

C.V. Mello

Mapping vocal communication pathways in birds with inducible gene expression

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Abstract Expression mapping of activity-dependent genes has been very useful to reveal brain activation patterns associated with specific stimuli or behavioral contexts. In addition, activity-induced neuronal gene expression is likely associated with neuronal plasticity and may be part of the mechanism(s) involved in long-term memory formation. Analysis of the immediate-early gene *zenk* has been used to generate high-resolution maps of brain activation associated with perceptual and motor aspects of vocal communication in songbirds and other avian groups. This molecular approach has generated novel insights into the organization of perceptual and motor control pathways for vocal communication in birds. Its impact on the neurobiology of birdsong will be reviewed here. Emphasis will be given to the caudomedial neostriatum, the area that shows the most robust *zenk* induction upon presentation of song to songbirds. Another focal point will be the comparative analysis of vocally induced *zenk* expression patterns across the avian orders that evolved vocal learning (i.e., songbirds, parrots, and hummingbirds). New research directions indicated by this molecular analysis will be discussed throughout.

Keywords Birdsong · Learning · Neural plasticity · *zenk* · Zif-268

Abbreviations ¹*AAc* central nucleus of the anterior archistriatum · *ACM* caudomedial archistriatum · *Ai* intermediate archistriatum · *area X* area X of the paleostriatum · *Av* nucleus avalanche · *cHV* caudal hyperstriatum ventrale · *clHV*, *cmHV* caudolateral and caudomedial hyperstriatum ventrale · *DM* dorsomedial nucleus of ICo · *ex* extensions of LPOm · *HVoc* complex including the oval nucleus of the hyperstriatum ventrale and surrounds · *ICo* intercollicular nucleus of the mesencephalon · (*field*) *L* primary telencephalic auditory area; L1, L2a, L2b, and L3, field L subdivisions · *IAHV* lateral nucleus of the anterior hyperstriatum ventrale · *IAN* lateral nucleus of the anterior neostriatum · *LH* lamina hyperstriatalis · *LPO* parolfactory lobe · *LPOm* magnocellular nucleus of the parolfactory lobe · *IMAN*, *mMAN* lateral and medial subdivisions of the magnocellular nucleus of the anterior neostriatum · *MLd* dorsal part of the lateral mesencephalic nucleus · *NAoc* complex including the oval nucleus of the anterior neostriatum and surrounds · *NCM* caudomedial neostriatum · *Ndc* dorsocaudal neostriatum · *NIDL* neostriatum intermedium pars dorsolateralis · *NIf* nucleus interfascialis · *NLc* central nucleus of the lateral neostriatum · *nXIIIts* tracheosyringeal subdivision of the hypoglossal nucleus · *Ov* nucleus ovoidalis of the thalamus · *Pc* caudal paleostriatum · *RA* robust nucleus of the archistriatum · *VA* vocal nucleus of the archistriatum · *VAH* vocal nucleus of the anterior hyperstriatum ventrale · *VAN* vocal nucleus of the anterior neostriatum · *VAP* vocal nucleus of the anterior paleostriatum · *VLN* vocal nucleus

C.V. Mello
Neurological Sciences Institute (NSI),
Oregon Health Sciences University (OHSU),
West Campus – Building 1, 505 NW 185th Ave.,
Beaverton, OR, USA
E-mail: melloc@ohsu.edu
Tel.: +1-503-4182650
Fax: +1-503-4182501

¹ A substantial revision of the avian brain nomenclature that supersedes the current system has been recently agreed upon by a team of experts in avian and comparative neuroanatomy. The new system will retain some of the current abbreviations, eliminate several errors and inconsistencies, and facilitate interchange with researchers in other vertebrate groups. The final set of recommendations will soon be available in a published format and at the Avian Brain Nomenclature Exchange web site (<http://jarvis.neuro.duke.edu/nomen/index.html>)

of the lateral neostriatum · *VMH* vocal nucleus of the medial hyperstriatum ventrale · *VMN* vocal nucleus of the medial neostriatum

Introduction

Songbirds are among the few animals that developed the ability to learn their vocalizations based on auditory input (Thorpe 1958; Marler and Peters 1977). The learning and the production of learned vocalizations in songbirds are under the control of a set of interconnected brain nuclei known as the song control system (Nottebohm et al. 1976, 1982). The study of the neurobiology of birdsong and the song control system has led to significant insights into fundamental aspects of brain function, including the neuronal basis of vocal production and vocal learning, the effect of sex steroids on the brain and behavior, and the processes of neuronal plasticity and neuronal replacement during adulthood (see reviews in Brenowitz et al. 1997 and other articles in this volume).

The use of molecular biology tools and methods has allowed researchers to begin to identify molecular mechanisms underlying the processes above. The pioneering work by Clayton et al. (1988) established some of the basic methodology for the isolation and expression analysis of genes of interest in the songbird brain. Two major approaches have been subsequently utilized, to identify genes involved in various aspects of song perception, learning and production. First, cloning and expression analysis of genes whose expression is enriched or modulated in brain areas controlling vocal communication and learning. This approach is exemplified by the identification of synuclein (George et al. 1995) and of *zRaldDH*, a retinoic acid-synthesizing enzyme (Denisenko-Nehrbass et al. 2000), in some key song control nuclei. Second, identifying genes that are strong candidates or known regulators of neuronal plasticity and learning in other systems, and testing for the possible involvement of such genes in the neurobiology of birdsong.

This latter approach is well exemplified by the study of immediate early genes (IEGs). IEGs are usually expressed at low basal levels in the brain in the absence of sensory stimulation, but their expression in neuronal cells rises rapidly and transiently in response to neuronal activation (Morgan and Curran 1989; Sheng and Greenberg 1990; for a recent review see Clayton 2000). Analysis of induced IEG expression can thus be very useful for mapping patterns of brain activation in response to specific stimuli or behavioral paradigms (Chaudhuri 1997; Tischmeyer and Grimm 1999). In addition, several IEGs encode transcriptional regulators and are thought to integrate a genomic cascade leading from neuronal activation to long-lasting changes in neuronal properties (Goelet et al. 1986; Morgan and Curran 1989; see also Clayton 2000). As such, they are good candidates for genes involved in the formation of experience-related long-term memories (Goelet et al. 1986).

This review will focus on studies that have used IEG expression analysis to map brain areas involved in perceptual and motor aspects of vocal communication and learning in songbirds and other avian groups, as well as to study the functional organization of these areas. The main emphasis will be on *zenk*, the IEG that has been most extensively studied in birds. *zenk* expression is highly sensitive to song stimulation and singing behavior, and analysis of its expression has allowed a refined mapping of vocal and auditory systems in several avian species, revealing new phenomena and characteristics that are of relevance to understanding auditory processing of song, auditory-vocal interactions, and the motor control of song production.

The *zenk* gene

The avian homologue of *zif-268*, *egr-1*, *ngfi-a* and *krox-24* (Milbrandt 1987; Christy et al. 1988; Lemaire et al. 1988; Sukhatme et al. 1988; Mello 1993) has been referred to as *zenk* (the notation *zenk* will be used in this review when referring to the gene or the mRNA, and ZENK when referring to the protein). *zenk* is expressed in neurons and considered an activity-dependent gene, as its expression is highly sensitive to membrane depolarization (Milbrandt 1987; Sukhatme et al. 1988). It is also considered an IEG due to its rapid and transient induction kinetics upon neuronal cell activation. The *zenk* gene encodes a zinc-finger transcription factor that binds to a DNA motif present in the promoter region of several genes (Christy and Nathans 1989; Pavletich and Pabo 1991; Swirnoff and Milbrandt 1995), thereby potentially exerting a broad-ranging transcriptional regulatory action. The low expression levels of *zenk* during rest and its high responsiveness to membrane depolarization render *zenk* a highly sensitive marker for neuronal activation (Worley et al. 1991; Mello et al. 1992; Chaudhuri 1997). Its expression has not been detected in glial cells (Bartel et al. 1989; Cole et al. 1990).

Most brain regions in birds and mammals contain large numbers of neurons that express *zenk* in response to depolarization. This is clearly shown by administration of the GABAergic antagonist metrazole, which leads to widespread depolarization and associated brain *zenk* expression (Saffen et al. 1988; Mello and Clayton 1995). *zenk* is not a universal marker of neuronal activation, though, as some neuronal populations do not show an induction of *zenk* expression in response to activation. Nevertheless, this dissociation between neuronal activation and *zenk* induction has only been observed for very few specific brain areas (Mello and Clayton 1994, 1995; Mello et al. 1995) and likely reflects a downregulation of signal transduction components (such as specific membrane receptors, calcium-binding proteins or kinases) that couple membrane depolarization to the nuclear gene expression response (Sheng and Greenberg 1990).

zenk induction in the brain represents an early regulatory event in a genomic cascade triggered by neuronal activation and thought to lead to long-lasting changes in neuronal cells (Goelet et al. 1986; Sheng and Greenberg 1990; Mello 1998; Clayton 2000). *zenk* induction is often seen in the context of neuronal plasticity. For example, it occurs in association with morphological changes triggered in the rat cortex by exposure to a novel enriched environment (Wallace et al. 1995). *zenk* induction has also been linked to hippocampal long-term potentiation (LTP), a model of activity-dependent synaptic plasticity (Cole et al. 1989). Among several IEGs, *zenk* induction best correlates with LTP induction (Wisden et al. 1990; Abraham et al. 1993; Worley et al. 1993; Roberts et al. 1996). A recent study using targeted disruption of the *zenk* (*zif-268*) gene in mice provides more direct evidence linking *zenk* to long-lasting LTP and memories (Jones et al. 2001).

The cloned avian *zenk* homologues shares about 70% overall identity at the amino acid level to the mammalian counterparts (Mello 1993; Long and Salbaum 1998). Importantly, the zinc-finger DNA-binding domain is 100% conserved between birds and mammals, indicating that the same DNA motif is recognized by the ZENK protein (Christy and Nathans 1989; Mello 1993) and that the same or similar genes are likely to be its targets. *zenk* expression can be assessed by in situ hybridization or immunocytochemistry (ICC) with a commercially available antiserum against the ZENK protein (Mello and Ribeiro 1998).

Basal *zenk* expression

The brains of songbirds that have not been exposed to the sound of birdsong and that have not been singing recently show very little *zenk* expression in auditory and vocal control regions (Mello et al. 1992, 1995; Mello and Ribeiro 1998). However, a significant percentage (3–5%) of neurons in auditory brain regions expresses *zenk* in this situation (Mello et al. 1995). They likely represent cells that respond to the sounds the birds make while moving or feeding in their isolation boxes. The number of such cells is higher by ICC than by in situ, likely reflecting the longer half-life of ZENK protein as compared to *zenk* mRNA. Higher numbers of *zenk*-expressing cells are found in lateral portions of the telencephalon known to respond to visual stimulation (Benowitz and Karten 1976; Karten and Shimizu 1989). Some basal *zenk* expression may thus relate to basal visual input during wakefulness.

Song-induced *zenk* expression: auditory

General aspects

A rapid, robust and transient *zenk* induction occurs in various brain regions when songbirds hear conspecific

song (Mello et al. 1992; Mello and Clayton 1994). This effect is observed both in males hearing song without responding vocally (“hearing only” birds), and in females, who usually do not sing, and it is abolished by deafening (Jarvis and Nottebohm 1997). The *zenk* induction response to song is thus related to the act of hearing song, and is likely modulated by perceptual, attentional and/or motivation factors associated with hearing. The areas showing *zenk* induction in response to song are closely associated with central auditory pathways (Vates et al. 1996; Mello et al. 1998b), forming a network of interconnected structures that seemingly represents a conserved avian auditory processing system (Fig. 1). This system precedes the song control system and is likely involved in various aspects of song auditory processing, perception and discrimination.

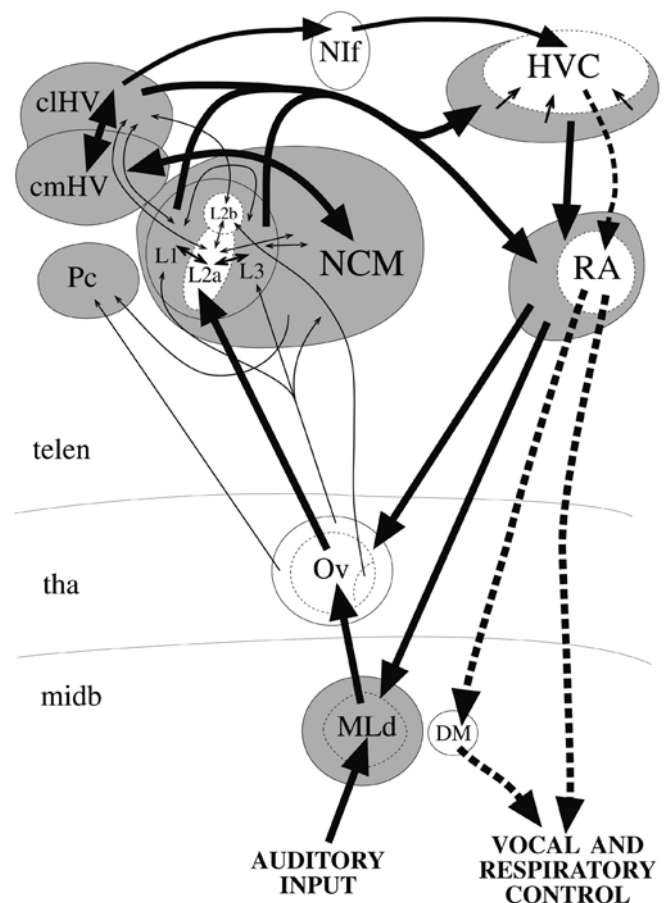


Fig. 1 Schematic representation of auditory pathways and associated *zenk* expression in songbirds. Areas in gray represent areas that show *zenk* induction in response to song presentation, areas in white represent areas that lack this response. The areas adjacent to song nuclei HVC and robust nucleus of the archistriatum (RA) correspond to the shelf and cup regions, respectively. *Thick solid arrows* represent the major ascending and descending projections of the auditory system; *thin solid arrows* represent either minor projections, or projections that have yet to be confirmed; *thick dashed lines* represent projections of the direct motor pathway for song production. *midbr* midbrain; *telen* telencephalon; *thal* thalamus; for others, see abbreviation list

Most *zenk*-labeled cells in song-stimulated birds have clear neuronal morphology (Mello et al. 1992; Mello and Clayton 1995), whereas cells with clear glial characteristics are not labeled. Thus, the *zenk* expression response to birdsong occurs primarily in neurons, conforming to observations in other systems (Worley et al. 1991). One should note that a small percentage of *zenk*-labeled cells are small and their identity difficult to determine based on Nissl staining. Although some of these cells could potentially be non-neuronal, they most likely represent small-sized neurons (for example, as shown by double-labeling with a neuronal tract-tracer, as in Jarvis et al. 1998).

A significant rise in *zenk* mRNA levels can be detected within minutes after stimulus onset (Mello and Clayton 1994; Kruse et al. 2000), but peaks around 30 min, returning to basal levels by 1 h. Thus, *zenk* mRNA levels measured at a given time reflect events that occurred during a relatively narrow time window before sacrifice. *zenk* levels at peak expression are proportional to the amount of song the bird heard (Mello and Clayton 1994). A single song presentation triggers a detectable rise in *zenk* mRNA levels, and stimulation beyond 10 min results primarily in increased variability of *zenk* expression levels across individuals (Kruse et al. 2000). As expected, the ZENK protein response is protracted relative to the mRNA increase, peaking between 1 h and 2 h and decreasing to unstimulated levels by 6 h after stimulus onset (Mello and Ribeiro 1998). Thus, although the ICC method provides high spatial resolution for ZENK expression mapping, ZENK protein levels at a given time reflect brain activation during a much longer preceding period compared with *zenk* mRNA levels.

NCM

The caudomedial neostriatum (NCM) presents the most robust *zenk* response to song playbacks (Mello et al. 1992; Mello and Clayton 1994) and is the brain area where *zenk* expression has been characterized in most detail. NCM is located immediately medial and caudal to field L (Mello and Clayton 1994), its boundaries being the ventricular zone dorsally, caudally and ventrally, and field L and the lamina hyperstriatalis rostrally. There are no obvious cytoarchitectonic boundaries laterally, but a clear lateral *zenk* expression boundary can be seen in frontal sections (Mello and Clayton 1994). NCM is not a discrete nucleus, but rather a large expanse of the caudal neostriatum that likely contains subdomains.

NCM receives dense projections from field L subfields L1 and L3 and a lesser projection from neurons in the shell around thalamic auditory nucleus ovoidalis (Mello 1993; Vates et al. 1996). The projections from L1 and L3 are most prominent to rostral NCM and sparser to caudal NCM. NCM also receives a dense projection from the medial part of the caudal hyperstriatum

ventrale (cmHV), another auditory area close to NCM (Vates et al. 1996). A projection to be confirmed from para-HVC, a subdomain of song nucleus HVC, may contribute an input of the song system to NCM (Foster and Bottjer 1998); a possible projection from the caudal paleostriatum (Pc) also remains to be confirmed (Mello 1993). NCM receives a significant noradrenergic input, as shown by ICC staining for noradrenergic fibers (Mello et al. 1998a). Although detailed studies are lacking, NCM subdomains appear to be interconnected by local projections forming a dense network (Mello 1993), consistent with the scarce Golgi data (Saini and Leppelsack 1981). The main known projection from NCM is to cmHV (Vates et al. 1996) and is likely topographically organized. The medial part of NCM projects to lateral NCM areas contiguous with the shelf under song nucleus HVC. A projection of lateral NCM to the shelf could provide a rather direct entry for auditory information from NCM into the song system, but such a pathway has not been demonstrated.

zenk induction in NCM is highest for same species (conspecific) song, lower for heterospecific song, and even lower or absent for synthetic tones (Mello et al. 1992). There is presently no evidence for sex differences in the *zenk* response to song in NCM (Mello 1993; Duffy et al. 1999). In starlings, where song-based female preferences have been studied in detail (Gentner and Hulse 2000), the *zenk* response in NCM of females is highest for their preferred conspecific stimuli, i.e., long songs (Gentner et al. 2001). This effect is observed even when taking into account total stimulus duration (for further discussion see Ball and Balthazart 2001). Overall, these observations indicate that NCM is tuned to auditory stimuli that are of high behavioral relevance to the recipient bird.

The *zenk* induction response in NCM, both at the mRNA and protein level, decreases upon repeated presentations of the same song (Mello et al. 1995), an effect referred to as “habituation”. This habituation is song specific, as presentation of a novel song re-induces *zenk* expression in habituated birds (Mello et al. 1995), providing further evidence that NCM participates in song discrimination. As a large percentage of neurons in NCM show a *zenk* response to any given conspecific song, it is likely that individual NCM neurons show independent *zenk* responses to multiple songs (Mello et al. 1995). This is consistent with the notion of ensemble coding for song representation, according to which the auditory representation of different songs or song elements involves the recruitment of distinct but partially overlapping groups of neurons from a given neuronal population (Ribeiro et al. 1998).

Electrophysiological responses to song are prevalent throughout NCM in zebra finches (Chew et al. 1995, 1996a, 1996b; Stripling et al. 1997; Ang 2001), confirming that auditory processing is a central function of this brain area. Auditory responses in NCM are less brisk and have longer latencies than those in auditory field L, and seem to lack the degree of selectivity for

specific song elements seen in song control nuclei (e.g., Margoliash 1983). These observations indicate that NCM occupies an intermediate position between the primary auditory telencephalic area and higher-order centers. They are consistent with early studies in starlings, where auditory responses were seen over a large portion of the caudal telencephalon, many units showing selectivity for complex stimuli (Müller and Leppelsack 1985).

The evoked auditory responses to song in NCM decrease quickly upon repeated presentations of the same song (Chew et al. 1995, 1996a; Stripling et al. 1997), as seen by comparing each response to the amplitude of the response to the first presentation of a given song. This electrophysiological habituation is song-specific and can last for hours to days. The habituation rate, a linear regression derivative of the habituation curve, provides an index of whether a given song is “remembered” by NCM neurons. While the response to familiar and remembered songs shows no habituation or low habituation rates, the responses to novel or “forgotten” songs have high habituation rates. Auditory responses in NCM are not abolished by habituation, but once below a certain threshold they are no longer capable of eliciting a gene expression response.

Injections of RNA and protein synthesis inhibitors in NCM close to the time of song exposure do not affect NCM’s auditory responses and habituation to song, but interfere with the long-term maintenance of the habituation (Chew et al. 1995). This effect is comparable to the properties described for hippocampal LTP (Nguyen et al. 1994) and is consistent with the idea that the gene expression triggered by neuronal activation is necessary for long-term neuronal modification (Goelet et al. 1986; Morgan and Curran 1989; Sheng and Greenberg 1990) and the establishment of long-lasting memories (Davis and Squire 1984). The time-course of song-induced *zenk* expression suggests that *zenk* is an early regulator in genomic events leading to long-term habituation to song in NCM (Mello 1998; Ribeiro and Mello 2000). Testing that possibility will require methods to selectively block *zenk* expression in NCM during specific time windows.

Analysis of song-induced *zenk* expression has also been used to study the internal functional organization of NCM in canaries (Waterslager strain). In this respect, canary song proved to be particularly useful due to its characteristic structure. Canary song is typically formed of phrases that are themselves repetitions of single syllables (Nottebohm and Nottebohm 1978; Güttinger 1985; Ribeiro et al. 1998). These syllables are simpler in their physical properties than complete songs (for instance, whistles are pure tone-like vocalizations), therefore representing more discrete stimuli. The ZENK expression patterns in NCM resulting from the presentation of these discrete auditory units can then be analyzed as global maps of neuronal activation using an automated mapping system (Cecchi et al. 1999).

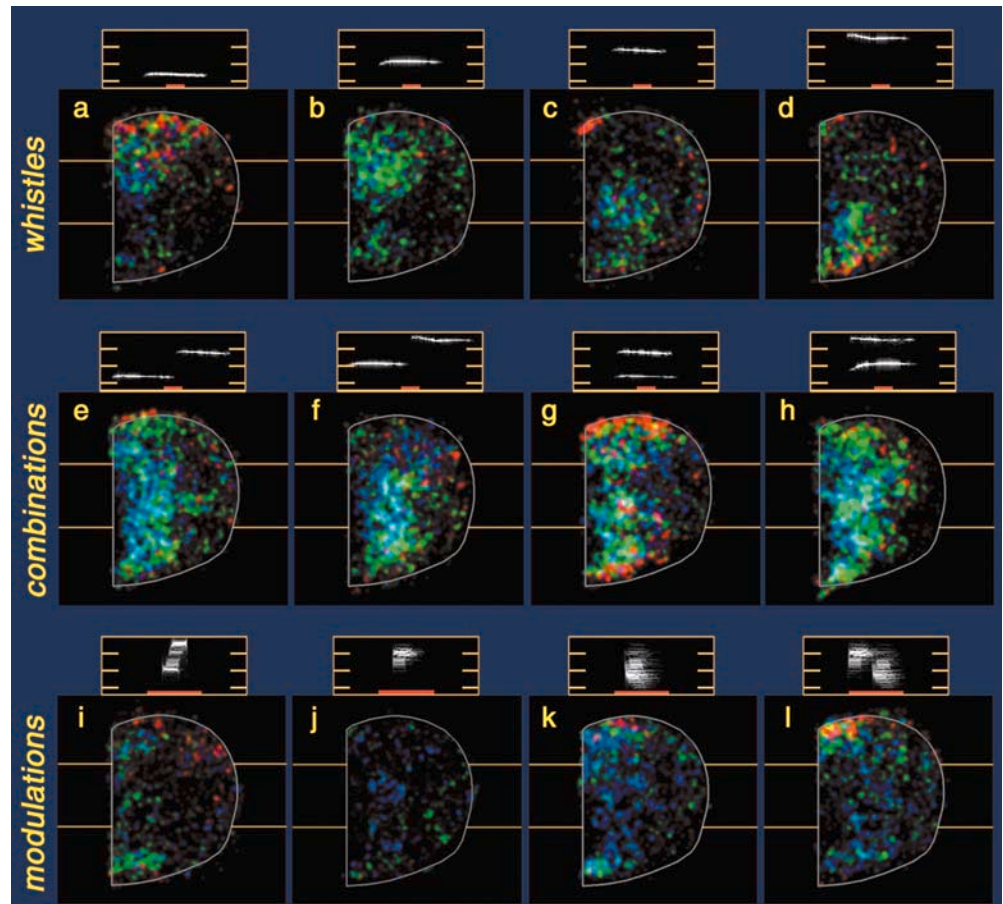
The results of this analytical approach have revealed several important features of the internal organization of

NCM (Fig. 2). Presentation of each syllable type normally present in the canary song (whistles, combinations, stacks and sequences, and fast frequency modulations) results in a distinct pattern of ZENK activation (Ribeiro et al. 1998). These patterns are more discrete than the patterns associated with whole or partial songs. For instance, whistles result in clusters of ZENK-expressing cells whose relative position along the dorsoventral axis of rostral NCM is a function of whistle frequency. The patterns associated with natural whistles are distinct from those resulting from artificial whistles of comparable frequencies, indicating that the organization of rostral NCM depends on yet unidentified features of natural vocalizations other than frequency. Whistle combinations result in patterns that are distinct from the sum of the patterns resulting from the individual component whistles, and syllables with fast frequency modulations result in distributed patterns with little activation of rostral NCM.

The patterns above can be discriminated using an index of clustering, an indicator of the spatial organization of ZENK-labeled cells. Further quantification through principal component analysis shows that the patterns associated with different syllable types can be distinguished from each other based on the location and labeling intensities of ZENK-expressing neurons in NCM. These patterns can also be classified into groups that accord to the different types of syllables used for stimulation. In other words, ZENK expression patterns contain sufficient information to classify them into distinct groups that accord to the acoustic properties of the different syllables studied. This finding has led to the proposal that the activation patterns resulting from different syllables provide a basis for an auditory syllabic representation in NCM (Ribeiro et al. 1998). It is also apparent that the patterns resulting from different syllables overlap, so that discrete NCM subdomains participate in the representation of various syllables. This observation is consistent with an auditory encoding of syllables based on the recruitment of neuronal ensembles from a large population of available units in NCM.

Consistent with data from rodents, *zenk* expression in NCM and other auditory areas is higher in juveniles than in adults (Mello 1993; Jin and Clayton 1997). No significant song-induced *zenk* expression is observed at 20 days of age (Jin and Clayton 1997), even though auditory responses in NCM are clearly present at that time (Stripling et al. 2001). Basal *zenk* expression then declines, and *zenk* induction by song presentation can be detected in NCM by day 30 (Jin and Clayton 1997; Stripling et al. 2001), within the initial period for song learning, increasing further thereafter. In another study, *zenk* expression in the NCM of adult males was tested in response to the songs heard during the song learning period (Bolhuis et al. 2000). *zenk* levels were found to correlate with the number of song elements the birds had copied from their tutors’ songs. Thus, significant changes in the organization of the auditory forebrain and in song-induced *zenk* expression patterns occur in

Fig. 2 Syllabic auditory representation in the canary brain (from Ribeiro et al. 1998, with permission). Panels (a–l) show maps of *zenk* expression in NCM associated with three song syllable types normally present in canary song, with the corresponding sonograms shown above each map. Each syllable type resulted in a family of *zenk* maps with distinct properties, as discussed in the text. For frequency scales in sonograms (1–4 kHz) and color keys in maps; see Fig. 1 in Ribeiro et al. (1998). Red scale bars represent 100 ms



NCM in parallel with song learning. Song-induced gene expression in NCM could thus be involved in the formation and/or storage of song auditory memories, and thereby impact song learning. It is still unclear, however, whether the song exposure protocols that trigger a *zenk* response also result in detectable song learning. In this regard, excessive exposure to song can be detrimental to learning (Tchernichovski et al. 1999). In addition, vocal learning is often affected by social context, arousal, motivation and hormonal state (for example, see reviews in Brenowitz et al. 1997), but the impact of such variables on song-induced *zenk* expression has not yet been assessed.

Other areas

Several other brain areas also show a *zenk* induction response to song (Mello and Clayton 1994; Mello and Ribeiro 1998), including the dorsal part of the lateral mesencephalic nucleus (MLd), the Pc, field L subfields L1 and L3, the caudal hyperstriatum ventrale (cHV), and the shelf and cup regions adjacent to song nuclei HVC and robust nucleus of the archistriatum (RA). MLd is a midbrain auditory station comparable to the mammalian inferior colliculus (Karten 1967, 1968; Mello et al. 1998b). It is the first nucleus in the ascending

auditory pathway where *zenk* induction by song has been detected, the preceding stations having yet to be examined. Although electrophysiological studies of MLd in songbirds are lacking, studies in non-oscine avian groups such as owls and pigeons suggest that this brain area may be involved in some aspects of sound localization (Moiseff and Konishi 1981; Lewald 1990). If this is also true for songbirds, MLd might play a role in determining the direction or source of a given song. Based on connectivity data, Pc could represent a sub-cortical telencephalic auditory station (Bonke et al. 1979a; Kelley and Nottebohm 1979; Durand et al. 1992; Mello 1993; Wild et al. 1993; Vates et al. 1996). This area has not been investigated electrophysiologically in songbirds or other avian groups.

Field L is the primary auditory thalamo-recipient zone in the telencephalon (Karten 1968; Kelley and Nottebohm 1979; Brauth et al. 1987) and has been studied in various avian species. The L2a core projects to the flanking L1 and L3 subdivisions, which then originate projections to other auditory telencephalic areas such as cHV, NCM and the shelf and cup (Kelley and Nottebohm 1979; Vates et al. 1996). Electrophysiological responses in L1 and L3 are of longer latency than in L2a and show more selectivity towards complex auditory stimuli (Müller and Leppelsack 1985; Heil and Scheich 1991; Sen et al. 2001). *zenk* expression in

response to song occurs in L1 and L3, but not in the L2a core (Mello and Clayton 1994; see also section Areas lacking a *zenk* response to song).

The cHV can be divided into medial and lateral parts (cmHV and clHV). cmHV is reciprocally connected with NCM and clHV, and clHV sends projections to the shelf and to song nucleus Nif (Vates et al. 1996). The latter projection provides the probable means for a direct entry of auditory information into the song control system. Electrophysiological responses in cHV are tonotopically organized and have longer latencies than those in field L, with a high number of units showing some selectivity for complex auditory stimuli (Müller and Leppelsack 1985; Heil and Scheich 1991). Lesions to cHV result in a disruption of normal song preferences in female zebra finches (MacDougall-Shackleton et al. 1998), indicating a participation of this area in auditory processing and discrimination.

The shelf and cup were initially identified as termination zones of projections from field L (Kelley and Nottebohm 1979). Recent studies (Fortune and Margoliash 1995; Vates et al. 1996) have established that these projections originate in L1 and L3 and that the shelf receives additional inputs from clHV. Neurons in the shelf then send discrete projections into HVC and originate a spatially organized projection to the RA cup (Mello et al. 1998b). The latter originates a descending projection that terminates onto nuclei of the ascending auditory pathway (Mello et al. 1998b), similar to projections described in the pigeon (Wild et al. 1993) and likely corresponding to descending projections from the mammalian auditory cortex. The shelf and cup regions are thus integral components of the central auditory pathways, likely representing high-order auditory processing centers. The shelf may provide auditory input to the song system and thereby play a role in sensorimotor integration (Nottebohm 1996), whereas the cup likely influences the flow of auditory information along the ascending auditory pathway.

Overall, *zenk* expression analysis combined with connectivity and some electrophysiological data indicate that a network of interconnected areas integrates a complex auditory processing system. This system likely participates in various aspects of the auditory processing of song, but the role of each component area remains to be determined.

Areas lacking a *zenk* response to song

A few brain regions where song-induced *zenk* expression might be expected do not show such a response. An example is nucleus ovoidalis (Ov), the main thalamic auditory nucleus in birds (Karten 1967, 1968; Kelley and Nottebohm 1979; Brauth et al. 1987; Wild et al. 1993; Vates et al. 1996; Mello et al. 1998b). Ov receives a major projection from MLD and is the main source of auditory input to the telencephalon through a projection to field L. Ov is activated by auditory stimuli such

as birdsong (Bankes and Margoliash 1993) but its neurons do not express *zenk* (Mello and Clayton 1994), providing a clear example of an uncoupling of electrophysiological activation and *zenk* induction. This may be a general characteristic of Ov, as *c-jun*, another song-inducible IEG, is also not induced in Ov (Nastiuk et al. 1994). The link between *zenk* and neuronal plasticity suggests that such uncoupling would prevent the physiological activation of Ov neurons to be followed by long-lasting changes in their response properties, thus preserving or stabilizing auditory representations in the thalamus. The mechanisms for this uncoupling are unknown, but could include the downregulation of membrane receptors or signal transduction elements necessary for the IEG response, upregulation of calcium-buffering systems, or direct repression at the promoter level.

zenk induction after song stimulation is also absent in L2a (Mello and Clayton 1994), in sharp contrast to the high expression seen in adjacent areas (Mello and Ribeiro 1998). This has been consistently observed with a variety of natural and synthetic auditory stimuli (Ribeiro et al. 1998). Data from several species show that L2a has a high frequency of spontaneous discharges, and that auditory responses in L2a are of short latency, are organized tonotopically, have low selectivity for complex stimuli, and do not habituate to repeated stimulation (Bonke et al. 1979b; Scheich et al. 1979; Müller and Leppelsack 1985; Heil and Scheich 1991, 1992; Chew et al. 1995; Stripling et al. 1997). Neurons in field L2a can be considered functionally equivalent to the cells in the granular layer of the mammalian auditory cortex that receive projections from the thalamus. L2a might thus serve primarily as a relay of auditory information to higher order areas in the telencephalon. As argued for Ov, the lack of *zenk* expression in L2a may result in the stabilization of auditory representations at this level of the pathway.

Interestingly, administration of the GABAergic antagonist metrazole causes widespread neuronal activation and *zenk* induction (Mello and Clayton 1995), except in the telencephalic termination zones of thalamic sensory projections: L2a (auditory), ectostriatum (visual), and nucleus basalis (somatosensory). This effect does not correlate with the known GABA or GABA receptor distributions (Müller 1988; Grisham and Arnold 1994) and is consistent with the notion that IEG expression has been uncoupled from neuronal depolarization at primary thalamo-recipient zones. Compared to the rest of the brain, L2a neurons have higher levels of calcium-binding proteins such as parvalbumin and calbindin (Braun et al. 1991). This may provide a means to down-regulate *zenk* induction in L2a neurons, as calcium entry is a necessary step in the signaling cascade that leads to IEG induction (Ghosh et al. 1994).

zenk expression in birds that passively hear song is also not observed in song control nuclei (Mello and Clayton 1994; Mello and Ribeiro 1998). This was surprising at first, since evoked electrophysiological

responses to song and other auditory stimuli in these nuclei have been well documented (Katz and Gurney 1981; Margoliash 1983; Williams and Nottebohm 1985; Doupe and Konishi 1991; Vicario and Yohay 1993; Volman 1996; Margoliash 1997). The lack of a *zenk* induction response suggested that a dissociation of gene expression from electrophysiological activation also occurred within the song system. However, it should be noted that *zenk* expression studies were conducted in awake, freely behaving animals, whereas most electrophysiological recordings of auditory responses were performed in anesthetized animals. Mapping *zenk* induction under conditions matching those for electrophysiology was not feasible, as the *zenk* expression response is blocked by anesthetic agents including nembutal, xylazine, ketamine, and urethane (C.V. Mello, unpublished observations).

Although the field is still controversial, recent studies with chronically implanted electrodes show that auditory responses in song nuclei are much weaker, if not absent, in awake as compared to anesthetized animals (Dave et al. 1998; Schmidt and Konishi 1998). Interestingly, robust auditory responses in song nuclei resembling those obtained in anesthetized birds can be recorded during sleep (Dave et al. 1998) and are likely under the control of modulatory (e.g., noradrenergic or cholinergic) systems that regulate sleep/wakefulness states. The significance of these observations is not clear, but they are inconsistent with the notion that auditory responses in song nuclei play a central role in song perception in awake animals. They resonate with *zenk* expression and lesion data implicating auditory processing areas that precede the song system in song

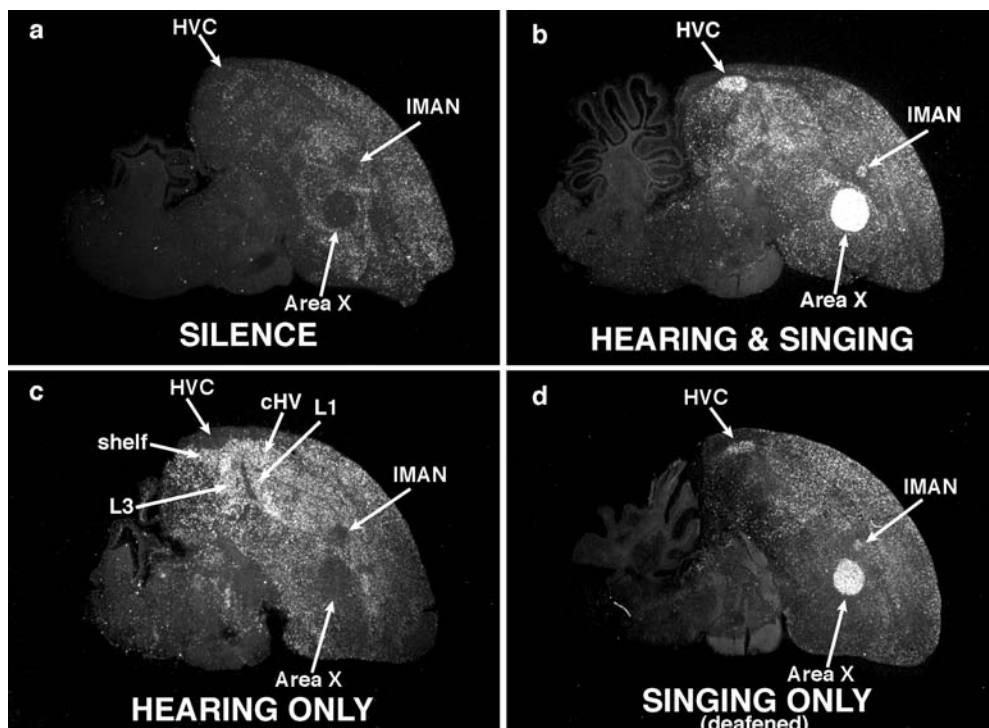
perception and discrimination (MacDougall-Shackleton et al. 1998).

Song-induced *zenk* expression: vocal-motor

zenk is significantly induced in song control nuclei in association with singing behavior (Jarvis and Nottebohm 1997; Jin and Clayton 1997; Jarvis et al. 1998; Mello and Ribeiro 1998). This can be seen by comparing *zenk* expression patterns between birds that sang before sacrifice ("hearing and singing") and birds that heard conspecific song but did not sing or were prevented from singing in response ("hearing only" birds) (Fig. 3). *zenk* expression follows singing activity, and its levels correlate positively with the number of song bouts produced before sacrifice. In addition, deafening greatly reduces or abolishes *zenk* expression in auditory areas (NCM, cHV, field L, and the shelf and cup) but does not affect *zenk* expression in song control nuclei of singing birds. *zenk* expression in song control nuclei is also seen in males that were muted by sectioning the left hypoglossal nerve and that attempted to sing a comparable number of times as intact males. Thus, *zenk* expression in song control nuclei is associated with the motor act of singing and not to auditory feedback.

DM, the dorsomedial nucleus of the intercollicular nucleus of the mesencephalon (ICo), a midbrain nucleus that shows singing-induced *zenk* expression, is thought to be involved in vocal and respiratory control in all bird species (Wild 1994, 1997), irrespective of vocal learning. DM projects to the tracheosyringeal subdivision of the hypoglossal nucleus (nXIIts) and to respiratory centers

Fig. 3 Auditory and vocalizing-induced *zenk* gene expression in the brain of male zebra finches (from Jarvis and Nottebohm 1997, with permission). Shown are dark field views of representative parasagittal sections hybridized with a radioactively-labeled *zenk* probe and counterstained with cresyl-violet (panels a–d). Notice that *zenk* expression (white-silver grains) in song nuclei only occurs in singing birds, and that deafening abolishes singing-associated *zenk* induction in auditory regions but not in song nuclei. For abbreviations see list



in the medulla (Wild et al. 1997a), and its stimulation results in vocalizations (de Lanerolle and Andrew 1975; Vicario and Simpson 1995). In songbirds, DM receives a projection from the dorsal-most portion of RA (Vicario 1991). Direct telencephalic projections to DM are thought to be absent in avian species that lack vocal learning (Wild et al. 1993; Wild 1994).

The other seven nuclei that show singing-induced *zenk* expression [HVC, RA, area X of the paleostriatum (area X), magnocellular nucleus of the anterior neostriatum (MAN), nucleus avalanche (Av), nucleus interfascialis (Nif), and oval nucleus of the hyperstriatum ventrale (HV0)] are telencephalic. Of these, six had been previously identified and linked to song behavior through techniques other than gene expression mapping. Nuclei HVC and RA constitute the direct pathway for vocal control (Nottebohm et al. 1982). They are necessary for song production (Nottebohm et al. 1976) and participate actively in the motor control of song (Yu and Margoliash 1996). Nif projects to HVC and its activation during singing precedes that of HVC (Nottebohm et al. 1982; McCasland 1987). Nucleus Av receives a direct projection from HVC (Nottebohm et al. 1982), but its functional role has not been investigated. IMAN and area X are part of the anterior forebrain pathway (Okuhata and Saito 1987; Bottjer et al. 1989) and are necessary for song learning (Bottjer et al. 1984; Sohrabji et al. 1990; Scharff and Nottebohm 1991). For the latter three nuclei, the data from *zenk* expression analysis represented a first functional demonstration of their activation in association with singing behavior. The other telencephalic nucleus that shows singing-induced *zenk* expression is in the anterior hyperstriatum ventrale, adjacent to IMAN. This location is similar to that of HV0, a vocal control nucleus in budgerigars (Brauth et al. 1997; Durand et al. 1997). HV0 was initially thought to be absent in songbirds, this being regarded as a basic difference in the organization of vocal control pathways between songbirds and parrots (Striedter 1994). The *zenk* mapping approach has provided evidence for the existence and functional activation of an HV0-like structure during singing in songbirds (Jarvis et al. 1998). The connectivity of this nucleus in songbirds is unknown.

While lesions to nuclei in the anterior forebrain pathway disrupt song learning when performed in juveniles, no deleterious effects to song structure are observed in adults (Bottjer et al. 1984; Sohrabji et al. 1990; Scharff and Nottebohm 1991). These observations argue that these nuclei participate in song learning but not in the motor control of song production. Contrary to that notion, however, the *zenk* mapping experiments provide clear evidence for the activation of area X and MAN during singing, arguing for an active participation of the anterior forebrain pathway in the control of song behavior. In fact, area X shows the most marked *zenk* induction in association with singing (Jarvis and Nottebohm 1997). Electrophysiological recordings with chronically implanted electrodes have indeed confirmed

the finding that nuclei of the anterior forebrain pathway are activated during singing behavior (Hessler and Doupe 1999).

A possible clue as to the role of the anterior forebrain pathway in singing behavior stems from the observation that *zenk* expression in area X of male zebra finches is context-dependent, occurring during singing in the presence of other males or in a solo context (undirected singing), but not during female-directed singing (Jarvis et al. 1998). The rather minor differences between female-directed and undirected song (Sossinka and Böhner 1980) seem insufficient to explain the strong contrast in gene expression in these two contexts. It is possible that this context effect on *zenk* expression could result from differences in arousal state or in the attention a bird pays to its own song in different contexts. Alternatively, it is important to note that female-directed singing is typically accompanied by a courtship dance (Sossinka and Böhner 1980; Jarvis et al. 1998). It is thus possible that area X is involved in coordinating singing with other behaviors that give contextual relevance to the song. Indeed, area X is part of the avian equivalent to the mammalian basal ganglia (Reiner et al. 1998), a structure that has been implicated in the learning and maintenance of sequential motor actions that depend on sensorimotor integration (Lidsky et al. 1985; Aldridge and Berridge 1998). Importantly, area X has combined properties of both the striatal and pallidal components of the basal ganglia (Farries and Perkel 2002). For instance, area X receives input from a cortico-like area and sends a direct GABAergic projection to the thalamus (Okuhata and Saito 1987; Bottjer et al. 1989; Luo and Perkel 1999). Furthermore, it contains cell populations that electrophysiologically resemble cells of the caudate-putamen and of the globus pallidum of mammals (Farries and Perkel 2002). It will be important to examine whether a comparable subregion or subdomain exists in the mammalian basal ganglia, and whether it is also involved in vocal control and potentially in vocal learning. Yet another alternative explanation for the context effect is that singing-induced *zenk* expression in area X is under the control of neuromodulatory systems involved in gene regulation (for instance, dopamine or noradrenaline – see Cirelli et al. 1996) that could be differentially active in the directed versus undirected contexts.

zenk expression mapping has revealed a medial extension of area X (Jarvis et al. 1998) that may have been spared in area X lesions in adults (Bottjer et al. 1984; Sohrabji et al. 1990; Scharff and Nottebohm 1991). Other song nuclei also appear to have medial and lateral domains, *zenk* expression in the former showing a more marked dependence on the behavioral context of singing (Jarvis et al. 1998). It remains to be determined whether such subdomains also differ in connectivity and electrophysiological properties. *zenk* expression analysis has also suggested a context-dependent effect in song motor nucleus RA (Jarvis et al. 1998) and provided functional

support to the existence of subdomains within this nucleus (Vicario 1991).

In search for correlations with song learning, *zenk* induction in association with singing behavior has also been investigated in juvenile zebra finches. Basal *zenk* expression is very low or absent in song nuclei of juveniles, but marked induction occurs upon singing (Jarvis and Nottebohm 1997; Jin and Clayton 1997). For several nuclei, including HVC, area X, MAN (both medial and lateral subdivisions), NIF and DM, this induction has a similar pattern as in adults, and is proportional to the amount of singing. In contrast, singing-induced *zenk* expression in anterior but not posterior RA is higher in juveniles than in adults (Jin and Clayton 1997). This suggests that singing-induced *zenk* expression levels in RA correlate with the degree of plasticity in the song produced, being higher during learning (sub-song and plastic song). However, as the amount of female-directed singing may also increase with age, a significant portion of this age effect may relate to the mode of singing rather than the degree of song plasticity. Singing-induced ZENK protein expression in RA of juveniles (assessed by ICC) is apparently dissociated from *zenk* mRNA levels, being lower at an early age and increasing as song matures (Whitney et al. 2000). Thus, post-transcriptional regulatory mechanisms may play a role in song development.

Wild songbirds

Song sparrows defend well-marked territories and females from competing males during the breeding season (Searcy et al. 1981; Wingfield 1985). Robust singing behavior can be promptly and reliably elicited by challenging a defending male with playbacks of an unfamiliar conspecific song. A comparison of *zenk* expression patterns in challenged birds versus unstimulated quiet controls demonstrates significant *zenk* induction in all auditory processing areas and song control nuclei revealed by *zenk* analysis in captive species (Jarvis et al. 1997). This proves the feasibility of using IEG expression analysis in a natural setting to map brain areas involved in complex behaviors not readily reproduced in a laboratory. Factors critical to field studies need to be closely observed: (1) it is necessary to quickly capture the animals after a given stimulation or behavioral context; the territorial behavior of song sparrows during the breeding season greatly facilitated locating, monitoring and capturing them; (2) to minimize basal *zenk* expression, it is important to only use animals that can be monitored for a long period preceding the experiment and eliminate those that engage in significant interactions during this period; and (3) assessment of IEG expression in field experiments is best done with *in situ* hybridization, as *zenk* mRNA levels are only affected by events occurring during a short period preceding sacrifice, as compared to protein levels. In summary, a detailed observation of the behavior of

interest, a careful definition of the stimulation/behavioral paradigm, and a correct choice of the method of detection are essential for mapping brain gene expression in wild animals.

Comparative analysis

Only three avian orders (parrots, hummingbirds and songbirds) are known to have developed vocal learning (Nottebohm 1972; Brenowitz 1997), i.e., the ability of animals to learn to produce their own vocalizations based on auditory input. These vocal learning orders are separated by several groups (such as suboscines, pigeons, cranes, and owls) that do not present the trait. It is therefore usually thought that the vocal learning orders evolved vocal learning and associated brain structures independently. Telencephalic nuclei that project to and control the activity of vocal and respiratory brainstem centers have been identified and characterized in vocal learners (songbirds and parrots) but appear to be absent in species that lack vocal learning (Brenowitz 1997). Thus, the presence of telencephalic vocal control nuclei seems to be a signature of the brain of vocal learners.

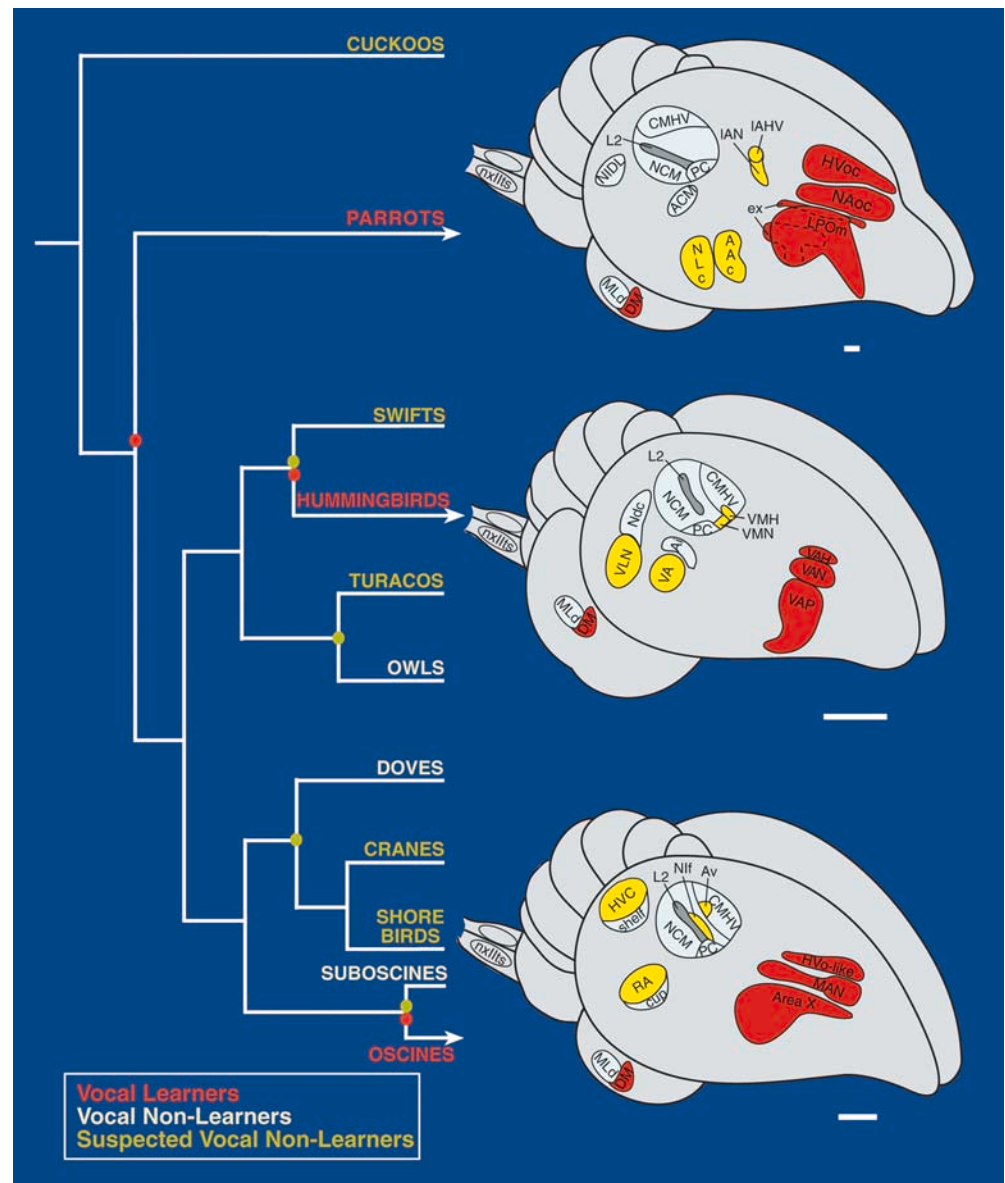
zenk expression analysis has been used to define and compare the brain systems for vocal communication in various avian orders. As discussed below, the comparative approach to *zenk* expression mapping has revealed striking similarities in the organization of brain systems for vocal communication in all avian vocal learning orders. Some of these common features (in particular the presence of seven telencephalic vocal control nuclei, their division into anterior and posterior clusters, and the participation of cortico-like and basal ganglia elements) may represent basic mechanistic requirements for a vocal control system capable of vocal learning (Fig. 4).

Parrots

The budgerigar, a small Australian parrot, is the most intensively studied bird in this group. Telencephalic vocal control nuclei have been identified in this species based primarily on tract-tracing (Paton et al. 1981; Striedter 1994; Brauth et al. 1987; Durand et al. 1997). These nuclei are in many regards similar to those described in songbirds, their projections converging onto brainstem nuclei that control the syrinx. As in songbirds, these nuclei also segregate into an anterior and a posterior cluster, although significant differences seem to exist in the details of their connectivity (Striedter 1994; Durand et al. 1997).

A comparison between "hearing only" (birds exposed to playbacks of budgerigar's warble song) and "quiet control" groups reveals *zenk* induction in the midbrain MLd, as well as the presence of several auditorily-activated areas in the caudo-medial telencephalon (Jarvis and Mello 2000). As in songbirds, the latter areas

Fig. 4 Comparison of *zenk* expression across vocal learning avian orders (from Jarvis et al. 2000, with permission). The diagram on the left represents phylogenetic relationships across extant avian orders, with one example common species name per order. Nodes representing possible independent gain or loss of vocal learning are indicated by red and green dots, respectively. The panels on the right represent hearing- and vocalizing-induced maps of *zenk* expression over semi-3-D renditions of the brains of vocal learners. Regions of hearing-induced *zenk* expression are depicted in blue, regions of vocalizing-induced expression are depicted in red (similar positions across orders) or yellow (different positions across orders). Scale bar: 1 mm; for abbreviations see list



surround a central core (L2a) that is negative for *zenk*. No evidence has been found for the activation of anterior telencephalic structures like nucleus basalis and its targets. This finding conflicts with studies indicating that such structures receive auditory projections and play a role in auditory processing and/or vocal learning and performance (Hall et al. 1993, 1994; Brauth et al. 1994; Striedter 1994; Farabaugh and Wild 1997; Wild et al. 1997b). A possible interpretation is that the activation of such structures has been uncoupled from *zenk* expression. Recording of auditory responses in awake animals will be necessary to clarify this point.

A comparison between “hearing and vocalizing” and “hearing only” birds reveals vocally-induced *zenk* expression in two brainstem (midbrain DM and thalamic DMm) and in seven telencephalic [HVo, oval nucleus of the anterior neostriatum (NAo), magnocellular nucleus of the parolfactory lobe (LPom), lateral

nucleus of the anterior hyperstriatum ventrale (NLC), central nucleus of the anterior archistriatum (AAC), lateral nucleus of the anterior neostriatum (IAN) and lateral nucleus of the anterior hyperstriatum ventrale (IAHV)] nuclei (Fig. 4). Among the telencephalic structures, the former five nuclei constitute the known vocal control pathways in budgerigars based on previous studies, the *zenk* mapping analysis providing a first functional demonstration of their activation in association with vocal production. The latter two nuclei had not been previously linked to vocal control. *zenk* mapping has also revealed novel features for some of the known vocal nuclei. A good example is LPom, for which several extensions were revealed by *zenk* expression analysis. The differences between *zenk* expression mapping and tract-tracing data may reflect the incompleteness of the latter approach, or alternatively represent evidence for functional/anatomical

subdomains within the vocal nuclei of parrots. LPOm in budgerigars, as area X in songbirds, is part of the basal ganglia, whereas the other telencephalic vocal control structures represent cortico-like elements.

Hummingbirds

Until recently, no data were available on brain mechanisms related to song production or learning in hummingbirds, the third avian group known to have developed vocal learning (Nottebohm 1972; Baptista and Schuchmann 1990; Brenowitz 1997). A comparison of *zenk* expression patterns among “hearing and vocalizing”, “hearing only” and “quiet control” birds led to the rapid identification and mapping of vocal control brain centers in two tropical hummingbird species studied in the field (Fig. 4), the sombre hummingbird and the rufous-breasted hermit (Jarvis et al. 2000). Comparable nuclei were then identified in two other species, thus covering the two existing hummingbird lineages.

Seven auditorily-activated brain regions were identified, one in the midbrain, one in the thalamus, and five in the telencephalon, all of which at very similar locations as in songbirds and parrots. In particular, structures comparable to NCM, Pc and cHV are located caudo-medially in the telencephalon and surround a *zenk*-negative core area (presumably L2a). The vocally activated regions consist of one midbrain (DM) and seven telencephalic structures [vocal nuclei of the anterior hyperstriatum ventrale (VAH), anterior neostriatum (VAN), anterior paleostriatum (VAP), medial neostriatum (VMN), medial hyperstriatum ventrale (VMH), lateral neostriatum (VLN) and archistriatum (VA)]. The latter can be divided into anterior, posterior-medial and posterior-lateral clusters. The vocally activated areas bear remarkable resemblance in cytoarchitectonic features and relative location to the vocal control nuclei of songbirds and parrots. For instance, VAP in hummingbirds, as area X in songbirds and LPOm in parrots, is part of the avian equivalent to the mammalian basal ganglia, providing evidence that the latter also participates in vocal control in hummingbirds. The other vocally activated telencephalic structures appear to correspond to cortico-like structures also present in songbirds and parrots. *zenk* expression levels in all vocally-activated brain nuclei are proportional to the number of song bouts the birds sang before sacrifice (Jarvis et al. 2000). The data above provide a first and complete anatomical and functional demonstration of vocal control nuclei in hummingbirds and provide a starting point for exploring the connectivity and physiology of their vocal control system. Some connectivity data on vocal control pathways have been recently obtained for the Anna hummingbird (Gahr 2000), but it remains to be seen how the nuclei thus identified relate to the *zenk* expression data.

Vocal non-learners

The identification of telencephalic vocal control areas in vocal non-learning avian orders would represent a significant challenge to the notion that such areas are present only in vocal learners (Brenowitz 1997). Nissl-staining and tract-tracing methods have failed to demonstrate such nuclei in vocal non-learning species such as chicken (Kuenzel and Masson 1988), pigeon (Karten and Hodos 1967; Wild et al. 1997a), the eastern phoebe, a sub-oscine (Kroodsma and Konishi 1991) and others (Gahr 2000). *zenk* expression mapping during vocal communication arguably represents a more sensitive test for whether telencephalic areas participate in vocal control, but such data are currently lacking in vocal non-learners. Interestingly, ZENK protein expression has been detected in portions of the telencephalon in the context of copulatory and appetitive behaviors in the Japanese quail (Ball et al. 1997) but whether the vocalizations associated with such behaviors played a role was not examined (for further discussion see Ball and Balzhart 2001).

Other genes

c-fos is another activity-dependent IEG extensively studied in mammalian systems (Morgan and Curran 1991; Herrera and Robertson 1996). As probes for *in situ* are not yet available in songbirds, studies of *c-fos* regulation by birdsong have relied on ICC. No significant *c-fos* induction has been observed in song control nuclei upon auditory stimulation (Kimpo and Doupe 1997). As argued for *zenk*, this can be interpreted as an uncoupling between the activation of song nuclei by song and IEG induction. More likely, though, it provides further evidence that song stimulation does not lead to the activation of song nuclei in awake birds. The reports on *c-fos* induction in NCM upon song presentation have been conflicting (Kimpo and Doupe 1997; Bolhuis et al. 2000). In contrast to song perception, *c-fos* is strongly activated in song control nuclei HVC and RA upon singing (Kimpo and Doupe 1997), independently of auditory feedback; the effect of singing context has not been examined. *c-jun* is usually co-expressed with *c-fos*, their protein products together comprising the AP-1 transcriptional activity (Sonnenberg et al. 1989). In songbirds, *c-jun* is strongly activated throughout the brain by depolarization (metrazole) and weakly so in NCM by song presentation (Nastiuk et al. 1994); its possible regulation in song control nuclei during singing has not been examined. It is also worthwhile mentioning that BDNF, a factor that acts both as a neurotrophin and a regulator of synaptic transmission, is induced in NCM by song presentation and in song control nuclei in association with singing behavior (Li et al. 2000). The time-course of its regulation by song is protracted relative to *zenk*, consistent with BDNF being a late effector song-

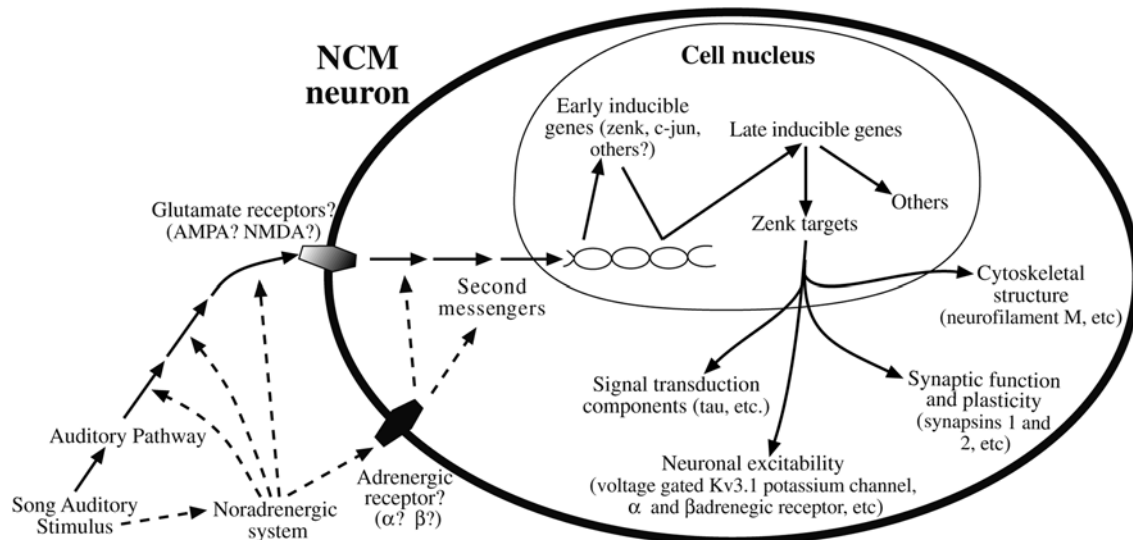


Fig. 5 Schematic representation of the gene induction response to song in a given caudomedial neostriatum (NCM) neuron. Indicated is the flow of activity triggered by song exposure and leading to the activation of NCM neurons, followed by the induction of early activity-dependent genes and subsequently of their transcriptional targets. *zenk* and its candidate targets (based on the identification of genes with a ZENK-binding motif in their promoter region from GenBank) are depicted as a representative example. Also notice that the activation of the noradrenergic system and associated receptors and signal transduction pathways is thought to affect song-induced gene expression, but the mechanisms are unknown

regulated gene. The functional consequences of BDNF regulation by song remain to be determined.

Summary and perspectives

zenk expression analysis has led to the identification of a set of brain areas distinct from the song control system that are activated in association with hearing song and that are likely involved in song auditory processing and perception, and possibly the formation of song auditory memories. Such areas form a system of interconnected nuclei whose organization resembles that of auditory cortical structures in mammals. Some of these auditory stations project to song control nuclei, providing potential sources of auditory input to the song system. They also potentially feed auditory information to brain areas that subserve non-vocal aspects of the bird's response to song. The data on induced gene expression argue against a direct participation of the song control system in perceptual aspects of vocal communication in songbirds.

Analysis of *zenk* expression in association with singing behavior has also generated new insights into the functional organization of the song control system. In particular, this approach has provided strong evidence for the active participation of the anterior forebrain pathway in the act of singing, as well as for the influence of the context of singing on brain activation patterns. The comparative use of *zenk* expression analysis across

several avian orders has helped identify features of the avian vocal control pathways that are likely critical for vocal learning. Overall, these studies illustrate well how a molecular approach can be used to map brain pathways associated with complex behaviors that are of evolutionary relevance and that are difficult to reproduce out of a natural context.

Induction of *zenk* is only one of several gene regulatory events that likely take place in the context of neuronal activation. A central working hypothesis is that *zenk* is an early regulator in a gene cascade leading from neuronal activation by song to the regulation of target genes representing various aspects of neuronal function, and eventually resulting in long-lasting changes to song-activated cells (Fig. 5; see also discussion in Clayton 2000). For a better understanding of the role of gene expression in avian vocal communication and learning, it will be essential to identify a larger set of song-induced genes. Initial screening efforts using differential cDNA library screening and differential display (Mello et al. 1997) indicate the existence of early song-inducible genes whose expression patterns differ considerably from that of *zenk* (C. Mello and R. Malcher, unpublished observations), but their identity has not been determined yet. It will be equally important to direct further efforts to the detection of late song-regulated genes, some of which may be direct targets of *zenk* or other early transcriptional regulators. A promising strategy is to test whether known or candidate ZENK targets (based on the presence of ZENK-binding motifs in their promoter regions) are indeed regulated by song stimulation in NCM. It will also be important to determine which neurotransmitter/neuromodulatory systems (glutamate?/GABA?/norepinephrine?) and respective receptors and signal transduction pathways regulate song-induced gene expression.

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