

Role of Gene Regulation in Song Circuit Development and Song Learning

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ABSTRACT: The songbird has emerged as an important model for study of brain–behavior relationships by virtue of its rich natural advantages and from the pioneering efforts of explorers using anatomical and behavioral approaches. Now, molecular biology is providing a new and complementary paradigm for discerning songbird brain organization and function. Here, I review the work over the last 10 years that has laid the foundation for approaching songbird biology from the molecular perspective. As a result of this work, specific hypotheses can now be framed and tested regarding the mechanisms behind song circuit formation, behavioral plasticity, and the boundaries of adaptability. Age-related changes in more than 15 molecules have been observed in the song system of juvenile zebra finches, and these changes seem to define specific phases in circuit development. In adult

songbirds, ordinary song-related activities such as singing and listening cause dramatic increases in gene expression in brain areas specific to each activity. The sensitivity of gene activation is modulated as a result of experience in adulthood and also changes during juvenile song learning. These studies have provided unexpected insights into the functional organization of the song circuit and the potential role of extrinsic modulatory systems in directing and limiting plastic change in the brain. With this rich base of knowledge, and techniques of gene manipulation on the horizon, answers to old questions seem within our reach: What sets the boundaries of neural plasticity? What limits learning? © 1997 John Wiley & Sons, Inc. *J Neurobiol* 33: 549–571, 1997

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INTRODUCTION

The dominant paradigm for analyzing the physical organization and function of the song system has so far been anatomical: measuring volumes, counting numbers of cells, lesioning brain regions, and determining patterns of connectivity via electrophysiology and tracers. The information gained by this approach is essential and invaluable—a prerequisite for making sense of function and organization in the nervous system. Indeed, the level of insight into

anatomical organization is one of the distinguishing advantages of working in this system. However, the anatomical paradigm has not yet led to an understanding of the big questions raised by the system. For example: How does this particular neuroanatomy give rise to a specific song? How does a bird choose what songs to copy? How does learning come to be limited to critical periods? How does singing behavior come to be different in different species?

Now another paradigm has emerged that promises a further advance in our understanding of function and regulation in the song control system. I shall refer to this as the paradigm of “molecular biology,” using the term not just in the narrow sense, as a set of tools, but to refer more broadly to a distinct and coherent system of thought for

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understanding how biological systems work. The paradigm is bottom up and begins with the idea that natural order flows from the dynamic interactions of molecules. From these interactions emerge cells, tissues—and organisms that behave. At the center of the paradigm is the realization that all living things share a common set of synthetic mechanisms organized around the gene, and it is through the expression and regulation of genes that the unique properties of each organism are determined, including the way it responds to experience. From roots grounded in this perspective, a major effort has been slowly building over the last decade to identify genes that are regulated in the song control system during its development and function. This article will review what we have learned so far from this approach, focusing in turn on five major questions that have driven this effort: (a) What, at the molecular level, distinguishes the nuclei of the song control system from the rest of the brain? (b) What molecular changes occur during development of the song system? (c) Does gene regulation have an active role in the perception of song? (d) Does gene regulation have an active role in operation of the song production system in adult birds? and (e) Is gene regulation involved in the restriction of song learning to specific critical periods in life?

THE SONG SYSTEM IS SPECIAL NOT SO MUCH IN THE GENES IT EXPRESSES, BUT IN HOW IT REGULATES THEM

The telencephalic nuclei of the song control circuit seem to have evolved only in songbirds (Brenowitz, 1997), and some of their properties set them apart from the surrounding brain in which each is embedded. Cell size and packing density are different, gross anatomical changes occur seasonally and between the sexes, and several of the nuclei contain relatively high levels of androgen receptors. Gene regulation in the song system could follow one of two different forms. Given the system's distinctive anatomy, physiology, and function, it is not unreasonable to suppose that a unique set of "song system-specific" genes is at work to determine these unique properties. Alternatively, the song system may express the same basic contingent of genes and proteins as the rest of the brain, but be constructed in a way that allows them to be combined and regulated so that distinct functional properties emerge.

The first exploration of gene expression in the

songbird brain was directed at this fundamental question (Clayton et al., 1988). By a combination of approaches, cDNA clones were isolated which represented a broad cross section of the genes expressed as messenger RNA (mRNA) in the adult canary telencephalon, including genes expressed at the lowest levels (i.e., their mRNAs comprised only a tiny fraction of all the mRNA in the forebrain). These mRNAs were then mapped to their sites of expression using *in situ* hybridization. Each of the RNAs was found to have a unique distribution across regions and cell types within the forebrain, but none was specific for any single brain region, song nuclei included. Although this result does not disprove the existence of song system-specific genes, it clearly indicates that region-specific gene expression is not the dominant mode of organization. To date, although many genes have been found that show distinctive patterns of regulation in the song control system (Table 1), none has been found that is expressed only in song nuclei.

Since that original investigation, various approaches have been taken to identify and study gene products that undergo some form of differential regulation in the song control nuclei compared to surrounding brain regions. These have included differential and subtractive hybridization (George and Clayton, 1992; George, 1993; George et al., 1995), differential-display polymerase chain reaction (PCR) (Denisenko et al., 1995; Siepka, 1977), and best-guess strategies to analyze the expression of likely candidate genes (Mello et al., 1992; Jin et al., 1994; Nastiuk and Clayton, 1995; Basham et al., 1996). Generation of monoclonal antibodies to song nucleus extracts led to a radial cell marker which proved to be vimentin (Alvarez-Buylla et al., 1987), a molecule independently identified in the first differential hybridization study (Clayton et al., 1988). Other approaches to molecular analysis of the song system have focused on detection of known proteins using immunocytochemistry and receptor autoradiography, as reviewed elsewhere (Ball, 1990; Balthazart and Ball, 1995).

Table 1 (also see Appendix) describes the stable of gene products that have been cloned and characterized in some depth in the adult song system. The table is dominated by proteins involved in intracellular signal transduction, which may be more a reflection of the brain in general than of the song system in particular. Proteins whose mRNAs are typically enriched in telencephalic song nuclei of adult birds include the androgen receptor, neurofilament (medium molecular weight subunit), and al-

Table 1 Cloned Gene Products Isolated from Songbird Brain

Name	Other Names, Abbreviations	Description	Enriched, Reduced	Development?	Identifying Reference
<i>n</i> -Chimaerin	HAT-2	Cytosolic signal transduction	<i>HVc, RA</i>	—	George and Clayton (1992)
Synelfin	HAT-3, NACP synuclein, PNP14	Presynaptic, lipid binding, ???	<i>HVc, IMAN, RA</i>	•	George et al. (1995)
MEK-1	HAT-5, MAPKK	Signal transduction kinase	<i>HVc</i>	—	George et al. (1994)
Canarigranin	HAT-14 (~RC3, neurogranin)	Dendritic signal transduction	HVc, RA <i>Area X</i>	•	Siepkala (1994)
GAP-43	B-50, F1, neuromodulin	Axonal growth/signaling	IMAN <i>HVc, RA</i>	•	Jin et al. (1994)
zenk	zif-268, egr-1, ngfi-a, krox-24	Transcription factor (IEG)	NCM <i>HVc, RA</i>	•	Mello et al. (1992)
c-jun		Transcription factor (IEG)	NCM <i>HVc, RA</i>		Nastiuk et al. (1994)
Aldehyde dehydrogenase	ALDH1	Retinoid acid production?	HVc, RA, IMAN	•	Denisenko et al. (1995)
Androgen receptor	AR	Steroid-regulated transcription	HVc, RA, IMAN	•	Nastiuk and Clayton (1995)
Estrogen receptor	ER	Steroid-regulated transcription	NCM	•	Jacobs et al. (1996)
c-myc		Transcription factor (IEG)	—	—	Collum et al. (1991)
n-myc		Transcription factor	—	—	Collum et al. (1991)
Neurofilament (medium MW)	NFm	Axonal cytoskeleton	HVc, RA, IMAN	•	Siepkala and Clayton (1995)
Myelin proteolipid protein		Myelination			Campagnoni et al. (1994)
Myelin basic protein	MBP	Myelination	HVc-RA tract	•	Siepkala et al. (1997)

cDNA clones for each of these molecules have been isolated from either canary or zebra finch (the androgen receptor clone was a genomic fragment).

Name: the protein/cDNA as referred to in this review.

Other names, abbreviations: as used elsewhere in the literature.

Description: brief summary of protein's function and/or localization.

Enriched/Reduced: Song system components where expression is increased relative to surrounding brain are shown in bold; regions where expression is reduced are shown in italics.

Development?: • developmental regulation has been observed within the song system (see Fig. 1); — no regulation was detected; blank indicates no data.

Identifying Reference: the first published report of the clone's isolation or expression in songbird brain. Where manuscripts are under review, abstracts from the Society for Neuroscience meeting are cited. Most of these gene products are listed in Genbank.

dehyde dehydrogenase (possibly involved in retinoic acid production). Expression of synelfin, in contrast, is notably and specifically reduced in adult song nuclei compared to surrounding telencephalon. Canarigranin and growth-associated protein (GAP)-43 show a mixed pattern of expression in the adult song system, increased in some nuclei and decreased in others. Understanding the song system from the molecular perspective will hinge on knowing how

(and why) these and other basic molecules of the nervous system are specifically regulated in the context of song circuit development and song learning.

GENE REGULATION OCCURS IN SEVERAL PHASES THROUGHOUT SONG CIRCUIT DEVELOPMENT

The song control system offers a unique opportunity to study the developmental construction of a func-

tional circuit in the central nervous system (Brenowitz et al., 1997; Nordeen and Nordeen, 1997; Bottjer and Johnson, 1997). The song control circuit is traditionally defined as a set of discrete, interconnected anatomical nuclei, organized into two pathways intersecting at nucleus RA in the telencephalon (e.g., Fig. 2). Nuclei in the anterior forebrain pathway (Area X, DLM, and IMAN) appear to be necessary for normal song development, but lesion of them in adulthood has little immediate effect on singing, whereas lesion of HVC or RA immediately disrupts song performance at any age (Nottebohm et al., 1976; Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). The details of song circuit development and function have been studied most closely in the zebra finch, where song learning and completion of the circuit both occur during a critical period in juvenile life (Immelmann, 1969; Arnold, 1975; Konishi and Akutagawa, 1988). HVC and RA emerge as discrete Nissl-stained structures in both sexes about 10 days after hatching (Braun et al., 1991), and RA becomes innervated by afferents from IMAN by day 15 (Mooney and Rao, 1994). At about day 20, young finches fledge from the nest and are believed then or soon thereafter to begin forming the memories upon which their own songs will eventually be based. HVC and RA only become synaptically linked after about 30 days of age (Konishi and Akutagawa, 1985), when young males first start to produce crude songlike vocalizations. Synapses from HVC form on the same RA dendrites that already bear terminals from IMAN (Canady et al., 1988; Herrmann and Arnold, 1991; Mooney and Konishi, 1991), and these IMAN terminals undergo a physical reorganization some time between day 25 and day 53 (Herrmann and Arnold, 1991). At 2 months of age, a male has developed a fairly stable song, and by adulthood (~90 days), his song has become quite stereotyped and does not change much for the rest of his life (Price, 1979; Williams and McKibben, 1992; Nordeen and Nordeen, 1993; Jones et al., 1996). Female zebra finches do not sing, nor do they normally form the synapses between HVC and RA (Konishi and Akutagawa, 1985; Williams, 1985).

All of the known gene products that show differential expression in the adult song system (Table 1) have been subjected to analysis of their regulation during this developmental process, and some interesting correlations have emerged. A timeline of developmental changes in gene expression is presented in Figure 1; also included here are several

specific proteins or enzymatic products that have not yet been studied at the level of gene regulation (mRNA) but which change in abundance in the developing song system based upon immunocytochemical or histochemical methods. The figure has been organized in temporal sequence so that events that occur earliest in development are at the top of the chart, and late events are at the bottom. Further descriptions and abbreviations for each molecule are presented in the Appendix.

The timeline (Fig. 1) vividly demonstrates that zebra finch song circuit development is a multiphasic process. Molecular changes seem to occur in three clusters. One phase occurs early, prior to days 35–40, and all of the known changes that occur at this time represent declines in the abundance of specific molecules (NOS, canarigranin, estrogen receptor in females, and synelfin). Each of these molecules has a pedigree implicating it in some aspect of synaptic plasticity (see Appendix for details), and the early timing of events in this group suggests involvement in the initial establishment of synaptic connections within the song control circuit. These early-phase events might also support or initiate the process of song memorization, which can be accomplished as early as day 35 (Böhner, 1990). Sensory learning can continue at least to day 65 under relatively normal conditions, however (Eales, 1985; Slater et al., 1991; Jones et al., 1996), and the early disappearance of these molecules makes it unlikely that they are directly involved in the learning process itself. Many aspects of song are species specific and apparently innate (Marler and Sherman, 1983; Searcy and Marler, 1987; Marler, 1997), and comparative analysis of these early-phase genes in different species conceivably might provide insight into how song circuits with different functional properties are constructed. For example, differences in the timing of early-phase synaptic gene regulation in different species might lead to the formation of different topographic mappings between song nuclei (Vicario, 1991; Johnson et al., 1995).

A second group of regulatory events takes place from about day 35 to day 50, and includes a mixture of developmental trajectories [increasing AR, NF immunoreactivity, and catecholamines; decreasing *N*-methyl-D-aspartate (NMDA) receptor and GAP-43 RNA; transitory increases in GAP-43 immunoreactivity, acetylcholine (ACh), aldehyde dehydrogenase (ALDH); more complex changes in *zenk*]. These changes occur shortly after song development commences and could either contribute to or result from the various cellular processes that are needed

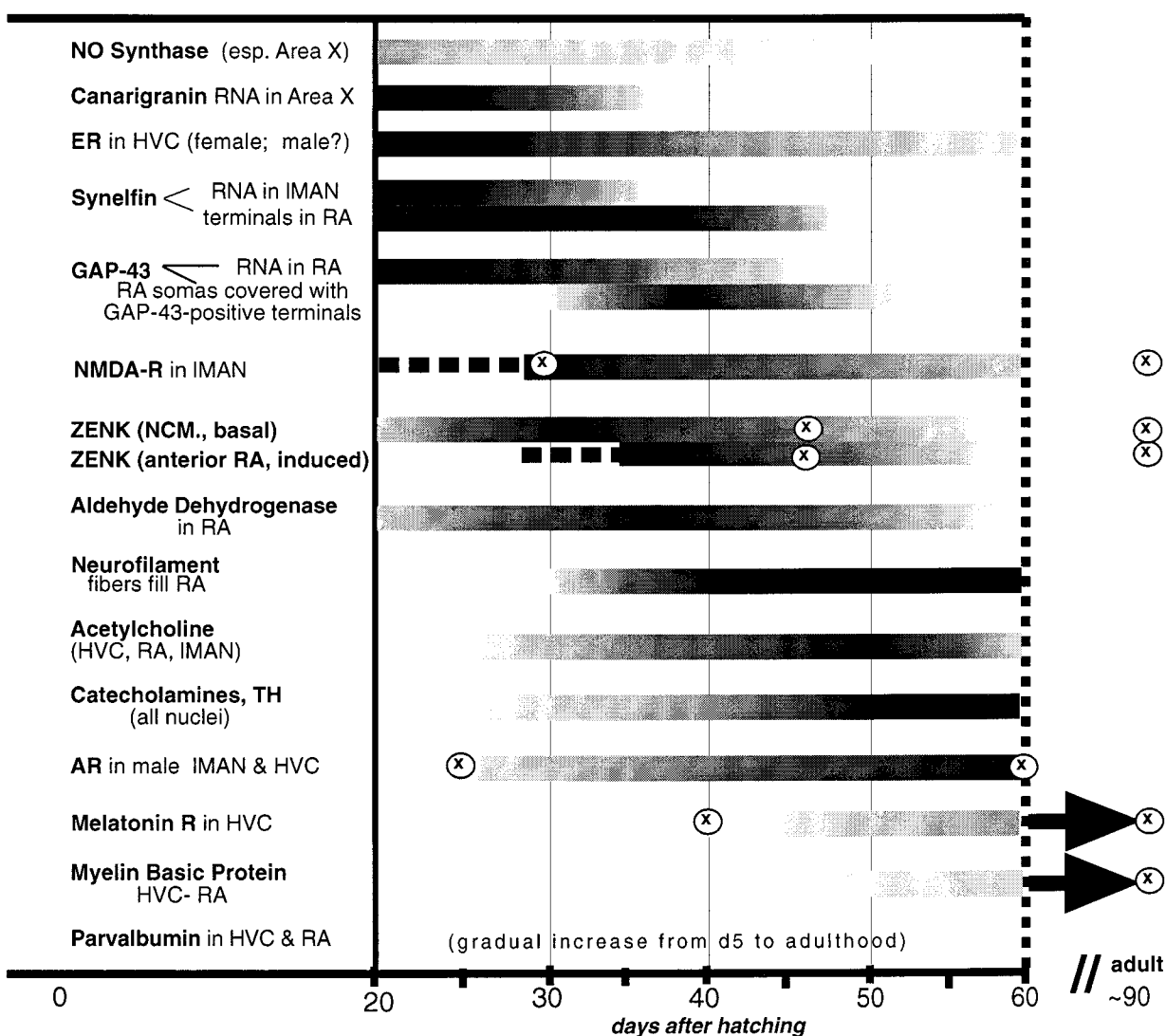


Figure 1 Timeline of developmental changes in gene expression in the zebra finch. Gene products (mRNAs and/or proteins) which have been shown to change in abundance or localization during development of the song system are included. The shaded bars represent the relative intensity of expression of the signal measured at different ages posthatching (indicated on the X-axis). Where potentially significant transitions are interpolated from widely separated data points, a symbol (circled X) has been placed to mark the nearest direct measurements. The timeline focuses on the ages between day 20 and day 60 because these are the ages when most of the molecular changes have been detected. Where significant data points have been collected at older ages, the circled X is placed to the right of the timeline. The two bold arrows (for melatonin receptor and myelin basic protein) indicate that most of the quantitative change in expression of these molecules takes place after day 60. The dashed lines (*zenk* and NMDA receptor) represent extrapolations to younger ages where direct measurements have not been made. References and further descriptions of each molecule and development process are given in the Appendix and Table 1.

for the selection of specific song elements and the gradual stabilization of the bird's own song. These processes may include changes in cellular excitabil-

ity triggered by extrinsic factors, such as androgens (mediated by changing levels of AR in the song nuclei), or catecholamines and ACh that presum-

ably reach the song nuclei via the diffuse modulatory systems of the brain. Meanwhile, specific alteration in proteins involved in intracellular signal transduction (such as NMDA receptor and *zenk*) could lead to adjustments in the efficiency of activity-dependent synaptic modification and consolidation, effectively opening and closing windows of plasticity (see the penultimate section of this article for further discussion).

The third wave of regulation does not occur until after day 55, as represented here by melatonin receptors and myelin basic protein. These changes may be associated with the final optimization of neural transmission in the mature pathway (myelination), and with placing circuit function under circadian and seasonal control (melatonin regulation). Myelin basic protein production in particular correlates closely with the timing of song crystallization and could represent a final step in circuit development that contributes to the stabilization and preservation of specific connections between neurons in different song nuclei. It will be interesting to test whether isolation paradigms that delay crystallization (Morrison and Nottebohm, 1993; Jones et al., 1996) will have effects on these late events, or whether aspects of myelination are different in open-ended and age-limited learners.

One other striking feature about these molecular changes is the way in which they are distributed among the song nuclei. Some gene products undergo specific developmental regulation in a single nucleus (e.g., *synelfin* RNA in IMAN, *GAP-43* RNA in RA). Others undergo concerted changes in more than one nucleus (e.g., *NOS*, androgen receptor). Each song nucleus is characterized by a unique profile of developmental regulation (Fig. 1) and adult gene expression (Table 1). Given this complexity, it seems doubtful that the distinctive development and plasticity of the song system will be fully explained by any single master regulatory gene or molecular process. Nevertheless, the information in Figure 1 and Table 1 begs the question of whether there are (a few) underlying regulatory processes, principles, or proteins that coordinate these diverse changes or otherwise account for specific phases of song circuit development or song-learning behavior. Promising insights in this direction have come from recent studies of the immediate-early gene (IEG) response in the songbird brain. But before returning to the issue of developmental plasticity, we must first take a detour to consider how mechanisms of gene regulation are engaged by neural activity in the adult.

ENGAGEMENT OF GENE EXPRESSION DURING SONG PERCEPTION AND ATTENTION

During the mid-1980s, it became apparent that neurons (like other cells) react to extracellular signals by activating the expression of specific genes in the cell nucleus. When nerve growth factor (NGF) is applied to certain tumor-derived cell lines, for example, increases in transcription of specific genes can be detected within 5 min, and the cells begin to develop an overt neuronal phenotype (Milbrandt, 1987). This rapid response to stimulation of cell-surface receptors is so robust and ubiquitous that it has come to be referred to under a general term, the IEG response (Sheng and Greenberg, 1990; Morgan and Curran, 1995). The number of genes that can potentially participate in this response has been estimated to be in the hundreds. The proteins they encode are varied in their specific cellular functions, although many of the best characterized ones are involved in regulating the transcription of other genes. It still is unclear to what degree a given cell type is capable of inducing different combinations of IEGs with different functional consequences, or whether a particular cell type usually expresses the same characteristic complement under most circumstances. In neurons, the IEG response seems to be coupled to processes of neuronal differentiation and adaptation. Compelling evidence has now accumulated that long-term memory formation specifically requires the expression of IEGs (Alberini et al., 1994; Bourtschuladze et al., 1994; Yin et al., 1994). This may account for the longstanding observation that a period of RNA and protein synthesis is necessary immediately following a training event for memories of that event to be retained (Davis and Squire, 1984; Goelet et al., 1986).

With these perspectives, I and my students at Rockefeller set out to monitor IEG responses in the songbird brain. The expectation then (and still valid now) was that IEG activation could serve as a molecular marker for sites in the brain where processes of neuronal adaptation are engaged by a particular behavioral stimulus. We cloned the canary homologue of one of the genes shown to be induced by NGF (*ngfi-a*) (Milbrandt, 1987). This gene encodes a protein built around the "zinc finger" motif, which confers the ability to bind to specific sequences of DNA and thereby modulate the transcription of other target genes. We also pursued another IEG (called *egr-1*) which had been shown to be induced by electrical stimulation of neurons

(Sukhatme et al., 1988). When it soon emerged that *ngfi-a* and *egr-1* were the same gene and had been independently discovered several additional times as well, we decided to refer to the canary homologue by an acronym for existing names—*zenk* (Mello et al., 1992). Later, we also cloned and studied the canary version of a second IEG, *c-jun* (Nastiuk et al., 1994). Among IEGs, *zenk* in particular has been implicated in memory consolidation, as it is induced in the mammalian hippocampus by stimuli that cause long-term potentiation (LTP), and both LTP and *zenk* induction are specifically blocked by NMDA receptor antagonists (Cole et al., 1989).

The canary *zenk* cDNA clone was then used to measure *zenk* mRNA levels in both canaries and zebra finches, by the technique of *in situ* hybridization. Basal levels of *zenk* mRNA in the brains of quiescent adults proved to be very low. As a simple if crude test of whether expression might be increased by physiological activity, adult canaries and zebra finches were then treated with metrazole, a γ -aminobutyric acid (GABA) antagonist which elicits a massive electrical discharge throughout the brain. In response, *zenk* mRNA levels rose dramatically throughout most of the forebrain (Mello and Clayton, 1995). In a more behaviorally relevant test, birds were then exposed to the sounds of either tape-recorded birdsong or pure auditory tones. The tones caused no detectable response, whereas the song playbacks activated robust *zenk* expression, but in a more restricted set of brain regions (Mello et al., 1992; Mello and Clayton, 1994).

Unexpectedly, no *zenk* response to song playbacks was observed in the nuclei of the traditional song control circuit, nor in Field L2, where auditory afferents from the thalamus terminate. Instead, the region of greatest response lay along the midline of the forebrain, in a portion of the caudomedial neostriatum (NCM) which had not been a subject of much study in songbirds (Mello and Clayton, 1994). Song stimulation also induced *zenk* in an immediately adjacent portion of the caudomedial hyperstriatum; in secondary processing areas of the avian auditory neostriatum (Fields L1 and L3); in the “shelf” beneath HVC and “cup” adjacent to RA which are believed to convey auditory information into the song control circuit (Kelley and Nottebohm, 1979); and in caudal paleostriatum, a possible homologue of mammalian amygdala (Mello and Clayton, 1994). Studies of *c-jun* revealed a similar pattern of response for this IEG as well (Nastiuk et al., 1994).

What is the purpose of this robust genomic re-

sponse to song, especially since it lies in parts of the brain outside the traditional song control circuit? Song perception is used by adult birds (of both sexes) in a variety of contexts ranging from territorial defense to mate selection (Catchpole, 1982; Kroodsma and Byers, 1991; Wiley et al., 1991; Beecher et al., 1996), and it makes sense that aspects of song perception might be mediated by brain regions distinct from those responsible for male-specific song production. Specific clues about the significance of the gene response in NCM have come from studies of how the response varies with different stimuli and contexts. One emerging theme is that the response is greatest under circumstances when the bird is most likely to be paying close attention to a complex auditory stimulus, as illustrated by four different sets of experiments. First, in a two-way comparison of adult male canaries and zebra finches, conspecific song induced twice the amount of *zenk* mRNA as did heterospecific song (Mello et al., 1992); conspecific song is presumably the more salient stimulus in this circumstance. Second, when a particular conspecific song was presented repeatedly (e.g., several times a minute over several hours), it no longer engaged any measurable response when presented again sometime later; yet the *zenk* response to other conspecific songs was not altered, suggesting that the bird had learned to discriminate (and perhaps disregard) the repeated song (Mello et al., 1995). Third, a novel song caused a greater response when paired with a foot shock than when presented alone, even though the foot shock on its own caused no *zenk* increase in NCM (Jarvis et al., 1995). (The pairing with a noxious foot shock would tend to focus the bird’s attention, one would think, on that particular stimulus.) Finally, a robust *zenk* response was induced in free unrestrained song sparrows in the field when they were presented with tape-recorded songs played through a speaker (Jarvis et al., 1997), a procedure which has also been shown to elicit defensive behaviors in free-ranging birds (Falls, 1982).

Further perspectives on the function of the changing IEG response in NCM have come from studies of its relationship to electrophysiological activity in NCM. Neurons in NCM increase their rate of firing during presentations of birdsong and other complex auditory stimuli, but are activated much less or even inhibited by simpler auditory stimuli (Chew et al., 1996a; Stripling et al., 1997). Individual neurons tend not to show strong preferences for individual songs (Stripling et al., 1997), yet the

response to a specific repeated song is rapidly modulated without affecting the response to other songs (Chew et al., 1995, 1996a,b; Stripling et al., 1997). This modulation follows a very characteristic form: The firing rate is highest during the first presentation of a song, and by the second presentation drops to a lower level (Stripling et al., 1997). After this rapid initial change, each successive presentation of the same song continues to elicit a more or less similar response (neurons in NCM typically increase their mean rate of firing about twofold during the song stimulus, relative to spontaneous firing before or after the stimulus). The rapidity of this initial change in the magnitude of the electrophysiological response is most evident with single-unit techniques (Stripling et al., 1997). The change is also evident using multiunit recording, which has the complementary advantage of providing mean population responses that can be compared in the same bird over several days (Chew et al., 1995, 1996a,b).

Given that the *zenk* response to a particular song is eventually eliminated completely when that song is repeated (Mello et al., 1995), what then happens to induced electrophysiological activity? The intriguing result is that NCM neurons do not stop firing in response to a song that has been recently repeated—but the rapid initial modulation of the firing rate disappears (Chew et al., 1995; Stripling et al., 1997). Hence, the complete habituation of the gene response with song repetition is not simply the result of a cessation of physiological activation. Rather, what is lost as the gene response fades is a relatively brief component of the electrophysiological response. Consistent with a relationship between initial response modulation and gene induction, white noise (which is ineffective at inducing a *zenk* response) induces a high rate of firing but little modulation (Stripling et al., 1997).

The repetition of a song stimulus thus invokes a complex sequence of changes in both electrophysiology and gene expression. To reiterate this sequence, the first change is electrophysiological (increased firing) followed almost immediately by a further change in the magnitude of the response (modulation). If stimulus presentation continues, *zenk* mRNA then begins to accumulate, first detectable at about 10 min and peaking at about 30 min after stimulus onset. Roughly coincident with the accumulation of *zenk*, the electrophysiological response then undergoes a more long-lasting change, so that subsequent presentation of the same song (e.g., after an interruption by presentation of other stimuli) no longer induces the rapid modulation.

Finally, if song stimulation is continued even longer, *zenk* mRNA spontaneously declines, and then if the same song is presented again after some pause, *zenk* is no longer induced (habituation). Presentation of a fresh song not recently repeated will activate the entire process once again. (Because of the difference in the time course and magnitude of the genomic and electrophysiological changes and the uncertain causal relationships between the two, it may not be appropriate to use the same term to refer to them both; here, I have employed “habituation” to refer to the wholesale loss of the *zenk* response, “modulation” to refer to the rapid initial change in the electrophysiological response, and “loss of modulation” to refer to what happens to electrophysiological activity after prolonged stimulus repetition. However, other terminologies have also been used.)

Given the abundant evidence that IEG responses are necessary for long-term memory consolidation, it seems possible that the gene response in NCM could be related to the eventual stabilization of the change in its electrophysiological response (i.e., loss of modulation). Supporting evidence for this has been provided by experiments in which RNA or protein synthesis inhibitors were injected into NCM prior to song repetition, and multiunit electrophysiological responses during song repetition were then measured. In this case, no loss of response modulation ever emerged (Chew et al., 1995). Thus, it seems that gene expression is actively required to achieve a flat or nonmodulated electrophysiological response in NCM. The causal linkages between increased IEG expression in NCM and a flattened electrophysiological response remain an important subject for future studies. For example, does an individual neuron change and then consolidate its electrophysiological response autonomously? This would imply that the induced gene products are focused on consolidating a change in the neuron’s dendritic synapses so that it fires in a different way to the same afferent input. This simple explanation seems unlikely, however, given that an NCM neuron typically responds to many different songs and the change in its firing is very specific for the single repeated song (i.e., how many individual songs can be represented by dedicated synapses on a single neuron?). The other possibility, to which I return below (also see Stripling et al., 1997), is that the neuron’s firing properties are being adjusted extrinsically via changes in the pattern of afferent excitation and/or modulation. If the extrinsic modulatory pathways depend themselves on the informa-

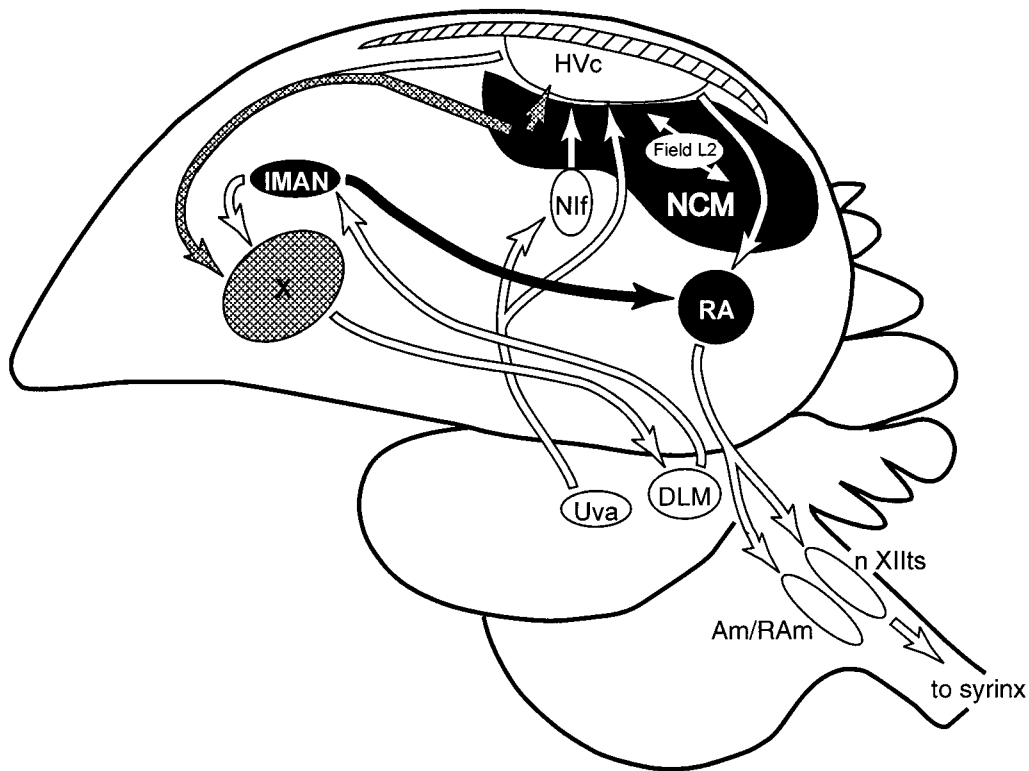


Figure 2 Key sites of molecular regulation in the song system. Modified from Brenowitz (1997) to show sites (in black) where robust changes in gene expression have been correlated with aspects of song circuit formation or song learning, as discussed in the text. These sites include the pathways connecting and including IMAN and RA, and the NCM region of the caudal telencephalon (added in a highly schematized rendering). The pathway leading from the gene-responsive regions in NCM to Area X has not yet been formally demonstrated, but abundant circumstantial evidence for it exists, as reviewed in the text.

tion processing that occurs in NCM neurons, then the function of the gene response may be targeted more at the NCM neuron's axonal synapses. This would allow a highly specific change to occur via extrinsic feedback pathways without requiring a high degree of intrinsic specificity on the part of the individual NCM neuron.

Consideration of the anatomical connectivity of *zenk*-responsive regions provides a final important piece of information that bears on the nature and significance of the gene response to song stimulation (Fig. 2). Auditory information from the thalamus first reaches the telencephalon at neostriatal Field L2, which has a tonotopic organization (Müller and Scheich, 1985; Fortune and Margoliash, 1992) and does not itself show a *zenk* response (Mello and Clayton, 1994). Neurons in Field L2 project to several sites that do show a *zenk* response, including neostriatal Fields L1 and L3 and the HVc shelf (Fortune and Margoliash, 1992). The region

of greatest *zenk* response (caudomedial neostriatum/hyperstriatum) receives auditory input from the Field L complex, and forms robust reciprocal connections internally (Vates et al., 1996) and also with other regions of the telencephalon including the caudal paleostriatum (Mello, 1993; Vates et al., 1996). Based on this anatomy, it seems unlikely that the highly selective and specific response modulations that occur in NCM are simply a passive reflection of changes in lower auditory afferents (i.e., Field L2, where neurons tend to have much simpler tonotopic responses). More likely is the possibility that functional modulation in NCM is mediated either via internal connections or reciprocal feedback from other processing centers in the brain. (Based on comparisons of published maps as well as unpublished observations, it is also likely that *zenk*-defined NCM includes the paraHVc region of the songbird neostriatum. This would suggest another kind of modulatory input to NCM, since paraHVc contains

a rich concentration of estrogen receptors, and is also notable since some neurons in paraHVC send projections to Area X, potentially providing a direct pathway from NCM into the song control circuit; see Nordeen et al., 1987; Johnson and Bottjer, 1993, 1995; Fortune and Margoliash, 1995; Foster and Bottjer, 1997).

The salient features of NCM's responses and anatomy can be summarized succinctly in four major points. (a) The *zenk* response to a specific song can be either potentiated or switched off based on the context in which the song is heard. (b) Activation of the *zenk* response is correlated not simply with an elevation in firing, but with the brief appearance of a specific component of the electrophysiologic response. (c) Individual neurons in NCM have complex auditory response properties, yet do not show much intrinsic selectivity in their responses to different conspecific songs; given precedents in other systems, it seems likely that NCM neurons could represent specific songs in the details of correlated population activity (e.g., Laurent, 1996). (d) *Zenk*-responsive regions are well positioned to provide highly processed auditory information to other higher centers in the brain, and to receive potential modulatory signals via reciprocal connections.

So what does all this mean for the behavioral significance of the changing IEG response in NCM? The most straightforward interpretation is that the gene response is engaged by processes related to the perception of significance, and the brief modulation in firing is the electrophysiological trace of this perception. Perception of significance may invoke or require systems outside NCM involved in, for example, affect, arousal and long-term memory storage. These systems may themselves depend on the auditory representations that are developed within NCM. The changing genomic and electrophysiological response properties in NCM could in turn arise from the influence of reciprocal feedback pathways from these other brain regions. Modulatory inputs would, of course, be represented by a set of synapses on NCM neurons distinct from those carrying the primary auditory signals from lower sensory afferents.

An interesting broader implication of this is that IEG induction might require coincident activity in two sets of inputs (sensory and modulatory). By such a mechanism, diffuse modulatory signals could selectively engage the IEG response only in those neurons whose activity is temporally associated with the significant event or context. As reviewed earlier, considerable evidence indicates that the IEG

response is necessary for long-term memory consolidation. Although the details of this process are not known, scenarios can be envisaged in which a short-term memory trace is represented by the reversible modification (e.g., phosphorylation) of molecular constituents at particular synapses; some of the IEG proteins or their downstream regulatory targets may be capable of interacting with these modified synaptic components to catalyze the formation of more permanent changes at that site (e.g., increasing the size or duplicating the synapse). The critical synaptic changes could be either efferent (axonal or presynaptic) or afferent (dendritic or postsynaptic) with respect to the cell nucleus in which the IEG induction occurs. Whether pre- or postsynaptic (or both), such a process would provide a global mechanism for adjusting the efficiency by which specific memories are committed to long-term storage.

GENE REGULATION PROVIDES CLUES TO THE FUNCTIONAL ORGANIZATION OF THE ADULT SONG SYSTEM

Despite the insights gained from study of NCM, the unique advantages of the songbird as a model system for molecular and cellular analysis are most obviously represented in the nuclei of the traditional song control circuit. These, however, express neither *zenk* nor *c-jun* in response to song playbacks, and even seizures result in little activation of these genes in the song nuclei (Mello and Clayton, 1995). Recently, however, three groups of investigators have shown that the song nuclei can indeed mount an IEG response, and do so when the bird sings.

Activation of the *zenk* gene occurs in the major telencephalic song control nuclei when a bird countersings to tape-recorded birdsong; and the more the bird sings, the greater is the response (Jarvis and Nottebohm, 1997). Activation also occurs in NCM in this behavioral paradigm, but this was attributable to the tape-recorded countersong and to auditory feedback, since NCM's response was specifically absent in deafened birds. Clayton and Jin (1997) confirmed the general result by monitoring *zenk* activity in normal birds producing spontaneous song, and further compared *zenk* induction patterns in singing adult and juvenile zebra finches (see the penultimate section of this article). Kimpo and Doupe (1997) also detected increased immunoreactivity for another IEG protein (*c-FOS*) in HVC and RA of singing zebra finches. There are some interesting differences in these three reports, but

they agree on the major conclusion: The normal act of singing causes a massive IEG response in song control nuclei of adult songbirds. Even the *attempt* to sing was sufficient to induce *zenk* in the song control nuclei in a bird whose song production was disrupted by tracheosyringeal nerve section (Jarvis and Nottebohm, 1997).

Earlier studies of auditory responses in anesthetized birds led to the suggestion that song perception might rely upon the same circuits that are responsible for song production (Williams and Nottebohm, 1985). Given the distinct patterns of gene activation observed during singing and listening, critical re-evaluation of this thesis now seems appropriate. More recent studies of the auditory response properties in the song nuclei of young birds have shown that the selectivity of these responses changes in parallel with the development of the bird's own singing ability (Volman, 1993; Doupe, 1997) and are selective for playback of the bird's own song as opposed to its tutor's song (Volman, 1993). Yet, juvenile finches clearly can remember specific song models to which they are exposed long before they can reproduce them (Böhner, 1990), and so the auditory responses measured in song nuclei do not seem to represent at least these aspects of song perception and memory. Auditory responses in the motor circuit might still serve a reinforcing function for maintenance of the bird's own song (Nordeen and Nordeen, 1992), but this hypothesis still must be reconciled with evidence that auditory activity is inhibited in the song nuclei at the moment the bird is singing (McCasland and Konishi, 1981; McCasland, 1987). Another piece of evidence often cited to support the idea that the song control nuclei have a role in song perception is the study of Brenowitz (1991), who lesioned HVC and regions immediately caudal to it in female canaries and observed effects on song discrimination ability in a behavioral assay. The interpretation of this study was drawn prior to the discovery of NCM's robust auditory properties, however, and the lesions that were most effective at disrupting song discriminations proved to be the ones that also included major portions of adjacent NCM as well as HVC (Brenowitz, 1991). It still remains possible, of course, that song perception depends in part on the song control nuclei but activates a set of different molecular mechanisms and biochemical cascades from those activated by song production. Further experiments are clearly needed to assess the physiological significance of the auditory responses observed in the song control nuclei.

In the two analyses of the *zenk* gene response during singing, the largest magnitude of activation was seen in Area X (Clayton et al., 1997; Jarvis and Nottebohm, 1997; Jin, 1997). This, too, must be reconciled with earlier results, which in this case have often been taken to imply a minimal functional role for the anterior pathway in adult singing. One earlier physiological study using multiunit recording failed to detect any change in activity in Area X or IMAN during singing (McCasland, 1987), and lesioning of Area X or its downstream partner in the anterior pathway (IMAN) has been reported to have a minimal effect on singing in adult birds (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). How can these observations be reconciled with the robust genomic response now observed in Area X in singing adults? No firm answer is immediately available, but again there are significant clues. One is the apparent homology of the brain region containing Area X with the basal ganglia of mammals (Karten and Dobbeldam, 1973; Casto and Ball, 1994; Bottjer and Alexander, 1995; Metzger et al., 1996; Bottjer and Johnson, 1997). The basal ganglia seem to be involved in the trial-and-error learning of motor tasks (Graybiel, 1995; Jueptner et al., 1997), much as a songbird must learn to sing by trial-and-error matching of vocal performance to a remembered template. In the basal ganglia, representations seem to be formed not via gross elevation of multiunit firing, but through brief phases of temporal synchronization of tonically active neurons (Graybiel, 1995). If Area X encodes representations in a similar way, this would be easy to miss using multiunit analysis of gross activity (McCasland, 1987).

More challenging is to reconcile the apparent lack of effects of adult Area X lesion with the robust *zenk* activation that must occur in this nucleus every time the bird sings. Since adult X lesions do not immediately disrupt song, Area X cannot be playing an immediate role in song production in the adult. This leaves two possibilities for the significance of the genomic/physiological activity that occurs there during singing: (a) Area X forms some sort of representations of song motor activity that are used in adults mainly for purposes other than the immediate support of singing. Although the focus of most research and thinking has been on the pathway from Area X through the thalamus and IMAN to RA, evidence for other possible output pathways has recently emerged (Bottjer and Johnson, 1997). (b) The physiological activity that occurs in Area X in

the adult may no longer have any functional consequence on singing, at least in zebra finches, because of constraints on plasticity further downstream in the song control pathway. The sites and mechanisms responsible for constraining adult song modification are not known. In the next section, however, I will develop the argument that those constraints may be placed primarily at the level of RA, at the convergence of the inputs from HVC and IMAN. If the circuit elements downstream of Area X are no longer capable of responding or adapting to its signals in the zebra finch, neither lesion nor activation of Area X may have any significant consequence in this species.

GENE REGULATION MAY DETERMINE THE BOUNDARIES OF THE CRITICAL PERIOD FOR SONG LEARNING

The work reviewed in the previous two sections implies a general model for the functional significance of the IEG response in the context of a neural circuit. To state the model succinctly, the IEG response in a given neuron is elicited by specific patterns or combinations of synaptic signals. The consequence of the gene response is to facilitate the cellular mechanisms by which labile, activity-dependent modifications of specific synapses are recognized and converted into more stable, long-lasting forms. Whether such a conversion occurs, and whether it has any detectable behavioral consequence, depends entirely on the position of the cell in the larger neuronal circuitry and on the particular input–output organization of its connections. With this model in place, we are now prepared to return to the point reached at the end of the second section: What sort of regulatory mechanisms could account for the changing levels of plasticity evident in the initial development and eventual crystallization of song in age-limited learners such as the zebra finch, or the seasonal changes in song stability evident in an open-ended learner such as the canary?

If IEG responses are indeed centrally involved in synaptic consolidation and sensitive to modulatory influences, then some change in IEG expression should be evident in the song system associated with the changing status of juvenile song-learning ability. To test this, Jin (1997) recently completed a major study of *zenk* expression in the juvenile zebra finch brain, following from the beginning of song memorization through sensorimotor learning. She monitored *zenk* mRNA levels throughout the

brain under three conditions: basal (quiescent or unstimulated), during song listening (to either tape recordings or a live adult male), and during song production. Significant and interesting developmental changes in *zenk* expression were observed under all three conditions, with implications for mechanisms that may govern both the juvenile acquisition of song model memories and the eventual stabilization of the mature adult song (Jin, 1997; Clayton et al., 1997).

First, basal *zenk* expression (measured in birds after 24 h alone in a soundproof chamber) was substantially elevated in NCM of juvenile male finches during the period of song memorization. Peaking at 30 days of age, mean basal expression at that time was almost as high as the mean level reached in adults only after stimulation by song playbacks. Basal expression was lower but still elevated at 45 days of age, when finches are still capable of overwriting their initial song memories with new ones (Slater et al., 1991). The basis for this age-related change in gene expression is not known, although elevated *zenk* expression has also been observed during the critical period for visual cortex formation in the mammal (Kaplan et al., 1995, 1996; Wallace et al., 1995). The constitutive elevation of *zenk* (and perhaps other IEGs) might shift neurons into a state where any synaptic modification is efficiently consolidated, enhancing the initial memorization of the tutor's song.

Second, relative to the increased basal expression, *zenk* could still be further induced by song playbacks in zebra finches 30 days of age or older. Unexpectedly, however, no *zenk* response to song playbacks occurred in birds at 20 days of age. At this age, zebra finches may still be too young to form sensory memories of song (the onset of song memorization has never been satisfactorily determined experimentally). To learn whether the delayed emergence of the *zenk* response was a function of chronological age or song-listening experience, finches were then reared in soundproof chambers with their mothers alone and tested at either 30 or 40 days of age. No *zenk* response was observed in these birds, either, suggesting that the ability to induce *zenk* depends on prior experience. This is quite the opposite of the phenomenon of *zenk* habituation in adult birds, in which the recent experience of hearing a song repeated abolished the response to that song (Mello et al., 1995). If *zenk* induction depends on mechanisms of selective attention as proposed in the third section, then it may simply be that birds raised in extreme social/acous-

tic isolation are initially incapable of recognizing and attending to a song the first time they hear it. It will be interesting to examine the electrophysiological responses in NCM in these birds, to determine whether the correlation between response modulation and *zenk* induction observed in adults also holds here.

Finally, developmental changes were also observed in the anatomical pattern of *zenk* activation obtained during singing, and these changes have implications for the mechanisms that may constrain the modification of song patterns in adult zebra finches. In juveniles, singing induced robust *zenk* activation throughout all of RA. In adults, however, the response was mostly limited to the posterior quarter of the nucleus. Does this mean that the anterior RA in the adult is no longer receiving the signals necessary to induce *zenk*? This possibility is suggested by evidence that in IMAN (RA's afferent), substantial synaptic pruning (Nixdorf-Bergweiler et al., 1995; Wallhauser-Franke et al., 1995b) and loss of MK-801 binding to NMDA receptors (Aamodt et al., 1992) both occur at the time a stable song is emerging in the juvenile. Alternatively, the anterior portion of RA may no longer be capable of responding by activating the gene or may have a higher threshold for activation, even if the proper synaptic signals are generated. In either case, constraints on plasticity of song performance could effectively be determined at the level of RA, especially since the details of song structure seem to be represented in the pattern of firing within RA (Yu and Margoliash, 1996). One indirect test of this hypothesis may already have been performed. Jarvis and Nottebohm (1997) reported high *zenk* induction in RA of adult canaries during singing, in contrast to the very restricted expression Jin (1997) observed within RA of adult zebra finches. If so, this would suggest a specific mechanistic basis for the observation that canaries retain substantial motor plasticity in adulthood, whereas zebra finches do not. The canary RA may be capable of converting transient changes in synaptic strengths into more permanent forms, allowing for gradual changes in the mapping of specific HVC afferents onto its dendrites, and thus leading to changes in the song produced. With the *zenk* response in RA suppressed or reduced in the adult zebra finch, it may not be possible to consolidate any synaptic changes even if they transiently occur.

SUMMING UP AND LOOKING AHEAD

The songbird has demonstrated its richness and power as a model system for exploring brain-be-

havior relationships, as the other articles in this special issue clearly show. In the last few years, molecular biology has also demonstrated its richness and power as a paradigm in which to conduct this exploration. By "letting the molecules speak," they have given us unexpected insights and new hypotheses about the biological organization of this system. These insights include:

- The implication of NCM as a primary site for sensory aspects of song processing (via *zenk*)
- Recent evidence of a functional compartment within RA which could be involved in setting the boundaries of song plasticity (via *zenk*)
- Evidence of physiological activity in the anterior pathway of the circuit during singing, even in adult birds (via *zenk*)
- Mechanistic bases for responses to steroids [via estrogen receptor (ER) or AR] and other modulatory signals (via NOS, NMDAR1, and melatonin receptor)
- A sharper description of the timing of major structural changes in the developing circuit (via GAP-43, neurofilament, myelin basic protein, and ER)
- The discovery that a novel protein involved in Alzheimer's disease is also a key target of regulation in song circuit development (synelfin)
- Intimations of a neural substrate for selective attention (via *zenk*, ACh, and catecholamines).

However, the story is far from over, and the role of gene regulation in brain development and function is far from understood. I close by identifying some of the broad issues and opportunities to be addressed in coming years:

- What are the signals responsible for directing HVC-RA synapse formation in the male zebra finch? How are molecules that support synaptogenesis and pathfinding regulated in this context? In what way can activity in IMAN influence the formation or function of connections between HVC and RA? Work in progress suggests that it may be possible to observe aspects of song circuit formation *in vitro* (Holloway and Clayton, 1997), which would make them much more accessible to direct experimental manipulation and analysis in a defined context. It should also be possible to deliver proteins or DNA constructs into specific nuclei in the

song circuit *in vitro* and then assay effects on circuit development and synaptic physiology.

- What are the specific synaptic signals responsible for inducing IEG responses in NCM and the song nuclei? And how does this induction influence mechanisms of long-term synaptic consolidation, such as site-specific synaptogenesis or pruning? In general, the large functional consequences of IEG expression are easy to imagine but difficult to prove. A model system is needed for study of this basic issue, which may be as fundamental to neuronal cell biology as the generation of the action potential. The advantages of the songbird model (large discrete nuclei, defined wiring diagram, altricial development, functional modulation via hormones and other signals, and behavioral assays) may be especially relevant here.
- How do processes responsible for global modulations of brain state or function give rise to specific localized effects on sensory processing and memory formation (e.g., selective attention)? The songbird literature is rich with evidence that social context and motivational factors have a major influence on song learning and other measurable behaviors. In this review I described evidence that these factors may impinge directly on processes of gene regulation in the brain. In coming years, a growing focal point for neuroscience research will be to understand the integration of organism-, circuit-, cellular-, and molecular-level processes in the nervous system. For this, the songbird is unparalleled as a model system for study.

APPENDIX

Catalog of Molecular Regulation in Song Circuit Development

Gene products (mRNAs and/or proteins) which have been shown to change in abundance or localization during development of the song system are described here. This listing follows the organization of the developmental timeline in Figure 1.

Nitric Oxide (NO) Synthase. NO is a diffusible gas with apparent functions as a neurotransmitter and modulator of synaptic plasticity. Relative to surrounding telencephalon, the song nuclei tend to display increased neuropil staining but a decreased density of labeled cell bodies for the rate-limiting

enzyme in NO production, NO synthase (NADPH diaphorase). This dichotomy would seem to imply that cells in the song nuclei may be sensitive to modulation by NO via inputs that arise outside the song circuit. The incidence of NO-positive cells decreases steadily, however, in HVC, RA, and Area X from day 21 to adulthood (Wallhauser-Franke et al., 1995a).

Canarigranin. Canarigranin is a close relative and possible homologue of RC3/neurogranin, a mammalian protein which is enriched in postsynaptic terminals, binds calmodulin under regulation by protein kinase C, and has been shown to be an active phosphorylation substrate in mammalian long-term potentiation (Watson et al., 1990; Baudier et al., 1991). Thus, this protein may be important in mediating activity-dependent changes in synaptic strength (Malenka et al., 1989; Malinow et al., 1989; Klann et al., 1992). The RNA for canarigranin is relatively abundant in HVC, RA, and IMAN, but virtually absent in the adult Area X. In juvenile males, however, canarigranin RNA is increased in Area X at days 15 and 25, but then declines sharply and specifically within Area X by day 35 (Siepkala et al., 1994, J. George and D. Clayton, unpublished data).

ER. Estrogen-binding capability in adult male HVC is found in neurons that project to Area X, but not in those projecting to RA (Nordeen et al., 1987; Johnson and Bottjer, 1995). In females, exposure to estrogen in the first weeks of life increases the size of song nuclei, stimulates the formation of synapses between HVC and RA, and instills the capacity for song production (Gurney and Konishi, 1980; Simpson and Vicario, 1991a,b). In a developmental study of ER immunoreactivity beginning at day 20, the number of labeled neurons in HVC of females peaked at day 30, dropped fourfold by day 45, and was negligible in the adult female HVC (Gahr and Konishi, 1988). Consistent with this developmental time course, neurons in HVC and RA showed morphological responses to estrogen when the hormone was implanted in juvenile females of various ages, but estrogen responsiveness declined steadily from day 30 to day 45 and was negligible after that (Konishi and Akutagawa, 1988). An explicit analysis of ER in developing males has not been reported.

Synelfin. Synelfin, an intracellular protein enriched at presynaptic terminals, is homologous to the non-amyloid- β component precursor purified from se-

nile plaques of Alzheimer's disease patients (George et al., 1995). The cellular function of the protein is not yet known, but its highly conserved structure predicts it should bind membrane phospholipids reversibly and with high specificity, suggesting a possible role in organization and function of synaptic membranes (George and Clayton, 1996). The synelfin protein is notably decreased in adults in HVC, RA, IMAN, and Area X relative to the rest of the telencephalon (George et al., 1995). In the juvenile zebra finch prior to HVC-RA ingrowth, the RNA is initially expressed at high levels in IMAN and the protein is detected in RA, consistent with increased synthesis in IMAN and transport to presynaptic terminals upon RA. RNA levels decline within individual IMAN neurons from days 25 to 30 and even more so from days 30 to 35, as revealed by *in situ* hybridization (George et al., 1995). Protein levels in the terminals in RA decline about a week later, between days 40 and 45 (Jin and Clayton, in press).

GAP-43. GAP-43 is an intensively studied growth-associated protein that is enriched in growth cones and also a presynaptic substrate for phosphorylation in mammalian long-term potentiation (Skene, 1989). In adult zebra finches, the RNA for GAP-43 is decreased detectably in HVC and even more notably in RA relative to surrounding telencephalon. IMAN neurons contain the RNA at a high level, whereas both Area X and the surrounding LPO have little. During juvenile development, this pattern changes specifically with respect to RA: The RNA is abundant in RA at both days 15 and 25, begins to decline by day 35, and reaches its minimum by day 45 (Jin et al., 1994). No developmental change in GAP-43 gene expression was detected in other song nuclei. In an independent study, a monoclonal antibody to the rat GAP-43 protein was used to localize apparent GAP-43-immunopositive terminals within RA in adult and juvenile male zebra finches (Sakaguchi and Saito, 1996). The number of neuronal soma covered with these terminals was negligible before day 30, then increased explosively at days 33-35 and dropped abruptly at days 38-40. These results were interpreted as indicating the arrival of axons from HVC and the abrupt formation of initial synaptic contacts on RA cell bodies between days 30 and 40, followed by redistribution of terminals to dendritic neuropil over the subsequent weeks leading to song crystallization (Sakaguchi and Saito, 1996). Although this idea is plausible and attractive, the fact that the RNA is more abundant in

IMAN than in HVC allows the formal possibility that the immunoreactivity might represent terminals of projections from IMAN. Similarly, the elevated RNA level within the juvenile RA allows the possibility that the immunoreactivity could represent interneuronal connections which are abundant within RA (Canady et al., 1988). Further study of this intriguing developmental phenomenon is clearly warranted.

NMDA Receptor. The NMDA receptor mediates glutamatergic transmission at some synapses and has been intensively studied for its possible role as a primary agent in synaptic plasticity (see Nordeen and Nordeen, 1997, for further discussion). Specific binding of the noncompetitive NMDA receptor antagonist MK-801 can be detected in HVC, RA, IMAN, and Area X of adults, but at levels slightly below the surrounding telencephalon in most cases (Aamodt et al., 1992). In a comparison of MK-801 binding in adults versus juvenile males at day 30, the only age-related difference was in IMAN, where binding was increased at day 30 relative to adults (Aamodt et al., 1992). Scatchard and saturation analyses indicated that this difference was due to a twofold increase in receptor number in the juveniles, but the juvenile receptors had a significantly lower affinity for MK-801 (Aamodt et al., 1995). An additional developmental analysis of NMDA receptor binding in IMAN showed a large decrease between days 30 and 60 and a smaller decrease from day 60 to adult levels at day 80. A preliminary report indicates a similar profile of expression of the mRNA for the NMDAR1 subunit of the receptor, detected by *in situ* hybridization (Basham et al., 1996).

ZENK. ZENK protein is a DNA-binding transcription factor implicated in the regulation of neuronal growth-related genes and induced in the adult songbird brain in response to a variety of stimuli, as discussed in detail below. Two features of the developmental profile of *zenk* gene regulation are included on this chart: the transient elevation of basal expression peaking around 30 days of age, and the marked decline in its responsiveness to singing in a subcompartment of RA as behavioral development progresses from subsong toward crystallization (Jin, 1997; Clayton et al., in press).

ALDH. Aldehyde dehydrogenase is a cytosolic enzyme involved in retinoic acid synthesis. It was detected as a song nucleus-enriched mRNA in an anal-

ysis using differential display PCR, and then shown to be expressed at high levels in HVc and IMAN at days 20, 38, 50, and 60 and adulthood. In RA it was developmentally regulated so that it peaked at day 38, declined by day 50, and was gone by day 60 (Denisenko et al., 1995, 1996). Retinoic acid is a signaling molecule that may be especially important in embryonic development of tissues including the nervous system (e.g., Tole et al., 1995) and works through a receptor that is a member of the steroid receptor family.

Neurofilament. Neurofilaments are critical cytoskeletal components of axons, and the medium-weight subunit (NF-M) is present in both growing and mature axons. Antibodies to NF-M brightly stain the projection from HVc to RA; HVc and RA themselves are also brightly labeled in adult males but not females (Siepk and Clayton, 1995). Immunoreactivity is not evident within the interior of juvenile male RA until day 30. (The absence of any signal despite the presence of terminals from IMAN is not well understood, but may be related to the angle of approach taken by IMAN afferents and the parasagittal plane of section used in these experiments.) Fibrous immunoreactivity indicative of axons and terminals begins to accumulate in RA by day 35, assumes an adultlike pattern by days 40–45, and continues to increase until it reaches the robust levels of the adult by day 60. These changes appear to be mediated entirely post-transcriptionally, as no change in mRNA levels was detectable in HVc, RA, or IMAN between 25 and 55 days of age (Siepka, 1997).

ACh. Acetylcholine is a neurotransmitter that (among other roles) acts as a diffuse neuromodulator in the central nervous system and may influence cellular excitability and mediate attentional activation (Cox et al., 1994; Pennartz, 1995). ACh is present in adult HVc, RA, and IMAN, as indicated by direct biochemical detection (Sakaguchi and Saito, 1989) and by the presence of significant immunoreactivity for the synthetic enzyme choline acetyltransferase (ChAT) in neuropil of HVc, RA, and IMAN (Sakaguchi and Saito, 1991). ChAT-immunoreactive cell bodies are not detected in these areas, however (nor in DLM or the afferents to HVc), although immunoreactive cell bodies are found in Area X (Sakaguchi and Saito, 1991). Thus, the ACh detected in most parts of the song circuit seems likely to originate in the diffuse projection systems that may serve neuromodulatory roles (e.g.,

Hasselmo and Barkai, 1995). In adults, muscarinic receptors have been detected by autoradiography in HVc, but few are present in RA and IMAN (Ryan and Arnold, 1981), nor does adult RA appear to contain significant nicotinic receptors (Watson et al., 1988). Synaptosomes prepared from RA in 50-day-old juveniles, however, will respond to carbachol (an ACh agonist) with an increase in phosphoinositide turnover (Sakaguchi and Saito, 1989). In juvenile males, acetylcholine and ChAT are low in the HVc, IMAN, and RA at day 30, but each increases to a maximum by day 50 (Sakaguchi and Saito, 1989, 1991). Thus, regulation of or through cholinergic neuromodulatory pathways could conceivably have direct effects on both HVc and RA at or shortly after the time of ingrowth.

Catecholamines, Tyrosine Hydroxylase (TH). Similar to ACh, catecholamines (dopamine and norepinephrine) have well-established roles as diffuse neuromodulators that can influence learning, attention, and arousal (Di Chiara et al., 1994; Pennartz, 1995), and norepinephrine has been specifically implicated in the formation of social memories (i.e., recognition of specific individuals) (Griffin and Taylor, 1995). Several song nuclei in adult males contain high levels of dopamine and significant norepinephrine that presumably arise from diffuse extrinsic modulatory inputs (Barclay and Harding, 1988, 1990; Sakaguchi and Saito, 1989). Immunoreactivity for TH, the rate-limiting enzyme for the synthesis of both dopamine and norepinephrine, is dense in terminals and fibers in adult male HVc, IMAN, Area X, and RA (ventral edge) (Soha et al., 1996), but this is not seen in females (Bottjer, 1992). Mature levels of TH immunoreactivity in the male song nuclei are apparently achieved sometime between day 35 and day 50—one study found that TH levels are low and fairly constant in these nuclei between day 20 and day 35 and reach adultlike levels by day 60 (Soha et al., 1996); another study found no difference in TH immunoreactivity in a comparison of day 50 birds and adults (Bottjer, 1992). High-performance liquid chromatographic analysis of catecholamines detected an increase in dopamine at day 40 relative to day 30 in HVc, but—in slight contrast to the developmental pattern of TH immunoreactivity just described—found stable or even declining levels in IMAN and RA at days 30–90 (Sakaguchi and Saito, 1989). In any case, the potential for influence of the catecholamines on song circuit function seems to continue into adult-

hood, unlike the more restricted developmental role suggested for ACh.

Androgen Receptor (AR). Androgens have been clearly implicated in both the development and adult expression of song behavior (Arnold, 1997; Bottjer and Johnson, 1997; Schlinger, 1997). Almost half the cells in HVC and IMAN of adult males contain AR proteins, and they are also present in RA. This has been demonstrated now by autoradiography (Arnold and Saltiel, 1979; Arnold, 1980; Nordeen et al., 1986, 1987; Bottjer, 1987), immunocytochemistry (Balthazart et al., 1992), and *in situ* hybridization (Nastiuk and Clayton, 1995). Both of the efferent projections from HVC (to RA and Area X) include a substantial proportion of androgen-binding neurons (Sohrabji et al., 1989). The capacity of HVC and IMAN to bind androgen increases in juvenile development in males. From day 25 to day 60, the percentage of androgen-binding cells in male HVC and IMAN approximately doubles to adult levels (Bottjer, 1987). Adult females have few androgen-binding cells in HVC and IMAN (Arnold and Saltiel, 1979). In contrast to males, there is no difference in females in the percent labeled neurons in IMAN at day 20 versus adults, and the percentage of androgen-binding cells in HVC drops by half between these two ages (Nordeen et al., 1987). Androgen binding is increased to male-like levels when females are implanted with estrogen or testosterone shortly after hatching (Nordeen et al., 1986, 1987), and these masculinized females are capable of producing songlike vocalizations in adulthood (Gurney and Konishi, 1980; Simpson and Vicario, 1991a).

Melatonin Receptor. Melatonin is of potential relevance to the song system because it has been implicated in other species in the control of various circadian, periodic, and sexual behaviors (Gahr and Kosar, 1996). A high density of binding sites for melatonin specifically in HVC among song nuclei emerges sometime between day 40 and adulthood (Gahr and Kosar, 1996), suggesting that melatonin might indeed have a role in crystallization or modulation of activity in the mature system, but probably not in the initial formation of the circuit.

Myelin Basic Protein. In a study just completed in my laboratory, the technique of differential-display PCR (Liang and Pardee, 1992) was used to amplify and clone mRNAs enriched in nucleus RA versus surrounding archistriatum (Siepka, 1997). One of

the candidates was found to encode myelin basic protein, a major component of myelin synthesized by oligodendrocytes (Siepka et al., 1997). HVC, RA, and IMAN (as well as the connecting fiber tracts) are all enriched for myelin basic protein-containing cells in adults, as assayed by *in situ* hybridization. The protein is absent, however, in HVC and the RA fiber tract as late as day 50. It begins to accumulate there significantly by day 70 and only reaches adult levels by about day 90. These molecular-level results complement earlier studies using histologic stains, which showed that myelination increases dramatically in the song circuit but not until late relative to the initial formation of the HVC-RA projection (Herrmann and Bischof, 1986; Kafitz et al., 1992).

Parvalbumin. Parvalbumin is a neuron-specific calcium-binding protein abundant in the auditory system and in other areas where spontaneous electrophysiologic activity is high (Braun et al., 1985). In the rat, parvalbumin has been shown to increase in the telencephalon associated with functional maturation of neurons (Delecea et al., 1995). In a developmental study of zebra finch HVC and RA, parvalbumin-positive cells and neuropil were detected in the area of RA as early as day 5 and in HVC by day 8, even before the borders of the nuclei had begun to emerge in Nissl stain (Braun et al., 1991). By day 28, dense parvalbumin-immunoreactive neuropil started to define the boundaries of these song nuclei, and by adulthood, the density of parvalbumin-positive immunoreactivity had increased massively.

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