc signaling in the higher auditory system of the zebra finch brain*

The CYP26B1 expression pattern (together with the RalDH expression pattern) revealed that the higher auditory regions CMM/CLM and NCM may be targets of complex RAc signaling. (Besides, CYP26B1 is, to our knowledge, the first molecular marker for the avian CM.) The gradient-like expression patterns of CYP26B1 in CM and of RalDH in NCM suggest the formation of a ventro-dorsal RAc gradient in CM, and of an antero-posterior RAc gradient in NCM. To our knowledge, no similar gradients of RalDH, RAc, or RAc degrading enzymes

[6] These target genes might for instance include neurogranin (RC3), neuromodulin (GAP43), or ionotropic glutamate receptors, which have all been shown to be regulated by RAc (Iniguez et al., 1994, Etchamendy et al., 2003, Bain et al., 1996).

have been described to date for adult mammalian brains. But interestingly, retinoid signaling might play a role for the early postnatal development of the mouse auditory cortex which is thought to functionally correspond to songbird CM and NCM: RalDH 3 is transiently upregulated in this region during an early postnatal phase (Wagner et al., 2002, Wagner et al., 2006). Whether or not the mouse CYP26B1 is also subject to regulation at this site and time window is not known. In the *adult* auditory mouse cortex, neither CYP26B1 nor any of the RalDHs seem to be expressed, nor any of the other RAc degrading CYP26s (see Wagner et al. (2002), and Allen Brain Atlas; http://mouse.brain-map.org), which is thus in contrast to the adult bird's higher auditory areas. CYP26B1 expression in the adult mouse brain is limited to the amygdala, parts of the hippocampal formation, and the adjacent subiculum (see Allen Brain Atlas), and thus differs greatly from the avian CYP26B1 expression we found.

### *4.4.1 A role for RAc in prolonged auditory plasticity in birds?*

Could the prolonged RalDH expression in zebra finches' higher auditory areas, as opposed to their rodent counterparts, point to prolonged auditory plasticity in birds (since the plasticity related role of RAc signaling in adult brains has been so strongly emphasized; see section 4.1)? While expression of the immediate early gene ZENK induced by auditory song exposure in adult zebra finches' higher auditory areas is long known and suggests that plasticity related processes may take place there (Mello et al., 1992, Mello and Clayton, 1994, Mello and Clayton, 1995, Vignal et al., 2005), a very recent study is the first one to prove adult neuronal plasticity directly within these areas using electrophysiology (Terleph et al., 2008): "Tuning" of NCM neurons – i.e. the sound frequencies they respond best to – is shown here to be influenced by recent auditory experience. Zebra finches housed with canaries for a certain time, and vice versa, developed an NCM tuning than clearly differed to that of control birds who had been housed with conspecifics. These results suggest that by changing spectral tuning, the adult songbird's auditory system may rapidly adjust when adapting to novel acoustic or social environments. Adaptation of auditory tuning might be the basis for a recently observed natural modification in adult zebra finch song production, consisting in a slight, gradual "drifting" of syllables when birds are housed with cagemates who sing different song syllables (Funabiki and Funabiki, 2008). Another form of (experimentally induced) adult song modification might also depend on this NCM plasticity: Adult Bengalese finches which have been transiently deafened are able to acquire new song material from other birds during their phase of auditory recovery (Woolley and Rubel, 2002). Interestingly, this could not be confirmed for adult zebra finches (Zevin et al., 2004). It would be interesting

to investigate whether this difference between species is correlated with different expression patterns or strengths of RalDH/CYP26B1 in higher auditory areas.

### *4.4.2 Possible role for a RAc gradient in higher auditory areas*

The persistent gradient-like expression of retinoic acid pathway players in higher auditory areas suggests a role of retinoid signaling for higher auditory development and/or function.

What could the role of a RAc gradient in NCM or CM be? The auditory areas NCM and CM receive input from the thalamo-recipient field L2, which is thought to be comparable to the thalamo-recipient layer IV of the mammalian primary auditory cortex. Auditory output coming from CM, NCM, and field L2 reaches other telencephalic areas, such as the shelf beneath HVC and the cup area anterior to RA (Mello et al., 1998). NCM and CMM show high immediate early gene expression after the awake, behaving bird has heard song, and these regions are likely to be involved in song memorization required for song learning and perceptual discrimination (for a review, see Bolhuis and Gahr, 2006).

*Gradients* of signaling molecules are in particular known from embryonic development, where they are fundamental for numerous pattern formation processes, providing cells with positional information and determining different cell fates in a concentration-dependent manner. RAc acts as a graded morphogen in the patterning of the central nervous system of the vertebrate embryo, where it is required to establish the anterior-posterior axis by specifying domains of differential Hox gene expression dependent on its local concentration, and induces neuronal differentiation (Boncinelli et al., 1991, Glover et al., 2006, Maden, 2007, White et al., 2007). Embryonic RAc gradients are formed by the coordinated expression of both RalDH and the RAc degrading cytochromes (Reijntjes et al., 2004, Hernandez et al., 2007, White et al., 2007). Interestingly, the songbird's NCM and CM are tonotopically organized, with higher frequencies being represented more ventrally (Muller and Leppelsack, 1985, Ribeiro et al., 1998, Terleph et al., 2006). An interesting possibility is that a RAc gradient in this region might play a role in establishing, or maintaining, the tonotopically graded tuning of the auditory neurons. However, isofrequency lines have been reported to run as a continuum through NCM, field L, and CM (Muller and Leppelsack, 1985), while the distribution of RalDH and CYP26B1 transcript does not seem to be suited to create an equally homogenous gradient all over this large area, which casts a direct relationship between RAc gradients and frequence tonotopy into doubt. Yet, a number of other auditory features more complex than frequency have been proposed to be coded by NCM and CM neurons, such as

familiarity of song stimuli (Gentner and Margoliash, 2003), recent auditory experience (Sockman et al., 2002), "surprise" (as a measure of how *different* a sound stimulus is from conspecific song as the bird knows it (Gill et al., 2008), categorization of songs into functional classes according to species, individuals, (George et al., 2008), social context of song (directed vs. undirected; Woolley & Doupe (2008)), or according to the sex of the vocalization producer (Avey et al., 2008). These features might be differentially represented across subregions NCM and CM (Gentner et al., 2004, Terleph et al., 2006, Avey et al., 2008, Woolley and Doupe, 2008), and their representation could be subject to adaptive changes, as pointed out above. RAc is a molecular candidate to influence their local representation by concentration-dependent differential gene expression.

This example of a non-developmental RAc gradient may be an interesting target for future reseach. As to our knowledge, this is the first time a retinoid gradient has been claimed for the postnatal brain. The graded distribution of RAc in the songbird's auditory system could relatively easily be experimentally manipulated, e.g. by direct infusion of RAc, to then examine whether disturbing the RAc gradient affects, for instance, electrophysiological properties such as frequency tuning of NCM/CM neurons, or auditory perception as could be measured with behavioral sound discrimination tasks.

### *4.5 A plasticity related role for retinoid signaling in the AFP?*

We showed here that the AFP, similar to the posterior premotor pathway, underwent extensive RalDH trafficking resulting in presence of the enzyme in the large striatal song nucleus Area X, which does not contain RalDH transcript (Denisenko-Nehrbass et al., 2000). Sources of the enzyme are – as for RA – most likely the pallial nuclei HVC and lMAN. It should be noted that this is in contrast to RalDH trafficking in the mouse brain: The mouse striatum also receives high amounts of RalDH enzyme, but their source is not pallial, but the ventral tegmental area (VTA) of the midbrain (Wagner et al., 2002). There is no evidence for RalDH expression in the VTA of birds, so that RalDH transported to the striatal Area X must be of pallial origin.

Area X is a basal ganglia structure that is necessary for normal song development (Sohrabji et al., 1990, Scharff and Nottebohm, 1991): Juvenile birds with Area X lesions maintain high variability in their song output and are not able to develop crystallized song. In the adult bird, Area X has been implicated in the control of social context-dependent song production. Area X shows increased neural firing and activity-dependent gene expression when birds sing, and

the level of activation is higher and more variable during undirected relative to directed song (Jarvis et al., 1998, Hessler and Doupe, 1999), while dopamine levels in Area X are *lower* during undirected relative to directed song (Sasaki et al., 2006). Interestingly, dopamine and RAc act as neuromodulators with complementing roles in the retina (Weiler et al., 2001): While dopamine conveys information about fast changing light conditions, RAc is a slightly slower signal for ambient light conditions, integrating over a certain period of time (in the range of at least several minutes). It has been suggested that both signaling systems need to work together to affect the site-specific formation of spinules in horizontal cells, the retinoid component triggering and the dopamine component stabilizing spinule formation (Dirks et al., 2004). This results in a high density of spinules in the light-adapted retina. In Area X, RAc and dopamine are unlikely to convey signals about light conditions, but their cooperation could be necessary in an analogous way to integrate pallial with midbrain inputs. For instance, spinule formation (or other forms of plasticity) could be induced only under the condition that certain levels of dopamine and RAc and specific activity patterns[7] temporally coincide. Such Area X plasticity might help matching the motor output to memorized tutor song during learning; in the adult bird, it might be a prerequisite for the still existing residual ability to use trial-to-trial variability in song output to dynamically optimize performance (see section 4.1; Tumer & Brainard (2007)). Given that dopamine input into Area X is regulated by social context of singing, an integrated dopamine/RAc plasticity-inducing signaling system could allow for more plasticity induction when the bird sings exploratory undirected song, relative to the more stereotyped, performance-style directed song, dependent on the different dopamine levels. Whether RAc is also regulated in Area X, similar to dopamine, and by which variables it might be, we do not know. Mechanistically, there are different possibilities of regulation – RalDH enzyme transport to the presynaptic terminals might be regulated, or release of RAc itself. In addition, the effects within Area X might be of high local specificity (as observed in the retina; Weiler et al., 2001, Dirks et al., 2004) depending on the source of RalDH – HVC or lMAN – and thus could affect different cell types in different ways.

Spinule formation is just one example of possible RAc effects on plasticity within Area X. As specified for song nucleus RA, there are a number of other mechanisms in which RAc may act; transcriptional as well as non-transcriptional ones (see section 4.3). Complex transcriptional, paracrine effects via nuclear retinoid receptors are very likely to take place within Area X, given the all retinoid receptors are being expressed in this nucleus to either

[7] The activity patterns reaching Area X from HVC are well characterized, and they have been shown to convey integrated auditory-vocal information of song (Prather et al., 2008).

particularly high levels, as RARα, or with particular cell-type specificity, as RXRγ (Jeong et al. (2005) and data presented in Chapter II).

Preliminary data from lesion experiments indicated that in juvenile birds, the sources of RalDH enzyme for the medial and lateral portions of Area X differed. Medial Area X mainly received its RalDH from HVC, while lateral Area X must have had an additional RalDH source, probably lMAN. Only little is known about different functions of the medial and lateral portions of Area X. A gene expression study showed that singing induces the immediate early gene ZENK in a subdivision-specific pattern in Area X, and that social context affects ZENK expression only in lateral Area X (Jarvis et al., 1998). Whether the different origin of RAc in the two subcompartments (medial RAc from HVC, lateral RAc from a different source) could be *causal* for this differential pattern of ZENK expression,[8] or whether ZENK and RAc could act together to regulate plasticity in a subcompartment-specific manner, are interesting questions to be investigated in the future.

In sum, we describe in this chapter that RAc and RalDH enzyme localization in the zebra finch brain revealed the entire telencephalic song system as a target of RAc signaling, and that large-scale RalDH enzyme trafficking took place there. In addition, RalDH and CYP26B1 expression patterns suggest that complex RAc signaling takes place in higher auditory areas. These observations are consistent with RAc playing a major role for vocal plasticity. RAc signaling could be the prerequisite for both juvenile plasticity, which enables the young bird to copy a heard tutor's song, and residual plasticity in the adult bird, which helps him to stably maintain his acquired song.

[8] Evidence for RAc inducing ZENK exists from the human skin and different non-neuronal rodent cell lines (Larsen et al., 1994, Suva et al., 1991, Edwards et al., 1991). How could RAc originating from HVC or lMAN possibly induce differential patterns of ZENK expression? Synaptic terminals of different origin could release RAc to different amounts, or under different conditions. If connectivity of lMAN- versus HVC-originating projection neurons is not uniform across Area X, this could lead to different spatial micro-environments of the synaptic terminals. These different environments could either be promoting or suppressing ZENK expression in the target cells.

# IV A link between retinoic acid and neurogenesis in the zebra finch brain?

## 1 Introduction

In the previous chapters, we have shown that various molecules of the RAc pathway are specifically enriched in the song control system and higher auditory areas of the juvenile and adult zebra finch brain, suggesting that retinoid signaling plays a crucial role for song development as well as adult song. It is unknown to date what retinoid signaling does to the various parts of the adult song control and auditory system. However, we do have some insights into its role in the juvenile HVC: Blocking RalDH locally in HVC impairs song acquisition, resulting in song with unusually variable note and pause duration, poor note morphology, and enhanced syntactic variability (Denisenko-Nehrbass et al., 2000). How can locally decreased RAc signaling lead to such an effect – or, in other words, how does RAc in HVC normally contribute to the acquisition of adult song?

RAc signaling has been implicated with different aspects of plasticity in postdevelopmental brains (see discussion of Chapters II and III), and one particularly prominent form of plasticity characterizing song nucleus HVC is the incorporation of new neurons, which has in other systems been implicated with RAc signaling (see below). The population of HVC>RA projection neurons is constantly replaced throughout the life of the male bird, but turnover rates are highest in the juvenile bird while he is learning his song, and slowing down with age (Kirn and Nottebohm, 1993, Wang et al., 2002). The molecular mechanisms to guide the replacement of this neuronal population are poorly understood. Neurogenesis includes different phases from proliferation, which takes place in the subventricular zone, over migration of the newborn cells to their final site, differentiation to a neuronal phenotype, incorporation into the existing network, and survival of the new neuron (for reviews, see Scharff, 2000, for reviews, see Doetsch and Scharff, 2001). Recruitment of new neurons into song nucleus HVC has been linked with song plasticity (see Nottebohm, 2004 for a review), although so far only correlative data is available: Recruitement rates are highest during the phase of song development; they increase again in adult birds which change their song every year, such as canaries, during seasonal phases of high song instability (Kirn et al., 1994, Alvarez-Buylla and Kirn, 1997). Moreover, species differences in (experimentally induced)

song instability are accompanied by according differences in neurogenesis rates (Scott et al., 2000).

Retinoids play a well established role for embryonic neurogenesis (reviewed by Maden, 2002), and there is growing evidence from rodents that RAc is also involved in the control of postdevelopmental neurogenesis: Besides correlational evidence – the specific pattern of retinoid signaling occurring at sites of adult neurogenesis in the CNS, the olfactory bulb and the hippocampus (Corcoran and Maden, 1999, Thompson Haskell et al., 2002), also causal relationships have been suggested: In retinoid-deficient mice (which have been fed on a vitamin A-depleted diet), hippocampal neurogenesis is impaired in the phase of early differentiation and survival (Jacobs et al., 2006), and in cultured slices, RAc is a potent mitogen for the proliferation of neuroblasts in the mouse subventricular zone, which provides the olfactory bulb with new neurons (Wang et al., 2005). In addition, if combined with environmental enrichment, RAc administration seems to promote rat subventricular zone neurogenesis after induced stroke (Plane et al., 2008). However, the link between RAc and neurogenesis does not remain undisputed – as neither alimentary retinoid deprivation with all its systemic effects nor the above *in vitro* experiments can provide direct proof of speficic and locally confined RAc function *in vivo*. In addition, a recent study reports that the boost in hippocampal neurogenesis induced by physical exercise is *independent* of retinoid receptor activation (Aberg et al., 2008), and the picture gets even more complicated by the findings of Crandall et al. (2004) who report that systemic administration of the retinoid 13-cis-RAc, or Accutane, *decreases* hippocampal neurogenesis (opposing the trend of the above findings) and, along with this, impairs spatial learning when applied in a clinical dose over a prolonged period.

The zebra finch with its specific pattern of RAc production in the nicely accessible song nucleus HVC is a particularly attractive model to investigate the role of RAc for the incorporation of new neurons *in vivo*: RalDH activity can be locally blocked by Disulfiram administration relatively easily in the free ranging animal (Denisenko-Nehrbass et al., 2000), and HVC neurogenesis is well-described (for reviews, see Nottebohm, 2002, Nottebohm, 2004, Wilbrecht and Kirn, 2004).

We aimed at testing the hypothesis that the learning-affecting influence of local RalDH-blocking in HVC, as reported by Denisenko-Nehrbass et al. (2000), is due to disturbed neurogenesis regulation. Incorporation of new neurons into HVC is particularly high during song development and slowing down with age, but the replacement of HVC>RA projection

neurons seems to never completely stop (Alvarez-Buylla et al., 1988, Kirn et al., 1991). A potential role for RAc in HVC neurogenesis control could therefore account for both the observation that blocking RalDH strongly affects juvenile song learning (but not adult song), and the finding that RAc signaling is still prevalent in the the adult brain: While interfering with the high juvenile neurogenesis rates could strongly affect HVC architecture and thereby impair learning, interfering with the lower neurogenesis rates of adult zebra finches might not be enough to detectably affect song performance within the relatively short time span of one month considered by Denisenko-Nehrbass et al. (2000).

To test whether RAc is involved in HVC neurogenesis control, we locally blocked RalDH in HVC and subsequently analyzsed neurogenesis rates. For blocking of RalDH enzymatic activity, we used implants which slowly released the drug disulfiram, similar to the blocking experiment of Denisenko-Nehrbass et al. (2000). If RAc supports the incorporation of new neurons into HVC, blocking RAc signaling should decrease neurogenesis rates, as seems to be the case for the hippocampus of retinoid deprived rodents (Jacobs et al., 2006). As onset age for disulfiram treatment, we chose post-hatch day (PHD) 30-35, the age at which RalDH blocking in HVC affects song development, as shown by Denisenko-Nehrbass et al. (2000) (see fig. 28). At this young age, we would also expect to observe a particularly strong effect on neuronal incorporation into HVC, due to the generally high juvenile neurogenesis rates, if RAc is involved in their control.

Which aspects of neurogenesis do we expect to interfere with, if blocking RalDH in HVC? Proliferation should remain unaffected, as it does not take place in HVC, but in the subventricular zone. The subventricular zone does not express RalDH and is therefore unlikely to be affected by a RalDH blocker in its surroundings. Incorporation into HVC and survival of the newly arriving neurons, however, could be regulated by local RAc signaling in HVC, and should thus be affected by interfering with their molecular control. Incorporation rates into HVC have been shown to be highest around 15 days after labeling with a cell division marker in canaries (Kirn et al., 1999), suggesting that newborn cells need up to two weeks to migrate from their origin in the ventricular wall to HVC. To maximize the expected effect of RalDH blocking on neurogenesis, we chose a time difference of 16 days between labeling of newborn cells with the DNA-synthesis marker BrdU (5'-bromo-2'-deoxyuridine, a thymidine-analog which, if administered systemically, gets incorporated into the DNA of newly synthesized cells) and analysis of new neuron numbers (fig. 28). The onset of disulfiram treatment was placed in the middle between BrdU-injections and analysis, so that

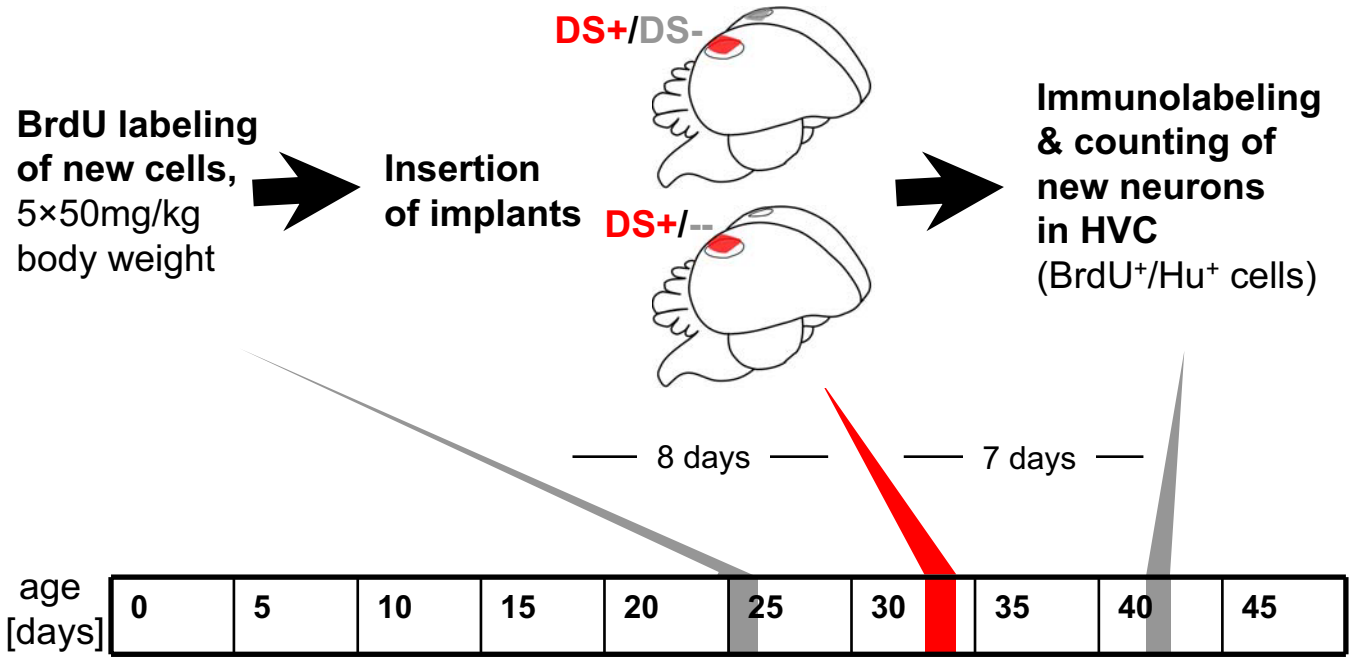


**Figure 28: Time course of treatment.** Juvenile birds were administered 5 injections of BrdU (50mg/kg per intramuscular injection) on a single day. 8 days later, implants releasing the RalDH blocker disulfiram were unilaterally placed on top of song nucleus HVC, the contralateral hemisphere served as control, receiving either an empty implant, symbolized in upper drawing or no implant (lower drawing). After 7 more days, birds were sacrificed, brains were dissected, cut, and immunolabeled for BrdU and the neuronal marker Hu. New neurons (BrdU+/Hu+ cells) were counted in HVC. The time point for implant insertion was chosen as to match the time point when the same treatment was shown to affect learning (PHD 30-35; Denisenko-Nehrbass et al., 2000). The time point for sacrifice (PHD 37-42) was chosen as to be 16 days after BrdU injections (PHD 22-27), which is around the time when the number of new neurons detectable in HVC is highest in canaries (Kirn et al., 1999). Being placed in the middle between BrdU labeling and sacrifice, the disulfiram treatment could affect incorporation and/or survival of new neurons in HVC, but not proliferation or early phases of migration.

disulfiram could affect both incorporation and survival of new neurons in HVC. We applied the disulfiram treatment unilaterally, leaving the other hemisphere as control which either received implants without any drug, or was left intact and did not receive any implant (see fig. 28).

We found that the number of newly incorporated HVC neurons was significantly reduced by blocking RAc production. However, we observed at the same time that in two birds, tissue damage had occurred adjacent to the disulfiram releasing implants. Although damaged tissue parts were excluded from analysis, and the reduced new neuron incorporation upon RalDH blocking was significant for apparently healthy parts of HVC, we cannot be sure that this is actually due to a physiological role of RAc for neuronal recruitment *in vivo.* Even though older neurons in healthy looking tissue were not affected by disulfiram treatment, the apparent toxicity of disulfiram might differentially affect young and old cells, and is therefore an alternative explanation for decreased incorporation that must be considered.

# 2 Materials & Methods

## *2.1 Animals*

Male zebra finches (Taeniopygia guttata) were obtained from our breeding colony at the Free University and the Max Planck Institute for Molecular Genetics, Berlin. Birds are housed in family or group cages in a breeding room with a 12:12h light-dark cycle.

In total, 18 male zebra finches received BrdU injections and disulfiram implants. However, 10 of them had to be discarded because of improper placement of one or both implants (see below).

Two more animals received disulfiram implants unilaterally without prior BrdU injections. The brains of these birds were subjected to RAc detection using the RAc reporter cell assay described in Chapter III, section 2.5, to test for the efficiency of the disulfiram implants.

For age of the birds at the different steps of the treatment, see following sections.

## *2.2 BrdU injections and time schedule of treatment*

New cells were labeled with the DNA synthesis marker 5'-bromo-2'-deoxyuridine (BrdU; Sigma). BrdU was administered intramuscularly (50mg/kg of body weight per injection, dissolved in 0.007 N NaOH solution with 0.9% NaCl). Each bird (22-27 days of age) received 5 BrdU injections in one day (one every 2h). Eight days later, the birds received disulfiram and control implants, respectively (at 30-35 days of age; details see below); after seven more days, they were sacrificed (at 37-42 days of age).

## *2.3 Disulfiram administration in vivo*

Disulfiram was administered locally to HVC by placing a drug-infused implant of the ethylene-vinyl acetate copolymer Elvax on top of the song nucleus.

The implants were prepared 1-6 days before being used. Elvax-40W beads (a gift from DuPont) were washed by immersion in 95% ethanol for at least one week and then dissolved in ten times their volume of methylene chloride (Sigma) in a covered glass tube. The tube was placed in a lukewarm waterbath on top of a gently agitating shaker to accelerate dissolving. Disulfiram crystals (Sigma) were added to the solution at a concentration of 9% (w/v) and

quickly dissolved by pipetting with a glass pipette. The solution was poured in a large glass Petri dish from which methylene chloride was allowed to slowly evaporate under a hood as the Elvax polymerized over night. The resulting layer of disulfiram-infused elvax was about 0.3mm thin and could be cut with a scalpel to rectangular implants of about 1.2×0.8mm. Control implants were prepared in the same way, without adding disulfiram. If not used immediately, implants were stored at 4°C for up to five days.

To insert implants, the juvenile birds of 30-35 days of age were analgesized with 0.1 mg/kg Meloxidyl, anesthetized with an intramuscular injection of ketamine (50mg/kg) and xylazine (10mg/kg), and fixed in a stereotaxic apparatus as described in Chapter III, section 2.7. The skull was opened with the help of a needle and a Delicate Bone Scraper (Fine Science Tools), and implants were inserted through a small cut to the hippocampus, and placed upon the ventricular surface of HVC, as described in Denisenko-Nehrbass et al. (2000). Insertion coordinates for the implants were medial/lateral 3.0mm and anterior/posterior 1.8mm (fronto-lateral tip of HVC) to medial/lateral 0.9 and anterior/posterior -0.2mm (medial-posterior end of HVC). Disulfiram implants were applied unilaterally, the other hemisphere either receiving a control implant (nine animals, seven of which had to be discarded) or no implant (nine animals, three of which had to be discarded). After surgery, birds were allowed to survive seven more days before being sacrificed by decapitation under ketamine/xylazine anesthesia (see section 2.2.1 of Chapter II). Brains were quickly dissected, hemispheres were separated and frozen over liquid nitrogen, cut sagitally at 16µm with a cryostat (Leica), and stored at -75°C until processed.

### *2.4 Test of disulfiram implant efficiency using a RAc reporter assay*

To test the efficiency of disulfiram releasing elvax implants on RalDH enzymatic activity in song nucleus HVC, we unilaterally implanted two otherwise untreated animals with disulfiram implants. Eight days later, the animals were killed, and RAc production in song nucleus HVC was determined with the RAc reporter assay as detailed in Chapter III, section 2.5.

### *2.5 Immunohistochemistry*

To assess the number of new neurons in song nucleus HVC after disulfiram treatment, frozen brain sections were immunostained for BrdU to label new cells, and for the neuronal marker

HuC/D to label neurons (Barami et al., 1995). Primary antibodies used were rat anti-BrdU (1:200, ImmunologicalsDirect) and mouse anti-HuC/D (1:200, Chemicon). Both primary antibodies were applied to the sections after permeabilization with 0.2% triton/PBS (PBST; 30 min at room temperature), DNA denaturation with 2N HCl (Sigma) for 30 min at 37°C, and saturation in PBST/4% BSA (Roth) for 30min at room temperature. Sections were incubated with primary antibodies in PBST/4% BSA for 48h at 4°C; excess antibody was washed off with three PBS washes. Primary antibodies were then detected with Alexa 488-labeled rabbit anti-rat and Alexa 568-labeled goat anti-mouse secondary antibodies in PBST/4% BSA (dilution of antibodies 1:200 and 1:500, respectively; both from Molecular Probes) for an incubation time of 2h at room temperature. After three washes in PBS, all sections were counterstained with 1µg/ml DAPI (4',6-Diamidino-2-phenylindole·2HCl·$H_2O$; Serva) in PBS and mounted with Mowiol (Roth).

### *2.6 Quantification of new neurons in song nucleus HVC*

Immunofluorescent sections were analyzed with a 63× oil objective, using a Zeiss axiovert S100 microscope with appropriate filters for Alexa 488 and Alexa 568 green and red fluorescence (also from Zeiss). HVC could be easily identified by its large neuron clusters standing out from the surrounding nidopallium in the Hu staining. All birds with improperly placed implants (i.e., less than 50% of the implant overlapping with HVC) were discarded from further analysis. In sections of the remaining birds, $BrdU^+$ cells in HVC were phenotyped for the presence or absence of Hu. The sections of 16µm thickness were thin enough to allow for a reliable mapping of Hu (red) and BrdU (green) label of single cells by eye, when each potentially double-labeled cell was observed at different focal planes. The number of BrdU/Hu double labeled cells was counted in the entire HVC of every $10^{th}$ section (144µm apart), or, if the resulting number of HVC containing sections available for analysis was less than seven, of every tenth section plus the according number of additional intermediate sections (resulting in distances of 64µm minimum between two sections). To correct for different sizes of HVC, the number of new neurons was divided by the area of HVC analyzed, as determined by tracing the HVC outline using the *outline spline*-function of the Zeiss axiovision 4.7 software on photographs of every section's HVC taken through a 10× objective. The resulting number indicates the average number of new neurons per $mm^2$ of analyzed HVC area (at a thickness of 16 µm).

Neurogenesis rates of HVCs of the two different control treatments, empty implant versus no implant, were tested for statistically significant difference with a Student's t-test performed in Excel, and shown to not significantly differ. Therefore, birds from the two groups could be pooled for further analysis. Differences between neurogenesis rates in disulfiram treated versus control HVCs were tested for statisctical significance with a one-tailed paired Student's t-test, performed in Excel.

# 3 Results

The requirement of RAc synthesis in HVC to develop normal song, along with its ongoing synthesis in the adult HVC (Denisenko-Nehrbass et al., 2000, Denisenko-Nehrbass and Mello, 2001), suggest that RAc might be involved in the incorporation of new neurons into HVC. We tested this hypothesis by locally blocking RalDH's enzymatic activity and analyzing the effect on HVC neurogenesis rates in juvenile male zebra finches. We used the aldehyde dehydrogenase blocker disulfiram, which has been shown previously to efficiently inhibit the enzymatic acitivity of both recombinant RalDH and HVC homogenates (Denisenko-Nehrbass et al., 2000, Denisenko-Nehrbass and Mello, 2001). Disulfiram irreversibly inhibits the enzyme by competing with the cofactor nicotinamide adenine dinucleotide for binding sites on RalDH, ultimately reducing RalDH's oxidation rate, leading to a five- to tenfold reduction of the oxidation product RAc (Dirks et al., 2004).

Disulfiram was administered via slow-release implants consisting of the ethylene-vinyl acetate copolymer Elvax. Elvax is biologically inert and has been used before to release substances like NMDA, muscimol, buprenorphine, bicuculline methiodide, etc. into tissues *in vivo* (Aamodt et al., 2000, Clowry et al., 2004, Kleppner et al., 2006, Heck et al., 2007). We placed the implants on the ventricular surface of HVC of juvenile male birds (PHD 30-35; see fig. 28). To minimize the influence of individual differences in neurogenesis rates on the results, we applied the disulfiram treatment unilaterally, using the other hemisphere for control, so that new neuron incorporation numbers of the disulfiram-treated hemisphere could be compared directly to the control hemisphere of the same individual. Control hemispheres either received implants without any drug, or were left intact and did not receive any implant (see fig. 28). The use of these two different controls enabled us to assess whether the physical intervention of inserting an implant on top of HVC had an influence on neuronal incorporation rates. A comparison of HVC neurogenesis rates under the different control conditions showed that they did not significantly differ (Student's t-test, two-tailed; $p=0.9$; $n=5$ for empty implant; $n=7$ for intact hemisphere). We therefore pooled the two groups for the following analysis.

The effectiveness of disulfiram released from the implants was tested using the RAc reporter assay presented in Chapter III, section 2.5. Two animals were unilaterally implanted with a disulfiram-releasing implant, eight days before their brains were cut and used for the reporter

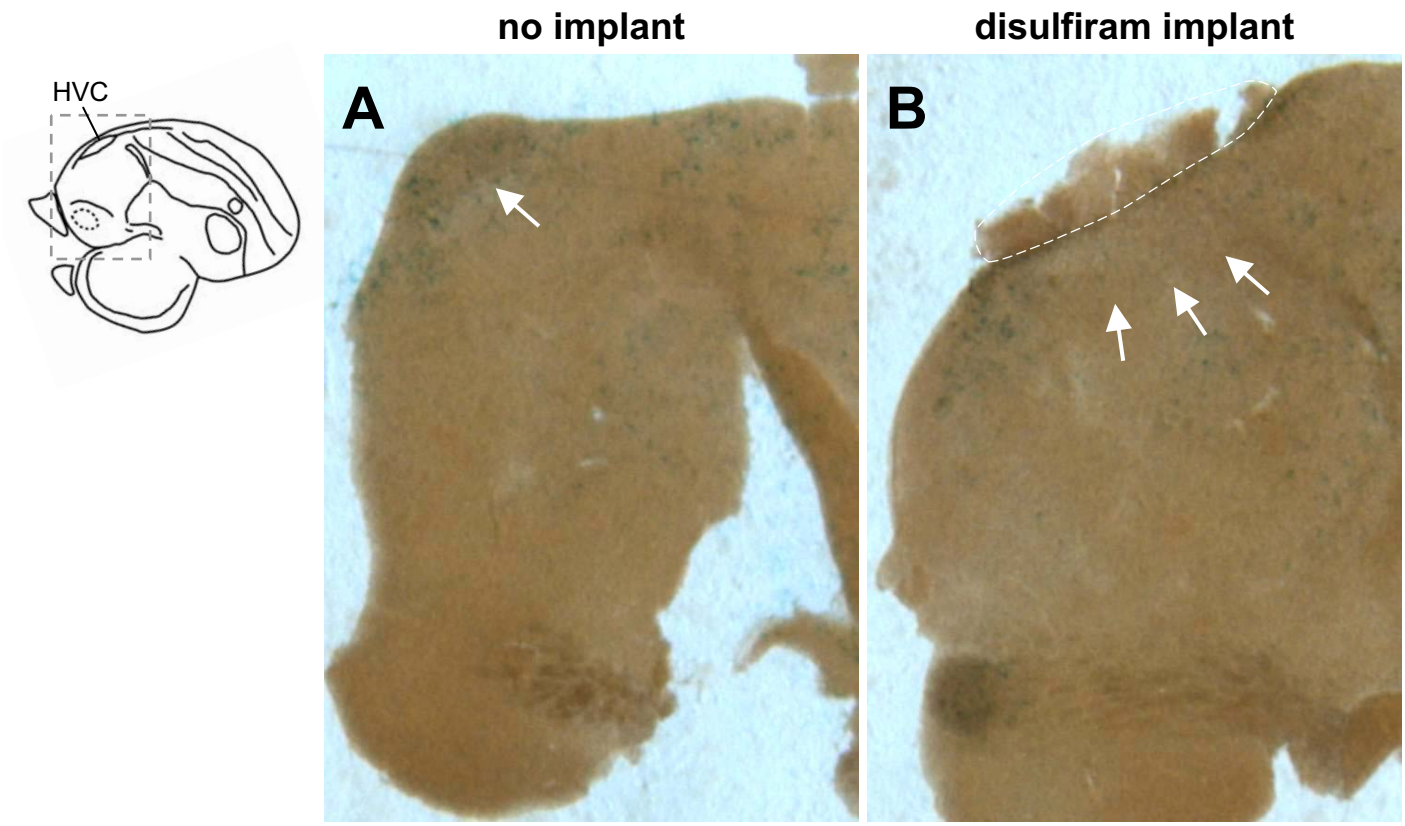


**Figure 29: Disulfiram releasing implants are able to decrease RalDH activity in song nucleus HVC.** The left drawing depicts the posterior region shown in A and B. Photos to the right show posterior parts of zebra finch brain sections that have been co-cultured on a layer of RAc reporter cells. Blue LacZ signal in the cell layer indicates local release of RAc from the brain section. Photos are taken from below, so that brain sections are viewed through the reporter cell layer. **A:** Local production of RAc in song nucleus HVC (arrow) is indicated by blue LacZ signal. **B:** A section of the contralateral hemisphere which received a disulfiram-releasing implant on top of the HVC region (implant surrounded by dashed line) shows considerably less LacZ staining beneath the implant, indicating that RalDH activity in HVC was blocked by disulfiram. The approximate HVC position is indicated by arrows.

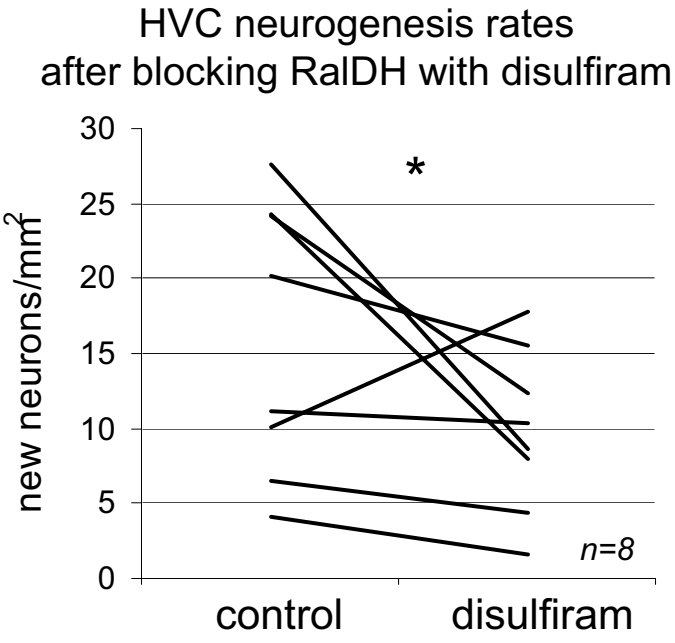


**Figure 30: Local RalDH blocking in song nucleus HVC decreases numbers of new neurons in HVC as compared to control hemispheres of the same animals.** The number of new neurons per $mm^2$ (in brain sections of 16 µm thickness) was significantly decreased in HVC under the influence of a local implant releasing the RalDH blocker disulfiram (right data points), if compared to control hemispheres which had received an empty implant or no implant (left data points). One out of 8 animals showed an opposite tendency for unknown reasons, having incorporated more new neurons in the disulfiram treated HVC. Paired Student's t-test, one-tailed; p=0.04.

cell assay, the intact hemisphere serving as control. RAc production in song nucleus HVC as determined by LacZ staining of the reporter cells was considerably reduced under the influence of a disulfiram-releasing implant (see fig. 29). This indicates that disulfiram released from an elvax implant was able to locally block RalDH enzymatic activity. Furthermore, apparent RAc presence at a (small) distance from the implant shows that the effect of disulfiram was locally constricted in a fairly precise manner (fig. 29).

We analyzed the incorporation rates of new neurons into HVC immunohistochemically using antibodies against the neuronal marker Hu and the cell-division marker BrdU. We identified new neurons in song nucleus HVC by their colocalized labeling for BrdU and Hu. The boundaries of HVC are easily identifiable by HVC's distinct cellular structure, characterized by large clusters of neurons, which are revealed by the Hu labeling.

We compared the numbers of new neurons in the HVC with blocked RalDH enzymatic activity to its counterpart, the contralateral control HVC of the same animal. Ten animals had to be discarded because of bad positioning of the implant, leaving only those animals for analysis where more than 50% of the implant actually covered HVC. Blocking RalDH reduced the number of newly incorporated HVC neurons by 39% on average (n=8). Seven out of eight animals had less new neurons incorporated into the disulfiram-treated HVC relative to the control HVC (fig. 30). We do not have an explanation for the one animal that showed the opposite tendency. The decrease of incorporated neurons under the influence of the RalDH blocker was statistically significant (paired Student's t-test, one-tailed; $p=0.04$).

This result seems to suggest that RAc signaling in HVC plays a role for the control of new neuron incorporation, or survival. However, another observation made us reluctant to assume this hypothesis: In two animals where disulfiram implants were actually a bit larger than the average implant, we could observe histological abnormalities in the tissue adjacent to the implant. Labeling for Hu revealed that cells were damaged, possibly indicating a general toxic effect of disulfiram. No such histological abnormalities were apparent in control hemispheres with empty implants, which makes physical damage rather unlikely to account for the tissue damage. Cell damage in the affected regions was so gross that it was impossible to determine numbers of $BrdU^+$ and $Hu^+$ positive cells; we therefore counted only cells in the remaining unaffected parts of HVC. In these apparently unaffected parts, we determined neuron density in disulfiram treated and control hemispheres as identified by $Hu^+$ labeling, to examine whether disulfiram toxicity also damaged neurons here, albeit in a less apparent way. No significant difference in neuronal densities could be detected (unpaired Student's t-test;

P=0.432). Hence, there is a chance that – despite the observed tissue damage – our observation of decreased neuronal incorporation rates into *intact* HVC tissue under the influence of disulfiram does point to an actual biological role of RAc in neurogenesis control: If general disulfiram toxicity (instead of a physiological role of RAc) was decreasing the number of new neurons, it would be likely to affect older neurons as well, which did not seem to be the case. However, we cannot exclude that non-specific disulfiram toxicity affects new and old neurons differentially. Therefore, we consider the insecurity brought to the experiment by disulfiram-induced tissue damage too severe to conclude that RAc regulates neuronal incorporation into HVC.

# 4 Discussion

To develop normal song, juvenile male zebra finches need to synthesize RAc in song nucleus HVC (Denisenko-Nehrbass et al., 2000). This nucleus is a site of massive growth and reconstruction processes during the period of vocal learning (Foster and Bottjer, 1998), including the incorporation of new HVC>RA projection neurons in high numbers, which then continue to undergo constant turnover in the mature bird. These neurons are crucial to develop stereotyped, learned vocalizations (Nottebohm et al., 1976, Simpson and Vicario, 1990), and exhibit a sparse firing pattern which is precisely time-locked to the song output (Yu and Margoliash, 1996, Hahnloser et al., 2002). The postdevelopmental incorporation and ongoing replacement of such a specialized population of projection neurons is striking, and purpose and mechanisms are still poorly understood. Our aim was to shed light into the molecular control of new neuron recruitment by investigating a potential role of RAc for its control. We focused here on juvenile birds, where on the one hand neuronal recruitment in HVC is particularly high, and on the other hand, the specific RAc production in HVC has been shown previously to exert a behaviorally relevant effect. The way in which RAc produced in HVC influences song acquisition might be by controlling the incorporation of new neurons. The investigation of juvenile postdevelopmental neurogenesis is very likely to be insightful for adult neurogenesis, too – although experimental confirmation in the adult bird would certainly be required – while promising stronger effects of experimental treatment, due to the higher juvenile neurogenesis rates. To explore a possible role of RAc in neuronal recruitment, we blocked RAc synthesis in HVC by means of locally inserted implants which released the RalDH blocker disulfiram, and quantified HVC neurogenesis. Our results are, with reservations, consistent with a potential role of RAc as a regulator for neuronal incorporation: The number of new neurons in HVC was significantly decreased when RalDH enzymatic activity was blocked. However, unexpected methodical problems – i.e. an apparent toxic effect of the drug disulfiram used to block RAc synthesis on tissue – make us reluctant to assume the hypothesis of RAc controlled neurogenesis in HVC based on our data. We cannot rule out that a particular effect of disulfiram toxicity on new neurons might account for our results.

In the following, we will first discuss a potential role of RAc for HVC neurogenesis, with which our observations would be in line, despite the toxicity problem. We will then discuss why toxicity might have occurred, in contrast to previous, similar experiments (Denisenko-

Nehrbass et al., 2000), and how the proceeding could be modified to eliminate this problem and deliver more reliable results.

The reduced recruitment of new neurons in HVC which we observe upon blocking RalDH would be consistent with a role of RAc for incorporation and/or survival of new neurons, and possibly the latest phase of migration. To reach HVC from their sites of origin in the ventricular zone, it takes new neurons up to two weeks of migration in canaries (Kirn et al., 1999). We started the disulfiram treatment one week after BrdU injections, at a time when some labeled cells have presumably already arrived in HVC (Kirn et al., 1999), while others were still on their way. Disulfiram could thus have affected the survival of some already incorporated new neurons, as well as the incorporation of those just arriving in HVC. This is in line with the finding that (systemic) RAc depletion affects early phases of neurogenesis in the adult rodent dentate gyrus, including differentiation and early survival, but not proliferation (Jacobs et al., 2006). Interestingly, incorporation and survival of new HVC neurons have been shown to be influenced by brain-derived neurotrophic factor (BDNF), and the receptor for BDNF, trkB, is a target of RAc transcriptional activation (Kaplan et al., 1993, Rasika et al., 1999). In various cultured cell types, RAc seems to modify BDNF dependent differentiation, growth, or survival via trkB regulation (Kobayashi et al., 1994, Lucarelli et al., 1994, Fryer et al., 1997). Another target gene of RAc, the insuline-like growth factor II (IGF-II; Melino et al., 1993, IGF-II; Gabbitas and Canalis, 1997) shows a complex expression pattern in the songbird HVC, where it has been proposed to regulate neuronal survival (Holzenberger et al., 1997). This would also be in line with IGF-II regulated growth and survival found in other systems, such as cultured chick peripheral ganglion cells, or the frog sciatic nerve (Recio-Pinto et al., 1986, Edbladh et al., 1994, Zackenfels et al., 1995). By regulating genes like trkB or IGF-II, RAc could play a modulatory role in neurogenesis control, possibly determining which neurons become incorporated into the existing network and survive, while others are replaced.

Migration has been suggested to depend on RAc signaling in the mouse subventricular zone (SVZ)/olfactory bulb pathway which shows high rates of adult neurogenesis (see Zhao et al., 2008 for a recent review): New cells born in the subventricular zone migrate to the olfactory bulb, which can be prevented in cultured brain slices by the expression of a dominant-negative retinoid receptor in the SVZ (Wang et al., 2005). However, the situation in the bird is somewhat different, since RAc is not produced at the site of proliferation, but only at the target site of neuronal incorporation. Only an influence of RAc on the latest phase of migration, the entry of new cells into HVC, would seem plausible for the songbird HVC,

where RAc might act as a sort of chemoattractor for the newly arriving cells. RAc has actually been shown to play a somewhat related role in the snail *Lymnaea stagnalis*, where it acts as a chemoattractor for the growth cones of neurites (Dmetrichuk et al., 2006). It would be interesting to investigate whether new neurons entering HVC, or migrating in its proximity, express retinoid related receptors which could mediate an effect of chemoattraction through RAc.

Another interesting question to explore in the future is whether (or when) new HVC neurons produce RAc themselves. In the previous chapter, we have shown that HVC>RA projection neurons contain RalDH enzyme (see section 3.9). If new HVC cells produce RAc, it might help them to grow out neurites, as suggested for spinal cord neurons: In mammalian embryos, RAc is necessary for neurite outgrowth from the spinal cord to the periphery (see Clagett-Dame et al., 2006 for a review), and adult spinal cord neurons, which normally lost their capability of growing neurites, can be enabled to do so again by transfection of the RARβ2-gene (Corcoran et al., 2002), a receptor which is also present in the zebra finch HVC (Jeong et al., 2005). Whether new neurons incorporated into HVC produce RAc and/or express RAc receptors before they connect to RA could be examined using BrdU/RalDH immunolabeling in combination with retrograde tracers and *in situ* hybridization for receptor transcript. RAc would, in this case, be a promising candidate regulator for axonal growth of new HVC>RA neurons.

Another particularly interesting mechanism by which RAc might ultimately influence new neuron survival in HVC could be via gap junctions. Gap junctions are found between the somata of tightly clustered HVC neurons (Burd and Nottebohm, 1985, Gahr and Garcia-Segura, 1996, see also Gahr et al., 2002 for a review). In other systems, they have been shown to serve as channels for the transfer of trophic molecules (reviewed by Guthrie and Gilula, 1989) and apoptotic signals (Lin et al., 1998). This way, older neurons within an HVC cluster could influence the growth or survival of newly incorporated cells by directly providing them with trophic input. In addition, electric coupling of older and new neurons via gap junctions could determine the newly incorporated cell's activity pattern, sort of "teaching" them when to fire. Ultimately, the survival of the new cell could then depend on its successful teaching. Those neurons which fail to electrically couple with the existing network might not be able to successfully integrate and could be prone to cell death – which is (in adult canaries) the fate of about one third of the newly incorporated neurons within the first three weeks after they are born (Kirn et al., 1999).

How could RAc come into play in this context? One possibility which would be different from the "canonical" transcriptional control of target genes could be a relatively fast, modulatory effect: In vertebrate retinae, RAc modulates electrical coupling between horizontal cells in relatively short time spans (Weiler et al., 1999, Weiler et al., 2001). By reducing the conductance of gap junctions between neighboring cells, RAc can modify the amplitudes of horizontal cell photoresponses. Since RAc production in the retina is light dependent (RAc is produced as a side effect of the rhodopsin transduction process in bright illumination), this modification of photoresponses is related to the ambient light conditions in a timely manner. Besides such a fast, neuromodulatory effect of RAc on gap junctional conductances, RAc has been shown to regulate the transcription of connexins (the trans-membrane proteins which make up gap junctions) in other systems (Liu et al., 2005, Tanmahasamut and Sidell, 2005). In HVC, either way of RAc influencing electric coupling could ultimately influence neuronal survival.

We would like to remind, though, that not only these mechanistic scenarios, but the fact of RAc influencing neurogenesis is speculative so far, since our results (which are in general in line with this idea) are derogated by the observation of treatment toxicity. Why did we observe a disulfiram-induced degradation of neuronal tissue beneath the implants, while Denisenko-Nehrbass et al. (2000) did not? A difference between their and our treatment was that they used agarose as substrate material for the implants, while we used elvax. However, elvax has been used before for brain implants in different organisms and was shown to be biologically inert, and our control implants which did not contain disulfiram corroborate this. The tissue damage occurred only with disulfiram implants, indicating that disulfiram was the toxic agent. We observed that only the largest disulfiram implants affected the tissue, suggesting that disulfiram toxicity was dose-dependent. Due to the different substrate of our implants, disulfiram release rates from our and Denisenko-Nehrbass's experiments are hard to compare: Denisenko-Nehrbass et al. incorporated disulfiram crystals into the agarose which were still visible after a period of several weeks to three months when the animals were perfused; the size and number of the crystals were presumably somewhat variable, but disulfiram release may have been considerably lower than from our elvax implants. In addition, our implants were larger in size (1.2×0.8×0.3mm versus 0.5×0.5×0.3mm). Disulfiram is used for treatment of alcoholism in humans, and neurotoxic effects of disulfiram are well described for high systemic doses of the drug over a prolonged period (see, for instance, Laplane et al., 1992, Simonian et al., 1992, Vaccari et al., 1998, Karamanakos et al., 2001). To prevent from neurotoxicity, we would suggest lowering the concentration of

disulfiram in the implants. In addition, implant size might be reduced; however, we demonstrated here with RAc localization using a reporter cell assay that the scope of disulfiram released from our implants is not unreasonably high (RAc was decreased only in the proximity of the implant, see fig. 29). This suggests that an implant size of approximately the surface of HVC, as we used, is a good choice.

In this chapter, we have presented an experiment to explore RAc function in the songbird brain, after having extensively investigated the localization of the different components of the RAc signaling pathway in the previous chapters. Here, we investigated a possible effect of RAc for postdevelopmental neuronal recruitment in song nucleus HVC. Our results are in line with a role of RAc for regulation of new neuron incorporation and survival, but due to methodological complications – i.e. toxicity of our treatment – this hypothesis cannot be accepted without reservations on the basis of our data. An improved methodological setup would be required to overcome the toxicity and confirm the results.

To elucidate the (presumably manifold) roles of retinoic acid in the different parts of the song control system and its effects on song acquisition and behavior, clearly more functional investigations are needed. Given the specific distribution of the retinoid pathway players, it seems promising to focus on local manipulation of retinoid signaling as has been done by Denisenko-Nehrbass et al. (2000) and also tried here. Such local manipulation could use – besides drugs against RalDH enzymatic activity – receptor (ant)agonists, drugs against RAc degrading cytochrome activity, or molecular knockdown and overexpression techniques (locally applied e.g. via a lentiviral system, as done by Haesler et al. (2007)), to modify availability of the different pathway players in different sites. Such treatments can be given at different ages, to explore the functional roles of RAc signaling during different phases of song development. A plasticity inducing signaling molecule like RAc might be responsible for numerous different reconstruction processes in the brain which mediate the acquisition and maintenance of song as a complex learned behavior.

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