THE GENOMIC RESPONSE TO SONG AND THE REPRESENTATION OF COMPLEX SOUNDS IN THE AUDITORY SYSTEM OF ZEBRA FINCHES

by

Tarciso Andre Ferreira Velho

A DISSERTATION

Presented to Neuroscience Graduate Program

and the Oregon Health & Science University

School of Medicine

in partial fulfillment for the degree of Doctor of Philosophy

June 2008

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. dissertation of
TARCISO ANDRE FERREIRA VELHO
has been approved

Advisor, Claudio V. Mello

Member, Curtis Bell

Member, Robert Duvoisin

TABLE OF CONTENTS

1 Introduction…… 1

1.1 The neurobiology of vocal communication in songbirds: the substrates for song perception, production and learning…….. 4

1.2 Birdsong and activity-dependent transcription……. 7

1.2.1 Gene regulation by song stimulation…… 7

1.2.1.1 Stimulus dependence…….. 10

1.2.1.2 Context dependence……. 12

1.2.1.3 Experience dependence…….. 13

1.2.1.4 Coupling of auditory activation and gene expression.. 15

1.2.2 Gene regulation by singing behavior……. 16

1.3 Regulation of song-induced gene expression in auditory neurons…….. 18

1.4 Possible functions of song-induced gene expression in auditory and song control areas ……. 22

1.5 Plasticity and song memorization…….. 23

1.6 The representation of birdsong in the NCM…… 25

2 The Early Response to Song…….. 35

2.1 Introduction…… 36

2.2 Methods……..... 39

2.3 Results……. 48

2.4 Discussion…….. 58

3 The Late Response to Song……. 78

3.1 Introduction…… 79

3.2 Material and Methods…….. 82

3.3 Results…… 94

3.4 Discussion…….. 104

4 Regulatory Mechanisms of the Early Response to Song…….. 126

4.1 Introduction…… 127

4.2 Material and Methods…….. 130

4.3 Results……. 144

4.4 Discussion…….. 152

5 The representation of complex sounds in the zebra finch brain…… 170

5.1 Introduction…… 171

5.2 Material and Methods…….. 174

5.3 Results……. 181

5.4 Discussion…….. 189

6 Discussion…….. 210

6.1 Song-induced gene expression: functional consequence…….. 213

6.2 Towards an understanding of norepinephrine's role in

memory consolidation…….. 218

6.3 Summary and future directions…….. 222

7 References……. 229

LIST OF ILLUSTRATIONS

Figure 1.1 …….. 27

Figure 1.2 …….. 28

Figure 1.3 …….. 30

Figure 1.4 …….. 31

Figure 1.5 …….. 33

Figure 2.1 …….. 65

Figure 2.2 …….. 66

Figure 2.3 …….. 67

Figure 2.4 …….. 69

Figure 2.5 …….. 70

Figure 2.6 …….. 71

Figure 2.7 …….. 73

Figure 2.8 …….. 75

Figure 2.9 …….. 77

Figure 3.1 …….. 111

Figure 3.2 …….. 112

Figure 3.3 …….. 113

Figure 3.4 …….. 114

Figure 3.5 …….. 116

Figure 3.6 …….. 118
Figure 3.7 …….. 119
Figure 3.8 …….. 120
Figure 3.9 …….. 121
Figure 3.10 …….. 122
Figure 3.11 …….. 124
Figure 4.1 …….. 161
Figure 4.2 …….. 162
Figure 4.3 …….. 163
Figure 4.4 …….. 164
Figure 4.5 …….. 166
Figure 4.6 …….. 168
Figure 4.7 …….. 169
Figure 5.1 …….. 199
Figure 5.2 …….. 200
Figure 5.3 …….. 201
Figure 5.4 …….. 203
Figure 5.5 …….. 204
Figure 5.6 …….. 205
Figure 5.7 …….. 206

Figure 5.8 …….. 207

Figure 5.9 …….. 209

Figure 6.1 …….. 228

LIST OF ABBREVIATIONS

A - arcopallium

Ad - dorsal arcopallium

Bas - nucleus basorostralis

Cb - cerebellum

E- entopallium

GP - globus pallidus

H - hyperpallium

HB - habenular nuclei

Hp - hippocampus

HVC - nucleus HVC of the nidopallium

L1 - subfield L1 of field L

L2a - subfield L2a of field L

L3 - subfield L3 of field L

LoC - coeruleus

LMAN - lateral magnocellular nucleus of the anterior nidopallium

M - mesopallium

MLd - dorsal part of the lateral mesencephalic nucleus

N - nidopallium

NC - caudal nidopallium

NCM - caudomedial nidopallium

NIf - nucleus interface of the nidopallium

OMv - ventral part of the oculomotor nerve nucleus

OT - optic tract

Ov - nucleus ovoidalis

RA - robust nucleus of the arcopallium

Rt - nucleus rotundus

S - septum

St - striatum

TeO optic tectum

X area X of the medial striatum

ACKOWLEDGEMENTS

First and foremost I would like to thank my supervisor Claudio Mello for the opportunity to conduct my research under his guidance. Claudio has played a fundamental role in my scientific formation and the reasons for thanking him are too many to be all listed here. Nonetheless, I would like to thank Claudio for the most rigorous, tireless, and caring guidance that I could possibly get. I cannot thank him enough for his infinite patience and dedication. I would like also to thank Claudio for his friendship.

I would like to thank my thesis committee: Curtis Bell, Christine Portfors, Robert Duvoisin and Claudio Mello for their guidance throughout this period. The product of this thesis (and my scientific training) contains a significant contribution of each one of them. I also would like to thank Matt Frerking, for his prompt willingness to join us in one of the last steps of this journey.

I also would like to thank Dr David Vicario. David gracefully gave me the opportunity to visit his lab at Rutgers, where he taught me surgical procedures that are a fundamental part of this work. I also would like to thank David for enlightening discussions and continuous scientific input throughout the years. The thanks also extend to Kai, his graduate student and post-doc, with whom we have successfully collaborated in the long-term adaptation experiments.

I would like to thank Dan Iancu. The great collaboration we have established with Dan represents an integral part of this thesis and is embodied by Chapter 5. His contribution, however, extends far beyond the work we have done together. Our constant scientific discussions over the years have shaped many of the thoughts I have expressed throughout this dissertation.

I also would like to thank Pat Roberts. Pat's expertise was crucial to the design and interpretation of the experiments I describe in Chapter 5.

I would like to give special thanks to Bill Wood and Jin Jeong, for their scientific input, technical support and friendship. Bill and Jin covered distinct years in the Mello lab and their help was also fundamental for the development of this work.

I would like to thank my classmates, Ilia Halatchev, Joseph Aslan and Sean Wolfson. Our regular meetings after work were an essential part of my graduate school career.

I would like to thank my former mentors Maristela Azevedo and Aldo Dillon for all they taught me.

I would like to thank my non-scientific friends Benjamin Sturtz, Jay Kobak, Curtis Teigen, Karl, Bill Wood, Brett Meyer, Melissa Barber, Eric Washburn, Jin Jeong, Illia Halatchev, and Andrea Wolfe that helped make my time in Portland even more memorable.

I thank Andrea Wolfe for her amazing support during the closing chapters of my graduate school career and during the first steps towards my next journey.

I thank Melissa Barber and Brett Meyers for their long-lasting friendship. I would like to thank Brett for constantly willing to make the “sacrifice” runs so I could score, for willingly reading and editing this dissertation’s introduction even though his background is in computer sciences.

I thank all Mello lab members.

I thank the NSI staff, Jane and Gary, for their help.

I would like to thank Liz Lawson.

Special thanks go to my parents, Maria and Dercilio, and siblings, Miro, Nei, Lizi and Arlei, for everything I am.

I would like to thank Doina Moldovan for her continuous support for all these years.

Finally, I would like to thank the beautiful game for being my stress reliever, source of exercise, and means by which I met some of my best friends.

ABSTRACT

The consolidation of long-lasting sensory memories requires the activation of gene expression programs. However, to fully understand the contribution of genomic events necessary for sensory learning, it is necessary to characterize the components involved in this response, their induction kinetics, and the possible functional interactions among individual components. Birdsong represents one of the best natural behaviors to study gene expression induced by auditory stimulation in awake, freely behaving animals. The present work addresses the properties and functional consequences of the genomic response to birdsong in zebra finches (*Taeniopygia guttata*), with a specific focus on the caudomedial nidopallium (NCM), a cortical-like auditory area involved in song auditory processing and memorization. We found that song induces a well-orchestrated cascade of gene expression that includes early and late genes. Early genes are co-expressed by NCM neurons and their induction is regulated by the mitogen-activated protein (MAP) kinase pathway. In addition, we also found that the induction of early genes is locally modulated by norepinephrine. We have also identified two late genes, *syn2* and *syn3*, whose induction is repressed by early song-induced proteins genes in NCM. Functionally, we show that blockade of this response by α-adrenergic antagonists disrupts the long-term maintenance of long-lasting neuronal changes triggered by song. Finally, we

show that the pattern of early song-induce gene expression in NCM is stimulus dependent. These patterns also indicate the existence of functional subdomains of more or less selectivity towards conspecific songs. Overall, these results help us understand the functional significance of the genomic response to song and the functional organization of sound representation in a higher order auditory area of the songbird brain.

Chapter 1

INTRODUCTION

One of the central questions in neurobiology concerns how the brain translates behaviorally-relevant sensory information into modified behavioral activity. In other words, how does sensory learning occur? In evolutionary terms, the environment can certainly influence the genome of a given species' through natural selection, and thus deeply affect behavior. On a shorter time scale, however, animals can show fast behavioral adaptations in response to a rapidly changing environment independent of permanent changes in its genome. These behavioral adaptations are enabled by the nervous system. Although short-term changes in behavior can also occur without permanent modifications in neuronal circuits, long-term changes, whether or not beneficial, in general are thought to require the environment or the behavioral activity to influence gene expression programs[1] (Goelet et al., 1986), which in turn can then lead to long-term changes in neural circuits that underlie the modified behavior (Fig 1.1). The general questions that guided this thesis concern how a specific behavior, vocal communication in songbirds, regulates changes in gene expression in auditory brain areas, and how such programs are regulated at the local circuitry level. I focused on the zebra finch, a representative songbird species that has been

[1] There are cases, however, of autoimmune diseases and spinal cord injuries in which behavioral modifications may not require prior changes in gene expression.

extensively studied, from the point of view of both auditory and motor representation of song. In the Introduction to this Dissertation, I first discuss briefly some basic aspects of birdsong neurobiology, focusing on the substrates for perceptual and motor aspects of vocal communication in songbirds. I then discuss our current knowledge of the influence of stimulus type, behavioral condition and context on transcriptional regulation by song, as well as the mechanisms regulating induced gene expression in song-responsive neurons. Next, I present how my experimental work led to the identification of novel early song-induced genes (**Chapter 2**), of the first known late song-induced genes (**Chapter 3**), and of regulatory and modulatory mechanisms involved in the genomic response to birdsong (**Chapter 4**). I have also made use of early song-induced genes as markers of neuronal activation to investigate how complex sounds are represented in areas involved in auditory processing in the zebra finch brain (**Chapter 5**). The relevance of these findings for the understanding of auditory processing, possible formation and maintenance of auditory memories, and for a better understanding of sensory-induced transcriptional regulation in the vertebrate brain in general will be discussed in the final chapter of this dissertation (**Chapter 6**).

1.1 The neurobiology of vocal communication in songbirds: the substrates for song perception, production and learning

Birdsong is a complex learned behavior used extensively by songbirds for individual recognition, territorial defense and attracting potential reproductive partners (Catchpole and Slater, 1995 and Kroodsma and Miller, 1996 for reviews). Whereas many animals use a wide variety of signals to communicate, vocal learning, i.e. the ability to learn their vocal communication signals from exposure to a model, is a rare trait shared only by a few groups (humans, cetaceans, possibly bats and elephants, and three orders of birds: songbirds, parrots and hummingbirds) (Nottebohm, 1972; Brenowitz, 1997; Brenowitz et al., 1997a). The processing and memorization of complex auditory signals is essential for the learning and maintenance of vocalizations and for perceptual aspects of vocal communication in all vocal-learners, including humans (Doupe and Kuhl, 1999). Yet the underlying brain pathways and mechanisms are for the most part unknown. In contrast to other vocal learners, the brain circuits controlling song learning, production and perception in songbirds have been identified and are well characterized (Brenowitz et al., 1997a; Zeigler and Marler, 2004 for reviews), placing birdsong as an excellent model for understanding the neuronal basis of vocal communication and vocal learning.

Vocal communication and learning in songbirds require two systems of brain pathways, the auditory system and the song control system. The avian ascending auditory pathways are quite similar, in their general organization, to

the auditory pathways of other vertebrates (Butler and Hodos, 1996). Although the brainstem auditory pathways have not been studied in detail in songbirds, they are thought to consist of a series of cochlear, lemniscal, and midbrain nuclei that carry auditory input from the cochlea to the thalamic auditory nucleus ovoidalis (Ov; Fig 1.1A, left panel). Auditory information then reaches the telencephalon through the projection from Ov to the primary thalamo-recipient zone field L, more specifically its L2a subdivision (Kelley and Nottebohm, 1979; Vates et al., 1996). L2a sends projections to adjacent subfields L1 and L3, which in turn project to the main pallial targets of L, namely the caudomedial nidopallium (NCM), the caudomedial mesopallium (CMM), and the shelf and cup areas, the latter two in close proximity to nuclei of the song control system (Fig 1.2A). This auditory system is common to both males and females (Vates et al., 1996; Mello et al., 1998b), and is necessary for the acquisition of song in juveniles and for its maintenance during adulthood (Konishi, 1965; Marler and Peters, 1977; Nordeen and Nordeen, 1992; Leonardo and Konishi, 1999; Woolley and Rubel, 2002). It is also necessary for the perceptual processing and memorization of birdsong in the context of identification and discrimination of other conspecifics (Catchpole and Slater, 1995; Kroodsma and Miller, 1996). Auditory pathways similar to those described in songbirds are thought to be present in other avian groups regardless of whether they learn their vocalizations, indicating that this is a highly conserved pathway among birds (Karten and Hodos, 1967; Karten, 1968; Bonke et al., 1979; Brauth et al., 1987; Brauth and McHale, 1988; Wild, 1993; Metzger et al., 1998).

The song control system comprises two sets on interconnected nuclei, the direct vocal-motor and the anterior forebrain pathways (Fig 1.2B, left panel). The direct vocal-motor pathway (Fig 1.2B, black arrows) consists of projections from nucleus HVC (used as a proper name) to the robust nucleus of the arcopallium (RA), and from RA to midbrain and medullary centers involved in the control of vocal and respiratory function (Nottebohm et al., 1982). This direct pathway is actively engaged during singing behavior (Yu and Margoliash, 1996; Jarvis and Nottebohm, 1997; Olveczky et al., 2005) and is required for song production (Nottebohm et al., 1976). The anterior forebrain pathway (Fig 1.2B, grey arrows) consists of projections from area X in the medial striatum to the medial part of the dorsolateral thalamic nucleus (DLM), from DLM to the lateral magnocellular nucleus of the anterior nidopallium (LMAN), and from LMAN back to area X (Bottjer et al., 1989; Johnson et al., 1995; Vates et al., 1997; Luo et al., 2001). This anterior pathway is connected with nuclei in the direct motor pathway via HVC-to-X and LMAN-to-RA projections (Fig 1.2B) and is essential for vocal learning (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991) and vocal plasticity (Olveczky et al., 2005). The song control system is typically more developed in males and is under the strong influence of sex steroids (Nottebohm and Arnold, 1976; Arnold, 1980; Arnold et al., 1986; Wade and Arnold, 2004). In some species, such as canaries, the song system shows marked seasonal fluctuations in neuronal cell numbers and morphology that are associated with vocal plasticity (Nottebohm, 1981; Nottebohm et al., 1986; Brenowitz and Arnold, 1990; Brenowitz, 2004). Such observations originated the

discovery of marked neuronal replacement during adulthood (Goldman and Nottebohm, 1983; Paton and Nottebohm, 1984).

It is important to note that the avian brain is currently considered to be more similar to the mammalian brain in its general organization than previously thought. Although birds lack the layered organization typical of the mammalian brain, it is clear that most of the avian telencephalon is pallial in its origin, and thus homologous as a field to the mammalian cortex (Jarvis et al., 2005). In addition, individual pallial and basal ganglia structures of the avian brain share many neurochemical markers and patterns of connectivity with their presumed mammalian counterparts (e.g. Fig 1.2A and B, compare right and left panels) (also see (Jarvis et al., 2005). This new view of the avian brain has been consolidated with a comprehensive nomenclature change in order to better reflect the known homologies between avian and mammalian brain structures (Reiner et al., 2004).

1.2 Birdsong and activity-dependent transcription

1.2.1 Gene regulation by song auditory stimulation

When songbirds hear song, the expression of activity-dependent transcription factors is induced in discrete areas along the auditory pathway (dark grey areas in Fig 1.2A) (Mello et al., 1992; Mello and Clayton, 1994; Mello and Ribeiro, 1998; Kruse et al., 2000; Velho et al., 2005). This phenomenon is most prominent in two major telencephalic areas, the caudomedial nidopallium (NCM)

and the caudomedial mesopallium (CMM). Based on their connectivity, these areas are arguably analogous to supragranular layers of the mammalian auditory cortex as they are targets of the primary thalamo-recipient zone field L (Fig 1.2A, right panel). More importantly, these areas are thought to participate in various aspects of song processing and discrimination, and perhaps in the consolidation and/or storage of auditory memories (Mello, 2002; Mello et al., 2004). The genomic response to song includes the early inducible transcription factors (ITFs) *zenk* (a.k.a. **z**if268, **e**gr1, **n**gfi-A, and **k**rox-24), *c-fos* and *c-jun* whose products are thought to exert an indirect and protracted effect through the regulation of the expression of downstream target genes (Fig 1.3; we use italics for the genes and normal lettering for the protein, for example *zenk* and ZENK). The activation of song-induced gene expression programs is a representative example of activity-dependent gene regulation, a conserved feature of the nervous system, from invertebrates to humans, that has been postulated to represent a link between neuronal activation and long-lasting plasticity events in the brain (Fig 1.3; Goelet et al., 1986; Bailey et al., 1996).

The song-induced expression of ITFs in auditory areas is rapid and transient (Mello et al., 1992), appearing within minutes after stimulus onset, peaking at 30 minutes, and declining thereafter (Mello et al., 1992; Mello and Clayton, 1994; Mello and Ribeiro, 1998; Kruse et al., 2000; Velho et al., 2005). The expression of *zenk* mRNA returns to levels close to unstimulated controls by one hour after stimulus onset (Mello and Clayton, 1994; Velho et al., 2005). Importantly, the expression levels of these activity-dependent genes, as

measured by either *in situ* hybridization or immunocytochemistry, is practically absent in auditory areas of unstimulated quiet controls (Mello et al., 1992; Mello and Ribeiro, 1998), indicating that the expression observed in song-stimulated birds results from activation at the transcriptional level. The translated protein products of song-inducible ITFs (ZENK and c-Fos) have the expected nuclear distribution, as revealed by ICC (Kimpo and Doupe, 1997; Mello, 1998), and have been extensively used to map song-induced neuronal activation in several species and contexts (Mello, 2002).

Neuronal activation can also lead to the expression of direct effectors. In contrast to ITFs, these proteins act directly by altering cellular properties independently of further RNA and protein synthesis. In rodents for example, the early genomic response to neuronal depolarization, estimated to involve 40 immediate early genes, is thought to include about 25-40 direct effectors (Lanahan and Worley, 1998). Thus, we hypothesize that in addition to genes encoding transcription factors, song also triggers the expression of genes encoding effector proteins in a well-orchestrated cascade that results in altering neuronal properties of song-responsive cells (Fig 1.3). We have tested this hypothesis by determining, through in situ hybridization, the extent to which several activity-regulated early genes known to be involved in neuronal plasticity, such as *Arc*, *c-fos*, and *narp*, are induced by birdsong auditory stimulation (Chapter 2), and whether song-induced genes are co-expressed and ultimately co-regulated in NCM auditory neurons. We also identified late song-induced genes, and examined their relationship with early-induced proteins (Chapter 3).

1.2.1.1 Stimulus dependence

The quality of the song, which directly influences the behavioral responses to song in females (mating choice), modulates the song-induced genomic response in auditory areas. Females often show behavioral preferences for particular features present in conspecific songs. For instance, starling (*Sturnus vulgaris*) females prefer males singing longer bouts (Gentner and Hulse, 2000). Similarly, canary (*Serinus canaria*) females prefer songs containing more complex syllables, with rapid frequency modulations and higher repetition rates (Vallet et al., 1998), and zebra finch females prefer direct over indirect songs (Woolley and Doupe, 2008). Stimulation of females with preferred songs induces higher levels of ZENK in the canary NCM and in the starling and zebra finch CMM when compared with non-preferred songs (Gentner et al., 2001; Leitner et al., 2005; Woolley and Doupe, 2008). These observations give strong indication that the strength of the *zenk* response to song in NCM and CMM correlates with acoustic properties of the male song.

Further evidence that the genomic response to song is tuned to specific acoustic components of the stimulus detecting subtle variations in spectral/temporal features, comes from work in canaries. Auditory stimulation with whistles (i.e., syllabic elements from canary song that resemble pure tones) triggers gene expression in discrete subregions of NCM (Fig 1.4; Ribeiro et al., 1998). Specifically, high-frequency whistles induce *zenk* expression in ventral portions and low-frequency whistles in dorsal portions of rostral NCM, in a

graded and tonotopic fashion (Fig 1.4A) (Ribeiro et al., 1998). However, a comparison between the *zenk* response to naturally occurring canary whistles versus synthetic stimuli of the same frequencies but that lack subtle variations in amplitude and frequency reveals markedly different patterns of activation in terms of both intensity and spatial distribution of responsive cells (Fig 1.4B and C) (Ribeiro et al., 1998). The patterns of activation elicited by individual whistles, combination of whistles and modulations (all naturally occurring syllables in canary songs) can be separated by principal component analysis (PCA), indicating that particular song features are represented differently in the NCM. Thus, the syllabic representation in canary NCM is tuned to specific acoustic elements present in canary song.

The amount of stimulation also influences the expression levels of song-induced genes. Contrary to stimulus type, the amount of stimulation has only a small effect on gene induction measured as the number of responding cells. For example, stimulation with a single song presentation (~2 seconds) is sufficient to recruit a large number of *zenk*-expressing cells in NCM. The number of responding cells peaks with a few further (4-10) song repetitions, but does not increase with even further stimulation (Kruse et al., 2000). In contrast,, the mRNA levels of song-induced genes increase continuously with increasing stimulation, peaking after about 30 minutes of song presentation (10-12 song repetitions per min), but decline thereafter, regardless of further stimulation (Mello et al., 1995; Velho et al., 2005). Thus, while the number of song-responsive neurons rises sharply after a brief stimulation, the amount of song-induced mRNA rises more

gradually before reaching a plateau and declining. These observations likely reflect the very nature of the genomic response to neuronal excitation, where the size of the neuronal population recruited depends primarily on properties intrinsic to the stimulus (such as spectral/temporal features of song), whereas the total amount of resulting transcription is determined by the recruitment of signal transduction pathways, which in turn is determined by the amount of stimulation.

Finally, the strength of the genomic response observed in NCM also depends on the category of the stimulus presented. Early studies showed that stimulation with conspecific song results in larger amounts of *zenk* mRNA compared to birds stimulated with heterospecific song or pure tones for similar durations and intensities (Mello et al., 1992). This observation suggests that NCM is more responsive (tuned) to acoustic (spectral and/or temporal) features present in conspecific song. It is not yet clear whether this effect, based on densitometric analysis of autoradiograms, reflects differences in the mRNA amounts per cell or in the total numbers of activated cells across stimuli. To gain further insights into the auditory representation of complex sounds in zebra finches, we have used *zenk* expression to determine the pattern of activation triggered by different stimulus categories in the auditory system (Chapter 4).

1.2.1.2 Context dependence

The strength of the genomic response observed in NCM and CMM is also modulated by the context in which the stimulus is presented. For instance, the associative pairing of conspecific song stimulation with a foot shock, in an active

avoidance paradigm, enhances the *zenk* response in NCM and CMM, whereas shock alone or shock uncorrelated with song stimulation has no modulatory effect (Jarvis et al., 1995). Similarly, pairing the song stimulus with salient visual stimulation, such as images of conspecifics or simply with an enriched light environment (multi-colored lights) results in higher levels of ZENK protein expression in these auditory areas (Kruse et al., 2004; Avey et al., 2005). Modulatory effects on transcriptional activation can also be observed by manipulating the distance to the stimulus source and/or intensity (i.e. sound pressure level) (Kruse et al., 2004). Experimental conditions that alter the arousal state of the bird, such as restraint, can also affect the amount of *zenk* expression when compared to that seen in freely-behaving birds (Park and Clayton, 2002). Such procedure-induced increase in gene expression can be reversed or attenuated by acclimation sessions consisting of brief and repeated (4-5) exposures to the experimental condition or apparatus (Jarvis et al., 1995; Velho et al., 2005). The mechanisms underlying the context dependence of song-induced gene expression most likely reflect changes in the behavioral state of the bird, particularly in attention. As discussed later in this chapter, this effect may involve catecholaminergic modulatory systems.

1.2.1.3 Experience dependence

The expression of *zenk* in NCM and CMM is also heavily influenced by experience. Repeated presentations of the same stimulus lead to a progressive decline of the *zenk* response, a phenomenon that has been termed adaptation

(formerly called habituation Mello et al., 1995). This reduction in *zenk* activation is accompanied by a similar reduction in firing rates of NCM neurons (Chew et al., 1995; Stripling et al., 1997; Terleph et al., 2006), and it can be relatively long-lasting, according to the amount of adapting stimulation used. Both the firing rates and the transcriptional induction of *zenk* in song-adapted NCM neurons can be reinstated by presentation of a novel song stimulus (Chew et al., 1995; Mello et al., 1995; Stripling et al., 1997). Importantly, adaptation can be induced in response to multiple songs independently (Figure 5A). Therefore, the degree of adaptation has been suggested as an index of the extent to which a specific song is remembered by song-responsive circuits (Chew et al., 1995). In other words, adaptation appears to reflect a physiological memory trace of a given song. Furthermore, adaptation to song in NCM may play a significant contributing role to song discrimination.

Spaced brief pre-exposure to particular songs or categories of songs also regulates the strength of the transcriptional activation. In song sparrows, familiar songs, i.e. songs to which the birds have been previously exposed to (30 min per day for seven days), induce ZENK expression in a smaller number of neurons in the ventral NCM when compared to novel songs (McKenzie et al., 2006), suggesting that some long-term adaptation has occurred in this area. In starlings, pre-exposure to long-bout songs results in higher ZENK expression in both NCM and CMM in response to a novel long-bout song when compared to a novel short-bout song (Sockman et al., 2002). Intriguingly, pre-exposure to short-bout songs increases Fos immunoreactivity in response to a novel short-bout song in

these areas (Sockman et al., 2004), suggesting in this case different modulatory mechanisms for ZENK and c-Fos.

In addition to recent experience (adaptation), the birds' past experience also influences the magnitude of the genomic response to song. In female zebra finches, the expression levels of *zenk* mRNA triggered by stimulation with the father's song are higher than the expression levels triggered by a novel conspecific song, demonstrating a modulatory effect of the bird's previous experience (Bolhuis et al., 2000; Terpstra et al., 2006). In addition, the number of ZENK-expressing cells in response to the tutor song (i.e. the song copied during the song learning period) correlates positively with how well that song was copied (Bolhuis et al., 2000). This indicates that the genomic response in NCM is tuned to acoustic properties in the tutor's song and may be linked with an auditory representation, or memory, of that song. Consistent data have recently been obtained by recording the electrophysiological response of NCM to learned tutor songs (Phan et al., 2006). In this study, a familiarity index, based on the adaptation rate of NCM in response to presentation of the tutor song, correlates with the number of syllables the bird learned to copy from that tutor song.

1.2.1.4 Coupling of auditory activation and gene expression

Some auditory areas do not show song-induced transcription of *zenk* or other inducible genes (Mello et al., 1992; Velho et al., 2005), even though they are obligatory stations in the ascending auditory pathway (Fig 1.2A, left panel) and are thought to be activated by song. These include the thalamic nucleus Ov

and the telencephalic subfield L2a. The latter is the main entry site for auditory information into the telencephalon and precedes NCM and CMM in the auditory pathway (Vates et al., 1996). The fact that L2a does not show song-induced transcription suggests that neuronal activation and activity-dependent gene expression are uncoupled in this area. In fact, even widespread telencephalic depolarization triggered by drug-induced seizures does not lead to *zenk* induction in L2a (Mello and Clayton, 1995). A possible function for the uncoupling would be the need to turn off activation-induced plasticity in L2a, preserving the fidelity of the auditory information that is relayed to higher-order auditory areas. Accordingly, in contrast to NCM and CMM, L2a neurons show strong phasic responses to auditory stimulation that do not habituate with repeated presentations (Terleph et al., 2006), indicating the absence of experience-dependent plasticity. Although the mechanism of this uncoupling between electrical activation and transcriptional regulation is unknown, it could be due to the down-regulation or absence of critical components of the signal transduction pathway leading to induced-gene expression or, as occurs in the rodent hippocampus (Weaver et al., 2004), by permanently altering the chromatin through methylation, which renders activity-dependent gene promoters unavailable to activating transcription factors.

1.2.2 Gene regulation by singing behavior

When birds sing, activity-dependent genes are markedly induced in the song control system (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997;

Velho et al., 2005). Similar to the auditory system, the song control nuclei of birds that remain silent prior to sacrifice show little or no expression of known song-inducible genes (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Velho et al., 2005). In contrast, when birds sing, a genomic response is triggered in nuclei that control singing behavior, more specifically in song nuclei HVC and RA (Fig 1.1B gray areas) (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Mello, 1998; Velho et al., 2005). This induction is also observed in the auditory processing areas discussed above (NCM, CMM, L1, L3, and the shelf and cup areas). In the latter regions, however, singing-induced expression is abolished by deafening, while it remains unaltered in song control nuclei. This demonstrates that the singing-induced transcriptional response in song control nuclei is motor-driven rather than auditorily triggered (Jarvis and Nottebohm, 1997).

Interestingly, singing-induced gene regulation is also dependent on the context of the singing behavior, as clearly demonstrated in zebra finches (Jarvis et al., 1998). For example, when the bird sings in a solo context, or performs “undirected singing”, i.e. song not directed to another male or female, *zenk* induction is observed in all telencephalic song control nuclei (HVC, RA, LMAN, and area X). In contrast, when the bird sings towards another male or female, i.e. performs “directed singing”, as during courtship behavior, *zenk* induction is not observed in striatal area X, revealing a context-dependent regulation of expression in the anterior forebrain pathway. This context-dependent regulation is also associated with differences in neuronal firing rates in area X during singing, i.e. higher firing rates for undirected compared to directed singing

(Hessler and Doupe, 1999a). This difference in neural activity is possibly the result of different neuromodulatory mechanisms acting under different behavioral conditions, as discussed later in this thesis.

1.3 Regulation of song-induced gene expression in auditory neurons

The study of the transduction machinery that links neuronal activation by song to activity-dependent gene expression in songbirds is still incipient. Based mostly on studies in mammals, the induction of activity-dependent transcriptional regulation in neuronal cells is likely initiated by the activation of ionotropic and/or metabotropic membrane receptors leading to rises in intracellular Ca^{++} (Berridge, 1998). The precise nature of the synaptic input has not been determined in songbirds, but intracellular Ca^{++} elevation and second messenger recruitment through glutamate receptor activation is well-established in the mammalian brain (Wang et al., 2004). Although representatives of all classes of glutamate receptors are expressed in auditory and song- control areas of zebra finches (Wada et al., 2004), their functional role has not been directly tested. Alternatively, Ca^{++} could enter song-responsive neurons through activation of L-type calcium channels, also known to regulate the activation of transcriptional responses in mammals (Ghosh et al., 1994; Finkbeiner and Greenberg, 1998).

It is important to point out that the inhibitory neurotransmitter GABA may also shape the genomic response to song. A very large percentage of NCM neurons (about 50%) are GABAergic and a significant portion of these inhibitory cells shows song-induced *zenk* transcription, indicating their direct participation in

NCM's response to song (Pinaud et al., 2004). Furthermore, whole-cell patch-clamp recordings in NCM slices show very prominent spontaneous inhibitory post-synaptic potentials. This suggests that the GABAergic network tonically suppresses the activity of excitatory circuits in NCM at rest, and that song-induced activation of NCM likely involves the relief of such circuits from GABAergic suppression.

The initiation of activity-dependent transcriptional activation requires further the recruitment of intracellular signal transduction pathways. In zebra finch auditory neurons, transcriptional activation of the *zenk* gene is dependent on the activation of the mitogen-activated extracellular-signal-regulated protein kinase kinases 1 and 2 (MEK 1/2), whose main substrate is the extracellular-regulated kinase (ERK) activity (Fig.2). The local blockade of MEK 1/2 prevents song-induced expression of *zenk* in NCM (Cheng and Clayton, 2004; Velho et al., 2005). Once phosphorylated, ERK is thought to translocate to the nucleus, where it activates constitutive regulatory transcription factors (RTFs) (see review in Grewal et al., 1999). Interestingly, the *zenk* gene promoter in zebra finches has binding sites for at least two RTFs that are known ERK targets (Cheng and Clayton, 2004), namely the cAMP response element-binding protein (CREB) and ETS-1, a member of the ETS oncogene family (Claudio Mello personal communication).

The kinetics of second-messenger activation may determine some fundamental aspects of song-induced transcriptional regulation. For instance, the phosphorylation of ERK by MEK1/2 in the telencephalic auditory lobule (NCM,

CMM and field L combined) shows very similar properties when compared to song-induced gene expression. ERK phosphorylation (*i*) is stimulus specific, i.e. it is higher for conspecific sounds compared to tones and white noise; (*ii*) is rapid and transient, returning to basal levels 5 min after the end of stimulation; (*iii*) habituates to repeated presentations of the same stimulus but is restored by presentation of a novel stimulus (Cheng and Clayton, 2004). Thus, even though neither the identity of ERK targets nor the duration of their activation is known, ERK phosphorylation mimics the genomic response to song and may determine some of its properties.

The fact that the transcriptional response to song is stimulus-dependent, regulated by experience, and influenced by the context in which the stimulus occurs, suggests that modulatory brain systems that alter the salience of the stimulus may regulate this response. A probable candidate is the noradrenergic system. In mammals, neuronal firing rates in the locus ceruleus (LoC), the main source of noradrenergic projections, are proportional to the behavioral relevance of the stimulus, decrease with repeated stimulus presentations, and are restored by a novel stimulus (Aston-Jones and Bloom, 1981a, b). In addition, LoC lesions disrupt the cortical induction of activity-dependent genes like *zenk* and *c-fos* (Cirelli et al., 1996; Cirelli and Tononi, 2000). Similar to mammals, the songbird telencephalon has a widespread noradrenergic innervation that includes pallial auditory processing and song control areas, but not the striatum, based on immunostaining for the noradrenaline-synthesizing enzyme dopamine-β-hydroxylase (DBH) (Mello et al., 1998a). The blockade of adrenergic receptors by

systemic injections of antagonists abolishes song-induced transcription of *zenk* in NCM, confirming a link between the noradrenergic system and song-induced gene expression (Ribeiro and Mello, 2000). Because these systemic injections do not reveal the site of noradrenergic action, we have performed intracerebral injections to determine the site of action and the potential roles of the noradrenergic system in the regulation of song-induced gene expression programs and long-lasting changes in the response properties of auditory neurons in NCM (Chapter **4**).

Another catecholamine, dopamine, may regulate the higher gene expression induced by undirected vs. directed singing in striatal area X of the song control system (Jarvis et al., 1998; Hessler and Doupe, 1999b). Similar to mammals, the songbird striatum, including area X, receives dense dopaminergic innervation from the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) in the midbrain, and expresses high levels of dopamine receptors (Lewis et al., 1981; Bottjer, 1993; Casto and Ball, 1996). Patch-clamp recordings in area X show that dopamine can modulate glutamatergic transmission in spiny neurons, reducing glutamatergic currents via activation of D1-like receptors (Ding et al., 2003). In addition, dopamine levels in area X measured by *in vivo* microdialysis in awake behaving birds increase above baseline during directed but not undirected singing (Sasaki et al., 2006). The increased dopaminergic input to area X in directed singing may decrease glutamate-dependent currents and therefore dampen the singing-induced transcriptional response in this nucleus. The singing context dependence of *zenk*

expression can also be altered by pharmacological ablation of the noradrenergic system in the zebra finch (Castelino and Ball, 2005). However, because the VTA receives a dense noradrenergic projection (Mello et al., 1998a), it is presently unclear whether the noradrenergic effect in area X is direct or mediated through the dopaminergic system (VTA-to-X projection).

1.4 Possible function of song-induced gene expression in auditory and song control areas

Activity-dependent transcription has been postulated to link neuronal activation to long-term changes underlying neuronal plasticity, learning and memory (Goelet et al., 1986; Clayton, 2000). Indeed, the induction of activity-dependent genes by birdsong occurs in a wide variety of circumstances associated with learning (e.g., when birds hear the tutor song during the song learning period or an unfamiliar song in adulthood), as well as retrieval and/or possibly reconsolidation (e.g. when birds hear tutor song or their own song during adult singing, or the songs of familiar conspecifics during adulthood). Because the strength of the response is differentially regulated by novel (new learning) vs. familiar songs (retrieval and/or reconsolidation), it is likely that activity-dependent transcription exerts multiple functions in song-responsive circuits, depending on age and context. The fact that known components of the genomic response to song have been linked to neuronal plasticity, as well as learning and memory consolidation and reconsolidation in mammals (Morrow et al., 1999; Guzowski et al., 2000; Jones et al., 2001; Lee et al., 2004) suggests

that these components may play similar roles in song-responsive circuits in songbirds.

Importantly, gene expression is required for the stabilization of activity-dependent changes in the properties of song-responsive neurons in the songbird NCM (Chew et al., 1995). More specifically, the long-term maintenance (several hours to days) of song-specific adaptation to repeated presentations of a given conspecific song (Fig 1.5A) requires local RNA and protein synthesis, as shown by injections of RNA and protein synthesis inhibitors in NCM (Fig 1.5B). (Chew et al., 1995). Two distinct *epochs* of *de novo* synthesis (i.e., the initial 2-3 hr period and the period between 5.5 and 7 hr after onset of song stimulation) are necessary for the stabilization of experience-dependent changes in NCM, as shown by varying the time of the pharmacological blockade relative to the training (song presentation) period (Fig 1.5B). In contrast, RNA and protein synthesis blockers do not affect the auditory responses to song or the initial acquisition of the habituated state in NCM neurons (Chew et al., 1995).

1.5 Plasticity and song memorization

NCM shows an experience-dependent plasticity of its response properties that is a possible substrate for song-specific memory. As mentioned above (section1.2.1.3), the amplitude of evoked multiunit responses in this area decreases rapidly with repeated song stimulation (Fig 1.5A), but is reinstated by introducing a novel song stimulus (Chew et al., 1995; Stripling et al., 1997). This “adaptation” is long-lasting and song-specific, occurring to multiple conspecific

vocalizations (songs or calls) independently (Chew et al., 1995; Chew et al., 1996). Furthermore, the rates of adaptation for tutor song (a song that young birds heard and copied while developing their own song) are lower compared to other songs, suggesting a higher degree of familiarity to that song (Phan et al., 2006). Importantly, responses in field L2a (the main point of entry for auditory information into the telencephalon, thus preceding NCM, do not habituate (Terleph et al., 2006). Thus, NCM exhibits several neural correlates of auditory memories.

Recent lesion and pharmacological experiments have provided even more compelling evidence of NCM's involvement in perceptual processing and memorization of songs. For instance, lesions targeting dorso-lateral NCM abolish the preference of adult female zebra finches for their father's song over that of other conspecifics. This preference which is acquired during development, represents a good example of a perceptual song memory (Gobes and Bolhuis, 2007). Moreover, local pharmacological blockade of map kinase kinase (MEK1/2) activity in NCM during exposure of juvenile male zebra finches to tutor song disrupts normal song-development (London and Clayton, 2008). This signaling pathway is required for the song-induced expression of *zenk* and other activity-dependent genes, suggesting an involvement of NCM's gene expression programs in vocal learning. Thus, in addition to its involvement in the sensory processing and/or discrimination of song, NCM exhibit a neural correlate of a stimulus-specific auditory memory trace (i.e. song-specific adaptation) and appears to be required for normal song-development in zebra finches.

1.6 The representation of birdsong in NCM

The ascending auditory system of songbirds has been under intensive investigation for the past two decades in the context of auditory perception. In addition to the gene expression studies discussed above, electrophysiological, pharmacological, and lesions studies also indicate that telencephalic structures such as NCM are likely candidates to represent the neural basis for song recognition, discrimination and perhaps formation and/or storage of auditory memories (Mello et al., 1992; Stripling et al., 1997; Grace et al., 2003; Woolley et al., 2005; Phan et al., 2006; Gobes and Bolhuis, 2007; Woolley and Doupe, 2008). Accordingly, electrophysiological responses in NCM show some selectivity for conspecific vocalizations. Specifically, responses amplitudes in this area are higher for zebra finch calls and songs compared to heterospecific songs, white noise and tones (Chew et al., 1996). Response properties also appear significantly different than the preceding auditory stations such as field L2. Larger numbers of NCM units are significantly more selective to complex sounds than in L2 (Muller and Leppelsack, 1985). Moreover, NCM responses to tones appear to have broader tuning curves and lower amplitudes (in both canary and zebra finch) compared to field L responses (Terleph et al 2007). Accordingly, an increasing selectivity for natural sounds is also observed form L2 to subfields L1 and L3 that also precede NCM (Amin et al 2007). NCM properties thus appear to be more complex, more selective to complex sounds, and to be integrating a wider range of spectral features contained in a song (i.e. broader frequency

range and more sustained responses in NCM) compared to the preceding station field L. NCM is thus well equipped to play a significant role in the sensory representation of conspecific communication signals. To test this hypothesis, we compared the levels and patterns of NCM activation triggered by conspecific song, spectrally and temporally decomposed songs, and other complex sounds, and analyzed the distribution of cells in response to two distinct songs in the same bird at the cellular level of resolution (Chapter 5).

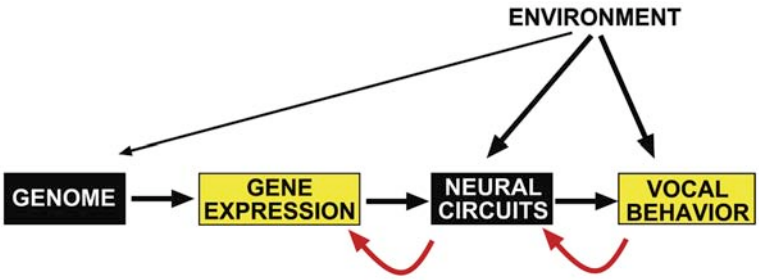
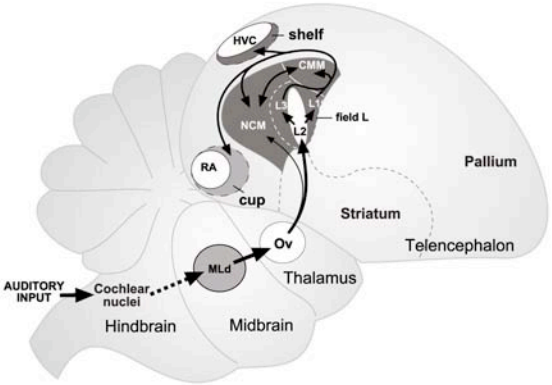
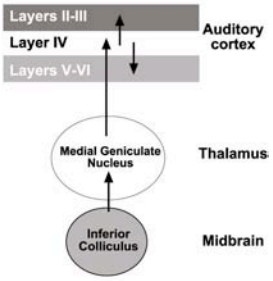


Fig 1.1. Schematic representation of experience-dependent long-lasting changes to neuronal circuits.

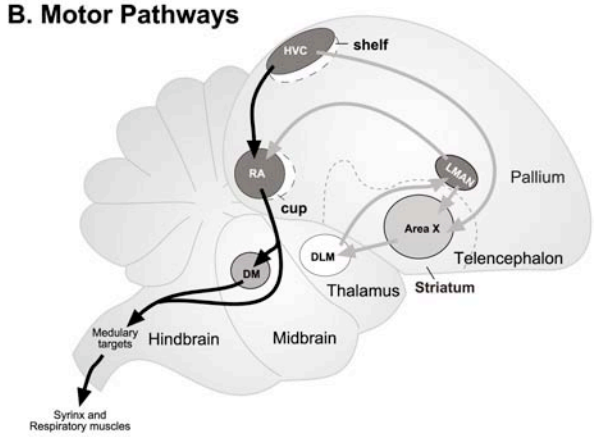
Songbirds **Mammals**

A. Auditory Pathways





B. Motor Pathways



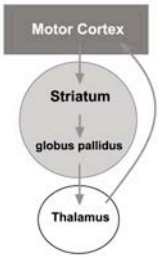


Fig. 1.2. Schematic representation of *auditory* (A) and *song control* (B) pathways and associated genomic responses to song in songbirds and the equivalent pathways in mammals. Areas in grey indicate areas that show activity-dependent transcription in songbirds upon song-stimulation (auditory pathway) and singing (motor pathway). Areas in white indicate areas that lack this response. In B, black arrows indicate the direct vocal-motor pathway and grey arrows indicate the anterior forebrain pathway. CMM, caudomedial mesopallium; DLM, dorsal lateral nucleus of the medial thalamus; DM, dorsal medial nucleus; L1, L2 and L3, fields L1, L2, and L3; LMAN, lateral magnocellular nucleus of the anterior nidopallium; MLd, dorsal lateral nucleus of the anterior mesencephalon; NCM, caudal medial nidopallium; Ov, nucleus ovoidalis; RA, robustus nucleus of the arcopallium.

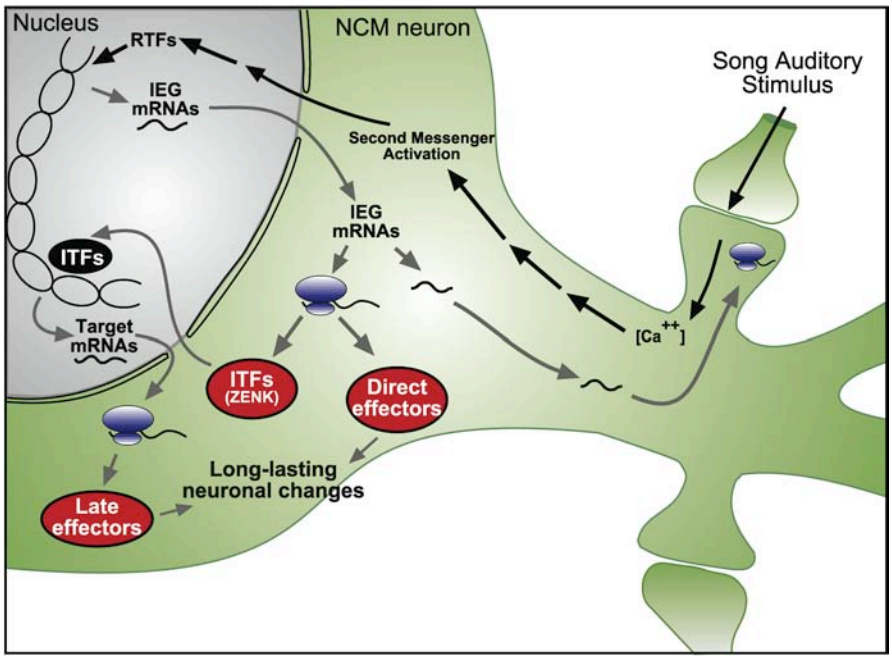


Fig. 1.3. Song-induced transcriptional response in auditory neurons. Black arrows indicate the second messenger signaling cascade triggering transcriptional activation; grey arrows indicate the genomic response to song stimulation; dashed arrows indicate possible modulatory mechanisms. ERK, extracellular-signal regulated kinase; ERK^{P}, phosphorylated ERK; MEK1/2, mitogen-activated extracellular-signal-regulated protein kinase kinase 1 and 2; IEG, immediate early genes; ITFs, inducible transcription factors; NA, noradrenaline; RTFs, regulatory transcription factors.

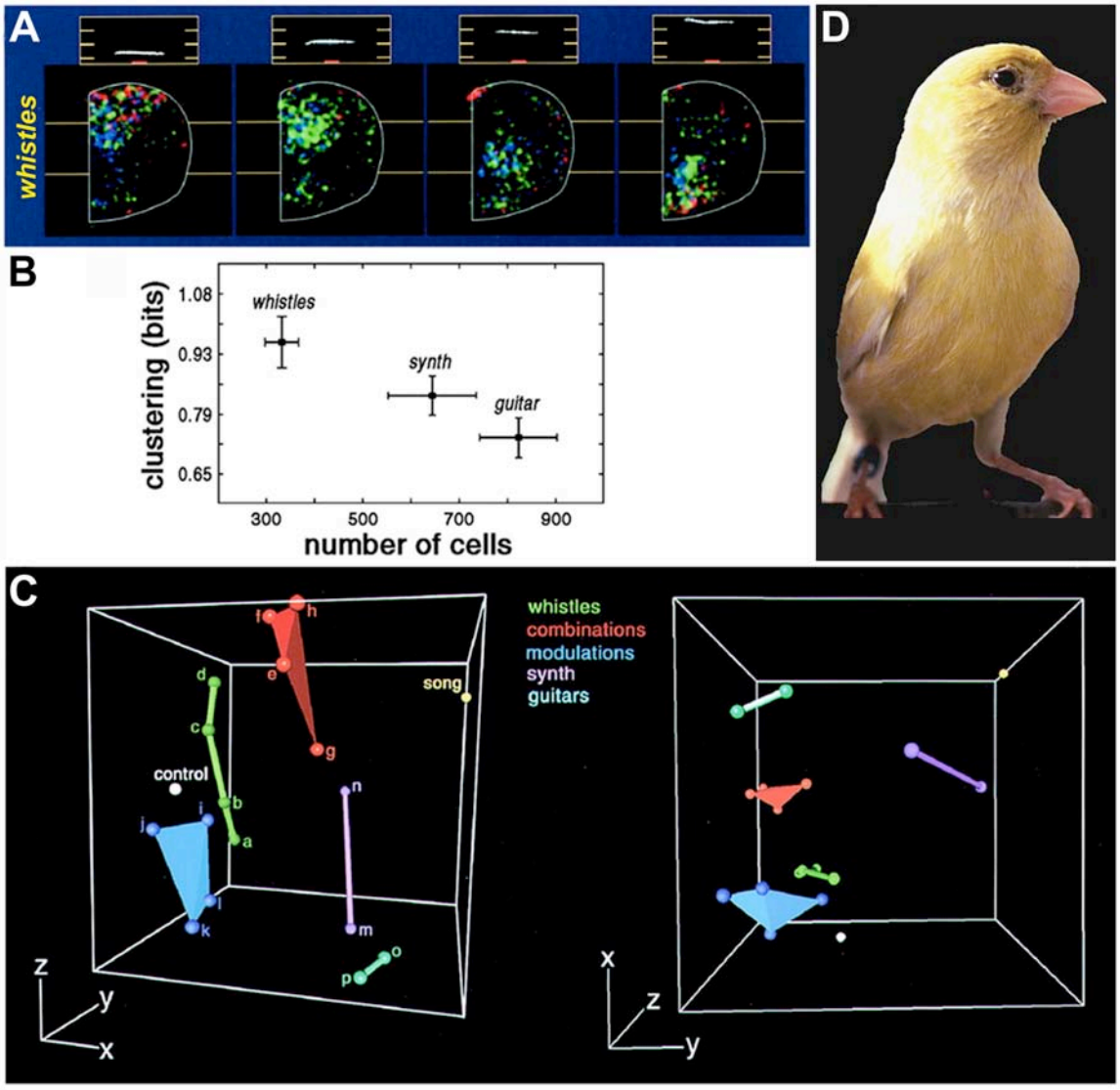

A
whistles
B
clustering (bits)
1.08
0.93
0.79
0.65
whistles
synth
guitar
300
500
700
900
number of cells
D
C
whistles
combinations
modulations
synth
guitars
song
control
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
z
y
x
x
z
y

Fig. 1.4. Transcriptional activation analysis of syllabic representation in canary NCM (modified from (Ribeiro et al., 1998). A) ZENK expression maps in the NCM of adult female canaries resulting from the presentation of natural canary whistles. The sonograms (top) show the respective frequencies (left to right), namely 1.4, 2.2, 2.8 and 3.5 kHz. B) Patterns elicited by natural whistles and artificial stimuli of the same frequencies can be clearly separated by quantifying total cell number and spatial clustering. C) Principal Component Analysis of ZENK expression maps in NCM. The first three components (plotted on the x, y and z axes) provide for a clear separation of the ZENK patterns that accords to the various groups of stimuli in this study, indicating that a syllabic representation of canary song is present in NCM. D) Female canary (*Serinus canaria*).

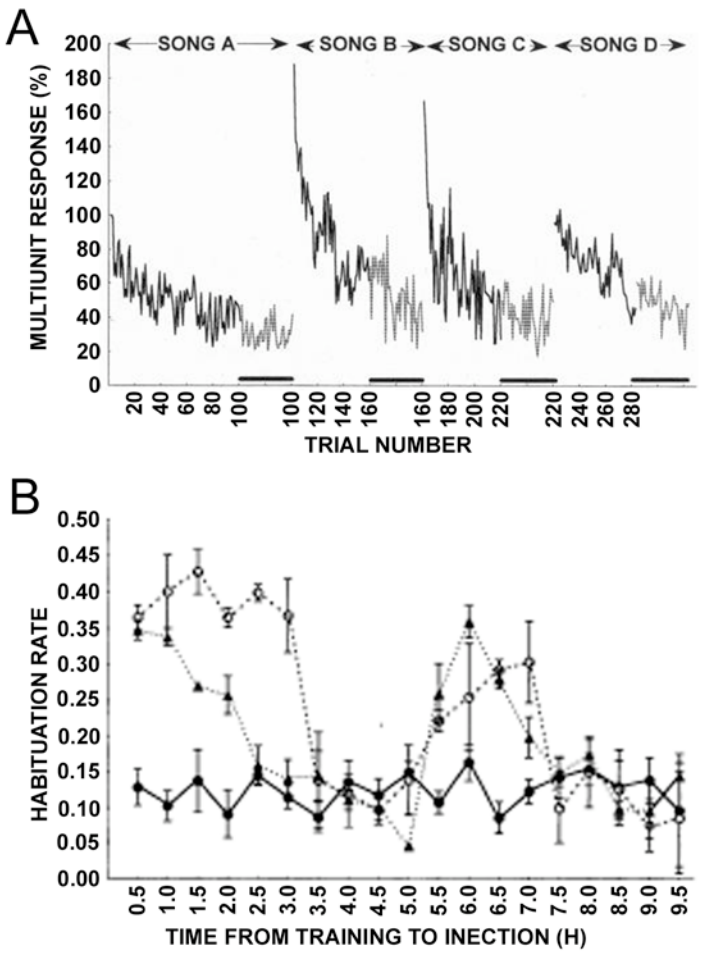

A
SONG A
SONG B
SONG C
SONG D
MULTIUNIT RESPONSE (%)
TRIAL NUMBER
B
HABITUATION RATE
TIME FROM TRAINING TO INECTION (H)

Fig. 1.5**.** Song-evoked electrophysiological responses of NCM neurons in zebra finches (Chew et al., 1995). A) Electrophysiological adaptation induced by repeated presentations of conspecific songs; the response amplitude from multiunit recordings were normalized to the initial response to the first song presented. Dark bars on the x axis indicate the responses during test trials after all four songs were presented, showing that the habituated state is maintained even after training with different songs. The adaptation rate represents the linear regression slope between normalized response magnitudes and iteration number. B) Effect of local injections of synthesis inhibitors on the long-term maintenance of the habituated state in NCM. Plotted are the adaptation rates as a function of the interval from training onset to injection of the protein synthesis inhibitor cyclohexanmide (open circles), of the RNA synthesis inhibitor actinomycin (triangles), and of saline (solid circles).

Chapter 2

The Early Genomic Response to Song:

Co-induction of activity-dependent genes in songbirds

INTRODUCTION

Neuronal activation induces genes encoding effector proteins that directly influence neuronal properties, and transcription factors that regulate downstream targets (Nedivi et al., 1993; Lanahan and Worley, 1998). These activity-dependent genes are thought to participate in a cascade that couples neuronal activation to long-term cellular changes underlying plasticity, learning and memory (Goelet et al., 1986; Clayton, 2000). This hypothesis assumes that genes linked to different aspects of neuronal physiology are co-expressed in activated cells. Although genes encoding effector proteins (*Arc* and *homer1*) are known to be co-expressed in hippocampal cells (Vazdarjanova et al., 2002), the co-localization of inducible transcription factors and effector proteins in response to a specific stimulus has not been directly demonstrated.

When songbirds hear song, a natural learned behavior, the activity-dependent genes *zenk*, c-Fos (protein) and *c-jun* are induced in the caudomedial nidopallium (NCM) and mesopallium (CMM) (Mello et al., 1992; Mello and Clayton, 1994; Nastiuk et al., 1994; Bolhuis et al., 2000; Bailey and Wade, 2003; Sockman et al., 2004), auditory areas considered analogous to portions of the mammalian auditory cortex (Vates et al., 1996). In contrast, during singing, *zenk*

and *c-fos* are induced in the song system (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997), a set of nuclei essential for song production and learning (Brenowitz et al., 1997a; Zeigler and Marler, 2004). *zenk* encodes a zinc-finger transcription factor that regulates targets such as synapsins and neurofilament (Pospelov et al., 1994; Thiel et al., 1994; Petersohn et al., 1995) and that has been linked to synaptic plasticity in rodents (Jones et al., 2001; Lee et al., 2004). Other known song-inducible genes (c-Fos, *c-jun*) also encode transcription factors, but there is currently no evidence that effector genes are regulated by song. Thus, the potential impact of song-induced gene expression on song-responsive circuits remains unclear.

The activity-regulated cytoskeletal-associated (*Arc*) gene encodes an early effector rapidly induced by seizures, hippocampal long-term potentiation (LTP) induction, enriched environment exposure, spatial navigation, and learning (Link et al., 1995; Lyford et al., 1995; Guzowski et al., 1999; Guzowski et al., 2001; Montag-Sallaz and Montag, 2003). *Arc* is required for hippocampal synaptic plasticity and long-term memory in hippocampal-dependent learned tasks (Guzowski et al., 2000). Both *Arc* mRNA and protein localize to recently activated sites along dendrites (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). The protein interacts with cytoskeletal and signal transduction elements associated with the postsynaptic density, indicating a specific action at synapses (Husi et al., 2000; Steward and Worley, 2001a; Guzowski, 2002).

Here, we show that *1) Arc* is induced by song stimulation and singing with distribution and stimulus specificity properties that match those of *zenk*; *2)* song-

induced *Arc* expression co-localizes cellularly with *zenk* and *c-fos*; and 3) induction of the three genes above depends on activation of MAP kinase signaling. These findings demonstrate *Arc* induction by a natural learned behavior. They are consistent with the hypothesis that gene induction by song is a well-orchestrated response that may contribute to synaptic plasticity and memory in the songbird brain.

METHODS

Arc cloning*:* PCR primers (forward: 5'tggaagaagtccatcaaggc3'; reverse: 5'ttgcgccagaggaactggtc3') were designed based on conserved domains within *Arc* mRNA sequences from several species available in GenBank. Standard PCR reactions were performed with an annealing temperature of 56.8°C. The amplified product was excised from an agarose gel, eluted using Qiagen Gel Extraction Kit® (Qiagen Inc, Valencia, CA, USA), inserted into pPCRScript (Stratagene Inc., La Jolla, CA, USA) and used to transform bacterial cells following standard procedures. Insert identity was confirmed by sequencing and analysis using DNAStar software (DNAStar Inc., Madison, WI, USA).

c-fos isolation*:* cDNA clones representing the zebra finch *c-fos* homologue (SB02023A2C08 and SB03001B2H03) were obtained from a collection of annotated cDNAs derived from a normalized zebra finch brain cDNA library (ESTIMA; for details on this collaborative consortium headed by David Clayton at UIUC, see http://titan.biotec.uiuc.edu/songbird). Clone SB02023A2C08 contained the 5' untranslated region (UTR) and part of the coding sequence (nucleotides –149 to 293), while SB03001B2H03 contained 445 bp of the coding sequence and the full 3' UTR (spanning a total of 1145 bp) (Fig 2.7A). At the nucleotide level, SB03001B2H03 showed 87% and SB02023A2C08 showed 80% identity with the

chicken homologue (GenBank Accession number NM 205508); at the level of the predicted aminoacid residues, the identities with the chicken homologue were 96% and 88%, respectively. *In situ* hybridization showed identical results for both clones; the data presented here are from the larger clone (SB03001B2H03).

Probe labeling*:* Riboprobes were used for *in situ* hybridization and Northern blot analyses. Plasmid DNA was isolated using Qiagen Miniprep Kit (Qiagen Inc. Valencia, CA, USA), linearized with the appropriate restriction enzymes, and purified with Qiagen PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Sense and antisense ^{33}P-labeled riboprobes for all genes analyzed in this study were synthesized as described elsewhere (Mello et al., 1997). The *zenk* and *hat2* probes were derived from the cloned canary homologues described previously (George and Clayton, 1992; Mello et al., 1992). For digoxigenin- (DIG-) and biotin-labeled riboprobes, we added 1.0 µl of DIG or biotin labeling mixes (Roche Diagnostics Corp., Mannheim, Germany) to a 10 µl reaction containing 1 µg of linear plasmid DNA, 10 µg of BSA, 10 mM of DTT, 20 units of RNAse inhibitor, 8-10 units of RNA polymerase, and 2.0 µl of a transcription buffer consisting of 50 mM DTT, 250 mM Tris-HCl pH 7.4, 30 mM MgCl2, 50 mM NaCl, and 10 mM spermidine in DEPC-treated water; otherwise, we followed (Mello et al., 1997). All riboprobes were purified in Sephadex G-50 columns and analyzed using a liquid scintillation counter or by visual inspection of a denaturing formaldehyde-agarose gel.

Northern blot analysis*:* Total RNA from zebra finch adult brain was extracted using the Trizol method (Life Technologies Inc., Rockville, MD, USA) and

quantified by spectrophotometry. Ten μg of total RNA were separated by electrophoresis in 1% MOPS/formaldehyde agarose gel, and transferred with 10X SCC to nylon membranes (Hybond-N^+ Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) as described in detail elsewhere (Mello et al., 1997). The membranes were incubated overnight in hybridization solution containing the ^{33}P-labeled antisense riboprobe (10^6 cpm/ml), followed by washes at 65°C and exposure to a KODAK storage phosphorscreen (Molecular Dynamics Inc., Sunnyvale, CA, USA), which was read by a phosphorimager (Typhoon 8600, Molecular Dynamics Inc., Sunnyvale, CA, USA).

Song stimulation and singing behavior*:* Male and female adult zebra finches were from our own breeding colony or obtained from local breeders. Birds were isolated overnight (16-20 hr) in sound-attenuated chambers (≈76 X 31 X 28 cm) under a 12:12 light:dark cycle (same as the aviary; lights on at 7:00 AM). On the following day, birds were exposed to a playback of a recorded conspecific song stimulus consisting of a medley of three representative non-familiar songs presented in blocks of 15 seconds followed by a 45-second silent interval. To determine the kinetics of *Arc* induction, birds were exposed to this stimulation for 30 min and killed after varying survival intervals. To determine the effect of stimulation time on gene expression, birds were exposed to the stimulation above for varying periods, followed by immediate sacrifice. To determine the stimulus specificity of the *Arc* induction response, additional birds were exposed for 30 min to heterospecific song, white noise or synthetic tones. The heterospecific stimulus consisted of a medley of three canary songs arranged to

match the duration of the conspecific stimulus. The non-song stimuli consisted of 15 seconds of white noise or of a combination of tones matching the frequency range of the conspecific songs followed by a 45-second silent interval. All stimuli were presented at comparable intensities (70dB mean SPL at 35 cm from the speaker). Females were used in all quantitative experiments above, to avoid the confound of singing behavior; controls consisted of birds that were not stimulated. In the case of males, we examined the effect of song auditory stimulation and of singing behavior on auditory areas and song control nuclei in three groups where the birds were killed immediately after reaching the following criteria: 1) Hearing and Singing: consisted of birds that were either killed after singing spontaneously at least 10 song bouts (10 to 78 bouts range) during a 30 min period after lights were turned on in the morning, or that were induced to sing for 30 min upon presentation of a female after a 2 hr quiet period; thus, this group contained birds that sang both directed and undirected song; 2) Hearing Only: consisted of birds that were stimulated for 30 min with conspecific song playback (as above for females) after being quiet for a period of at least 2hr, and that did not sing in response to the playback; and 3) Unstimulated Quiet Controls: consisted of birds that were monitored and found not to sing for a period of at least 2 hr. For subcellular localization analysis, an additional subset of females (n=6 birds) received a short stimulation (5 min) of a single song and were sacrificed immediately or 30 min after stimulus onset. All procedures involving birds were approved by OHSU's Institutional Animal Care Use Committee (IACUC) and are in accordance with NIH guidelines.

Surgery and microinjections*:* To block activation of the MAP kinase signaling pathway, we performed intracerebral injections of UO126 (Cell Signaling Technology Inc., Beverly, MA, USA), a specific inhibitor of the mitogen-activated extracellular-signal regulated protein kinase kinase 1 and 2 (MEK1/2). We used a protocol for injections in awake restrained birds, as first described in Chew *et al* (1995), with some modifications. Briefly, adult female zebra finches (n=7) were first anesthetized with 50 µg/g of sodium pentobarbital, and placed in a stereotaxic apparatus. A metal fixation pin was affixed to the skull using Tylok-Plus dental cement (Dentsply International Inc., Milford, DE, USA), and a window was partially open by removal of the superficial layer of bone over NCM. Birds were allowed to recover for 24 hr in sound isolation boxes. The following day, the birds underwent 4 acclimation sessions (one every 2 hr), each consisting of gently restraining a bird, fixing it through the attached pin to an adapter in the stereotaxic apparatus (bird skull pin slide adapter; MyNeuroLab, Saint Louis, MO, USA), maintaining it in the apparatus for 10 min (in the presence of a researcher), and returning it to the isolation box. These sessions were performed to minimize procedural induction of activity-dependent genes in the brain. The following morning one additional acclimation session was performed and the birds were returned to the isolation boxes. After a 3-4 hr interval, the birds were again fixed to the stereotaxic apparatus through the metal pin. The remaining layer of bone was removed, and UO126 (10µg/µl in 50% DMSO in saline) was injected (50 nl/site over 2 min) into NCM (AP: 0.5 mm; ML: 0.65 mm; DV: 1.2 and 1.6 mm) of one hemisphere and vehicle in the contralateral hemisphere, using a

microinjector (Narishige International Inc., Long Island, NY, USA). To facilitate the identification of the injection site, the pipettes were coated with fluorescent latex beads (Lumafluor Corp., Naples, FL, USA.,). Birds were then released from the apparatus and returned to the isolation boxes; one hour later they were stimulated with conspecific song for 30 min followed by immediate sacrifice. The injection procedures were performed in a sound-attenuated room with minimal sound exposure.

Tissue preparation: All birds were sacrificed by decapitation. Their brains were quickly dissected from the skull, frozen in Tissue-tek (Sakura Finetek Inc., Torrance, CA, U.S.A) in a dry ice/isopropanol bath, and stored at -80°C. Parasagittal 10 μm brain sections were cut on a cryostat, thaw-mounted onto slides, and stored at -80°C until use.

Radioactive in situ hybridization (ISH)*:* ISH was performed using ^{33}P-labelled sense and antisense riboprobes followed by exposure to a KODAK phosphorscreen or emulsion autoradiography and Nissl counterstaining. The hybridizations and washes for all probes analyzed were performed at 65^{o}C, using essentially the same procedure as previously described (Mello et al., 1997).

Densitometry: Phosphorimager images were obtained with a Typhoon 8600 (Molecular Dynamics Inc., Sunnyvale, CA, USA) and quantified using NIH's Image software. Densitometric measurements taken over specific brain regions were subtracted from the slide background and resulting values averaged for two adjacent sections. For normalization, all densitometric values were divided by the

average values from unstimulated controls. For the MEK1/2 inhibitor experiments, densitometric values obtained in vehicle- and UO126-injected areas of song-stimulated birds were normalized by the respective values from vehicle- and UO126-injected areas in non-stimulated control birds. To examine the effect of post-stimulation survival time, stimulus duration and drug treatment, we used ANOVA followed by Fisher's PSDL *posthoc* tests for pairwise comparisons, and a probability level of <0.05 for significance.

Fluorescence ISH (FISH): FISH was performed essentially as above for ISH, except that biotin- or DIG-labeled riboprobes were used, followed by probe detection according to manufacturer's instructions (PerkinElmer Life and Analytical Sciences Inc., Boston, MA, USA), with some modifications. Briefly, after hybridization and washing steps identical to ISH, sections were initially incubated for 10 min in 0.3% H_2O_2 in TNT buffer (0.1 M Tris-HCl pH7.5, 0.15 M NaCl, and 0.3% Triton X-100) to inactivate endogenous peroxidase, rinsed 3X for 5 min in TNT buffer, and incubated for 30 min in blocking buffer (TNT containing 8.3 mg/ml of BSA). The sections were then incubated for 2 hr at room temperature (or overnight at 4^oC) with HRP-conjugated anti-DIG (1:200 dil; Roche Diagnostics Corp., Mannheim, Germany) or anti-biotin (1:500 dil; Vector Laboratories, Burlingame, CA, USA) in blocking buffer, washed 3X for 5 min in TNT buffer, followed by a 1-2 hr incubation at room temperature with tyramide coupled with Alexa Fluor 594 or Alexa Fluor 488 (TSA™ Molecular Probes, Eugene, OR, USA). Sections were then rinsed 3X for 5 min in TNT buffer, counterstained with Hoechst, and coverslipped with ProLong™ Antifade kit

(Molecular Probes, Eugene, OR, USA). Control reactions in which the probe or antibody were omitted were included in every experiment.

Double FISH (dFISH): For double-labeling procedures, sections were hybridized simultaneously with the two probes (biotin- and DIG-labeled) as described above for FISH and probe detection was performed sequentially after end of washes. After the detection of the first probe was completed, the sections were incubated for 45 min in 0.45% H_2O_2 in TNT buffer for peroxidase inactivation of the first antibody, followed by detection of the second probe. Controls for the effectiveness of this inactivation consisted of sections where the antibody for the second probe was omitted. Such controls resulted in no detectable signal for the second probe under the conditions utilized.

Microscopy and cell mapping*:* In brain sections processed for *Arc* FISH labeled cells were examined and mapped using a Nikon Eclipse E-600 microscope equipped with a motorized stage drive and Lucivid, and coupled to a PC running Neurolucida Software (MicrobrightField Inc., Colchester, VT, USA). Cell counts were performed in 250 μm^2 windows placed over NCM, subfields L1 and L3, the nidopallial shelf underneath HVC, the arcopallial cup area adjacent to RA, and song nuclei HVC and RA (robust nucleus of the arcopallium; see (Reiner et al., 2004) for nomenclature). Song nucleus NIf was not quantified because it could not be reliably identified in all birds. For co-localization analysis, dFISH-processed slides were mapped under a 40X objective. Four 250 μm^2 fields placed over rostral, caudal, dorsal and ventral NCM domains were counted separately and subsequently added (for a total of 1 mm^2) for each individual bird

(n=3-4 song-stimulated birds). Individual cells were identified based on nuclear (Hoechst) staining and then examined for signal under the two appropriate fluorescence channels. The cells were then assigned to single- or double-labeled categories according to their dFISH signal. The results are expressed as the percentage of double-labeled cells from the total number of labeled cells counted. The resulting maps were compared using NeuroExplorer Software (MicrobrightField Inc., Colchester, VT, USA), and the resulting counts were analyzed using descriptive statistics. Photomicrographs were acquired using a digital camera (DVC, Austin, TX, USA) and Adobe Photoshop was used to assemble photomicrographic plates. For analysis of subcellular localization of expression, sections were analyzed using both the fluorescence microscope and a laser-scanning (confocal) microscope (LSM 510 Meta, Carl Zeiss USA Inc., Thornwood, NY, USA). The latter was used for a precise definition of the subcellular distribution of the probes, avoiding ambiguities intrinsic to fluorescence microscopy.

RESULTS

Arc cloning

To obtain the zebra finch homologue of *Arc*, we identified conserved domains within homologous sequences of chicken, rat, and human and designed PCR primers to amplify a 650 bp fragment within the coding region of *Arc*. The predicted aminoacid sequence of the amplified fragment spanned residues 85 to 306 of the chicken sequence (GenBank Accession number AJ272062). The zebra finch *Arc* sequence had 97%, 72%, and 71% identity at the aminoacid level (Fig 2.1A), and 91%, 65%, and 63% identity at the nucleotide level with the chicken, human and rat sequences respectively. Northern blot analysis of zebra finch total telencephalic RNA revealed a single *Arc* band of about 5.1 kb (Fig 2.1B). The larger size of this transcript when compared to the brain transcript size of *Arc* in rodents (3.1 kb) suggests a significant species difference, most likely in the 5' and 3' UTR sequences; splice variants of *Arc* have not been described.

Arc is induced by song in auditory processing areas

To determine the expression of *Arc* following song stimulation, we first performed *in situ* hybridization of medial parasagittal brain sections of adult zebra

finches with a radioactively-labeled antisense riboprobe, followed by phosphorimager and emulsion autoradiography. Basal *Arc* expression was low throughout the telencephalon at this level, as evidenced by its distribution in unstimulated quiet controls (Fig 2.2A and B, Control). In contrast, birds stimulated with conspecific song showed high *Arc* expression in telencephalic auditory areas, namely the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM) (Fig 2.2A and B, Stimulated). *Arc* expression in stimulated birds was low in field L2a, indicating that *Arc* is not induced in this brain area (Fig 2.2B). Hybridization with sense strand probes did not yield any hybridization signal, demonstrating probe specificity (Fig 2.2A, insets). Song-induced *Arc* expression in the caudomedial telencephalon had a similar pattern (i.e. the same anatomical distribution pattern for *Arc* mRNA) in both males and females.

Densitometric analysis in females revealed that song-induced *Arc* expression in NCM reached a peak 30 min after stimulus onset and declined with further survival (Fig 2.3A, solid triangles). However, *Arc* mRNA levels remained above control levels for several hours after stimulation (Fig 2.3A). The effect of time was statistically significant by ANOVA (F (1.578)= 10.517, p <.0001, main effect of time). *Posthoc* analysis revealed differences in mean expression levels between controls (n=6 birds) and stimulated birds at all post-stimulation survival times tested (Fischer's PSLD, p <.0001, p <.0001, p=.0115, and p=.0004 for 0.5, 2, 4, and 6 hr respectively) except for 8 hr (Fischer's PSLD, p=.07, n= 4). The induction kinetics of *Arc* was similar to that of *zenk*, assessed in adjacent sections from the same birds (Fig 2.3A, solid squares). However, consistent with

previous observations (Mello and Clayton, 1994) *zenk* expression returned to unstimulated control levels by 2 hr after stimulus onset (Fischer's PSDL, $p \geq .44$ for 2 hr and all later time points, n= 3-5 birds/group). As a control for overall individual variability in mRNA levels, sections from all birds were also hybridized with a probe for *hat2* (the canary homologue of *n-chimaerin*) a gene that is enriched in the songbird forebrain yet not regulated by song (George and Clayton, 1992; Jarvis et al., 1995). The expression levels of *hat2* were unaffected by song stimulation (Fig 2.3A, solid circles; F(0.013)= 1.403, p= 0.257)**.** Significant song-induced expression of *Arc* was also observed in the NCM of males stimulated with conspecific song and sacrificed 30 min after stimulus onset, as compared to unstimulated controls (fold-induction= 1.6±0.1; F(0.564)=36.192, p=.003, n=3 birds/group).

We next tested the effect of duration of stimulation on *Arc* expression. For this purpose, we used a continuous stimulation protocol known to lead to habituation of song-induced *zenk* expression (Mello and Clayton, 1995; Ribeiro et al., 1998). We observed that *Arc* expression levels in birds stimulated for periods longer than 30 min do not show a further increase compared to 30 min, but rather decline to levels comparable to those seen in birds that had long survival times after stimulus onset (Fig 2.3B, empty triangles). The effect of time was significant by ANOVA (F (1.504)= 13.860, $p < .0001$), as were *posthoc* comparisons between control and several stimulation durations (Fisher's PLSD, $p <.0001$, p .038, and p=.0294 for 0.5, 4 and 6 hr, respectively; n=4-6 birds per group). When the two stimulation protocols above (survival after stimulus onset

vs. stimulation duration) were compared by ANOVA, no effect of the stimulation protocol was observed. This indicates that further stimulation beyond 30 min has little or no effect on *Arc* mRNA levels, and is consistent with a habituation of the *Arc* response to song. In contrast, *zenk* mRNA expression levels were higher than controls at 30 min, but were not significantly different from controls in any of the groups stimulated for longer periods (Fig 2.3B, open squares; F (.447)= 9.6, p=.0004, effect of time; Fisher's PLSD, p=.0001, p=.50, p=.57, p=.44 and p=.66 for 0.5, 2, 4, 6 and 8 hr respectively), whereas *hat2* expression did not change as a function of stimulation (Fig 2.3B, empty circles; F (.003)= .255, p= .93). *Arc* induction in CMM had similar induction kinetics as in NCM (Fig 2.3C). The effects of post-stimulation survival and duration of stimulation in this area were significant by ANOVA (F (0.562)=12.049, p<.0001 for varying survival times, and F (0.561)= 20.664, p<.0001 for varying stimulation durations), as were the *posthoc* comparisons between control and all time points in both curves (Fisher's PLSD, p<.05 for all points in both conditions).

To determine the extent to which *Arc* induction in NCM is specific to conspecific sounds, we compared its expression in adult females stimulated with other classes of stimuli, namely heterospecific (canary) song and non-song sounds (pure tones and white noise). We found that *Arc* mRNA expression is highest for conspecific stimulus, intermediate for heterospecific song, and very low for white noise or artificial tones (Fig 2.3D). Densitometric analysis of *Arc* mRNA in NCM showed that the effect of stimulus class was statistically significant by ANOVA (F (1.324)=83.163, p<.0001, main effect of stimulus).

Posthoc analysis revealed that both classes of song, conspecific and heterospecific, induced higher levels of *Arc* when compared to silent controls and non-song stimuli (Fischer's PSLD, p <.0001 for all comparisons), demonstrating specificity of the response, whereas comparisons between controls and non-song stimuli yielded no significant differences (Fischer's PSLD, p=.0679 and p=.073 for tones and white noise respectively). A similar effect of stimulus class on *Arc* expression was observed in CMM (not shown).

Arc is induced in song control areas during singing

To test whether *Arc* mRNA is expressed in song control nuclei, we examined brain sections containing song nuclei and adjacent areas from singing males (Singing & Hearing) compared with song-stimulated (Hearing Only) and unstimulated quiet control (Control) birds (n=3-4 males per group). An initial analysis using radioactively-labeled probes and phosphorimager autoradiography indicated marked differences in *Arc* expression across groups (not shown). To analyze this in greater detail, we performed FISH for *Arc* and mapped *Arc*-expressing cells in all groups. A comparison between the Control and Hearing Only groups (Fig 2.4B, middle and top panels) revealed that *Arc* is induced by song stimulation in several auditory areas, including field L subfields L1 (2.8±1.2 and 53.5±14.1 cells/mm^2 for Control and Hearing Only groups respectively) and L3 (8.9±7 and 84.4± 8.4 cells/mm^2 for Control and Hearing Only respectively), the nidopallial shelf area adjacent to song nucleus HVC (12.9±3.9 and 77.5±3.2

cells/mm^2 for Control and Hearing Only respectively), and the arcopallial cup area adjacent to song nucleus RA (3.8±1.9 and 43.2±4.2 cells/mm^2 for Control and Hearing Only respectively), as well as the dorsal part of the caudal striatum (not quantified), and NCM and CMM at more medial levels (not shown). No labeled cells were seen in L2a in either group. Comparisons between the Singing & Hearing (Fig 2.4B, bottom) and the other groups (Fig 2.4B, upper and middle panels) revealed that, in addition to the auditory areas mentioned above, *Arc* is induced during singing in song control nuclei HVC (1.3±1.2 and 113±58.6 cells/mm^2 for Control and Hearing & Singing groups respectively), RA (0 and 29.4±9.5 cells/mm^2 for Control and Hearing & Singing groups respectively), and the interface nucleus of the nidopallium (NIf; not quantified). An examination of rostral areas gave indication that *Arc* is also induced by singing in striatal song nucleus area X of the anterior forebrain pathway (not shown). A systematic analysis of this pathway, however, will require future studies that address a possible effect of context on *Arc* expression (Jarvis et al., 1998).

Arc **subcellular localization is time-dependent**

To determine whether the subcellular distribution of *Arc* mRNA varies as a function of time after stimulation, as has been seen in the mammalian hippocampus (Guzowski et al., 1999), we performed FISH for *Arc* in NCM of birds sacrificed at different times after a short (5 min) stimulation. In birds sacrificed immediately after stimulation, the vast majority of labeled cells in NCM and other song-responsive areas had a characteristic punctate pattern of labeling

(Fig 2.5A, top panel). This consisted of a pair of foci of nuclear localization (Fig 2.5A, bottom panels) that likely represent the sites of transcription of the two *Arc* alleles. In contrast, in birds that were sacrificed 25 min after the end of stimulation, labeled cells showed a different pattern consisting of both nuclear and cytoplasmic labeling (Fig 2.5B, bottom panels), the latter likely representing the accumulation of *Arc* mRNA at translation sites in the soma. The staining pattern in birds that received a longer stimulation (30 min) and were killed after an additional 1.5 hr showed a pattern that resembled that seen at 30 min. At close inspection, however, labeled cells typically contained a series of small varicosities aligned like beads on strings emanating from the soma (Fig 2.5C, arrowheads in bottom panels), consistent with a dendritic localization. Such pattern was never observed for *zenk* or *c-fos* (see below), or at earlier time points for *Arc*. The nuclear pattern of expression in birds sacrificed immediately after a short stimulation was also observed for *zenk* (see below).

Cellular co-expression of *Arc*, *zenk* and *c-fos* in NCM and CMM

Since *Arc* and *zenk* are induced by conspecific stimulation with similar kinetics in the same brain areas, we asked whether they are expressed by the same neurons. Analysis of sections hybridized simultaneously with probes for both mRNAs (dFISH protocol) showed a high degree of cellular co-localization of *Arc* and *zenk* signals in NCM (Fig 2.6, large panels). A close examination revealed that the mRNA signals for *Arc* and *zenk* localized to the same cells. These double-labeled cells typically display two distinct pairs of foci located within the nucleus (Fig 2.6, large panels, insets) that represent distinct

transcriptional loci for *Arc* and *zenk*, confirming their cellular co-localization. This staining pattern was confirmed by confocal microscopy (Fig 2.6D, panels on bottom right). A quantitative analysis showed that 99.4% of the neuronal cells in NCM express both *Arc* and *zenk* (a total of 170 cells were analyzed in 3 birds stimulated with conspecific song for 30 min).

We next tested whether *Arc* expression also co-localizes with *c-fos.* A molecular probe for *c-fos* had not been previously characterized in songbirds. Therefore, we first determined that an antisense riboprobe derived from the zebra finch *c-fos* homologue (cDNA clone SB03001B2H03 from the ESTIMA collection) (Fig 2.7A) hybridizes with a single transcript of about 2.5 Kb on a Northern blot of zebra finch total telencephalic RNA (not shown). *In situ* hybridization with this riboprobe revealed that the mRNA levels of *c-fos* in unstimulated control birds were low throughout the medial telencephalon (Fig 2.7B and C, Control). In song-stimulated birds, however, *c-fos* was markedly induced in both NCM and CMM, but not in L2a (Fig 2.7B and C, Stimulated). The sense strand riboprobe for *c-fos* did not yield any detectable hybridization (Fig 2.7B, insets). The kinetics of *c-fos* induction in NCM was similar to that of *zenk*, with *c-fos* mRNA levels reaching a two-fold induction by 30 min and returning to control levels by 2 hr after stimulus onset (not shown). ANOVA revealed a significant effect of time ($F(0.584) = 12.64$, $p = .0033$) and *posthoc* comparisons showed a significant difference between unstimulated controls and birds that were stimulated for 30 min followed by immediate sacrifice (Fisher's PLSD $p = 0.0016$). dFISH for *Arc* and *c-fos* mRNAs revealed a high degree of co-

localization in NCM (Fig 2.7D, large panels) and CMM (not shown) of song-stimulated birds sacrificed 30 min after stimulus onset. A closer examination under both conventional fluorescence (Fig 2.7D, large panels, insets) and confocal microscopy (Fig 2.7D, panels on bottom right) revealed that, like *zenk*, *c-fos* mRNA is transcribed in the same cells as *Arc*, as demonstrated by the presence of transcriptional foci for *Arc* within the same cells showing cytoplasmic labeling for *c-fos*. A quantitative analysis showed that 92±2% (means±S.E.M.) of the neuronal cells in NCM express both *Arc* and *c-fos* mRNAs (a total of 363 cells were analyzed in 4 birds stimulated with conspecific song for 30 min).

Song induction of *Arc*, *zenk*, and *c-fos* requires activation of the MAP kinase signaling pathway

Since *Arc*, *zenk*, and *c-fos* are induced by song in the same auditory neurons, we asked whether they are co-activated by a common intracellular signaling pathway. For this purpose we utilized UO126, an inhibitor of the mitogen activated and extracellular regulated protein kinase kinase (MEK1/2) known to block song-induced *zenk* expression in NCM (Cheng & Clayton, 2004). We observed that the injection of UO126 into the NCM of song-stimulated birds resulted in a marked local blockade of the induction of *zenk*, *Arc* and *c-fos*, whereas the induction of these genes was unaffected in the contralateral hemispheres injected with vehicle alone (Fig 2.8B). The blocking effect of UO126 was localized to an area surrounding the center of the injection site (indicated dashed circle in Fig 2.8A and B). Quantitative densitometry followed by ANOVA

revealed a significant effect of treatment on gene expression in NCM ($F(6.029)= 198.349$, $p=.0001$; $F(1.102)= 13.658$, $p=.0209$; and $F(1.18)= 19.381$, $p=.0117$ for *zenk*, *Arc* and *c-fos* respectively; Fig 2.8C). No significant effect was observed in the neighboring CMM, which was clearly outside of the area targeted by the injection (Fig 2.8B and 8D; no significant effect of treatment by ANOVA; $F(168)=2.914$, $p=.1630$; $F(0.075)= 2.184$, $p=.2136$; and $F(0.59)=.286$, $p=.6214$ for *zenk*, *Arc*, and *c-fos* respectively). Injections of UO126 had no significant effect on basal expression of inducible genes in the NCM of unstimulated controls (not shown). Together, these results clearly indicate that song-induced expression of *Arc*, *zenk* and *c-fos* depends on activation of the MAP kinase pathway.

DISCUSSION

Song is a naturally occurring learned behavior used extensively by songbirds for vocal communication. Processing and memorizing songs is essential for males to learn how to sing and for males and females to perform behaviors related to territoriality and mate selection (Catchpole and Slater, 1995; Kroodsma and Miller, 1996). Our present data show that *Arc*, a gene that acts at recently activated synapses and is involved in long-term synaptic plasticity in mammals, is induced by song stimulation in auditory areas and by singing behavior in song nuclei of zebra finches. This constitutes a first demonstration that a gene encoding an effector protein is induced by song in the songbird brain. We also show that *Arc* expression in song-stimulated birds co-localizes cellularly with the transcriptional regulators *zenk* and *c-fos*, and that the induction of these genes depends on the MAP kinase pathway. These observations provide a first direct demonstration that regulators and effector genes associated with distinct cellular processes are co-induced in the same neuronal cells in a vertebrate brain.

The expression patterns of known song-inducible genes (*Arc*, *zenk*, *c-fos* and *c-jun)* are remarkably similar. They are all induced by song auditory stimulation in areas that are part of the central auditory pathway (field L subfields

L1 and L3, NCM, CMM, and the shelf and cup areas), some of which have been implicated in the perceptual processing of song (Mello et al., 1992; Mello and Clayton, 1994; Nastiuk et al., 1994; Kimpo and Doupe, 1997; Bolhuis et al., 2000; Sockman et al., 2002; Bailey and Wade, 2003; Sockman et al., 2004). Moreover, *Arc* induction in NCM shows the same stimulus specificity properties previously shown for *zenk*, indicating that the *Arc* response is also tuned to particular acoustic features present in conspecific song.

In contrast, song stimulation does not activate gene expression in the thalamic auditory nucleus ovoidalis (Ov) and in the thalamo-recipient subfield L2a, both obligatory stations in the ascending auditory pathway and known to be activated by song stimulation (Karten, 1967; Kelley and Nottebohm, 1979; Muller and Leppelsack, 1985; Vates et al., 1996; Theunissen et al., 2000; Sen et al., 2001). The lack of an associated transcriptional response suggests that inducible gene expression has been dissociated from electrophysiological activation in these areas (Ov has not yet been tested for *c-fos* or *c-jun*). Accordingly, even widespread activation with a strong depolarizing agent (metrazole) does not induce *zenk* or *c-jun* in L2a (Nastiuk et al., 1994; Mello and Clayton, 1995). In song nuclei, robust evoked auditory responses to song are seen under anesthesia and during sleep, but less so during wakefulness (Margoliash, 1986; Doupe and Konishi, 1991; Margoliash and Fortune, 1992; Vicario and Yohay, 1993; Lewicki, 1996; Dave et al., 1998; Schmidt and Konishi, 1998; Cardin and Schmidt, 2003). Song stimulation during wakefulness does not induce *Arc*, *zenk* or *c-fos* in song control nuclei, yet all three genes are induced in these areas by

active singing (Mello and Clayton, 1994; Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997). This suggests that, upon auditory stimulation, the electrophysiological activation of song nuclei is not sufficient to induce *Arc*, *zenk*, or *c-fos*, or that modulatory systems that depend on the bird's state regulate the expression of these genes. The possibility that the areas above express other activity-dependent genes remains, but seems remote. Altogether, the similarities in the expression patterns of song-induced genes indicate that cell-type gene expression specificity (Chaudhuri, 1997) or gene-specific thresholds (Worley et al., 1993), argued as major caveats of the use of inducible gene expression as an activity marker (Chaudhuri, 1997), likely do not apply to song-induced gene expression.

Early immunocytochemical studies provided clear evidence for c-Fos protein induction by singing in song nuclei, but the apparent lack of expression in auditory areas suggested that different regions have different requirements for c-Fos and ZENK protein activation (Kimpo and Doupe, 1997). Later studies, however, showed that c-Fos is induced by song in NCM and CMM, with a significant correlation with the song learning process in NCM (Bolhuis et al., 2000; Bailey and Wade, 2003). Recent data suggest that c-Fos and ZENK immunoreactivity patterns in NCM may be differentially sensitive to different song features in starlings (Sockman et al., 2004). Our current study, the first to examine c-fos with an in situ probe specific to zebra finches, provides clear evidence that c-fos mRNA is induced by song stimulation in a pattern identical to those of zenk and Arc, including the lack of induction in L2a. All previously known

song-induced genes (zenk - a.k.a. zif268, egr1, ngfi-A and krox-24 -, c-fos and c-jun) encode DNA-binding proteins that can regulate downstream targets with the appropriate motif in their promoters (Curran et al., 1984; Sambucetti and Curran, 1986; Milbrandt, 1987; Angel et al., 1988; Christy et al., 1988; Sheng and Greenberg, 1990). In contrast, Arc represents an effector that can directly influence neuronal physiology, yet at an earlier time than the targets of inducible transcriptional factors (Steward and Worley, 2002).

Our present results demonstrate that song auditory stimulation induces Arc in the same NCM and CMM neurons that express zenk and c-fos. We also show that Arc, similarly to zenk and c-fos, behaves as an immediate early gene, as it is rapidly induced by song. Because Arc mRNA induction occurs before a significant accumulation of ZENK and c-Fos proteins (Morgan et al., 1987; Mello and Ribeiro, 1998), it is highly unlikely that Arc regulation by song is mediated by these inducible transcription factors. Furthermore, we show that the induction of these three genes depends on MEK1/2 activity, providing strong support to the hypothesis that song-induced genes are co-regulated in song-responsive cells (Fig 2.9). Song thus appears to trigger a coordinated response that includes early effectors such as Arc, and inducible transcription factors that can act on target genes encoding late effectors (Fig 2.9). Early and late effectors could then act in a concerted fashion to mediate long-term changes in song-responsive neurons and circuits.

Repeated song stimulation leads to a long-lasting habituation of the NCM response to song, whose long-term maintenance depends on local RNA and protein synthesis (Chew et al., 1995). The induced expression of known song-responsive genes occurs within the same time window when gene expression is necessary for long-term habituation in NCM, suggesting an involvement of these genes in this form of neuronal plasticity. At least two song-inducible genes, zenk and Arc, have been linked to long-term synaptic plasticity and memory maintenance in mammals (Guzowski et al., 2000; Jones et al., 2001; Lee et al., 2004). The subcellular localization of Arc in mammals indicates that it acts at recently activated synapses (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998; Steward and Worley, 2001a), through interactions with cytoskeletal (F-actin) and signal transduction (NMDA receptor complex and possibly calcium/calmodulin-dependent kinase II) elements associated with the postsynaptic density (Husi et al., 2000; Steward and Worley, 2001a; Guzowski, 2002). The synaptic action of Arc is thought to be required for the long-term maintenance of the potentiated state of those synapses (Guzowski et al., 2000). The mRNA distribution we observed after song stimulation is consistent with a dendritic localization of Arc in activated NCM neurons. This is of particular interest because the habituation shown by NCM neurons is song-specific, occurring to multiple songs independently and being fully rescued by a novel song (Stripling et al., 1997). This requires that different songs activate individual NCM neurons differentially, for example by activating different synaptic sites. The precedent in rodents suggests that the punctate Arc staining we observed might

correspond to recently activated synaptic sites. Arc and associated molecules could thus serve as molecular tags, allowing for activated vs. non-activated synapses in NCM neurons to be differentiated, and contributing to long-term song-specific habituation at the synaptic level in NCM.

Because Arc, c-fos and zenk promoters share several regulatory elements in mammals (Guzowski, 2002; Davis et al., 2003), it is plausible that converging transduction signals regulate their transcriptional activation by song. In fact, in the rodent hippocampus, NMDA receptor blockers interfere with the expression of Arc, c-fos and zenk (Cole et al., 1989; Worley et al., 1991; Worley et al., 1993; Steward and Worley, 2001b), and Ca^{2+}-regulated kinases such as protein kinase A (Shaywitz and Greenberg, 1999) and MAP kinase (Davis et al., 2000; Waltereit et al., 2001) regulate the expression of these three genes. In zebra finches, MEK1/2 was recently shown to regulate song-induced zenk expression through the activation of the extracellular regulated kinase (ERK), and this pathway has been suggested as an upstream regulator of the habituation of the zenk response to song in NCM (Cheng and Clayton, 2004). Our current data demonstrate that the song induction of Arc and c-fos are also dependent on MEK1/2 activity, indicating that the induction of these three genes depends on a common intracellular signaling pathway (Fig 2.9).

In sum, our data together with previous studies indicate that a well-orchestrated gene regulatory program is initiated by the activation of song-responsive brain circuits. They also provide clear evidence that early effectors

and transcriptional regulators are co-regulated in the same neuronal cells. Further investigations on the mechanisms and function of this program should provide further insights into the significance of song-induced gene expression.

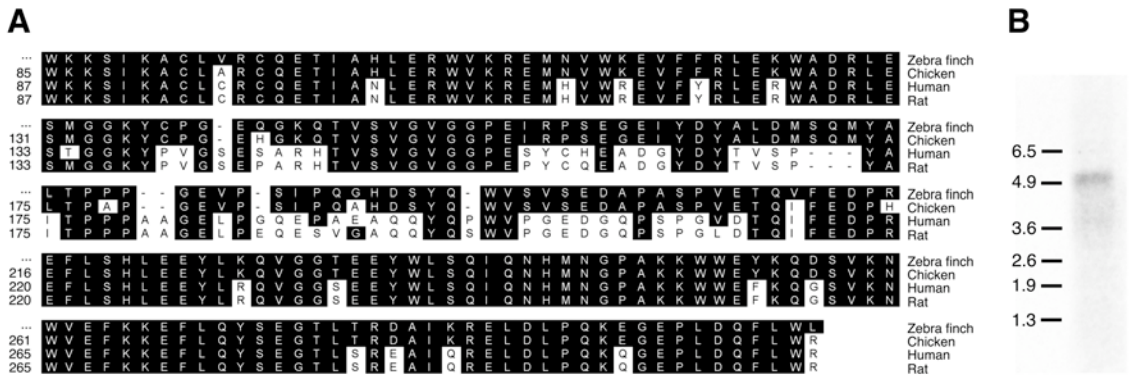


Figure 2.1. A zebra finch *Arc* homologue was obtained by PCR amplification of a brain cDNA library. A) Alignment of the predicted amino acid sequence of zebra finch *Arc* protein with homologues from other species. The numbers on the left indicate the relative position of *Arc* when compared to homologues. B) Northern blot of total zebra finch brain RNA hybridized with ^{33}P-labelled *Arc* antisense riboprobe. The molecular sizes of RNA markers are indicated on the left.

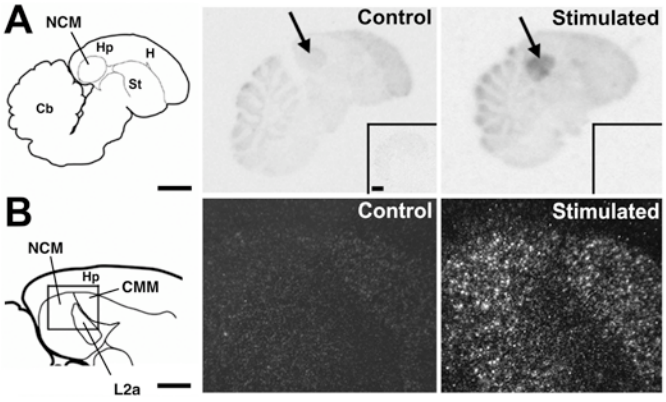


Figure 2.2. *Arc* is induced by song in auditory brain areas of zebra finches. A. Left panel, camera lucida drawing of a parasagittal section at the level of NCM (about 100 µm from the midline). Middle and right panels, phosphorimager autoradiograms of sections at the level shown in A, from control and song-stimulated birds, hybridized with a radioactively-labeled *Arc* riboprobe. Insets: autoradiograms of sections hybridized with sense strand riboprobes. B. Left panel, camera lucida drawing of a parasagittal section at the level of NCM, field L2a and CMM (about 500 µm from the midline). Middle and right panels, dark-field views of emulsion autoradiograms of sections from control and song-stimulated birds hybridized with a radioactively-labeled *Arc* riboprobe; the area shown is indicated by the rectangle in the panel on the left. All images are from females sacrificed 30 min after stimulus onset. Scale bars, 2 mm. Cb, cerebellum; H, hyperpallium; Hp, hippocampus; L2a, subfield L2a; St, striatum.

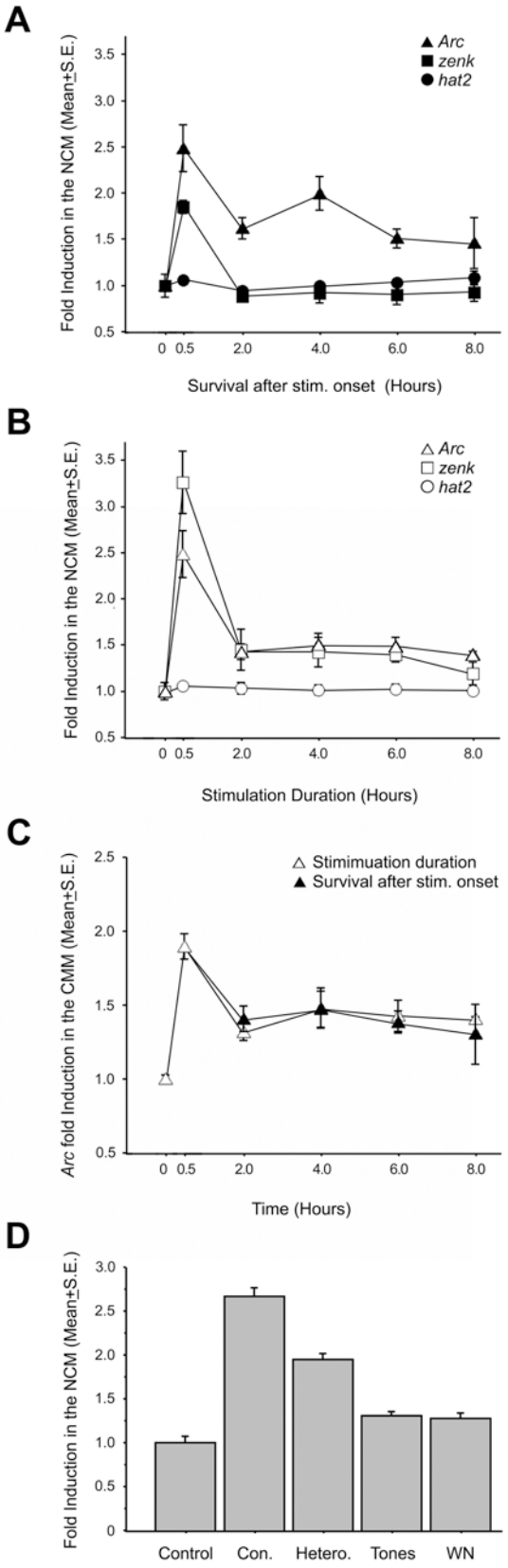

A
Arc
zenk
hat2
Fold Induction in the NCM (Mean±S.E.)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0.5
2.0
4.0
6.0
8.0
Survival after stim. onset (Hours)
B
Arc
zenk
hat2
Fold Induction in the NCM (Mean±S.E.)
Stimulation Duration (Hours)
C
Stimimuation duration
Survival after stim. onset
Arc fold Induction in the CMM (Mean±S.E.)
Time (Hours)
D
Fold Induction in the NCM (Mean±S.E.)
Control
Con.
Hetero.
Tones
WN

Figure 2.3. Quantification of *Arc* mRNA expression. A) Fold-induction in the NCM of birds stimulated for 30 min and sacrificed at various time points after stimulus onset (filled symbols; Survival after stim. onset); B) Fold-induction in the NCM of birds stimulated for various durations and sacrificed immediately thereafter (empty symbols; Stimulation duration); C) Fold–induction in the CMM of the same birds as in A (filled triangles) or as in B (empty triangles); D) *Arc* fold-induction in the NCM of birds stimulated for 30 min with conspecific song (Con.), heterospecific song (Hetero.), artificial tones (Tones), and white noise (WN) compared to unstimulated birds (Control). The values for the 0.5 hr time point are the same for both the A and B curves. For A-C, brains sections were hybridized with radioactively-labeled riboprobes for *Arc* (triangles)*, zenk* (squares), or *hat2* (circles). Autoradiographs were obtained with a phosphorimager and quantified using NIH's Image. Density measurements were normalized against values from the respective areas from unstimulated controls; n=3-6 birds per group. Plotted are means ± S.E.M. from adult females.

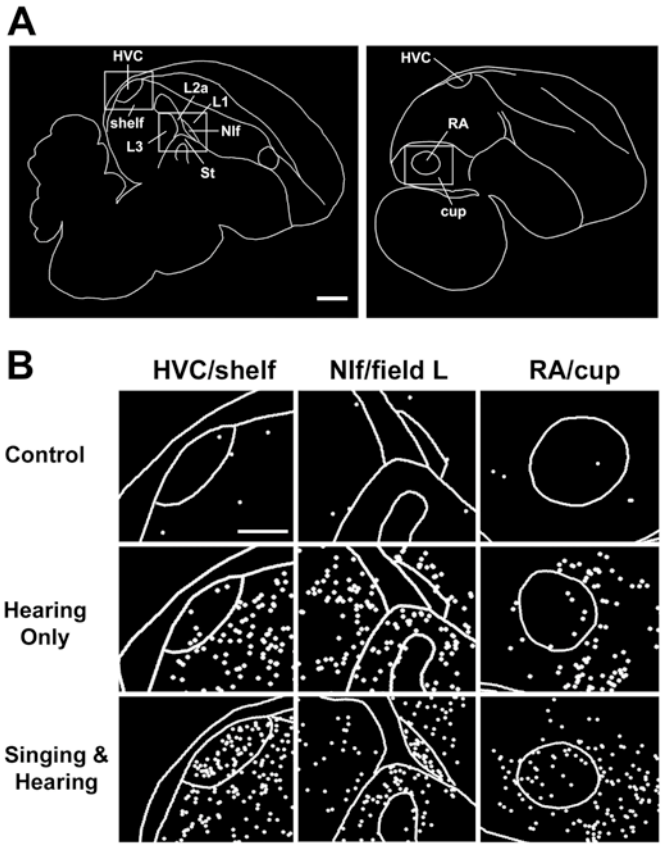


Figure 2.4. *Arc* is expressed in song control areas during singing. A) Camera lucida drawing of parasagittal brain sections from male zebra finches, at about 1.5 mm (left panel) and 2.4 mm (right panel) from the midline. The rectangles indicate the areas depicted in B. B) Camera lucida mapping of *Arc*-expressing cells (white dots) in Control (unstimulated quiet male), Hearing Only (a male that heard conspecific song for 30 min and did not sing back), and Singing & Hearing (a male that sang about 10 song bouts in 30 min upon presentation of a female) adult birds. Abbreviations: CSt, caudal striatum; L1, subfield L1; L3, subfield L3; Scale bars: 1 mm

(A) and 500 μm (B).

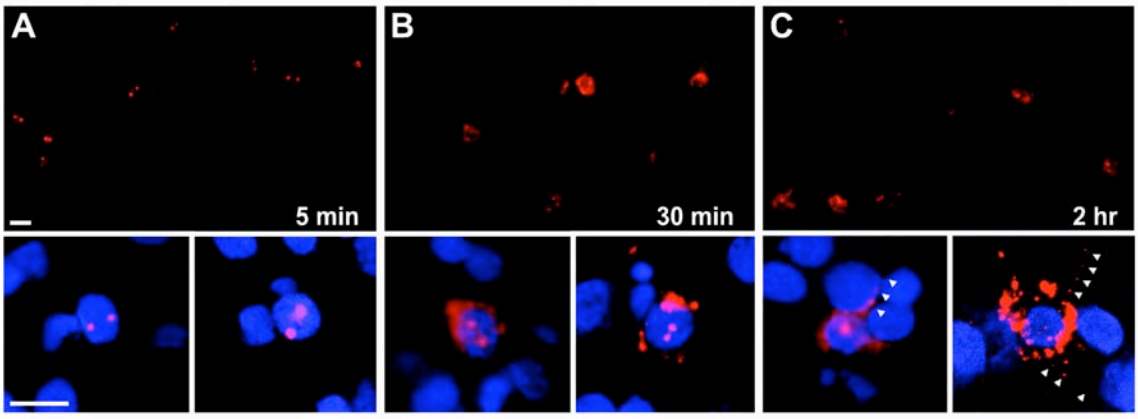


Figure 2.5. Subcellular localization of *Arc* mRNA in NCM auditory neurons is time-dependent. Images of FISH sections show *Arc* mRNA expression patterns in NCM from birds that were stimulated with conspecific song for 5 min and sacrificed immediately (A), 30 min after stimulus onset (B), or 2 hr after stimulus onset, the latter stimulated for 30 min (C). Insets: high power views of the subcellular distribution of *Arc* mRNA in individual cells; shown are representative examples of conventional fluorescence (left) and confocal (right) images for each time point, in sections counterstained with Hoechst (blue). Arrowheads in C (bottom panels) indicate bead-like labeling along neuronal processes. Scale bars: 10 μm.

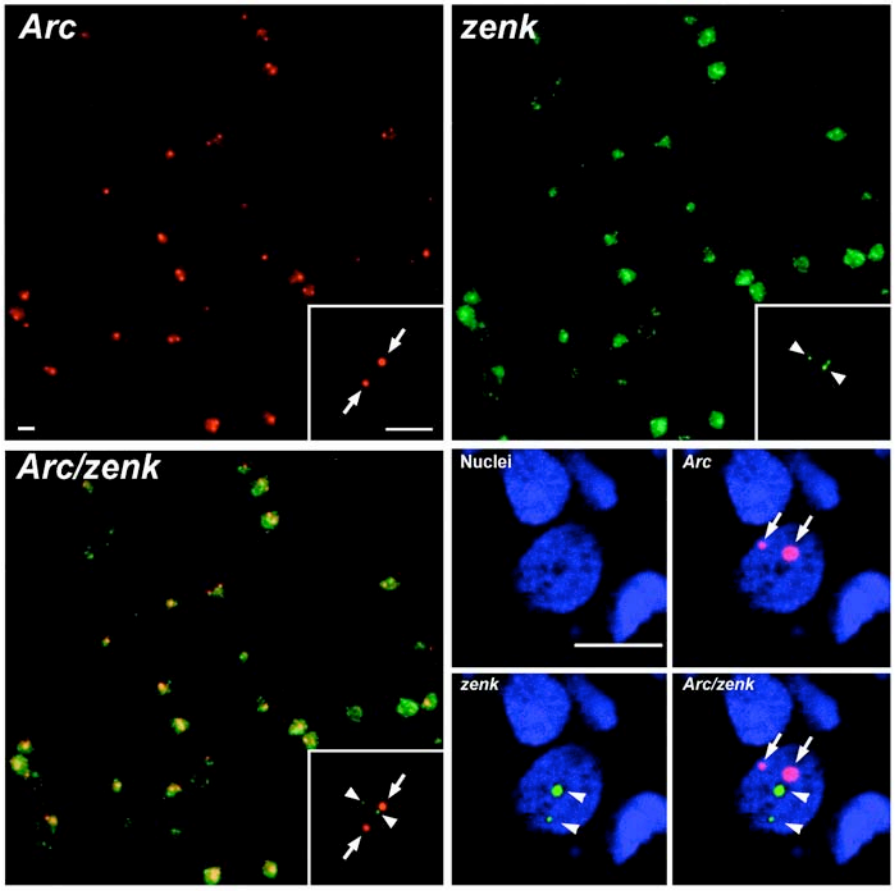

Arc
zenk
Arc/zenk
Nuclei
Arc
zenk
Arc/zenk

Figure 2.6. Co-localization of song-induced *Arc* and *zenk* mRNAs in NCM neurons. Top panels: images from a section subjected to dFISH visualized with the appropriate filters for the identification of *Arc*- (red; left panel) and *zenk*- (green; right panel) expressing neurons. Insets: high power views of a representative field containing nuclear foci for both probes. Bottom left panel: overlay of *Arc* and *zenk* signals demonstrating double-labeled cells (yellow). Inset: overlay of the inset images from the top panels, demonstrating both the proximity and separation of individual *Arc* and *zenk* foci in a double-labeled cell. Bottom right panels: detailed views of another double-labeled cell showing two distinct pairs of labeled nuclear foci representing *Arc* (red, arrows) and *zenk* (green, arrowheads) transcriptional sites within the nucleus, as indicated by Hoechst counterstaining (blue). Images in all insets and bottom right panels are confocal. Scale bars: 10 μm.

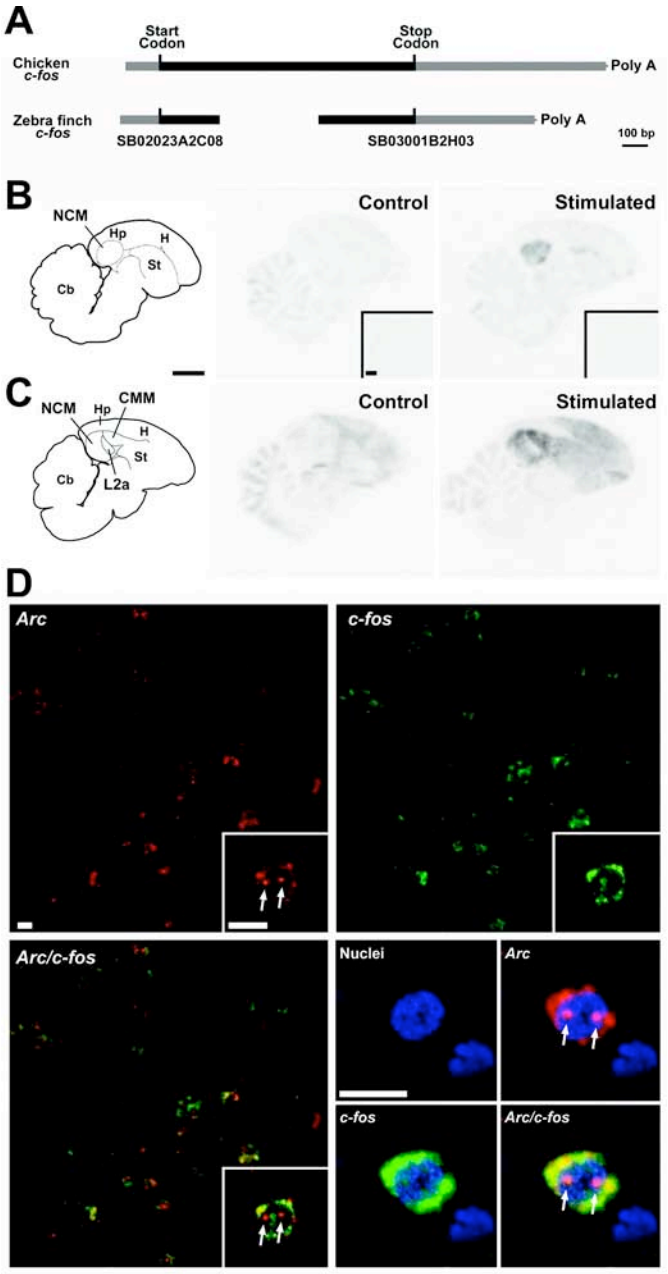

A
Start Codon
Stop Codon
Chicken c-fos
Poly A
Zebra finch c-fos
Poly A
SB02023A2C08
SB03001B2H03
100 bp
B
NCM
Hp
H
St
Cb
Control
Stimulated
C
NCM
Hp
CMM
H
St
Cb
L2a
Control
Stimulated
D
Arc
c-fos
Arc/c-fos
Nuclei
Arc
c-fos
Arc/c-fos

Figure 2.7. *c-fos* is induced by song in auditory areas and co-localizes with *Arc*. A) Schematic representation of two partial *c-fos* cDNAs (SB02023A2C08 and SB03001B2H03) from the zebra finch, and their relative position according to the chicken homologue. The 5' and 3' UTRs are represented in gray, and coding sequences in black. B) Left panel: camera lucida drawing of a parasagittal brain section from an adult female zebra finch at the level of NCM (about 100 µm from the midline). Middle and right panels: phosphorimager autoradiograms of sections at the level shown in A, from control and song-stimulated birds, hybridized with a radioactively-labeled *c-fos* riboprobe. C) Left panel: camera lucida drawing of a parasagittal section containing NCM, field L2 and CMM (about 500 µm from the midline). Middle and right panels: phosphorimager autoradiograms of sections from control and song-stimulated birds hybridized with a radioactively-labeled *c-fos* riboprobe. D) Co-localization of song-induced *Arc* and *c-fos* in NCM neurons. Top panels: identification of *Arc* (red)- and *c-fos* (green)-expressing neurons by dFISH. Insets: high power views of a representative field containing a single neuron labeled for both probes. Bottom left panel: overlaying of *Arc* and *c-fos* fluorescence signals shows a high degree of overlap. Inset: detailed view of a double-labeled neuron showing both *arc* and *c-fos* labeling. Bottom right panels: detailed views of another double-labeled cell showing a distinct pair of nuclear foci representing *Arc* (red, arrows) transcriptional sites within the nucleus and cytoplasmic labeling for both *arc* (red) and c-*fos* (green). Images in all insets and bottom right panels are confocal.

Scale bars: 2 mm (B and C) and 10 µm (D).

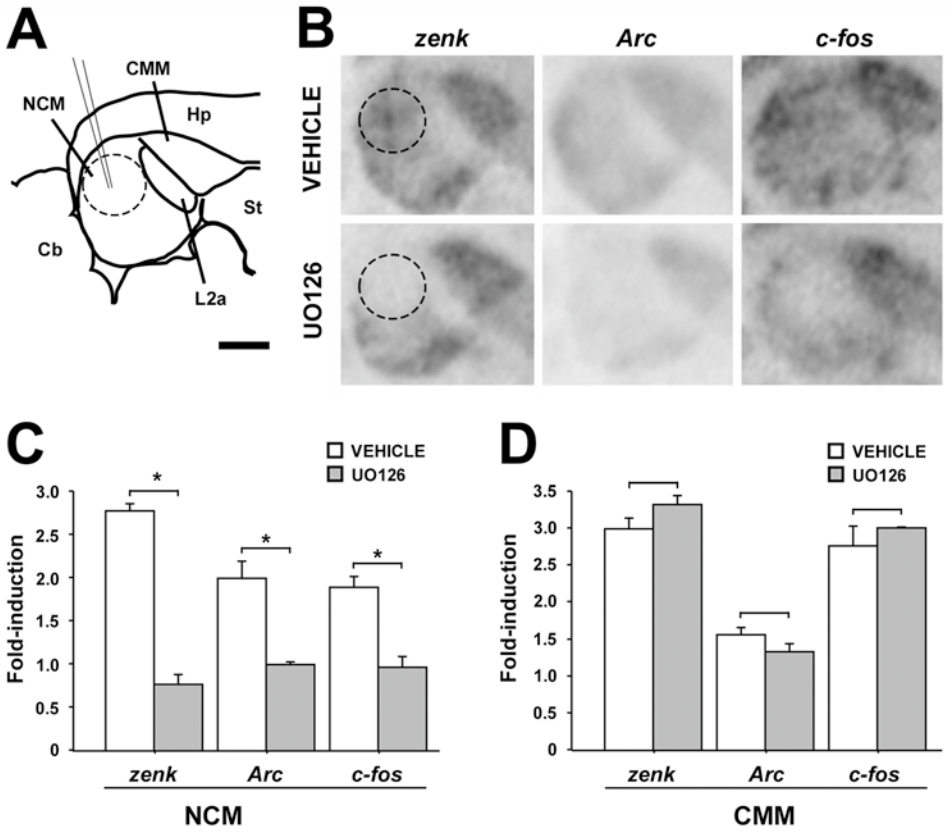

A
CMM
NCM
Hp
St
Cb
L2a
B
zenk
Arc
c-fos
VEHICLE
UO126
C
VEHICLE
UO126
Fold-induction
3.0
2.5
2.0
1.5
1.0
0.5
0
*
*
*
zenk
Arc
c-fos
NCM
D
VEHICLE
UO126
Fold-induction
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
zenk
Arc
c-fos
CMM

Figure 2.8. Song-induced gene expression is dependent on MEK1/2 activity. A) Camera lucida drawing of a parasagittal section containing NCM, field L2a and CMM (about 500 µm from the midline) indicating the site of injections (pipette and dashed circle). Scale bar: 1 mm. B) Phosphorimager autoradiograms of adjacent parasagittal sections from a representative bird that received an injection of UO126 or vehicle (opposite hemispheres) into NCM, hybridized with *zenk*, *Arc*, or *c-fos* riboprobes. C) Fold-induction of *zenk*, *Arc*, and *c-fos* in the NCM of vehicle- (white bars) and UO126-injected (grey bars) birds stimulated for 30 min with conspecific song. D) Fold-induction of *zenk*, *Arc*, and *c-fos* in the CMM of vehicle- (white bars) and UO126-injected (grey bars) birds stimulated for 30 min with conspecific song. All densitometric measurements in song-stimulated birds were normalized against the values from the respective areas from unstimulated controls (n=3-4 adult females per group). Plotted are means +/- S.E.M.

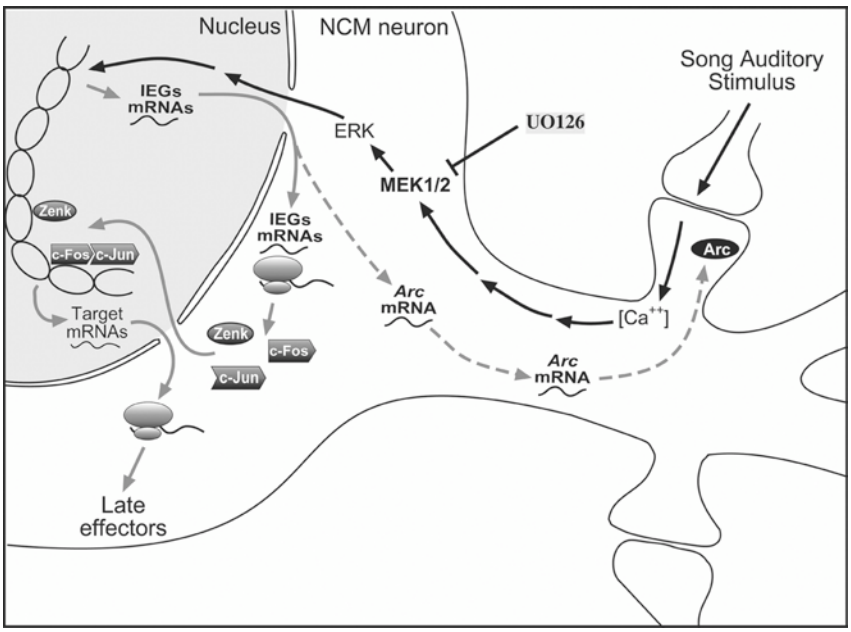


Figure 2.9. A song-induced gene expression program in NCM neurons. The activation of song-responsive auditory neurons in NCM activates intracellular signaling events (black arrows), including components of the MAP kinase pathway (MEK1/2 and ERK), that lead to the rapid induction of activity-dependent genes encoding early effectors and transcription factors, collectively known as immediate early genes (IEGs). Early transcription factors (e.g. *zenk*, *c-jun*, *c-fos*) act by regulating the expression of downstream targets that encode late effectors (grey arrows). Early effectors, in contrast, can have a more rapid effect on cell physiology. *Arc* (dashed grey lines) has a dendritic localization and is thought to act at the level of activated synapses. Abbreviations: MEK1/2, mitogen-activated and extracellular signal-regulated protein kinase kinase 1 and 2; ERK, extracellular signal-regulated kinase; UO126, MEK1/2 inhibitor.

Chapter 3

The late genomic response to song:

Synapsins are regulated by song stimulation in the brain of songbirds

INTRODUCTION

Brain activation triggers rapid transcriptional events thought to lead to long-lasting neuronal changes underlying long-term memories. This response comprises direct effectors that exert cellular actions independent of further RNA/protein synthesis, and inducible transcription factors (ITFs) that regulate late target genes (Curran and Morgan, 1985; Sheng and Greenberg, 1990; Nedivi et al., 1993; Lanahan and Worley, 1998; Clayton, 2000; Velho and Mello, 2007). Whereas early gene mapping has been instrumental to the analysis of brain activation by specific stimuli and behaviors (Chaudhuri, 1997; Mello, 2002), late effectors or targets are mostly unknown.

Synapsins are phosphoproteins associated with the synaptic vesicle membrane and thought to modulate different aspects of synaptic transmission (Greengard et al., 1993; Hilfiker et al., 1999). They are encoded by three genes (*syn1-3*) with multiple transcripts (Kao et al., 1999) that have been linked to schizophrenia (Chen et al., 2004; Lachman et al., 2005; Cavalleri et al., 2007), and their deletion leads to seizures (Li et al., 1995; Rosahl et al., 1995; Gitler et al., 2004). Changes in *syn1* expression occur in the retina after prolonged exposure to an enriched environment (Pinaud et al., 2002) and in the

hippocampus during the induction of long-term potentiation or prolonged exposure to stressors (Morimoto et al., 1998b; Alfonso et al., 2006; Iwata et al., 2006). Importantly, s*yn1-2* promoters respond to EGR-1 (an ITF, a.k.a. ZENK) in cultured neuronal-like cells (Thiel et al., 1994; Petersohn et al., 1995). Thus, synapsins are candidate ITF targets, but their *in vivo* regulation in a manner dependent on early protein synthesis has not been shown.

Brain gene regulation by natural stimuli is well documented in songbirds. Stimulation with song, a learned vocal communication signal, induces ITFs (*zenk*, *c-fos* and *c-jun*) in high-order auditory areas (Mello et al., 1992; Mello and Clayton, 1994; Nastiuk et al., 1994; Bolhuis et al., 2000; Velho et al., 2005), particularly the caudomedial nidopallium (NCM). ITF induction reflects the acoustic properties and novelty of the stimulus, and is modulated by attention/arousal, age, and experience (Clayton, 2000; Mello, 2002; Mello et al., 2004; Velho and Mello, 2007). Repeated stimulation habituates NCM's electrophysiological responses, an effect whose long-term maintenance depends on RNA and protein synthesis during distinct periods following song (Chew et al., 1995). Moreover, MAP-kinase signaling in NCM is required for ITF expression (Cheng and Clayton, 2004; Velho et al., 2005) and the auditory memorization of tutor song (London and Clayton, 2008). Thus, song-induced gene expression is required for long-lasting neuronal changes and song auditory memories.

We hypothesized that synapsins might be late song-regulated genes. In support, we found that the expression of synapsins in zebra finch NCM increases upon song stimulation with a protracted time-course compared to early genes.

This effect occurs primarily in excitatory neurons and is under a suppressive action by early song-induced proteins. We also show that ZENK protein binds *in vivo* to the *syn3* promoter. Thus, synapsins are an integral late component of the brain's response to stimulation, and a likely late effector of long-lasting changes in responsive neurons.

MATERIAL AND METHODS

Identification of zebra finch synapsins* (see Fig 3.1):* PCR primers (forward: 5'ccgcacaccgactgggccaa3'; reverse: 5'gtcctcatgtargccttgtagt3') were designed based on a conserved domain within synapsin 2 (*syn2*) mRNA sequences from several species in GenBank. Standard touch-down PCR reaction was performed with DNA from a zebra finch cDNA library (Holzenberger et al., 1997; Denisenko-Nehrbass et al., 2000) with annealing temperatures starting at 63°C minus 1 degree per cycle for 8 cycles, and 22 additional cycles at 55°C. The amplified product was excised from an agarose gel, eluted using Qiagen Gel Extraction Kit® (Qiagen Inc, Valencia, CA, USA), inserted into pPCRScript (Stratagene Inc., La Jolla, CA, USA) and used to transform bacterial cells following standard procedures. Insert identity was confirmed by sequencing and analysis using DNAStar software (DNAStar Inc., Madison, WI, USA). This amplified cDNA (GenBank accession # AY494948; Fig 3.1A) does not discriminate between the two *syn2* isoforms. We also identified a set of ESTs representing *syn2* from an EST collection derived from a normalized zebra finch brain cDNA library (ESTIMA collaborative consortium; see http://titan.biotec.uiuc.edu/songbird). These ESTs comprised a contig (PTA_05.6657.C1.Contig7234) encompassing most of the shared sequences of *syn2a* and *2b*.

A partial cDNA representing the zebra finch synapsin 2a (Fig 3.1A, *syn2a*) isoform (Fig 3.1A, *syn2a* 4-13) was PCR-amplified from the same cDNA library using a forward primer (5'gccggcatccccagcgtcaact3') based on exon 4 sequence from the previously obtained *syn2* clone (GenBank's AY494948) and a reverse primer (5'tgaaagctgtgggtgcgactgagg3') based on a conserved domain within exon 13 from sequences of several species. The resulting amplification product was cloned and analyzed as above. Additional *syn2a* clones obtained from ESTIMA comprise a contig (SB.929.C1.Contig 1194). One of the ESTs (GenBank CK234869) within this contig contains a poly-A tail, indicating we have identified the complete 3' UTR of this isoform. Partial ESTIMA cDNAs representing the zebra finch synapsin 2b (Fig 3.1A, *syn2b*) isoform (GenBank's CK301417 and CK303619) were also identified. The EST CK303619 contains a poly-A tail, indicating we have identified the complete *syn2b* 3'UTR.

PCR primers for *syn3* (Fig 3.1B) were designed based on conserved domains within synapsin 3 (*syn3*) mRNA sequences from several species available in GenBank for exons 3 and 13 (forward330: 5'ccaggggaagaaggtgaatg3', reverse1728: 5'cagaaaacaggctagcaaatgat3'). PCR amplification and cloning were performed as described above for *syn2*. Three additional cDNA clones representing mostly the 5'UTR and 3'UTR regions of the zebra finch *syn3* homologue (GenBank's DV949224, CK305874 and CK305733) were also identified in ESTIMA. Importantly, clone CK305733 had a poly-A tail, confirming this region as the likely 3' end of the transcript.

We used the sequences derived from the PCR-amplified clones and from ESTIMA clones to assemble full-length transcript sequences for all 3 synapsin isoforms. These sequences were then used for blast searches of the zebra finch genomic trace archives, which were then assembled to reconstruct the complete *syn2* and *syn3* genes. We also amplified a *syn2a*-specific fragment (grey bar over exons 12 and 13 on Fig 3.1A, *syn2a*) using a forward primer based on the 3' end of the shared region of *syn2a* and *b* (grey portion of exon 11 on Fig 3.1A, *syn2* gene; 5'agccaaacaaaatcccacctcag3') and a reverse primer based on exon 13 (5'ggactgcgacttgaaagctgtg3'), which is present in the *syn2a* isoform only. Sequence from one EST (GenBank's CK301417) was used to PCR-amplify a 200 bp *syn2b*-specific fragment (grey bar over exon 11 on Fig 3.1A, *syn2b*). These isoform-specific fragments were cloned into PCRScript and used to generate isoform-specific riboprobes for Northern and *in situ* hybridization. GenBank's clone AY494948 was used to generate a non-discriminating *syn2* probe.

Probe labeling*:* Riboprobes were used for *in situ* hybridization and Northern blot analyses. Plasmid DNA was isolated using Qiagen Miniprep Kit (Qiagen Inc. Valencia, CA, USA), linearized with the appropriate restriction enzymes, and purified with Qiagen PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Sense and antisense ^{33}P-labeled riboprobes for all genes analyzed in this study were synthesized as described elsewhere (Mello et al., 1997). The *hat2* probe was derived from the previously described canary homologue (George and

Clayton, 1992). For digoxigenin-(DIG-)labeled riboprobes, we added 1.0 µl of DIG labeling mix (Roche Diagnostics Corp., Mannheim, Germany) to a 10 µl reaction containing 1 µg of linear plasmid DNA, 10 µg of BSA, 10 mM of DTT, 20 units of RNAse inhibitor, 8-10 units of RNA polymerase, and 2.0 µl of a transcription buffer consisting of 50 mM DTT, 250 mM Tris-HCl pH 7.4, 30 mM MgCl2, 50 mM NaCl, and 10 mM spermidine in DEPC-treated water; otherwise, we followed (Mello et al., 1997). All riboprobes were purified in Sephadex G-50 columns and analyzed using a liquid scintillation counter or by visual inspection of a denaturing formaldehyde-agarose gel.

***Northern blot analysis**:* Total RNA from adult zebra finch brain was extracted using the Trizol method (Life Technologies Inc., Rockville, MD, USA) and quantified by spectrophotometry. Ten µg of total RNA were separated by electrophoresis in 1% MOPS/formaldehyde agarose gel, and transferred with 10X SCC to nylon membranes (Hybond-N^+ Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) as described in detail elsewhere (Mello et al., 1997). The membranes were incubated overnight in hybridization solution containing ^{33}P-labeled antisense riboprobes for *syn2*, *syn2a*, *syn2b* or *syn3* (10^6 cpm/ml), followed by washes at 65°C and exposure to a KODAK storage phosphorscreen (Molecular Dynamics Inc., Sunnyvale, CA, USA), which was read by a phosphorimager (Typhoon 8600, Molecular Dynamics Inc., Sunnyvale, CA, USA).

Birds and song stimulation*:* All birds in this study were adult zebra finches (*Taeniopygia guttata*) from our own breeding colony or obtained from local breeders. For song stimulation, females were first isolated overnight (16-20 hr) in sound-attenuated chambers (≈76 X 31 X 28 cm) under a 12:12 light:dark cycle (same as the aviary; lights on at 7:00 AM). On the following day, birds were exposed to a playback of a recorded stimulus consisting of a medley of three representative non-familiar conspecific songs presented in blocks of 15 seconds followed by a 45-second silent interval. For ISH (see below), birds were either exposed to this stimulus for 30 min and killed after varying survival intervals (Fig 1.3A), or exposed to different stimulus durations followed by immediate sacrifice (Fig 3.6A). For Western blots, birds were stimulated for 30 min and sacrificed at 6 hr after stimulus onset. For ChIP assays, birds were stimulated for 30 min and sacrificed 2 hr after stimulus onset. All stimuli were presented at comparable intensities (average 70 dB mean SPL at 35 cm from the speaker). Females were used in all quantitative song stimulation experiments to avoid the confound of singing behavior in males; controls consisted of birds that were not stimulated. We also analyzed the general brain expression of synapsins in a separate set of adult males (n=3). All procedures involving birds were approved by OHSU's Institutional Animal Care Use Committee (IACUC) and are in accordance with NIH guidelines.

Stereotaxic microinjections of cycloheximide: We performed intracerebral injections in awake restrained birds essentially as described in Velho et al.

(2005), with some modifications. Briefly, the birds were first anesthetized with 50 μg/g of sodium pentobarbital, and placed in a stereotaxic apparatus containing a bird skull pin slide adapter (MyNeuroLab, Saint Louis, MO, USA). A metal fixation pin was affixed to the skull using Tylok-Plus dental cement (Dentsply International Inc., Milford, DE, USA), and a window was partially opened by removal of the superficial layer of bone over NCM. Birds were allowed to recover for at least 48 hr. The birds were then restrained within a plastic tube and fixed to the stereotaxic apparatus by inserting the implanted metal pin into the pin adapter. The remaining layer of bone was removed, a glass pipette lowered into NCM (AP: 0.5 mm; ML: 0.65 mm; DV: 1.2 and 1.6 mm), and 2 μg/μl of cycloheximide (CYC), a dose previously proven to effectively block protein expression in NCM (Chew et al., 1995), were injected (50 nl/site over 2 min) into one hemisphere and vehicle (VEH) in the contralateral hemisphere, using a microinjector (Narishige International Inc., Long Island, NY, USA). To facilitate the identification of the injection site, the pipettes were coated with fluorescent latex beads (Lumafluor Corp., Naples, FL, USA.).

Injected birds were divided into two sets: song stimulated and unstimulated controls. Song-stimulated birds were injected with CYC into one hemisphere and VEH into the other, and then stimulated with song and killed at 6 hr after stimulation onset. Unstimulated controls were also injected CYC and VEH into the NCMs but did not receive any auditory stimulation. The drugs assigned to each hemisphere were alternated across birds.

Acclimation sessions: In order to minimize procedural induction of activity-dependent genes in the brain for the brain microinjections, the birds were subjected to several acclimation sessions after recovering from the pin implant surgery for at least 24 hr. Each session consisted of gently restraining a bird, fixing it through the attached pin to the adapter in the stereotaxic apparatus, maintaining it in the apparatus for 10 min (in the presence of a researcher), and returning it to the isolation box. Typically we performed 4 such sessions (once every 2 hr) on the day preceding the brain microinjections and one last session on the day of the injections. In addition, the birds were kept in acoustic attenuation boxes at all times after the pin implants, except during the acclimation sessions and during microinjections. Both the acclimation and microinjections were performed in a sound-attenuated room with minimal sound exposure.

Tissue preparation: For in situ *hybridization* and Western blots, birds were sacrificed by decapitation. Their brains were quickly dissected from the skull, frozen in Tissue-tek (Sakura Finetek Inc., Torrance, CA, U.S.A) in a dry ice/isopropanol bath, and stored at -80°C. Parasagittal 10 μm brain sections were cut on a cryostat, thaw-mounted onto slides, and stored at -80°C until use. For ChIP assays, sound-stimulated birds (n=3) were killed with an overdose of Nembutal and perfused transcardially with freshly prepared 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were then removed from the skull and the caudomedial telencephalic auditory lobules

(including the caudomedial nidopallium and mesopallium - NCM and CMM - and medial field L2a) dissected and frozen.

Radioactive* in situ *hybridization (ISH): ISH was performed using ^{33}P-labelled sense and antisense riboprobes followed by exposure to a KODAK phosphorimager screen or emulsion autoradiography and Nissl counterstaining. The hybridizations and washes for all probes analyzed were performed at 65^{o}C, using essentially the same procedure as previously described (Mello et al., 1997). *Syn3* ISH sections were treated with RNAse A (1 µg/mL) as previously described (Mello et al., 1997).

***Double ISH and Fluorescent* in situ *hybridization (FISH)*:** We used a double-labeling procedure combining ISH (for *syn2a*) and FISH (for *syn2b* or for the gabaergic marker *gad65*), followed by nuclear counterstaining (Hoechst). We followed the double-labeling procedures described in Velho et al., 2005 and Jeong et al., 2005.

Densitometry: Phosphorimager files were quantified using NIH's Image software. The slide background was subtracted from optical density (O.D.) measurements taken over specific brain regions, and the resulting values averaged for two adjacent sections. For normalization, all densitometric values were divided by the average values from unstimulated controls. To examine the effect of post-stimulation survival time, stimulus duration and drug treatment, we

used ANOVA followed by Fisher's PSDL *posthoc* tests for pair-wise comparisons, and a probability level of <0.05 for significance.

Cell counts: For quantitative analysis of our double ISH and FISH experiment, we used Neurolucida software (MicroBrightField Inc., Colchester, VT) and estimated the number of labeled cells in specific areas of brain sections through lateral NCM (~800 µm from the midline) of song-stimulated birds and unstimulated controls (n=3-6 per group). We first placed a counting square of 200 X 200 µm over dorsal or ventral NCM, equidistant from NCM's rostral and the caudal boundaries (L2a and the ventricle, respectively), or over the hippocampus. We then centered a counting circle (12 µm diameter) over each cell nucleus, identified based on the nuclear (Hoechst) counterstaining and excluding cells with clearly non-neuronal staining (small, homogeneously and strongly labeled nuclei). We next counted the number of emulsion autoradiography grains per labeled cell. We note that the typical nuclear diameter of most cells counted was in the 6-10 µm range and that the diameter of the counting circle was determined to be inclusive of the majority of grains associated with the cells. Cells with grain counts of at least 2 standard deviations above background levels (measured over the glass or over the neuropil between labeled somata – see Fig 3.6 for example) were considered *syn2a*-positive. Finally, we examined the mapped field under the appropriate fluorescence filter to determine how many *syn2a*-positive (emulsion autoradiography signal) cells were also labeled for *syn2b* or *gad65* (fluorescence signal). We did not observe

evidence of regional differences in the number of cells in NCM, thus dorsal and ventral measurements were averaged.

Western blots: The auditory lobules of song-stimulated or unstimulated control birds were homogenized in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM DTT, and 0.5% Triton X-100) plus 1X proteinase inhibitor (Calbiochem, NJ) and 30 µg of protein per sample were separated by SDS-PAGE and transferred to nylon membranes (Novax Inc., San Diego, CA, USA). The membranes were then incubated for 1 hr in blocking buffer (BB, consisting of 5% dry milk in TBST: 25 mM Tris, pH 7.4, 3.0 mM KCl, 140 mM NaCl and 0.05% Tween 20), incubated overnight at 4˚C with a mouse anti-synapsin 2 antibody raised against amino acid 348-449 of the human Syn2 (1:500 dilution in BB, Novus Biologicals Inc., CO, USA) or a mouse anti-actin antibody (1:500 dilution**,** Chemicon Inc.), washed 3 X 10 min in TBST buffer, incubated for 2 hr with a peroxidase conjugated goat anti-mouse IgG antibody (1:10000 dilution in BB, Kirkegaard & Perry Laboratories, Inc.), and washed 3 X 10 min in TBST buffer. Incubations and washes were all performed at room temperature. Protein expression was detected by chemiluminescence (Pierce Biotechnology; IL) autoradiography.

Promoter Analysis: The *syn3* promoter was assembled by sequentially retrieving and assembling sequences from the zebra finch genomic trace archives (NCBI, Genomic Biology) using Seqman software (DNAStar Inc., WI, USA). We reconstructed a genomic region of about 2.0 kB corresponding to the

5’ end of the *syn3* transcript and the upstream promoter region. The presence of putative transcription factor binding sites was examined based on matrix analysis (Transfac 8.0) using MatInspector software (Genomatix; http://www.genomatix.de/). A comparable region could not be retrieved from the trace archives for the *syn2* gene.

Chromatin immunoprecipitation assay (ChIP): The microdissected auditory lobules were processed using a commercial ChIP kit (Upstate, Temecula, CA), following manufacturer’s instructions. Briefly, the tissue samples (typically 20 mg wet weight per bird) were homogenized in SDS lysis buffer (at a 10-to-1 buffer-to-tissue volume ratio) containing a protease inhibitor mix (Complete Mini mix at 1X concentration; Roche Diagnostics, USA) with a microtube pestle, and incubated on ice for 10 min. Chromatin shearing was then performed by sonicating the samples in 6 X 15 sec bursts at 35% power, a protocol we previously determined to result in ~1-2 kB average DNA size in our samples. The homogenates were spun at 17000 g for 10 min and the supernatants were separated, diluted further and pre-cleared by agitation for 30 min with the proteinA/agarose/salmon sperm DNA slurry. The samples were then immunoprecipitated overnight with 10 µg of goat anti-Egr-1 (ZENK) sc-189 or mouse anti-parvalbumin antibodies (Santa Cruz Biotechnology, Inc, CA), followed by incubation for 1 hr with the proteinA/agarose/salmon sperm DNA slurry. After washes, the immunoprecipitated chromatin was eluted, de-crosslinked by incubation in 0.2 M NaCl for 4 hr, and treated with proteinase K for 1 hr at 45˚C. The resulting DNA

samples were then isolated using QIAGEN's PCR purification kit (QIAGEN, Inc, CA). All steps up to the elution were performed at 4˚C; further details were performed according to the manufacturer's recommendations. The immunoprecipitation was verified by spectrophotometric analysis of the resulting DNA product. Further analysis was carried out by performing PCR amplification with primers for the promoter regions of *syn3* (forward: 5'ggaggaagggcagaaaagtgtcat3', reverse: 5'gtgggaagagaatcaaagaaat3', corresponding to a 523 bp fragment between positions -508 and +15 of the promoter sequence relative to the putative transcription start site) and *eno2* (forward: 5'ggtctccctcccctgctgtctgt3'; reverse: 5'ttccccttttcactgccctcttctg3', corresponding to a 498 fragment within the promoter region). ChIP specificity was determined by comparing the products of DNA precipitated with the different antibodies used.

RESULTS

Identification of zebra finch synapsins

The zebra finch *syn2* homologue contains 13 exons and a *timp4* gene nested between exons 5 and 6 (Fig 3.1A), thus resembling closely the structure of its mammalian counterparts. We have identified transcripts corresponding to two isoforms, *syn2a* and *syn2b* (Fig 3.1A). Similar to mammals, these transcripts are likely derived through a combination of alternative termination and differential splicing from a common gene. The *syn2a* mRNA includes all 13 exons, whereas *syn2b* contains a longer exon 11 but lacks exons 12 and 13 (Fig 3.1A). In spite of the longer coding sequence of *syn2a*, the 3'UTR is much shorter in *syn2a* than in *syn2b*. Thus, the predicted sizes of these transcripts are ~2.9 and 3.9 Kb respectively. The predicted amino acid residue composition in comparison with chicken, human and rat homologues respectively shows 92.7, 76.1 and 75.1% identity for *syn2a* (Fig 3.2A), and 97.1, 83, and 81.5% identity for *syn2b* (Fig 3.2A-B). Interestingly, *syn2a* shows low conservation in the proline-rich domain (between residues 464 and 526 on Fig 3.2A).

The zebra finch *syn3* gene also had a conserved organization compared to mammals, consisting of 13 exons and including a *timp3* gene nested between exons 5 and 6 (Fig 3.1B). This gene encodes a long transcript (predicted at ~8.8 kb) with a very long (~6 kb) 3'UTR (Fig 3.1B). Interestingly, ESTs representing Fbox7, a gene located in the opposite orientation in the genomic sequence of both zebra finch and chicken, show partial overlap with the 3' end of clone CK305733, suggesting that a small domain may be shared between these two transcripts (not shown). The predicted Syn3a protein shows 93.1, 78.6, and 77.9% identity with the chicken (based on genomic sequence), human and rat homologues respectively (Fig 3.3).

Northern blot analysis using a riboprobe that targets the common region of the *syn2a* and 2*b* isoforms (clone AY494948) revealed two transcripts (of ~2.6 and ~3.6 Kb), with higher expression of the longer one (Fig 3.1C). The *syn2a*- and *syn2b*-specific probes, however, recognized respectively the shorter and longer transcripts, consistent with the sizes predicted from their sequences (Fig 3.1A). Northern analysis for *syn3* revealed a single transcript of ~8.8 kb (Fig 3.1D), consistent with the prediction of a single large transcript.

Comparative analysis between avian (zebra finch and chicken) and mammalian genomic sequences revealed high conservation in the regions adjacent to *syn2* and *3*, including large sinteny groups for both genes (not shown), supporting the conclusion that we have identified their true homologues. In contrast, despite extensive searches of chicken and zebra finch genomic sequences with coding and non-coding sequences from several species, we did

not obtain any significant hits for *syn1*, strongly suggesting that birds may lack this gene (Velho and Mello, unpublished data). In support of this possibility, blast searches for *timp1* and *a-raf1*, which are respectively nested within and in close proximity to the *syn1* locus in other vertebrates, also had no hits in avian genomes.

Expression of synapsins in the zebra finch brain

We next examined the regional and cellular expression of synapsins in the brain of adult zebra finches, the first such analysis in an avian species. We observed broad distributions for *syn2a* and *2b* (Fig 3.4B and C, respectively), including pallial areas (nidopallium, mesopallium and hyperpallium)and granule cell layer of the cerebellum, but much lower expression in the striatum and arcopallium, as well as in the auditory thalamo-recipient zone field L2a and the magnocellular nucleus of the lateral nidopallium (LMAN) compared to the surrounds (Fig 3.4B and C, bottom panels). The two isoforms had similar distributions, but *syn2b* expression was higher than *syn2a*, consistent with the Northern analysis. The *syn3* transcript had lower expression than *syn2*, and also had a broad but largely uniform distribution (Fig 3.4). Sense probes for all transcripts did not yield detectable hybridization.

Cellular analysis in NCM demonstrated *syn2a* and *2b* expression in cells with neuronal-like features such as large nucleus, clear nucleolus and Nissl substance (Fig 3.4E-G, arrows), whereas smaller cells with dark, homogeneously staining nuclei that represent glial and blood cells were clearly negative (Fig

3.6E, arrowheads), as were also the cells in the ventricular ependyma and in the subventricular zone (Fig 3.4G, arrowheads). Double-labeling ISH revealed that the majority of cells (~67%; n=3 females) co-express *syn2a* and *2b* (Fig 3.4H, white arrows), but single-labeled cells could also be seen (Fig. 3.4H, red arrow). For *syn3*, we also observed both positive and negative neuronal cells (Fig 3.4I, respectively small and large arrows), whereas glial-like cells were clearly negative (Fig. 3.4I, arrowheads).

Song stimulation increases synapsin expression in NCM

To determine whether synapsins are regulated by song, we examined their expression in parasagittal sections through medial and lateral NCM (~0.4 and 0.8 mm from the midline) from birds stimulated for 30 min and sacrificed at different post-stimulation times (Fig. 3.5A), using isoform-specific probes. Quantitative autoradiography showed that *syn2a* is significantly induced in lateral NCM (ANOVA, $p<0.01$; n=4-6 per group) at 2, 4, 6 and 8 hr after the start of a 30-min song stimulation period compared to unstimulated controls (Fig 3.5B, right; Fisher's PLSD, $p<0.05$, <0.01, <0.01, and <0.001, respectively). In contrast, we observed considerable variability that did not reach significance in medial NCM (Fig 3.5B, left; ANOVA $p=0.50$). Similarly, *syn2b* was significantly induced in lateral NCM (ANOVA $p<0.01$) at 2, 4, 6, and 8 hr after the start of the song stimulation compared to controls (Fig. 3.5C right; Fisher's PLSD, $p<0.01$, <0.05, <0.001 and <0.05, respectively) but not in medial NCM (Fig 3.5C, left; ANOVA

p=0.11). The expression of *syn3* also increased in lateral NCM (Fig 3.5D, right; ANOVA *p*<0.05), but was significantly higher than controls only at 6 and 8 hr after stimulation onset (Fisher's PLSD, *p*<0.05 and <001, respectively); a similar trend in medial NCM did not reach significance (Fig 3.5D, left; ANOVA *p*=0.06). In sum, song induces the expression of synapsins primarily in lateral NCM, with a protracted time course in comparison with early inducible genes.

We also examined birds killed immediately after hearing song for various time durations compared to unstimulated controls (Fig. 3.6A). We observed significant increases in *syn2a* expression in lateral NCM (Fig 3.6B, right; ANOVA *p*<0.01, n=4-6 per group) after stimulation for 0.5, 2, 4, 6 and 8 hr (Fisher's PSLD, *p*<0.05, *p*<0.05, *p*<0.05, *p*<0.001, and *p*<0.018, respectively) but again not in medial NCM (Fig. 3.6B left; ANOVA *p*=0.76). These results resembled closely those of the previous experiment where all birds were stimulated for the same duration (0.5 hr; Fig. 3.5B), suggesting that stimulus duration has little or no effect on *syn2a* expression levels. Indeed, we detected no significant effect of stimulation protocol (fixed vs. variable stimulus duration) when the data from both experiments were analyzed together (ANOVA, *p*=0.63). Thus, *syn2a* expression levels in lateral NCM are primarily determined by the first 30 min of song stimulation. In sharp contrast, *syn3* expression did not significantly increase after prolonged stimulation periods when compared to unstimulated controls in either medial or lateral NCM (Fig 3.6C; ANOVA *p*=0.7591 and *p*=0.7880 respectively). Indeed, we observed an effect of stimulation protocol (fixed vs. variable stimulus duration) when the data for lateral NCM from both experiments (compare Figs.

3.5D right and 3.6C right) were analyzed together (ANOVA, $p<0.05$). Thus, while *syn3* is clearly induced with a protracted time course by a short (30 min) stimulation period, further stimulation appears to suppress the initial inductive effect of song. Finally, in accordance to the known lack of *hat2* induction by song, we observed no evidence of regulation of that transcript in either the time course or differential duration experiments (not shown).

Song induces synapsin changes in a specific cell population

To examine the neurochemical identity of the cells in which synapsins are induced, we performed double ISH for *syn2a* and the gabaergic marker *gad65* in sections from birds killed at different times after the start of a 30-min stimulation period (n=4-6 per group). We first determined that there was a significant effect of song stimulation on the number of *syn2a*-positive cells in NCM (ANOVA, $p< 0.05$). We then found that the number of *syn2a*-positive cells that were also *gad65*-positive (presumably gabaergic neurons) did not change significantly with stimulation (Fig 3.7B; ANOVA, $p=0.53$). In contrast, the number of *syn2a*-positive cells that were *gad65*-negative (presumably excitatory neurons) increased significantly in song-stimulated birds compared to unstimulated controls (Fig 3.7A; ANOVA, $p<0.01$) at 6 hr (Fisher's PLSD, $p<0.01$). Thus, the increased synapsin expression in NCM appears to occur predominantly in excitatory cells. The number of *syn2a*-expressing neurons, regardless of their neurochemical identity, did not change in the adjacent hippocampus (Fig 3.7C and D; ANOVA $p=0.89$ and 0.21, respectively), indicating a regional specificity for the effect

observed in NCM.

Synapsin proteins are induced by song

To investigate whether synapsin proteins are regulated by song stimulation, we examined *syn2* expression on a Western blot of microdissected auditory lobules with an anti-synapsin 2 antibody. We detected two bands (~70 and ~58 kDa), likely representing the polypeptides encoded by *syn2a* and *syn2b* respectively. A comparison of unstimulated and song-stimulated birds showed that both isoforms are up-regulated by song (Fig 3.8A), consistent with our findings at the mRNA level. In contrast, no group difference was observed on the same blots using an anti-actin antibody (Fig 3.8B), providing a control for gel loading and further evidence for the specificity of synapsin up-regulation. We note that a recent study utilizing a proteomics approach (Pinaud et al., 2008a) also found evidence of song regulation of one synapsin peptide, which we interpret to have been Syn2a, based on molecular weight.

Synapsins expression in NCM is regulated by early song-induced gene expression

The song-induced regulation of synapsins is delayed compared to the expression kinetics of known early song-induced transcription factors (which peak 30 min after stimulus onset; (Mello and Clayton, 1994; Velho et al., 2005), consistent with the hypothesis that the expression of synapsins in NCM might be under the regulatory control of early song-induced genes. To test this hypothesis,

we examined whether the protein synthesis inhibitor cycloheximide (CYC) injected locally into NCM 10 min before a 30-min song stimulation period would affect song-induced synapsin expression in birds killed 6 hr after the start of the stimulation (Fig 3.9A). We targeted the injections to medial NCM (~500 µm from the midline) since this region shows a very robust expression of early song-inducible genes. We observed significant effects of drug treatment (higher expression in CYC- than VEH-injected hemispheres) for both *syn2a* and *syn3* in song-stimulated birds (Fig 3.9B and C, right columns; Anova, $p<0.05$, and paired t-test, $p<0.01$, n=5 birds per group), but not in unstimulated controls (Fig 3.9B and C, left columns; Anova, $p=0.36$). Consistent with the previous data (Fig. 3D, left), we did not observe an effect on *syn3* expression at a very medial NCM level (~200 µm from the midline; Fig 3.9D), arguing for the regional specificity of the CYC effect (Fig 3.9C). Importantly, the early song-responsive gene *Arc* was induced in NCM of both cycloheximide- and vehicle-injected hemispheres (Fig 3.9E; Anova $p=0.01$ and $p=0.03$ respectively), indicating that the early mRNA induction response to song was undisturbed by the local CYC injection and suggesting that the observed effects were specific for late song-regulated genes. In sum, the early genomic response to song appears to have a suppressive effect on the song-induced expression of synapsins in NCM.

ZENK binds to the synapsin 3 promoter

To further investigate the regulation of synapsins, we examined the promoter region of *syn3* (the promoter sequence for *syn2* is currently

unavailable) for the presence of conserved binding sites for known transcription factors using a matrix-based analysis (Genomatix's MatInspector). Based on the 5'-most zebra finch mRNA sequence available (clone DV949224 in Fig 3.1B) and comparisons with sequences from chicken and mammals, we identified a putative transcription start site (TSS; position 0 on Fig 3.10A). We did not find a canonical TATA element, but found a CAAT box and a CEBP (CAAT/enhancing binding protein) upstream of the TSS. We noted that the promoter region is decorated with multiple occurrences of the consensus binding sites for various factors, including general ones (e.g. SMARCA3, EVI1, Oct1, ETS1) as well as tissue-specific ones (e.g. HOXF/CRX, TAL1, LEF1). We also found that *syn3* contains 2 binding sites for ZENK at positions +276 and +444, and 2 for AP-1 (c-Fos/c-Jun complex) at positions -39 and -790 relative to the TSS (Fig 3.10A; one ZENK site coincides with the translation start site). We also noticed several occurrences of the binding site for E4BP4, a member of the CREB family that is generally considered to have an inhibitory effect on transcription, and the presence of neural-restrictive silencer elements (NRSE and NRSF) within the first exon.

To determine whether ZENK actually binds to its putative sites in the *syn3* promoter *in vivo*, we performed chromatin immunoprecipitation (ChIP) with an anti-ZENK antibody on dissected auditory lobules from song-stimulated birds perfused 2 hr after the start of a 30 min stimulation with conspecific song. This time corresponds to the peak of ZENK protein induction by song (Mello and Ribeiro, 1998). We then PCR-amplified the immunoprecipitated DNA with *syn3*-

specific primers and obtained a fragment of the predicted size within the *syn3* promoter region (Fig 3.10B, left panel, *syn3*/Zenk-IP lane; n=3). In contrast, these primers yielded no product when used to PCR amplify DNA immunoprecipitated using an anti-parvalbumin antibody (Fig 3.10B, right panel, *syn3*/Parv-IP lane), establishing the specificity of the immunoprecipitation with the anti-ZENK antibody. In both cases, the same fragment could be amplified from the input samples isolated prior to the immunoprecipitation (Fig 3.10B, Input lanes). To further determine the specificity of the immunoprecipitation with the anti-ZENK antibody, we designed primers targeting the reconstructed promoter region of the neuronal specific enolase gene (*eno2*), which we determined to lack conserved ZENK binding sites (not shown). PCR amplification using the *eno2*-specific primers resulted in no amplification product from the DNA immunoprecipitated with the anti-ZENK antibody, but robust amplification from the input sample (Fig 3.10B, left panel, *eno2* lanes).

DISCUSSION

We have shown that the genes encoding synapsins, a family of phosphoproteins associated with synaptic vesicles and thought to regulate their availability for synaptic release, are induced by song stimulation in a higher-order auditory brain area of songbirds. This induction is protracted relative to early song-inducible genes, occurs primarily in excitatory cells, and is under a suppressive action of early song-induced proteins. Our results support the hypothesis that synapsins are an integral part of the genomic response to sensory stimulation. More generally, synapsins appear to represent late effectors that could mediate the long-term effects of sensory experience on the regulation of neuronal properties.

Regulation of synapsins

The expression of synapsins increases in brain tissue after tetanic electrical stimulation, or prolonged sensory stimulation and behavioral training (Morimoto et al., 1998a; Gomez-Pinilla et al., 2001; Pinaud et al., 2002). It is unclear, however, whether such changes are directly associated with the sensory activation of responsive neurons, or secondary to the chronic stimulation or overtraining. In our paradigm, synapsins were induced after a brief auditory

stimulation with birdsong, a natural learned vocal communication signal. Song processing and memorization is essential for songbirds to learn their own song and to identify other individuals during territorial defense and courtship (Kroodsma and Miller, 1996). Thus, our data show that synapsins can be transcriptionally regulated in the brain of freely-behaving animals, by a stimulus of established behavioral relevance.

Our data also imply that synapsins can be regulated by activation of cortical-like circuitry. NCM, a telencephalic region, receives input from the primary thalamo-recipient field L and is interconnected with other auditory areas (Vates et al., 1996), its position in the auditory pathways being analogous to supragranular layers of the auditory cortex. NCM is part of the avian pallium, considered homologous to the mammalian neocortex and parts of the amygdala and claustrum (Reiner et al., 2004; Jarvis et al., 2005). Electrophysiological, gene expression, and lesion studies indicate that NCM plays central roles in song perceptual processing and memorization (Mello et al., 1992; Mello et al., 1995; Velho et al., 2005; Phan et al., 2006; Gobes and Bolhuis, 2007; London and Clayton, 2008), akin to postulated roles of the mammalian auditory cortex (Ghazanfar and Hauser, 2001). We predict that synapsins may also be regulated by complex, behaviorally relevant stimuli in the auditory cortex, and/or by other sensory modalities in the corresponding cortical areas.

Synapsins are late song-induced genes

We have found that synapsins are constitutively expressed at high levels,

and significantly induced only hours after the onset of song stimulation. This contrasts with the early song-induced transcription factors (ITFs) *zenk*, *c-fos* and *c-jun*, and the early effector *Arc*, which have low basal expression and an early induction peak at 30 min after stimulus onset (Mello and Clayton, 1994; Nastiuk et al., 1994; Mello and Ribeiro, 1998; Velho et al., 2005) and singing-induced genes (Poopatanapong et al., 2006; Wada et al., 2006). Thus, *syn2* and *syn3* are late song-induced genes, regulated by both constitutive and modulatory components.

The induction of synapsins by song occurred primarily in lateral NCM, in contrast to ITFs, whose induction is most pronounced medially. This suggested that synapsin induction does not simply reflect the up-regulation of early genes. Indeed, the evidence from CYC injections indicates that: *1)* song stimulation exerts a positive effect on synapsin expression that is independent of early song-induced proteins; and *2)* early song-induced proteins appear to have a suppressive effect over the song-induced synapsin expression. Importantly, the lack of CYC effects on synapsin expression in unstimulated birds indicates that the suppressive effect in song-stimulated birds is over the song-regulated component rather than on the constitutive expression of synapsins. In sum, song appears to exert a direct, positive action on synapsin expression, and an indirect, suppressive action through early-inducible genes (Fig 3.11A). Our evidence adds to an increased awareness of anatomical and functional NCM subdomains (Terpstra et al., 2004; Pinaud et al., 2006).

Several *syn3* promoter elements may relate to its regulated expression, in

particular the binding motifs for the early ITFs ZENK/EGR-1 and AP1. We show that ZENK protein binds to this promoter in song-stimulated birds, consistent with a direct regulatory action. This is important, given that the presence of binding sites does not guarantee that a given transcription factor binds to a promoter *in vivo* (Cha-Molstad et al., 2004; Impey et al., 2004). Indeed, we observed no evidence of song-induced regulation of other candidate late genes such as NF-M and Kv3.1 (not shown), whose promoters contain ITF binding sites (Velho et al., 2007). We also note that mammalian *syn1* and *2* promoters contain ZENK/EGR-1 binding sites and are responsive to ZENK/EGR1 over-expression in PC12 cells (Thiel et al., 1994; Petersohn et al., 1995). However, whereas *syn1* and *2* are up-regulated based on a gene reporter assay (Petersohn et al., 1995), the native *syn2* gene is down-regulated by ~30% in a similar paradigm, based on microarray analysis (James et al., 2006). This suggests that the construct used in the reporter assay did not include all regulatory elements, and that ZENK actually exerts a suppressive effect on synapsin promoters in a normal cell context. Our finding that song-induced synapsin expression is least apparent where ZENK induction is most robust (i.e. medial NCM), and vice-versa, is consistent with this emerging view of ZENK action.

Other ITFs such as AP-1 may also modulate the induced expression of synapsins. In contrast, constitutive factors such as the activity-regulated Elk-family factor ETS1 are more likely to mediate the positive effect of song on synapsin expression, since the latter persists under CYC. Finally, the suppressive effect of prolonged stimulation on *syn3* but not *syn2* suggests

different promoter organizations, a possibility that awaits the *syn2* promoter sequence. In sum, the induced expression of synapsins is under complex regulation, including transcriptional activators and suppressors; ZENK is most likely among the latter.

Potential roles for synapsin regulation

The time-course of synapsin upregulation overlaps with the late phase (5-7 hr after song) of NCM's long-term habituation dependence on song-induced gene expression (Chew et al., 1995). This contrasts with the early dependence phase, which largely coincides with the induction of the early ITFs *zenk* and *c-fos* (Mello et al., 1992; Nastiuk and Clayton, 1995; Velho et al., 2005). Thus, synapsins could be a late component of the gene expression program associated with long-lasting habituation. However, based on the apparent suppressive effect of early song-inducible proteins, it seems unlikely that synapsins simply mediate the effect of early ITFs on long-term habituation.

Synapsins are associated with the synaptic vesicle membrane and thus locate to pre-synaptic terminals. Their over-expression leads to an increase in the frequency of synaptic contacts (Han et al., 1991; Zhong et al., 1999), whereas their knockout decreases the number of synaptic vesicles (Gitler et al., 2004; Gaffield and Betz, 2007), suggesting that synapsins are important determinants of synapse formation and/or maintenance. By inference, synapsin upregulation by song may increase the number of synaptic contacts and/or vesicles in song-responsive neurons. Both gabaergic and non-gabaergic NCM

neurons are song-responsive (Pinaud et al., 2004), but the present data indicate that the induced synapsin expression occurs primarily in non-gabaergic cells. We thus predict that the effects of synapsin upregulation will be associated mainly with excitatory cells. These could include local interneurons or projection neurons, resulting in predicted modulation of excitatory synapses locally or at NCM's projection targets such as CMM (Fig 3.11B). A combined approach with tract-tracing will be required to settle this issue.

Current evidence indicates that synapsins modulate different aspects of synaptic transmission, such as the recruitment of synaptic vesicles to a reserve pool, the modulation of the kinetics of membrane fusion, the stabilization of synaptic vesicles, and/or the regulation of the late steps of endocytosis (Hilfiker et al., 1999; Humeau et al., 2001). Thus, although increased synapsin proteins will likely accumulate in pre-synaptic terminals of activated neurons, it is difficult to predict the exact consequences to synaptic physiology *in vivo*. Interestingly, injections of synapsin proteins to goldfish Mauthner axons result in reduced quantal release (Hackett et al., 1990), suggesting that synapsin overexpression might actually downregulate synaptic efficacy. Such an effect, if occurring in interneurons, would be consistent with a role of synapsins in the habituation of NCM's response to song.

The consolidation of experience-dependent changes underlying long-term memory requires the activation of gene expression programs in different models and organisms (Goelet et al., 1986; Bailey et al., 1996). In songbirds, recent evidence implicates MAP kinase signaling in NCM and adjacent areas in the

acquisition of tutor song memory during song learning (London and Clayton, 2008). Since the expression of early song-induced genes depends on MAP kinase activation (Cheng and Clayton, 2004; Velho et al., 2005), song-inducible genes in NCM may be involved in the memorization of tutor song. Our current data establish that synapsins are an integral part of NCM's genomic response to song, suggesting that they could also participate in the formation and/or consolidation of song-related memories, a hypothesis requiring future tests.

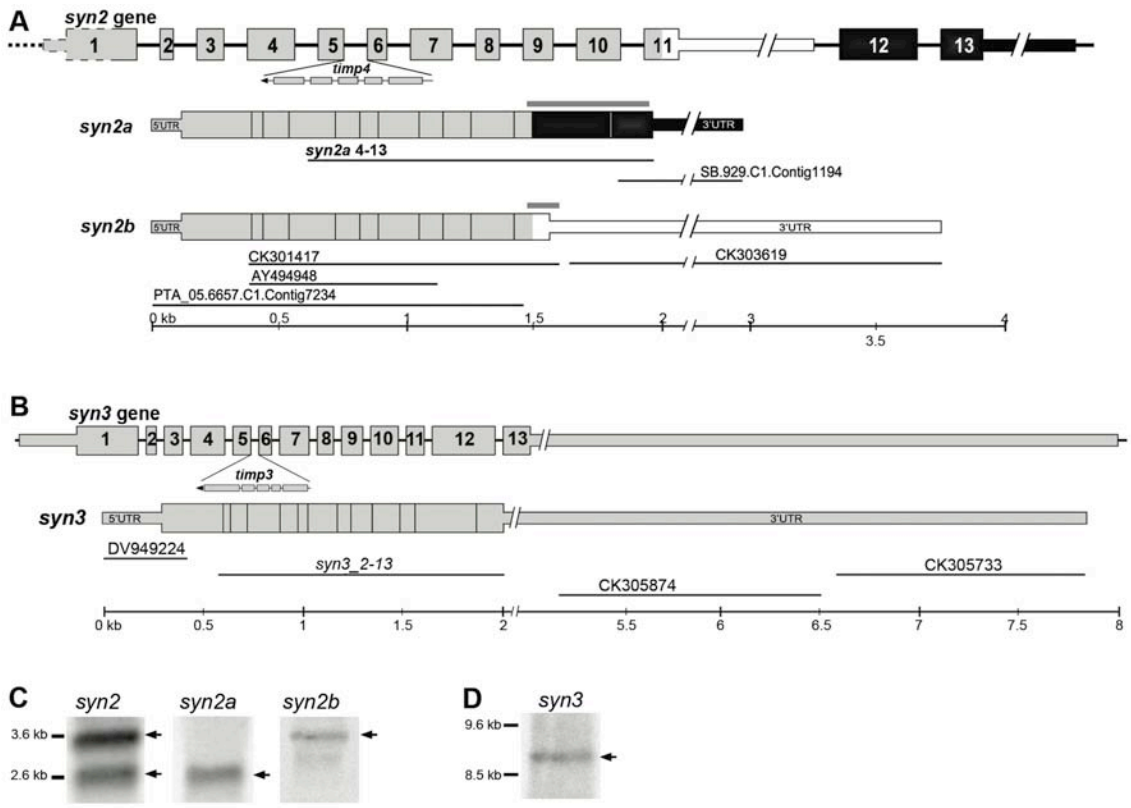


Fig 3.1. The *syn2* and *syn3* zebra finch homologues. A) Top, structure of the *syn2* gene in the zebra finch; middle, *syn2a* and *2b* transcripts. B) Top, structure of the *syn3* gene in the zebra finch; middle, *syn3* transcript. In A and B, numbers denote exons in the gene and the introns are not to scale. The lines underneath the transcripts indicate the various ESTs, contigs and PCR products used to derive the gene structure, as detailed in Methods, and the bottom line represents scales in kilobases. UTR indicates 5' and 3' untranslated regions. Notice the nested *timp* genes between exons 5 and 6 in both genes. C-D) Northern blots of total zebra finch brain RNA hybridized with ^{33}P-labelled *syn2*, *syn2a syn2b* (C), and *syn3* (D) antisense riboprobes. Molecular sizes of RNA markers are indicated on the left; arrows point to synapsin transcripts.

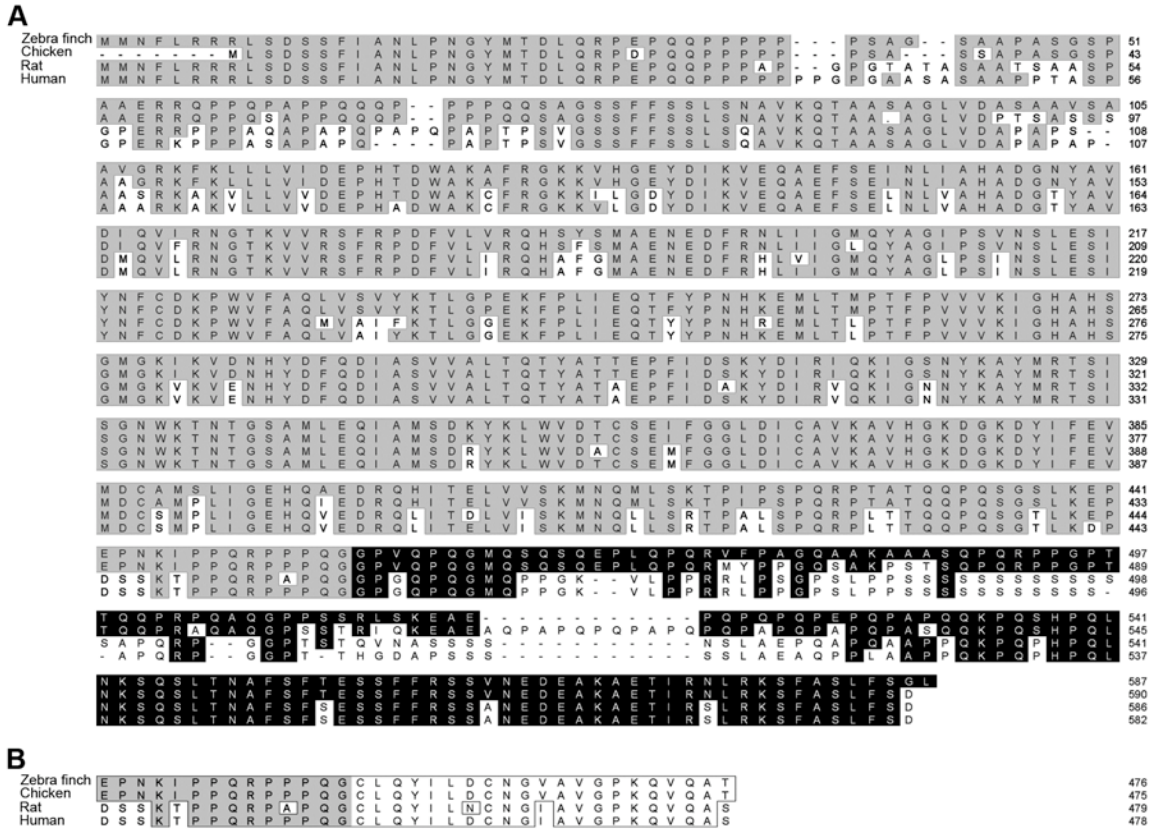


Fig. 3.2. Predicted aminoacid sequences of Syn2 isoforms. A) Alignment of the predicted amino acid sequence of zebra finch Syn2a protein with homologues from other species. B) Alignment of the predicted amino acid sequence of the Syn2b-unique domain with homologues from other species. The numbers on the right indicate the relative position when compared with homologues. Boxed domains represent conserved residues: grey represents regions common to *syn2a* and *2b*, black represents the *syn2a*-specific region, and white represents the *syn2b*-specific region.

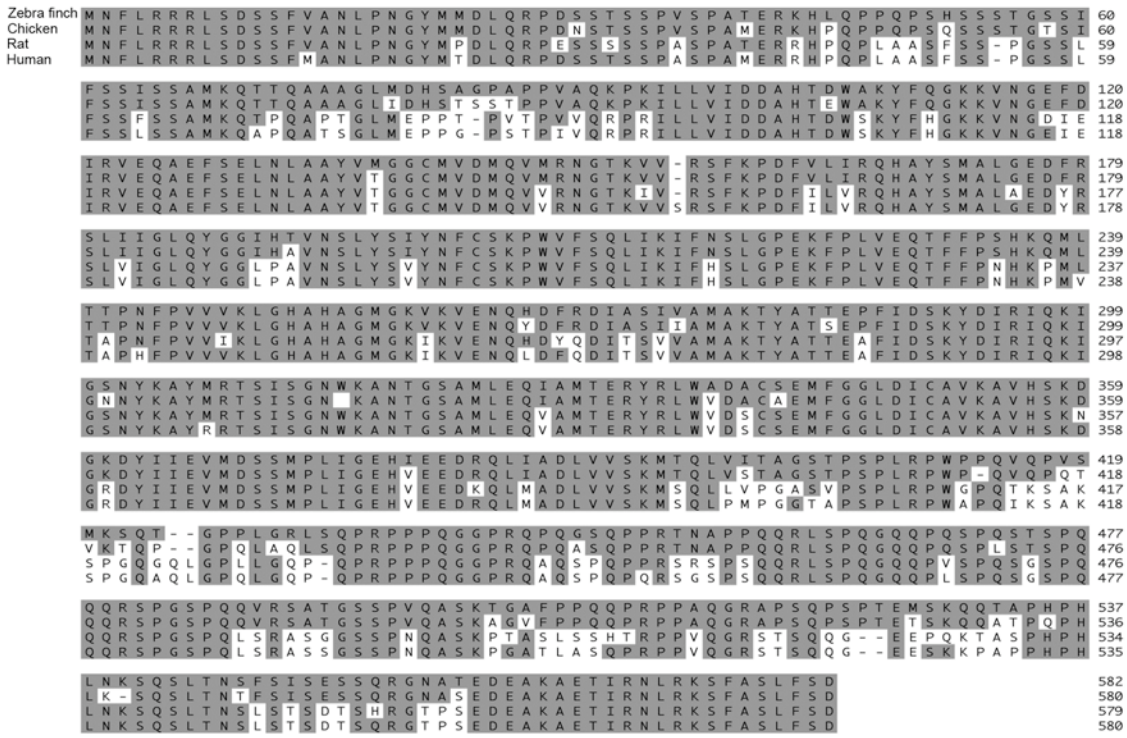


Fig 3.3. Predicted aminoacid sequence of the Syn3 protein. Alignment of the predicted amino acid sequence of zebra finch Syn3 protein with homologues from other species. Numbers on the right indicate the relative position when compared with homologues, grey represents conserved residues.

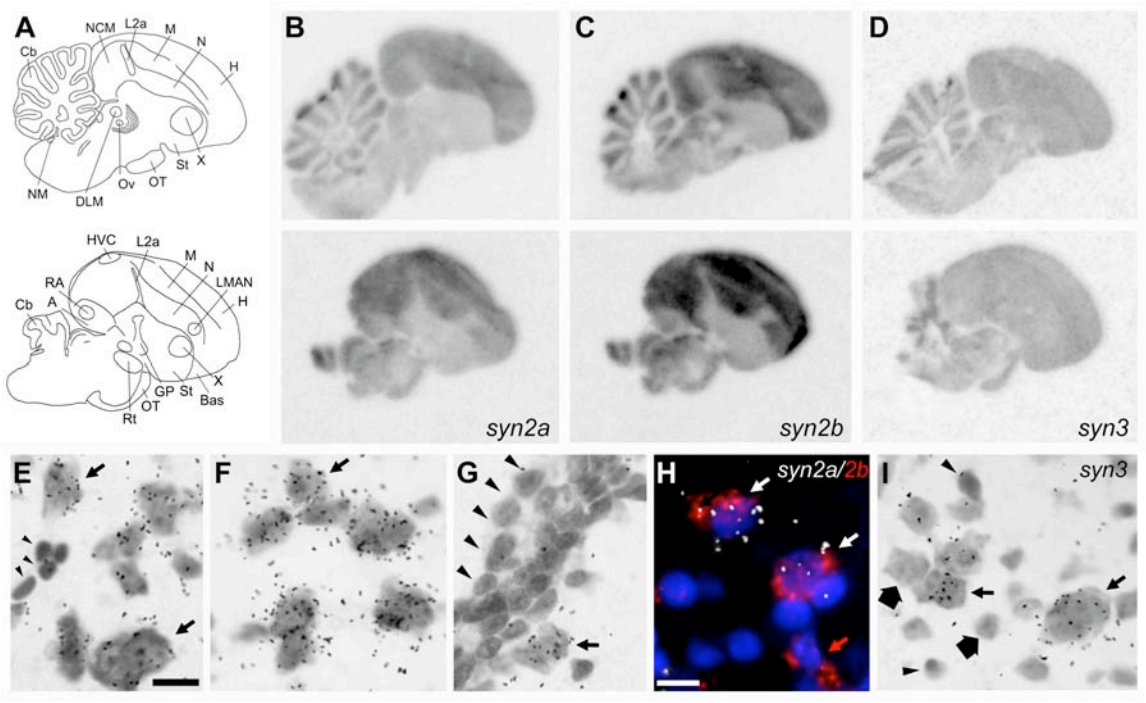

A
NCM
L2a
M
N
H
Cb
St
X
NM
DLM
Ov
OT
HVC
L2a
M
N
LMAN
H
RA
A
Cb
GP
St
X
OT
Bas
Rt
B
C
D
syn2a
syn2b
syn3
E
F
G
H
syn2a/2b
I
syn3

Fig 3.4. Expression of synapsins in the zebra finch brain. A) Camera lucida drawings of parasagittal brain sections at the level of NCM (top panel, ~800 μm from the midline) and at the level of the song control system (bottom panel, ~1800 μm from the midline). B-D) Autoradiograms depicting the expression of *syn2a*, *2b* and *3*, respectively. E-G) High-magnification bright-field view of emulsion autoradiography depicting cellular expression of *syn2a* and *b* in NCM and the lack of *syn2b* expression in the ventricle wall, respectively. H) High-magnification view of double *in situ* hybridization for *syn2a* (emulsion grains) and *b* (red fluorescence); white arrows point to double-labeled cells and red arrow indicates a single-labeled *syn2b*-positive cell. I) Emulsion autoradiography showing cellular expression of *syn3* in NCM. Small arrows indicate *syn3*-positive neurons, large arrows indicate *syn3*-negative neurons, and arrowheads indicate non-neuronal cells. A, arcopallium; Bas, nucleus basorostralis; Cb, cerebellum; CMM, caudomedial mesopallium; DLM, medial part of the dorsolateral thalamic nucleus; GP, globus pallidus; H, hyperpallium; HB, habenular nuclei; Hp, hippocampus; HVC, nucleus HVC of the nidopallium; L2a, subfield L2a of field L; LMAN, lateral magnocellular nucleus of the anterior nidopallium; M, mesopallium; MLd dorsal part of the lateral mesencephalic nucleus; N, nidopallium; NCM, caudomedial nidopallium; NM nucleus magnocellularis; OT, optic tract; Ov, nucleus ovoidalis; RA, robust nucleus of the arcopallium; Rt, nucleus rotundus; St, striatum; X, area X of the medial striatum. Scale bars: 10 μm.

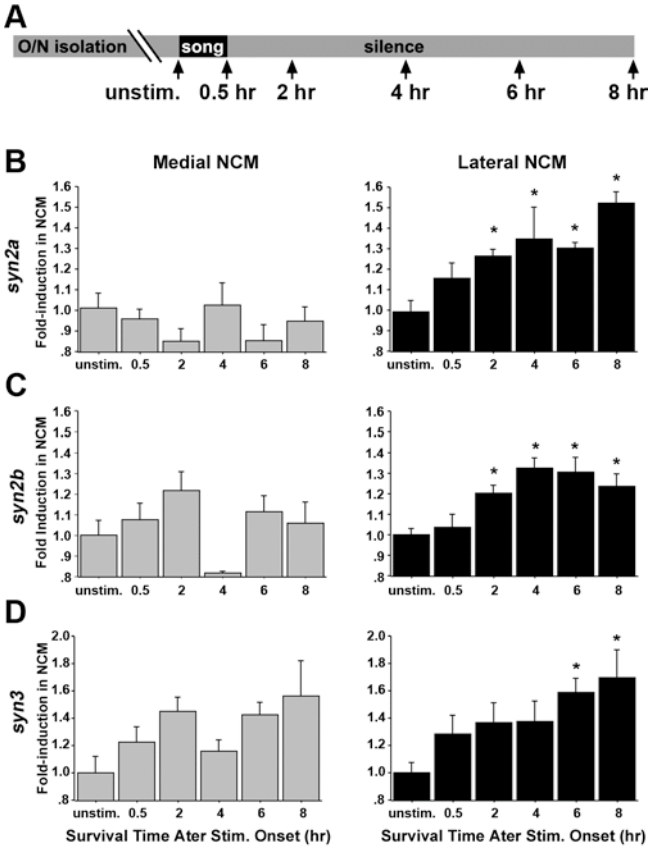

A
O/N isolation
song
silence
unstim.
0.5 hr
2 hr
4 hr
6 hr
8 hr
B
Medial NCM
Lateral NCM
syn2a
Fold-induction in NCM
C
syn2b
Fold Induction in NCM
D
syn3
Fold-induction in NCM
unstim. 0.5 2 4 6 8
Survival Time Ater Stim. Onset (hr)

Fig 3.5. Song regulation of the expression of synapsins in NCM. A) Schematic representation of the experimental design, arrows indicate time of sacrifice. B-D) Fold-induction values of *syn2a* (B), *syn2b* (C) and *syn3* (D) in the NCM of birds stimulated for 30 minutes and sacrificed at various time points after stimulus onset compared to unstimulated controls. Optical Density (O.D.) measurements were normalized against values from the NCM from unstimulated controls; n=3-6 birds per group. Plotted are means ± SEM from adult females. Medial and lateral NCM correspond to approximately 400 and 800 μm from the midline respectively. O/N, overnight; unstim., unstimulated controls; asterisks indicate statistically significant differences, for values see text.

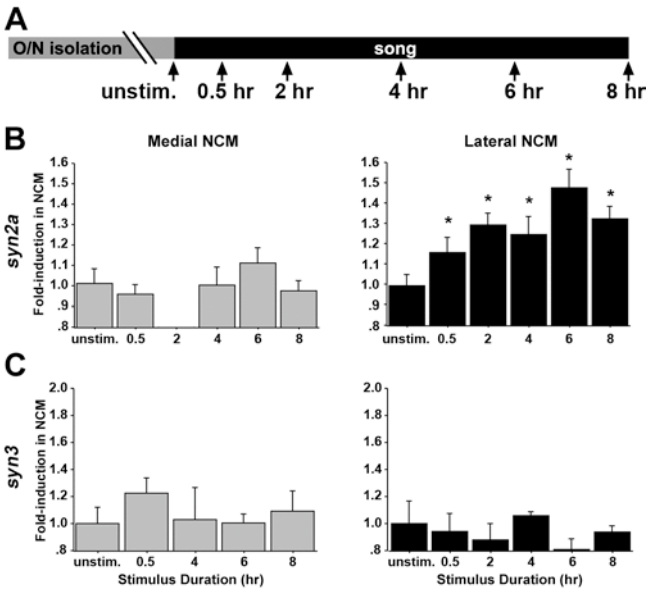


Fig 3.6. Expression of synapsins in birds exposed to increasing periods of stimulation. A) Schematic representation of the experimental design, arrows indicate time of sacrifice. B-C) Fold-induction values of *syn2a* (B) and *syn3* (C) in the NCM of birds stimulated for various stimulus durations and sacrificed immediately thereafter. Optical Density (O.D.) measurements were normalized against values from the NCM of unstimulated controls; n=3-6 birds per group. Measurements for the 2 hr time point at medial levels are not included due to accidental loss of material. Plotted are means ± SEM from adult females. Medial and lateral NCM correspond to approximately 400 and 800 µm from the midline respectively. O/N, overnight; unstim., unstimulated controls; asterisks indicate statistically significant differences, for values, see text.

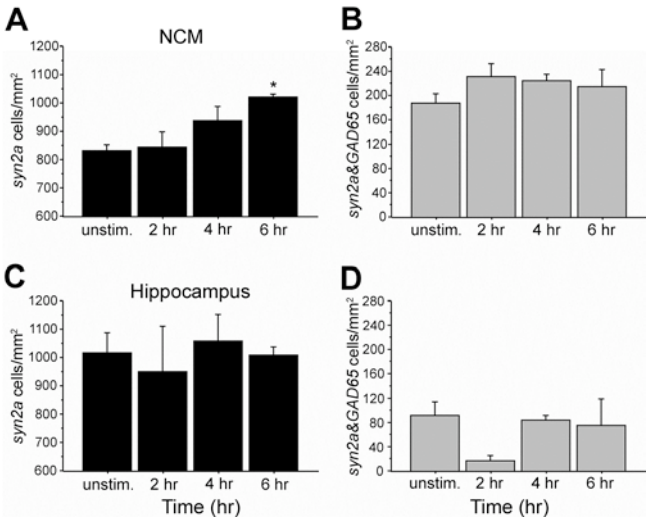


Fig 3.7. Changes in *syn2a*-expressing cells after song stimulation. A-B) The number of *syn2a*-expressing in NCM cells that were *gad65*-negative (A) or –positive (B) counted in birds killed after several intervals after the onset of a 30-min song stimulation period. (C-D) The same cells were counted in the hippocampus (C and D respectively). Plotted are means ± SEM from adult females; asterisks indicate statistically significant differences, for values, see text.

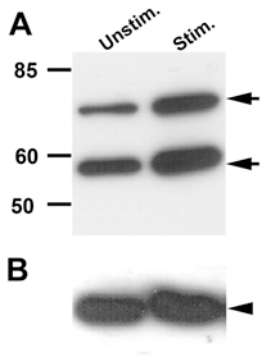


Fig 3.8. Song-induced changes in Syn2 proteins. Western blot of protein extracts from dissected auditory lobules from unstimulated controls and birds stimulated for 30 min and sacrificed 6 hr after stimulus onset, immunostained using anti-synapsin (A) and anti-actin (B) antibodies. Molecular sizes of protein markers are indicated on the left. Arrows indicate Syn2a and 2b proteins.

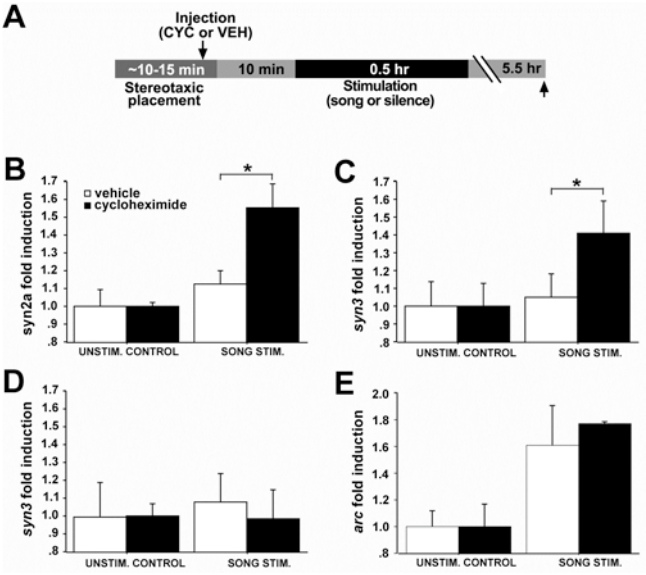
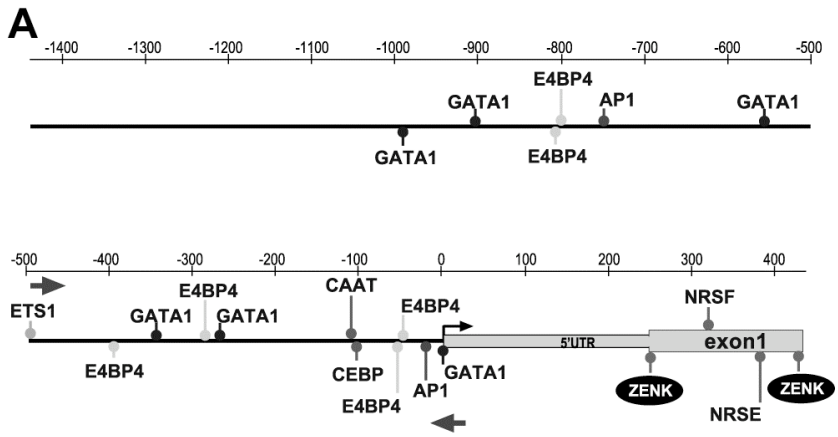


Fig 3.9. The regulation of synapsins by song is protein synthesis-dependent. A) Schematic representation of the experimental design, arrow points to time of sacrifice. B-E) Fold induction values of *syn2a* (B), *syn3* (C and D), and *Arc* (E) in vehicle- and cycloheximide-injected hemispheres from unstimulated controls and from birds stimulated with song for 30 min and sacrificed at 6 hr after stimulus onset. D depicts expression levels away (medially) from the injection sites. Plotted are means ± SEM from adult females; asterisks indicate statistically significant differences, for values, see text.


A
-1400
-1300
-1200
-1100
-1000
-900
-800
-700
-600
-500
E4BP4
GATA1
AP1
GATA1
GATA1
E4BP4
-500
-400
-300
-200
-100
0
100
200
300
400
ETS1
E4BP4
GATA1
GATA1
CAAT
E4BP4
NRSF
5'UTR
exon1
E4BP4
CEBP
AP1
GATA1
ZENK
ZENK
E4BP4
NRSE

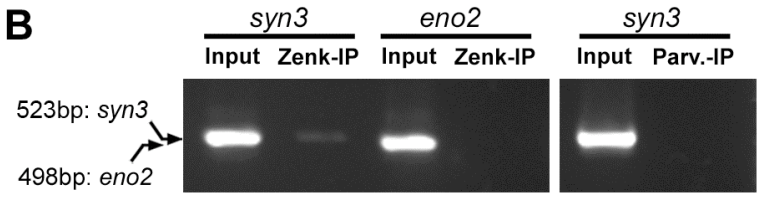

B
syn3
eno2
syn3
Input
Zenk-IP
Input
Zenk-IP
Input
Parv.-IP
523bp: syn3
498bp: eno2

Fig 3.10. Structure and *in vivo* interactions of the *syn3* promoter in the zebra finch. A) Schematic drawing of the *syn3* promoter region depicting the location of selected consensus motifs. Small arrow at bp 0 indicates putative transcription start site. B) Chromatin immunoprecipitation (ChIP) from the auditory lobule of adult females sacrificed 2 hr after the onset of a 30-min song stimulation period. Fragments representing portions of the *syn3* (523 bp, indicated by arrows in A) and *eno2* (498 bp) promoter regions could be amplified from total cross-linked chromatin before immunoprecipitation (Input samples). After ChIP, the *syn3* but not the *eno2* promoter fragment could be amplified from samples immunoprecipitated for ZENK (ZENK-IP), and the *syn3* could not be amplified from the sample immunoprecipitated for Parvalbumin (Parv-IP). AP1, Activator protein 1; CAAT1, Avian C-type LTR CCAAT box; CEPB2, CCAAT/enhancer binding protein; CREB/E4BP4, E4BP4, bZIP domain, transcriptional repressor; ZENK, early growth response 1.

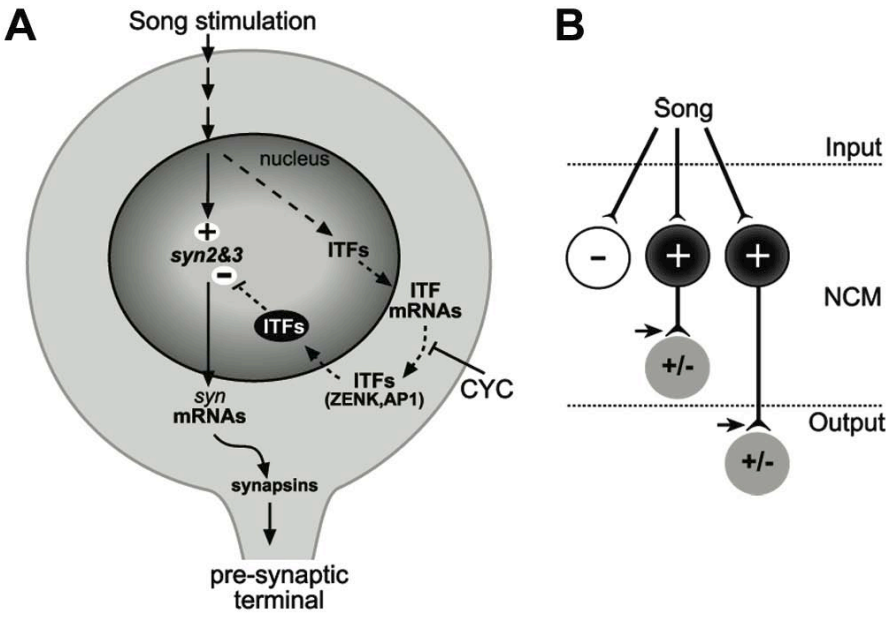

A
Song stimulation
nucleus
+
syn2&3
-
ITFs
ITF
mRNAs
ITFs
ITFs
(ZENK,AP1)
CYC
syn
mRNAs
synapsins
pre-synaptic
terminal
B
Song
Input
-
+
+
NCM
+/-
Output
+/-

Fig. 3.11. Models of song-regulated synapsin expression in NCM. A) Cellular model depicting direct and indirect pathways of synapsin regulation. The activation of song-responsive auditory neurons in NCM triggers the activation of intracellular signaling cascades (arrows) that upregulate the expression of the *syn2* and *3* genes (direct pathway) and of genes encoding inducible transcription factors (ITF; indirect pathway, indicated by dashed arrows). Song-induced ITFs (possibly including ZENK and AP1 - c-Fos and c-Jun) act by negatively regulating the expression of *syn* genes. The blockade of synthesis of song-induced proteins by cycloheximide (CYC) relieves the suppressive effects of ITFs on *syn* expression. B) Circuit model depicting cells that show song-induced *syn* upregulation. Song stimulation results in an upregulation of synapsins by song in excitatory NCM neurons, likely resulting in an increase in their concentration at pre-synaptic terminals of local NCM synapses or in NCM projection targets (output) such as CMM. + and – indicate excitatory and inhibitory neurons respectively; +/- indicate a generic target cell.

Chapter 4

Noradrenergic transmission regulates gene expression and long-term neuronal adaptation evoked by learned vocalizations

Introduction

The noradrenergic system modulates attention and alertness (Aston-Jones and Bloom, 1981b) (Foote et al., 1980), and noradrenergic transmission is required for the consolidation and recall of long-term memories (Murchison et al., 2004 McGaugh, 2000; Aston-Jones and Cohen, 2005; Ramos and Arnsten, 2007 McGaugh and Izquierdo, 2000), but the mechanisms are not clear. Importantly, projections from the locus coeruleus (LC), the main source of noradrenergic input to the brain, are required for sensory-induced daily fluctuations in the cortical expression of immediate-early genes (Cirelli et al., 1996; Cirelli and Tononi, 2000). Furthermore, protein synthesis blockers prevent hippocampal long-term synaptic potentiation induced by LoC stimulation (Stanton and Sarvey, 1985; Walling and Harley, 2004), implicating the noradrenergic system as a modulator of the gene expression that follows neuronal activation. We have therefore hypothesized that a fundamental role of the noradrenergic system is to couple neuronal activation to activation-induced gene expression. Such coupling would then initiate long-lasting changes in neuronal and circuit properties that may underlie long-term memories.

To examine this hypothesis, we have investigated the role of noradrenergic transmission in song-induced gene expression and long-term

electrophysiological changes of song-responsive neurons in a high-order auditory brain area of zebra finches. The zebra finch is a representative songbird, one of the few animals that learn their vocalizations based on auditory feedback (see Brenowitz et al., 1997b for a review). When finches hear song, activity-dependent genes like *zenk* and *arc* are rapidly and transiently induced in the telencephalon (Mello et al., 1992; Velho et al., 2005), particularly the caudomedial nidopallium (NCM), an area analogous to supragranular layers of the mammalian auditory cortex. The *zenk* gene encodes a transcription factor (Christy and Nathans, 1989) required for memory consolidation (Jones et al., 2001), while the *Arc* gene encodes an activity-regulated cytoskeleton-associated protein with a key role in synaptic plasticity (Guzowski et al., 2000). The induction of activity-dependent genes in NCM is higher for conspecific songs than for sounds of lower behavioral relevance (Mello et al., 1992; Velho et al., 2005), and is modulated by stimulus novelty, behavioral state, context and experience (see (Velho and Mello, 2007). NCM also has robust song-evoked responses that decrease upon repeated stimulus presentations (Chew et al., 1995). This decrease (originally called “habituation”) is a form of song-specific adaptation, since responses are readily restored upon introduction of a novel song. Adaptation is long-lasting (hours to days) depending on the stimulation protocol, and reflects a cellular trace of song perceptual memories. Importantly, local blockade of RNA or protein synthesis immediately after song stimulation disrupts long-term adaptation to song without interfering with its acquisition. This indicates that song-induced gene expression is required for long-term song adaptation, and possibly represents a critical link

between neuronal activation by song and long-term neuronal changes underlying song perceptual learning (Clayton, 2000; Mello et al., 2004). Nonetheless, regulatory pathways and mechanisms controlling song-induced gene expression and adaptation remain largely undefined.

The noradrenergic system is a likely regulator of song-induced gene expression, since the latter depends on novelty and attention. Here we show that *zenk*-expressing neurons in NCM are in close proximity to noradrenergic terminals and express α-adrenergic receptors. We also show that the antagonism of α-adrenergic transmission in NCM interferes with song-induced gene expression, without significant effects on spontaneous or evoked activity of song-responsive neurons. Moreover, local α-adrenergic antagonism during the period of adaptation to song disrupts the maintenance of the adapted state 20-22 hours later. These findings implicate noradrenergic transmission in coupling the electrophysiological and gene expression responses to song, and in the long-term adaptation of song-responsive NCM neurons. This effect could reflect the involvement of the noradrenergic system in the formation of a long-term perceptual memory for song and possibly other sensory stimuli at the cortical level.

MATERIALS AND METHODS

Animals All experiments used adult zebra finches (*Taeniopygia guttata*) that were obtained from our own breeding colonies at the OHSU and at Rutgers University, or from local breeders. The birds were kept under a 12:12 light:dark cycle; food and water were provided *ad libitum*. All procedures involving birds were approved by the Animal Care and Use Committees (IACUC) at the OHSU and Rutgers University, and are in accordance with NIH guidelines.

Song stimulation for gene expression analysis*:* Birds were isolated for about 18 hr in sound-attenuated chambers (≈76 X 31 X 28 cm). They were then stimulated with playbacks of a medley of three different conspecific songs presented in succession (each for 15 sec followed by 45 sec of silence), at 70 dB mean SPL at 35 cm from the speaker (essentially as in (Mello et al., 1992), for variable total durations according to the experiment. Controls consisted of birds that underwent the acoustic isolation and were not stimulated.

Tissue preparation for gene expression analysis: For *in situ* hybridization, the birds were sacrificed by decapitation 30 min after the start of the stimulation, and their brains were quickly dissected from the skull, frozen in Tissue-tek (Sakura

Finetek Inc., Torrance, CA, U.S.A) in a dry ice/isopropanol bath, and stored at -80°C. Parasagittal 10 μm sections were cut on a cryostat, thaw-mounted onto slides, and stored at -80°C until use. For immunohistochemistry, birds were sacrificed by Nembutal overdose 90 min after the start of the stimulation, and perfused transcardially with phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were then dissected out, washed overnight at 4°C in 0.1 M PB, cryoprotected by placement in 20% sucrose in 0.1 M PB at 4°C, and frozen in embedding medium (TissueTek; Sakura Finetek, Torrance, CA) in a dry-ice/isopropanol bath. Parasagittal 20 μm sections were cut on a cryostat, and thaw mounted onto Superforst Plus slides (Fisher Scientific, Pittsburg, PA, USA). Sections were dried out and stored at -80°C.

Immunohistochemistry (IHC): Brain sections were removed from the -80˚C freezer and dried overnight at room temperature. Slides were then equilibrated for 30 min at room temperature in 0.1 M PB, followed by incubation for 30 min in blocking solution (0.5% skim milk and 0.3% TritonX-100 in 0.1 M PB). The slides were then incubated for 15 min in avidin solution and for 15 minutes in biotin solution at dilutions recommended by the manufacturer (Vector blocking kit; Vector Laboratories, Burlingame, CA). Next, sections were incubated for 2 hours with one of the primary antibodies, either a rabbit anti-bovine-DBH (dopamine-beta-hydroxylase) antibody (1:1,000; Eugene Tech, Ridgefield Park, NJ), or a rabbit anti-egr-1 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA,

USA) that label respectively the DBH and ZENK proteins in the zebra finch brain (Mello et al., 1998a; Mello and Ribeiro, 1998), followed by a 2 hour incubation with a biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories). Slides were then incubated for 2 hours in avidin-biotin complex (ABC) reagent (Vector Laboratories). Each of the steps above was followed by three washes (10 minutes each) in 0.1 M PB, pH 7.4. The slides were then developed by incubation in 0.03% diaminobenzidine (DAB), 0.15% nickel-ammonium sulfate, and 0.001%H2O2 in PB followed by rinsing in 0.1 M PB, dehydration, and coverslipping with Permount. To assess the specificity of our detection system, ICC controls were run as described above but omitting incubations with either the primary or secondary antibodies. For the double-labeling procedure (DBH plus ZENK detection), the sections were reacted sequentially for the DBH or ZENK IHC, but the ABC reagent was inactivated by incubation in 0.3% H_2O_2 for 30 min**.** Importantly, the two antigens have very different cellular localizations and, therefore, their detection did not interfere with each other.

Clone isolation for in situ hybridization The *zenk* and *hat2* probes were derived from the cloned canary homologues described previously (George and Clayton, 1992; Mello et al., 1992). To isolate clones representing the zebra finch homologues of α−adrenergic receptors (ADRAs), we designed PCR primers for each ADRA subtype based on conserved domains within mRNA sequences from several species in GenBank and from sequences derived from the zebra finch genomic archives. The primers were used to amplify fragments from ADRA1a

(forward: 5’ggggctgattggttttggggtttt3’; and reverse: 5’ttgagcaggcgcacggagaagt3’), ADRA1b (forward: 5’tcttttcagccgtggatgtg3’; and reverse: 5’tctgggcagccgtggatgcg3’), ADRA1d (forward: 5’gtgggcgtgggggtctttcctg3’; and reverse: 5’ggcggcggcggctcctt3’); ADRA2a (forward: 5’gaccgctactggtccatcac3’; and reverse: 5’agcgtgtaggtgaaraagaa3’) ADRA2b (forward: 5’cagccagcgcccttcccaacac3’; and reverse: 5’tgcaccacatcccaccaaagta3’), and ADRA2c (forward: 5’cagaacctcttcctggtgtc3’; and reverse: 5’tasaggctgtagctgaagaa3’). Standard PCR reactions were performed with DNA from a zebra finch cDNA library (Holzenberger et al., 1997; Denisenko-Nehrbass et al., 2000). Two transcripts (ADRA1a and 1b) were not represented in our cDNA library and we instead used genomic DNA for the PCR amplification with primers targeting only the coding region of exon 1. The amplified products were excised from an agarose gel, eluted using Qiagen Gel Extraction Kit® (Qiagen Inc, Valencia, CA, USA), inserted into pPCRScript (Stratagene Inc., La Jolla, CA, USA) and used to transform bacterial cells following standard procedures. Insert identity was confirmed by sequencing and analysis using DNAStar software (DNAStar Inc., Madison, WI, USA) and BLAST searches.

Probe labeling for in situ hybridization*:* Plasmid DNA for all clones analyzed was isolated using Qiagen Miniprep Kit (Qiagen Inc. Valencia, CA, USA), linearized with the appropriate restriction enzymes, and purified with Qiagen PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). For radioactive procedures, we synthesized sense and antisense ^{33}P-labeled riboprobes as described in Mello et

al. (1997). For non-radioacive procedures, we prepared digoxigenin-(DIG) labeled ribroprobes as described in Velho et al. (2005). All riboprobes were purified in Sephadex G-50 columns and analyzed using a liquid scintillation counter or by visual inspection of a denaturing formaldehyde-agarose gel.

Radioactive in situ hybridization (ISH)*:* ISH using ^{33}P-labelled riboprobes for *zenk* and *hat2* was performed as previously described (Mello et al., 1997). Hybridized slides were exposed to a phosphorimager screen or processed for emulsion autoradiography followed by Nissl counterstaining. The hybridizations and washes for all probes analyzed were performed at 65^{o}C.

Double in situ hybridization (dISH): We combined the procedures for radioactive (for each *ADRA*) and fluorescent (for *zenk*) ISH, followed by nuclear counterstaining (Hoechst) and emulsion autoradiography to reveal the radiolabeled probe. The overall protocol was essentially as described in Jeong et al. (2005) and Velho & Mello (submitted).

Head post implants**:** For stereotaxic manipulations in the awake restrained preparation, birds were surgically implanted with head posts. For this purpose, birds were anesthetized with 50-55 mg/Kg IM of sodium pentobarbital, and placed in a stereotaxic apparatus containing a head post adapter. A round window was made on the skull by removal of the superficial layer of bone over the area that includes the bifurcation of the midsagittal sinus and NCM. A

chamber was formed and a head post was attached to the skull using dental cement (Dentsply International Inc., Milford, DE, USA). To allow for full recovery from anesthesia, further procedures were performed 24-48 hr after surgery.

Acclimation sessions: In order to minimize procedural induction of activity-dependent genes in the microinjection experiments for gene expression analysis, the birds were subjected to several acclimation sessions starting 24 hr after the head post implantation. Each session consisted of gently immobilizing the awake bird in a comfortable plastic tube, fixing the head post to the stereotaxic apparatus, maintaining the bird in the apparatus for 10 min (in the presence of a researcher), and returning it to the acoustic isolation chamber. Four such sessions (once every 2 hr) were performed on the day preceding the brain microinjections, and one last session on the day of the injections. The birds were kept in acoustic isolation chambers at all times, except during the acclimation sessions and microinjections. Both the acclimation and microinjections were performed in a sound-attenuated room with minimal sound exposure.

Brain microinjections: Four hours after the last acclimation session the birds were immobilized and their heads attached to the stereotaxic apparatus as in the acclimation session. The remaining layer of bone over NCM was then removed and a glass pipette lowered into NCM (AP: 0.5 mm; ML: 0.65 mm; DV: 1.2 and 1.6 mm). Phentolamine mezylate (300 mM in saline**;** Sigma Aldrich) was injected (50 nl/site over 2 min) into one hemisphere and vehicle in the contralateral

hemisphere, using a hydraulic microdrive (Narishige International Inc., Long Island, NY, USA). To facilitate the identification of the injection site, the pipettes were coated with fluorescent latex beads (Lumafluor Corp., Naples, FL, USA.). The birds were then released from the apparatus and returned to the isolation boxes. Starting 10 min after the injections, the birds were stimulated with conspecific song for 10 min and sacrificed by decapitation 30 min after stimulation onset. The brains were then processed for ISH for *zenk* and *hat2*.

Densitometry: Phosphorimager autoradiograms of sections hybridized for *zenk* and *hat2* were obtained with a Typhoon 8600 (Molecular Dynamics Inc., Sunnyvale, CA, USA) and quantified using NIH's ImageJ software. Densitometric measurements taken over specific brain regions were subtracted for slide background and resulting values averaged for two adjacent sections. For normalization, all densitometric values were divided by the average values from unstimulated controls. The values obtained in the injected areas of vehicle- and phentolamine-injected hemispheres of song-stimulated birds were normalized by the respective values from non-stimulated control birds. To examine the effect of drug treatment, we used ANOVA followed by Fisher's PSDL *posthoc* tests for pairwise comparisons, and a probability level of <0.05 for significance.

Cell counts: For quantitative analysis of our double ISH, we used Neurolucida software (MicroBrightField Inc., Colchester, VT) and estimated the number of labeled cells in specific areas of brain sections through NCM of song-stimulated

birds (n=3). We first placed a counting square of 200 X 200 μm over dorsal or ventral NCM, equidistant from NCM's rostral and the caudal boundaries (L2a and the ventricle, respectively). We then centered a counting circle (12 μm diameter) over each fluorescent-labeled *zenk*-expressing cell. We next counted the number of emulsion autoradiography grains per labeled cell. We note that the typical nuclear diameter of most cells counted was in the 6-10 μm range and that the diameter of the counting circle was determined to be inclusive of the majority of grains associated with the cells. Cells with grain counts of at least 2 standard deviations above background levels (measured over the glass or over the neuropil between labeled somata – see Fig 4.6 for an example) were considered *ADRA*-positive.

Electrophysiological recordings

One day after the head post implantation surgery, each bird was acoustically isolated for a day. Electrophysiological recordings then began 48 hr after the initial surgery, allowing for full recovery from anesthesia. Recordings were made in an acoustically isolated sound booth. The awake animal was immobilized in a comfortable tube and the head post was fixed to a stereotaxic device. A square grid with holes spaced at 250 μm intervals (Electron Microscopy Sciences, Fort Washington, PA) was cemented to the inner layer of the skull over a craniotomy that exposed the brain surface in order to define the position of electrode penetrations along the caudo-rostral and medio-lateral axes. Recordings were made at 7 sites (3 in the left hemisphere, 4 in the right) using

seven quartz-platinum/tungsten microelectrodes (1-4 MOhms impedance), controlled by a multielectrode microdrive (Eckhorn model, Thomas Recording, Giessen, Germany). White noise stimuli with the amplitude envelope of canary song were presented to search for responsive sites that indicated the dorsal border of NCM.

Testing the short-term effect of phentolamine microinjection on electrophysiological responses in NCM

After responsive sites were found, the bird was trained by presenting a set of 3 novel conspecific songs (recorded from animals that the subject bird had never heard before), each repeated 100 times in a block at an inter-stimulus interval of 8 sec. Multi-unit recordings (filters: high pass, 500 Hz, low pass 5 kHz) were made through the microelectrodes while song stimuli were played through a speaker (0.5 m from the bird's ears) at an amplitude of 70 dB SPL, under computer control (Spike2 version 5.18, Cambridge Electronic Design, Cambridge, UK).

After the training was completed, a glass micropipette (tip I.D. ~30 mm) was driven into the right hemisphere through an adjacent grid hole to approximately the same depth as the electrodes, and phentolamine (phentolamine mesylate, Sigma-Aldrich, 300 mM in distilled water) was administered with a microinjector system (Drummond Scientific Wiretrol, Broomall, PA) advanced by a hydraulic microdrive (Narishige). Each animal received an initial loading dose (30 nl injected over 1.5 min), followed by maintenance doses (2 nl every 1min) for the

duration of the auditory stimulus trials. Spontaneous activity was recorded continuously for several minutes before and after the first injection. Then, neural responses were recorded during playback of a set of 25 repeats of each of 6 conspecific songs (the 3 previously trained songs and 3 novel songs), presented in shuffled order.

At the conclusion of recording, three small electrolytic lesions were made in each hemisphere to enable histological reconstruction of recording sites. Animals were then given a lethal dose of anesthetic and perfused with saline, followed by paraformaldehyde. Their brains were cut into 50 μm sections that were stained with cresyl violet. The lesion sites were confirmed histologically and then the other recording sites were located through their relative distance to the confirmed lesion sites, using grid position and depth coordinates. NCM is located within the caudo-medial area of the forebrain and has larger, more loosely packed cells compared to field L, located rostral to NCM (Fortune and Margoliash, 1992). Recording sites found to be in field L2 and the transition areas between field L2 and NCM were excluded from analyses.

Testing the long-term effect of phentolamine microinjection on electrophysiological responses in NCM

After responsive sites were found, a glass micropipette filled with phentolamine was driven through a grid hole that targeted one of a cluster of responsive sites in one hemisphere to approximately the same depth as the electrodes. These experiments used a lower concentration of phentolamine (150

mM). Each animal received an initial loading dose (30 nl in 1.5 min), followed by maintenance doses (6 nl every 1 min) for 30 min. The same amount of vehicle was injected into the equivalent position in the contralateral hemisphere as control. Three birds received phentolamine in the right hemisphere and 3 received phentolamine in the left hemisphere. The bird was then returned to its cage and, after a 10 min interval, trained with playback of a series of 8 conspecific training songs, each repeated 200 times at an interval of 8 sec.

The next day, 20-21 hr after song training, the bird was again positioned in the stereotaxic apparatus. Electrodes were lowered into each hemisphere at the same grid positions as in the initial recordings. Recordings were made while playing 16 songs (8 training songs heard just after the injections on the previous day and 8 novel songs). Twenty-five repeats of each song in a shuffled order were presented in 4 groups of 4 songs each (2 training and 2 novel songs). At the conclusion of recording, three small electrolytic lesions were made in each hemisphere and the recording sites were confirmed histologically in NCM, as described above.

Electrophysiological data analysis

Multi-unit responses (typically 5-10 units) were recorded at each site in order to enable valid comparisons between conditions (before vs. during drug administration) because these responses are stable over a period of 1-2 hr. Single-units cannot easily be held for the necessary time period in the awake preparation. Multi-unit neural responses to song stimuli were quantified as the

difference between the root-mean-square (RMS) values obtained for each electrode during a response window (from stimulus onset to stimulus offset plus 100 ms) and the RMS during the control period of each trial (a 500 ms window occurring prior to stimulus onset). To compute the RMS, each digitized value is squared, the mean of these squares over the response interval is computed, and the square root of that mean is taken. This provides a method of rectifying the multi-unit activity and computing its average power. The RMS amplitude of the response to each stimulus was quantified as the average of 5 stimulus presentations; the 2^{nd} to 6^{th} repeats were used because the very first presentation of each stimulus had a high variance.

In short-term experiments, response amplitudes (averaged across sites in each hemisphere) to a novel set of songs heard before injection were compared to responses to another novel set heard during phentolamine injection. A two-way ANOVA used stimulus set (first vs. second) and hemisphere (control vs. phentolamine) as factors. A second analysis compared averaged response amplitudes in each hemisphere for a set of songs when they were novel (before injection) with amplitudes when they were recently familiar 1 hr after training (during phentolamine injection). A one-way ANOVA used hemisphere as the factor and the two tests with same stimuli as repeated measures.

The adaptation rate for each song at each site was determined by finding the slope of the regression between response amplitude and repetition number over the linear portion (repetitions 6-25). The slope was normalized by dividing it by the mean response amplitude over the same responses (repetitions 6-25) to

produce the adaptation rate (Phan et al., 2006). In effect the rate represents the percentage drop in response amplitude per stimulus repetition. These rates were compared between song sets and hemispheres and song sets using a two-way ANOVA as described for amplitude analyses above.

Spontaneous neural activity was measured at each site as the mean of the rectified multi-unit activity recorded over a 1min window at a fixed time before and during phentolamine injection in both hemispheres in each animal. The ratios of spontaneous activity before and during phentolamine injection were computed and averaged across each hemisphere, then compared with the Wilcoxon matched pairs test.

The strength of the neuronal memory for any song can be measured by presenting that song in a shuffled set with novel songs and computing a familiarity Index (FI). The FI for a given test song is the ratio of the mean adaptation rate for the novel songs to the adaptation rate for the test song at the same site (Phan et al., 2006). If the test song is not familiar, its adaptation rate will be similar to the rate for novel songs and the FI will be near 1.0. If the test song is familiar, its adaptation rate will be lower than rates for novel songs and the FI will be greater than 1.0 (Chew et al., 1995; Phan et al., 2006). In testing the long term effect of phentolamine injection in NCM during training, the FI for each training songs was computed at each site, then averaged across sites in each hemisphere. The Wilcoxon matched pairs test was used to compare adaptation rates and FI values between the hemispheres.

As described for each analysis above, non-parametric statistical tests were

used for most comparisons, due to the small sample of the electrophysiology experiments. In cases where a parametric method was required, normality of distributions and homogeneity of variances were verified with the Kolmogorov-Smirnov and Levene's test, respectively. The criterion for statistical significance was set at $p<0.05$, two-tailed. Statistical computations were carried out in Statistica (Statsoft).

Results

Noradrenergic innervation of NCM

We have previously used immunostaining for the noradrenergic marker dopamine β-hydroxilase (DBH) to show that NCM contains noradrenergic fibers (Mello et al., 1998a). To further investigate the noradrenergic innervation of NCM and how it relates to song-induced gene expression, we double immunostained serial parasagittal brain sections from song-stimulated birds using antibodies against the noradrenergic marker dopamine beta-hydroxylase (DBH) and ZENK protein. We observed that ZENK-expressing neurons as revealed by nuclear staining (Fig 4.1A, arrows) are in close proximity to DBH-stained fibers (Fig 4.1A, arrowheads). Camera lucida reconstructions of the DBH and ZENK staining revealed a relatively dense network of both noradrenergic fibers (Fig 4.1B red traces) and ZENK-expressing cells (Fig 4.1B, black circles) throughout NCM. The density of labeling for both markers decreased from medial to lateral levels (Fig 4.1B, left to right), particularly at a centro-rostral region corresponding to field L2a, which lacks song-induced ZENK expression.

Noradrenergic modulation of *zenk* expression in NCM

To examine whether the noradrenergic system modulates the song-induced

expression of activity-dependent genes in NCM, we administered α- or β-adrenergic receptor antagonists (phentolamine or propranolol, respectively) systemically to adult female zebra finches. Birds were sound-isolated overnight, received a first drug or vehicle (saline) IM injection 10 min prior to stimulation onset and a second dose 15 min after the onset of stimulation (Fig 4.2A), and were sacrificed at the end of the stimulation period (30 min). As expected, *in situ* hybridization (ISH) revealed marked *zenk* induction in the NCM of vehicle-treated birds (Fig 4.2C, compare top vs. bottom left panels). In contrast, *zenk* mRNA levels in song-stimulated phentolamine-injected birds were comparable to those in unstimulated controls (Fig 4.2C, compare top and bottom right panels of birds injected with 20 mg/kg of phentolamine), indicating that the systemic blockade of α-adrenergic receptors inhibits song-induced gene expression in the auditory telencephalon. A quantitative analysis showed a significant decrease in *zenk* induction in phentolamine-injected birds compared to saline injections (Fig 4.2D; ANOVA, $p<0.0001$). Importantly, this reduction is observed at relatively low doses (5 mg/Kg, n=3), and no significant differences are seen between control and stimulated birds at higher doses (Fisher's PSLD, $p= 0.83$ for 20 mg and 40 mg combined, n=4 respectively). For propranolol, we observed only a partial decrease of song-induced *zenk* expression (not shown; ANOVA, $p<0.001$) at a very high dose (3 μM, compared to effective doses in the 10-300 nM[2] range in different learning paradigms, including inhibitory avoidance in chicks; Gibbs &

[2] When systemically injected these concentration did not significantly block *zenk* expression.

Summers, 2002) indicating a lesser involvement of this receptor subtype in modulating song-induced gene expression. Importantly, these high doses of propranolol led to undesirable behavioral side-effects such as light-sedation and trembling that were absent in phentolamine injected birds.

To determine whether song-induced gene expression in NCM is dependent on local α-noradrenergic transmission, we injected phentolamine into the NCM in one hemisphere of awake-restrained birds (Fig 4.3B), and vehicle (saline) in the contralateral hemisphere. We then presented the injected birds with song playbacks (song-stimulated birds) or maintained them in silence (unstimulated controls). ISH revealed marked hemispheric differences in the expression of *zenk* and *c-fos* in song-stimulated birds (Fig 4.3C, left and middle, respectively). In contrast, expression of *hat2* (the canary homolog of n-chimaerin), a gene that is enriched in the songbird forebrain but not regulated by song (George and Clayton, 1992; Jarvis et al., 1995; Velho et al., 2005), showed no apparent differences between phentolamine- and vehicle-injected hemispheres in the same birds (Fig 4.3C, right panels). Densitometric analysis revealed that *zenk* and *c-fos* were induced normally in saline-injected hemispheres of song-stimulated birds, reaching ~2-fold induction when normalized to saline-injected hemispheres of unstimulated controls (Fig 4.3D, left and middle, white columns). In contrast, *zenk* and *c-fos* expression levels in phentolamine-injected hemispheres of song-stimulated birds did not show significant induction when normalized to the phentolamine-injected hemispheres of unstimulated controls (Fig 4.3D, grey columns; fold-induction ~1), indicating

that phentolamine injections abolished the song-induced expression of these mRNAs. In contrast, *hat2* expression analyzed in adjacent brain sections showed no induction in song-stimulated birds and no significant differences between vehicle- and phentolamine-injected hemispheres (ANOVA, p= 0.41).

α−adrenergic blockade in NCM does not affect auditory responses

To test for the immediate effects of α−adrenergic blockade on electrophysiological properties of NCM, we used methods previously developed to show drug effects on inhibitory neurotransmission in this brain area (Pinaud et al., 2008b). We inserted multiple microelectrodes into NCM in the left and right hemisphere of awake, restrained adult male zebra finches (n=7), and then recorded multi-unit activity while microinjecting phentolamine continuously near the electrodes in one hemisphere. When we compared the mean level of spontaneous bursting activity (typical of NCM) before and during phentolamine injection, we saw no change in activity in either hemisphere (Fig 4.4B). The ratio of activity at each electrode (amplitude before injection divided by amplitude during injection) did not differ between hemispheres (Control 1.02 ± 0.01 vs. Drug 1.01 ± 0.01, mean±S.E.; Wilcoxon, n=7 birds, Z=0.68, p=0.499).

In order to assess the effect of α−adrenergic blockade on song-evoked responses, we played a set of novel songs (Song set A) to the bird before the injection and then played those same songs together with another set of novel songs (Song set B) during phentolamine injection (Fig 4.4A). First, we compared the amplitude of multi-unit responses in both hemispheres to the novel set heard

before injection to the response amplitude of the other novel set heard during phentolamine injection (Fig 4.4C). An ANOVA showed that, although there was a significant difference in absolute responses between the two song sets (different songs elicit different response levels at any given NCM recording site), there was no difference between the hemispheres and no interaction between the song set and side of recording (Stimuli: $F=7.0$, $p=0.01$; hemisphere: $F=0.3$, $p=0.61$; Interaction: $F=0.02$, $p=0.88$). Second, we compared the amplitude of multi-unit responses in both hemispheres to the same set of songs when they were novel (before injection) and when they were recently familiar (1h later during injection). As expected, an ANOVA showed that the responses were significantly lower to the songs when familiar, demonstrating the immediate adaptation effect that is typical of NCM ($n=7$ birds; Stimuli: $F=1.83$, $p=0.02$; hemisphere: $F=1.12$, $p=0.31$; Interaction: $F=0.75$, $p=0.41$). Importantly, there was no difference between the hemispheres and no interaction between the familiarity of the song set and the side of recording (Fig 4.4D). We also compared the normalized adaptation rates for these same songs between the hemispheres before and during phentolamine injection, and found the same overall pattern of effects (not shown).

These results demonstrate that phentolamine injections do not significantly affect ongoing activity in NCM or auditory responses to song stimuli. Further, they show that the immediate process of stimulus-specific adaptation is normal in the presence of phentolamine: responses to repeated stimuli decrease and stay low for at least 1 hr. This suggests that the action of NE at α−adrenergic receptors does not make a significant contribution to ongoing

activity, auditory responses, or immediate adaptation.

α−adrenergic blockade in NCM affects long-term adaptation in NCM

To assess the contribution of α−adrenergic modulation to long-term adaptation in NCM, we employed procedures that were previously used to demonstrate that new RNA and protein synthesis in NCM are required for long-term adaptation to be maintained for 40 hr or more (Chew et al., 1995). We carried out preliminary recordings to locate NCM, then injected phentolamine into one hemisphere and vehicle into the other. We then presented a set of novel training songs. On the following day (20-21 hr later), we recorded from multiple microelectrodes placed in clusters around the injection site in each hemisphere while we played back the training songs together with a set of novel songs (Fig 4.5A).

Because stable recordings from the same multi-unit sites cannot be made over >20 hr to measure changes in response amplitude, we used the previously established method for assessing neuronal memory for songs, based on comparing the adaptation rates to test songs with rates for novel songs to compute a familiarity index (FI), as detailed in the Methods (Chew et al., 1995; Phan et al., 2006). An FI greater than 1.0 indicates that a test song is familiar in NCM. Using this method, we found that the FI for training songs in the control (vehicle-injected) hemisphere was significantly higher (Fig 4.5B) than in the experimental (phentolamine-injected) hemisphere (Control, 1.66 ± 0.28 vs. phentolamine, 1.26 ± 0.16, mean ± S.E.; n=6, Wilcoxon Z=2.20, p=0.028). The

adaptation rates to the novel songs used in computing the FI did not differ between hemispheres (Control -0.41 ± 0.08 vs. phentolamine -0.38 ± se0.07; Wilcoxon, n=6 birds, Z=0. 31, p=0.75) so the FI difference is due to the higher rates for training (familiar) songs in the phentolamine hemisphere. Training songs are clearly familiar for sites in the control hemisphere, but almost novel for sites in the phentolamine hemisphere. A hypothetical FI of 1.0 (indicating no familiarity) lies within the lower 95% confidence interval for phentolamine (0.862), suggesting that drug treatment may not only have reduced the long-term familiarity of the training songs but may have abolished it.

This result indicates that α−adrenergic blockade with phentolamine interferes with the maintenance of the initial adaptation to a repeated song over a long period. A necessary component of this process is located within the phentolamine diffusion zone that produces an effective blockade. We have not measured the extent of this zone, but at least it does not extend to the opposite hemisphere, 1-1.5 mm away. Taken together, the findings in the short and long term experiments are consistent with a role for α−adrenergic modulation in long-term memory formation.

Song-responsive NCM cells predominantly express α-1-adrenergic receptors

The experiments above clearly established that α-adrenergic transmission play important roles in the physiology of NCM. To start to address the cellular mechanisms associated with these actions, we next determined what α-

adrenergic receptors are expressed in NCM. For this purpose all known receptor subtypes (ADRA1a, 1b, 1d, 2a, 2b and 2c) were examined by *in situ* hybridization. Based on emulsion autoradiography, we found that ADRA1d is more strongly expressed in NCM compared to the other α-adrenergic subtypes (Fig 4.6B and C, ADRA1d and 1a, respectively). Cellular analysis using double *in situ* hybridization revealed that although some ADRA1d-expressing cells are *zenk*-negative (Fig 4.6D arrowhead), ADRA1d is mostly expressed in *zenk*-expressing (song-responsive) neurons (4.6D arrows). Quantitative analysis (n=3 birds) showed that on average 65% of the *zenk*-expressing cells in NCM also express the ADRA1d subtype. This level of cellular co-expression is much (at least ~3-fold) higher than those of all the other receptor subtypes (1a: 23%; 1b: 15%; 2a: 11%; 2b: 19%; 2c: 8%). Altogether, these data give strong indication that the local noradrenergic transmission in NCM occurs predominantly through ADRA1d.

Discussion

We have shown that noradrenergic transmission is required for the induction of activity-dependent genes triggered in central auditory neurons when songbirds hear conspecific song. This action is mediated mostly through α-adrenergic receptors and occurs in the absence of immediate changes in spontaneous or song-evoked electrophysiological activity. The local blockade of α-adrenergic transmission also prevents the establishment and/or maintenance of long-term (but not short-term) electrophysiological adaptation triggered by repeated song presentation. Altogether, these data indicate that the noradrenergic system couples song-evoked electrophysiological responses to activity-dependent gene expression, thereby resulting in long-lasting changes in the neuronal response to song stimulation. We propose that this action may represent a general mechanism whereby the noradrenergic system modulates the consolidation of long-lasting synaptic changes and the memorization of sensory events in the vertebrate brain.

Noradrenergic transmission is required for activity-dependent gene expression

The locus coeruleus (LoC) is the main source of NE in the vertebrate

central nervous system (Moore and Bloom, 1979). Chemical lesions of the LoC with the neurotoxic agent DSP-4 in rodents dramatically reduce the induced cortical expression of the activity-dependent genes *zenk* (*zif-268*) and *c-fos* during the awake period of the sleep-wake cycle (Cirelli et al., 1996). A similar manipulation disrupts singing-induced *zenk* expression in the basal ganglia nucleus of the song control system of male zebra finches (Castelino and Ball, 2005). These observations have suggested a role for NE in regulating activity-dependent gene induction in the brain. Due to the widespread connections of the LoC, however, it is unclear whether the effects of LoC lesions on inducible gene expression are due to a direct dependence on noradrenergic transmission or rather reflect changes in overall arousal/attention that could indirectly influence neuronal activity in the brain. Furthermore, other modulatory agents that interact with the noradrenergic system (e.g., dopamine) may be affected by noradrenergic depletion and could contribute to mediating the effects of LoC ablation.

The results of our local application of the antagonist phentolamine provide direct evidence that intact α-adrenergic transmission in an auditory telencephalic area (NCM) is required for normal song-induced expression of activity-dependent genes. Based on the presence of a dense noradrenergic innervation in NCM (and the lack of evidence for adrenaline releasing terminals in NCM), the observed effect was most likely due to the blockade of α-adrenergic receptor activation by NE released from noradrenergic terminals. Consistent with this possibility, we have observed that local application of NE itself can induce *zenk*

expression in NCM in a dose-dependent manner (not shown). Furthermore, even though we cannot entirely rule out a contribution of β-adrenergic transmission, the relative ineffectiveness of the systemic application of propranolol supports the view that the effects of NE in NCM are mostly exerted through local α-adrenergic receptors.

The blockade under phentolamine and the effect of NE application indicate that NE exerts a positive effect on song-induced gene expression in NCM. Such an action suggests that NE may act on α-1-adrenergic receptors in song-responsive NCM neurons. These receptors are coupled to $G_{\alpha q}$-protein, which is a known activator of phospholipase C (PKC) that leads to inositol triphosphate (IP3) and dyacylglycerol release. The activation of this signaling pathway could then increase intracellular Ca^{+2} levels or PKC activation, which could then result in the induction of activity-dependent genes. Alternatively, α-1-the activation of these receptors may also trigger the activation of the Src family of tyrosine kinases, which in turn activate ERK1/2 (Lindquist et al., 2000). Indeed, we found that ADRA1d is the most abundant α–-adrenergic subtype in NCM, and that α-2-adrenergic receptors, which are coupled to the inhibitory $G_{\alpha i}$-protein (Strosberg, 1993), are expressed at very low levels in this region (data not shown). More importantly, our cellular analysis showed that ADRA1d is the main subtype expressed in song-responsive cells. These results give a strong indication that locally released NE acts on α-1-d adrenergic receptors to induce gene expression in song-responsive NCM neurons.

Noradrenergic coupling of electrophysiological and gene expression responses to song stimulation

Phentolamine applied locally to NCM interferes with the gene induction response without affecting NCM's electrophysiological responsiveness to song stimulation. This observation indicates that noradrenergic transmission does not act by directly modulating the firing properties of NCM neurons. Rather, local α-adrenergic antagonism can uncouple the electrophysiological and genomic responses that normally occur in song-responsive neurons. The corollary is that the normal coupling of these two responses requires the activation of α-adrenergic receptors. We propose a simple model (Fig 4.7) whereby this could be accomplished. The model posits that inputs from the ascending auditory pathway and from noradrenergic projections, both of which are activated by song auditory stimulation, converge onto song-responsive NCM neurons and are required for song-induced gene expression. In support of this model, NCM receives ample projections from preceding centers in the auditory pathway (Vates et al., 1996), as well as a rich noradrenergic innervation in close proximity to *zenk*-expressing cells. In addition, *zenk*-expressing cells express α-adrenergic receptors (mainly ADRA1d). The fact that gene expression patterns reflect the acoustic properties of the song stimulus indicates that the auditory input is also an important determinant of induced gene expression and must somehow interact with the noradrenergic input, but the nature of this interaction is unclear.

The model also includes other known effects: the blockade of long-term

adaptation by RNA synthesis inhibition with actinomycin-D and the protein synthesis with cycloheximide (Chew et al., 1995). Although we did not study specific intracellular signaling pathways, previous studies have shown that song-induced gene expression in NCM requires the activation of MAP kinase (Cheng and Clayton, 2004; Velho et al., 2005). The latter could be downstream of adrenergic receptor activation through Ca^{+2}-dependent cascades or Src activation. In sum, we propose that noradrenergic inputs activated by song stimulation act in concert with input from the ascending auditory pathway to result in song-induced gene expression in central auditory neurons. Such a model would help to explain the effect of modulatory variables on song-induced gene expression (e.g., why a strong stimulus like a foot-shock can modulate song-induced gene expression but fails to induce activity-dependent genes in NCM in the absence of auditory stimulation (Jarvis et al., 1995). Similarly, NE release due to the activation of noradrenergic projections from the LoC would likely be maximal for novel stimuli due to their high attentional salience, resulting in the higher levels of induced gene expression for novel than familiar songs.

NE and the consolidation of song-induced cellular changes

NCM responses to familiar song stimuli adapt in a manner that is long-lasting, song-specific, and dependent on song-induced gene expression for its long-term maintenance (Chew et al., 1995). This phenomenon is a clear example of an experience-dependent cellular change in song-responsive circuitry, and a candidate cellular substrate for song auditory memories. However, the specific

synaptic mechanisms involved are presently unclear. Our current data clearly show that long-term adaptation is dependent on intact α-adrenergic transmission in NCM during the song stimulation that leads to the adaptation. Since phentolamine has no effects on spontaneous or song-evoked electrophysiological activity, this dependence is not due to a noradrenergic modulation of the immediate electrophysiological response to song in NCM. In addition, phentolamine does not exert a significant effect on NCM's short-term adaptation to song. Therefore, α-adrenergic transmission in NCM does not appear to be required for the initial phase of adaptation during song exposure. The most parsimonious interpretation of these data, taken together with the observed effects of phentolamine on gene expression, is that the blockade of α-adrenergic transmission during song stimulation prevents the gene induction events required for the long-term adaptation. In other words, the convergence of auditory and noradrenergic inputs triggers gene regulatory events that result in a stabilization of the adapted state in NCM (Fig 4.7). Of course, our present experiments cannot rule out the possibility that noradrenergic-dependent long-term electrophysiological adaptation is induced in parallel by song-stimulation. The dashed line from short- to long-term adaptation in our model indicates this possibility.

Our conclusion that noradrenergic transmission triggers gene regulatory events that lead to long-lasting changes in the electrophysiological response to stimulation is also supported by findings in the mammalian brain. For example, adrenergic transmission is required for the maintenance of the long-term

potentiation (LTP) that is observed after tetanic stimulation in synapses of the perforant pathway onto the dentate gyrus (Bramham et al., 1997) and of mossy fibers onto CA3 cells (Huang and Kandel, 1996) of the rodent hippocampus, as well as in the lateral amygdala (Huang et al., 2000). In addition, direct pharmacological activation of the LoC leads to hippocampal synaptic plasticity as measured 24 hr later (Walling and Harley, 2004). Moreover, the maintenance of NE-dependent synaptic changes in the hippocampus and amygdala require *de novo* RNA and protein synthesis (Huang and Kandel, 1996; Huang et al., 2000; Walling and Harley, 2004). These observations support the notion that distinct mechanisms underlie short and long-term synaptic plasticity, and that NE plays a more significant role in the retention (long-term) than in the acquisition (short-term) of these plasticity events. We note, however, that contrary to tetanic electrical stimulation, birdsong is a natural learned stimulus and that our gene induction paradigm used awake freely-behaving animals. Thus, our results enable us to more directly link noradrenergic transmission to the plasticity underlying the brain's response to a natural stimulus of behavioral relevance. We also note that, contrary to LTP, adaptation reflects a decrease in neuronal responsiveness. Our results thus, are more in line with long-term depression. Accordingly, NE acting through α-1-adrenergic receptors can induced depression of hippocampal synapses that last for ~40 min (Scheiderer et al., 2004). Importantly, this form of LTD requires both Src and ERK activity (Scheiderer et al., 2004). Thus, the noradrenergic system does not seem to determine the direction of the plastic change, but rather to play a facilitating role in long-term

neuronal plasticity, regardless of the direction of change.

NE and memory consolidation

NCM is thought to play important roles in the perceptual discrimination and memorization of song. It also appears to be important for the memorization of tutor song, which serves as a reference base for vocal learning in juveniles. Specifically, both electrophysiology and gene expression responses in NCM are influenced by exposure to tutor song during song learning, suggesting that a memory trace of that song is formed in NCM as a result of such experience (Bolhuis et al., 2000; Phan et al., 2006; London and Clayton, 2008). In addition, NCM lesions appear to affect tutor song recognition (Gobes and Bolhuis, 2007). Furthermore, when MEK1/2 activity, which is required for song-induced gene expression in NCM, is locally blocked during tutor song exposure, the normal development of song is markedly disrupted (London and Clayton, 2008). Given our current results linking NE to gene expression and changes in electrophysiological responses to song, and the known involvement of NE in learning and memory, the noradrenergic system may be an important regulator of the cellular NCM changes associated with tutor song memorization, a hypothesis that will require direct testing in future studies.

We believe our data are also of relevance to understanding the long-term effects of the noradrenergic system on learning and memory in a broad range of vertebrates (McGaugh, 2000; McGaugh and Izquierdo, 2000; Gibbs and Summers, 2002; Ramos and Arnsten, 2007). Specifically, we suggest that

noradrenergic input onto the neuronal cells that are activated during the acquisition phase of a given task modulates the induction of gene regulatory programs, which then helps to consolidate the neuronal changes underlying the task memorization (akin to the cellular diagram on Fig 4.7). Interestingly, in contrast to our data, the evidence in mammals points mostly to an involvement of β-adrenergic receptors in the context of learning and memory (McGaugh and Roozendaal, 2002). While this discrepancy could reflect a species difference, it could also be related to regional brain specializations. Specifically, while most of the mammalian data on the noradrenergic involvement in learning and memory derive from studies of the hippocampus and amygdala, NCM is a high-order avian auditory area considered analogous to supragranular layers of the mammalian auditory cortex. Our data suggest, therefore, that α-adrenergic transmission may play a more prominent role at the level of cortical-like circuitry than in the amygdala and/or hippocampus.

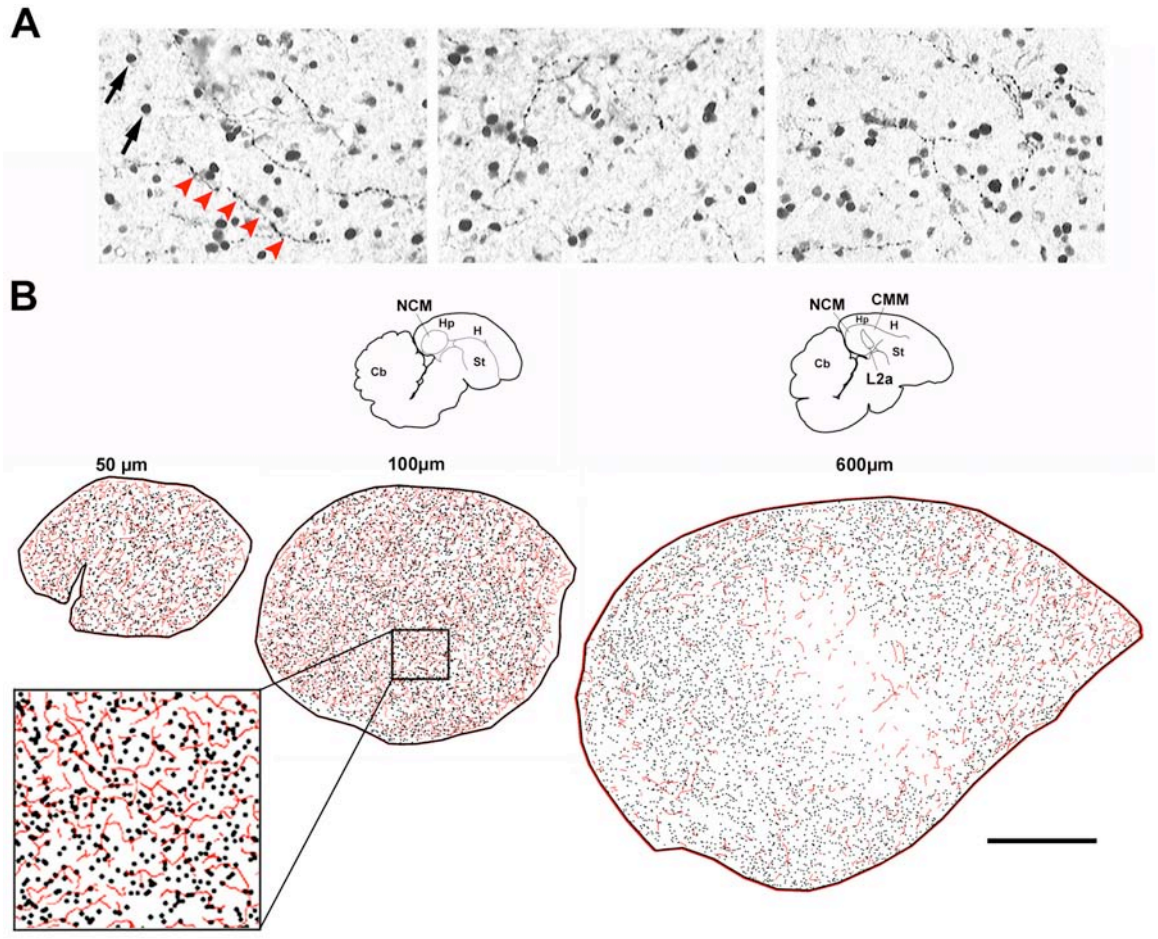


Figure 4.1. Noradrenergic innervation and sensory-activated neurons in the NCM. A) Double-immunostaining for ZENK (nuclear) and DBH (fibers) in parasaggital brain sections of song-stimulated zebra finches. B) Camera lucida drawings from the same brain sections depicting DBH-positive fibers (red traces) and ZENK-expressing neurons (black dots) at different medial to lateral NCM levels (in µm from the midline). Red arrows, DBH-positive fiber. Scale bar, 500 µm.

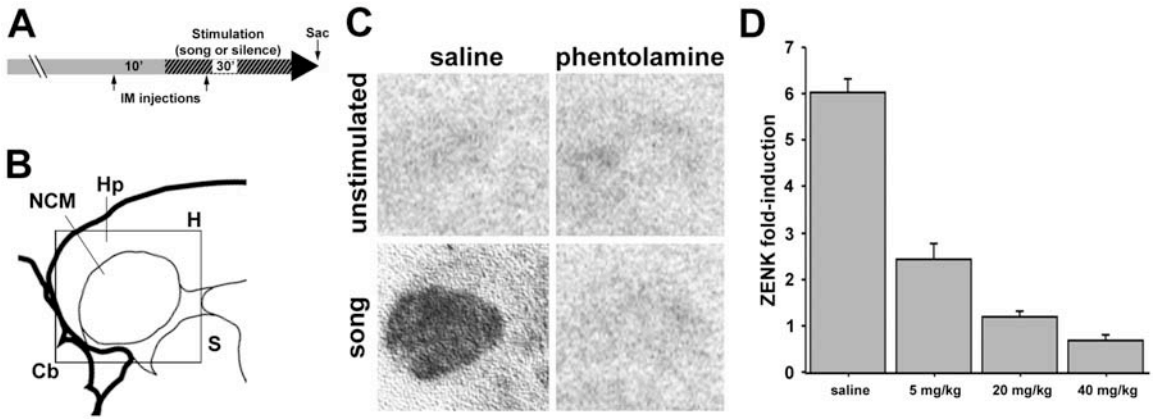


Figure 4.2. Systemic injections of α-adrenergic receptors antagonists block song-induced gene expression. A) Schematic representation of the experimental design depicting systemic injections and stimulation protocols. B) Camera lucida drawing of a parasagittal brain section containing the NCM at the level analyzed. C) Autoradiographic images of brain sections from unstimulated controls and song stimulated birds systemically injected with saline and phentolamine hybridized against *zenk* riboprobes. D) Fold-induction of *zenk* mRNA in birds injected with phentolamine compared to vehicle controls (saline).

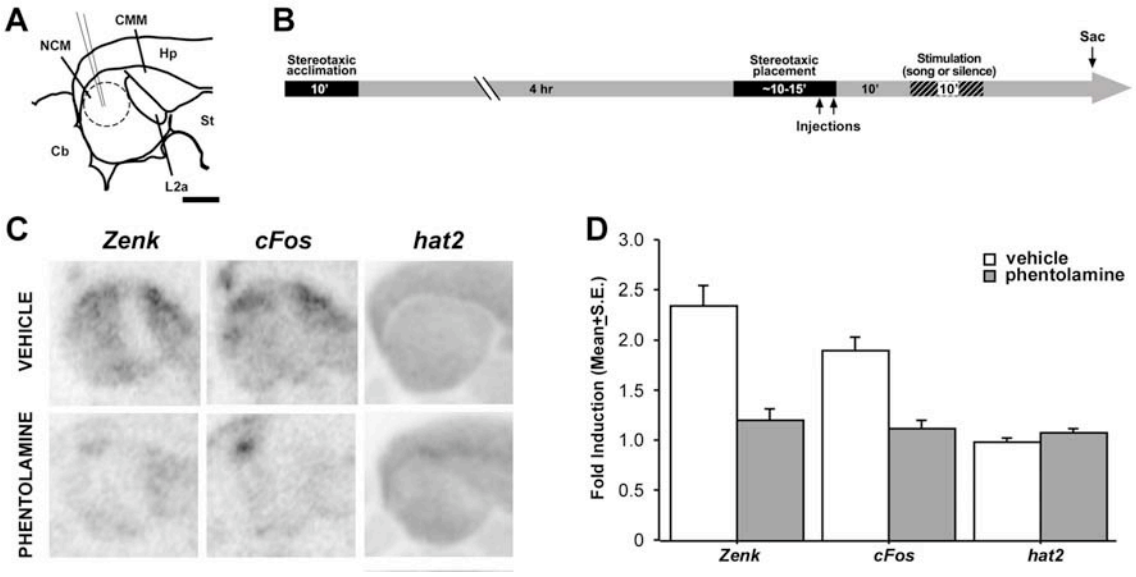


Figure 4.3. Local action of noradrenaline modulates song-induced gene expression in the NCM. A) Camera lucida drawing of a parasagittal section containing NCM, field L2a and CMM (about 500 µm from the midline), indicating the site of injections (pipette and dashed circle). B) Schematic representation of the experimental design depicting the stereotaxic injection into NCM of awake restrained birds. C) Phosphorimager autoradiograms of adjacent parasagittal sections from a representative bird that received an injection of phentolamine or vehicle (opposite hemispheres) into NCM hybridized with *zenk*, *c-fos* or *hat2* riboprobes. D) Fold-induction of *zenk*, *c-fos* and *hat2* in the NCM of vehicle- (white bars) and phentolamine-injected (gray bars) birds stimulated for 10 min with conspecific song.

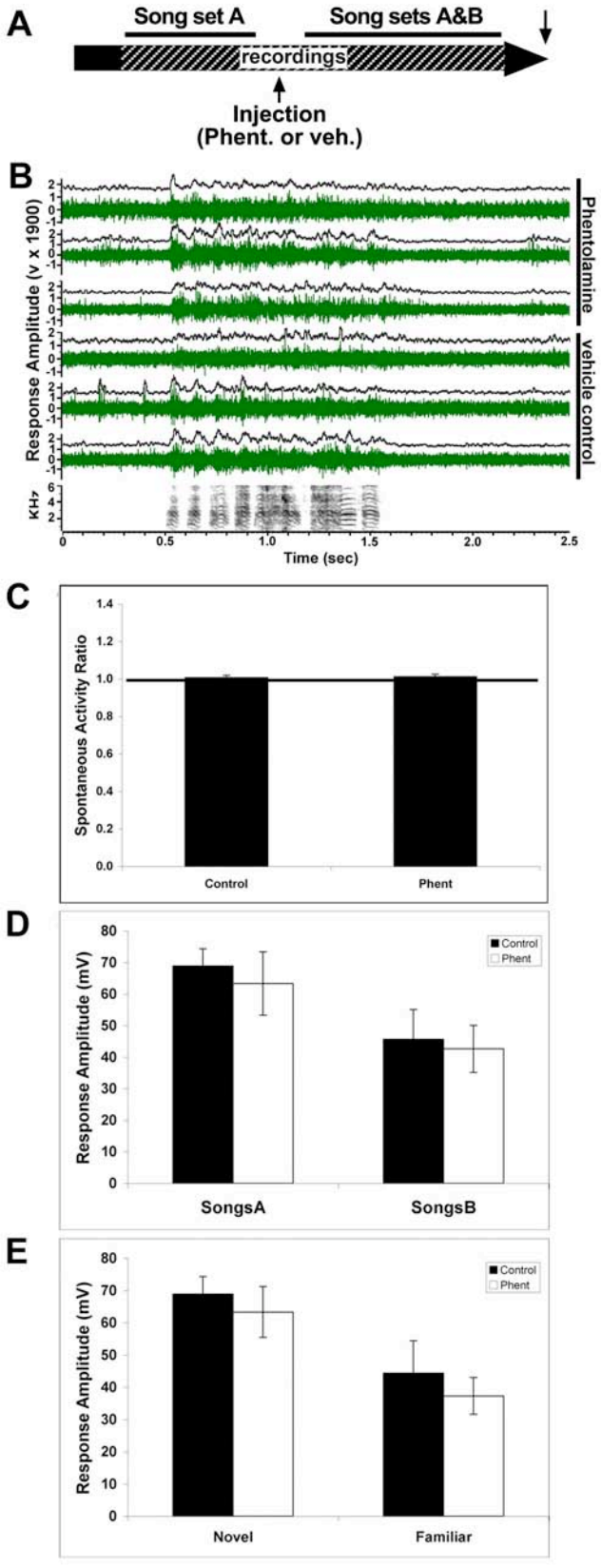

A
Song set A
Song sets A&B
recordings
Injection
(Phent. or veh.)
B
Response Amplitude (v x 1900)
Phentolamine
vehicle control
KHz
Time (sec)
C
Spontaneous Activity Ratio
Control
Phent
D
Response Amplitude (mV)
Control
Phent
SongsA
SongsB
E
Response Amplitude (mV)
Control
Phent
Novel
Familiar

Figure 4.4. Alpha-adrenergic blockade does not affect short-term responses in NCM. A) Schematic representation of the experimental design depicting electrophysiological recordings of NCM responses in awake restrained birds before and during local injections of phentolamine. B) Representative responses to a song stimulus shown as raw multi-unit activity (green), and as RMS (20 ms bins) for 6 simultaneously recorded sites (3 in the phentolamine hemisphere, 3 in the control hemisphere). C) The change in NCM spontaneous activity is plotted as the ratio of activity recorded before phentolamine injection to activity during injection for both the control and phentolamine-injected hemispheres. A ratio of 1 (solid line) indicates no change. D) Amplitude of NCM responses to song sets A and B in control- and phentolamine-injected hemispheres. E) NCM response amplitude to the same songs when novel before phentolamine injection and when familiar during injection in both control and phentolamine-injected hemispheres.

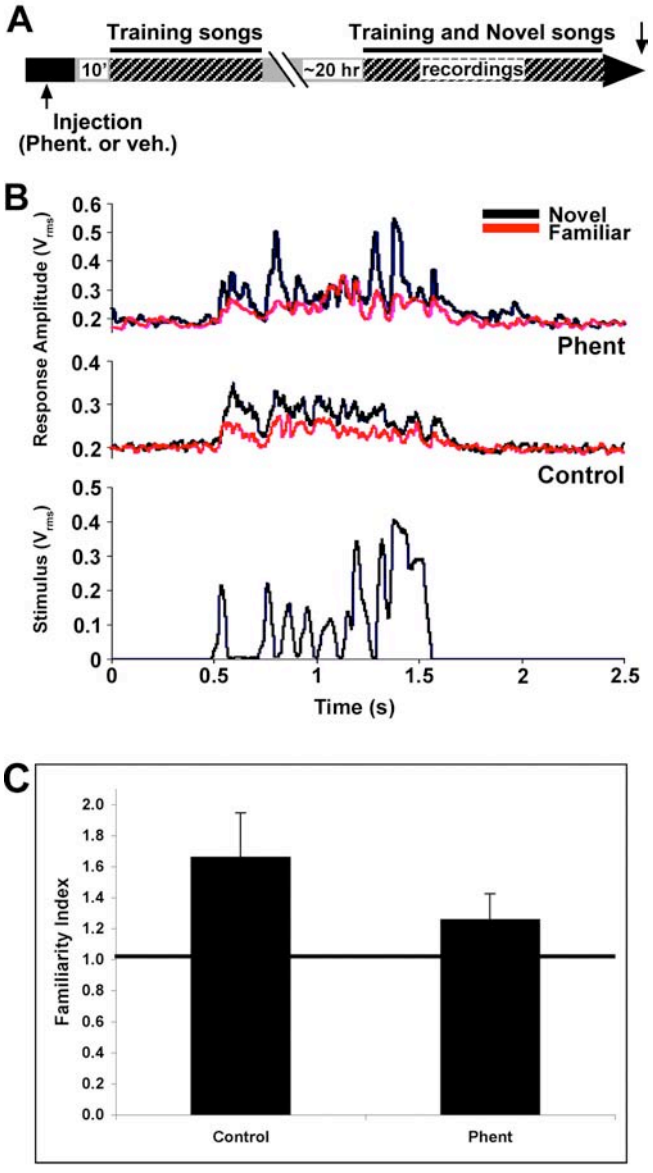

A
Training songs
Training and Novel songs
10'
~20 hr
recordings
Injection
(Phent. or veh.)
B
Novel
Familiar
Response Amplitude (V_{rms})
0.6
0.5
0.4
0.3
0.2
Phent
0.4
0.3
0.2
Control
Stimulus (V_{rms})
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
Time (s)
C
Familiarity Index
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Phent

Figure 4.5. Alpha-adrenergic blockade disrupts long-term adaptation in NCM. A) Schematic representation of the experimental design depicting electrophysiological recordings to novel and familiar songs in control and phentolamine-injected hemispheres. B) Phentolamine injection does not affect short-term adaptation in NCM. Averaged RMS responses (20ms bins, n=5) for the same song stimulus when novel before (black) phentolamine injection and when familiar (1h after training) during (red) injection in one site in each hemisphere. C) Familiarity indexes (the ratio between adaptation rates for novel songs and songs heard by the animal 20-21h earlier, see text) are shown for both the control and phentolamine-injected hemispheres.

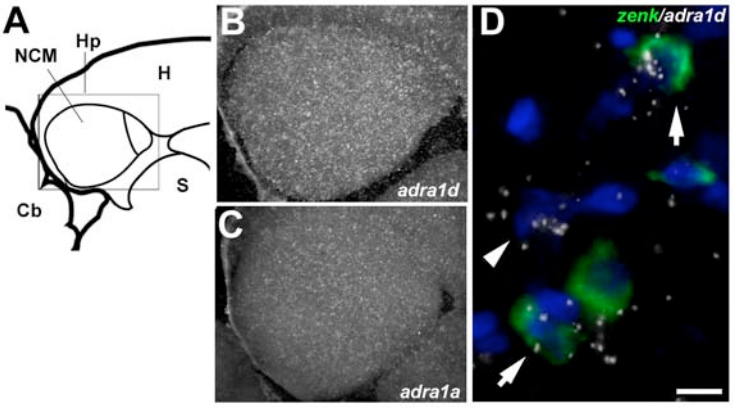


Figure 4.6. Expression of α-adrenergic receptors in the NCM. A) Camera lucida drawing of a parasagittal section containing NCM and CMM (about 100 μm from the midline), indicating the position of the photomicrographs depicted in B and C. B-C) Dark-field view of emulsion autoradiograms of brain sections hybridized with radioactively-labeled *ADRA1d* and *1a* riboprobes, respectively. D) High-magnification view of double *in situ* hybridization for *ADRA1d* (emulsion grains) and *zenk* (green fluorescence); arrows point to double-labeled cells and arrowhead indicates a single-labeled *ADRA1d*-positive cell.

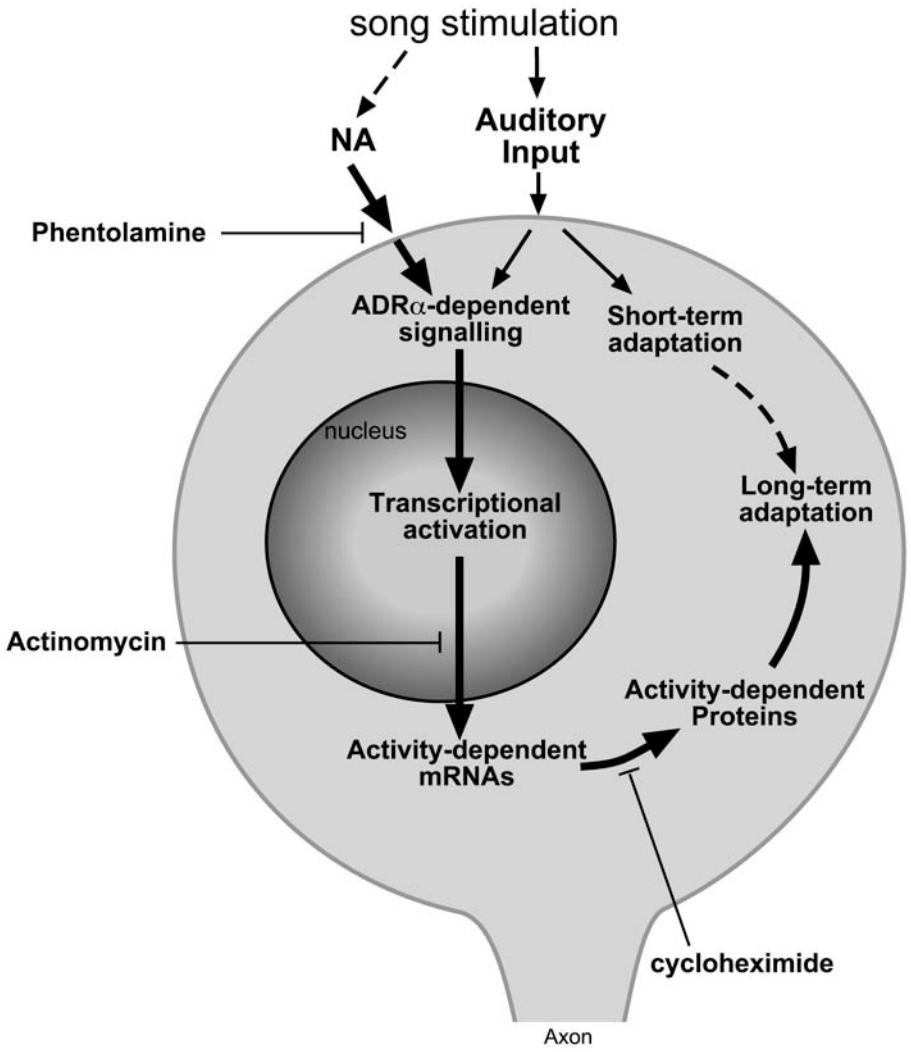


Figure 4.7. Noradrenergic modulation of song-induced gene expression and long-lasting changes in NCM auditory neurons. Undisturbed α-adrenergic transmission is required for song-induced gene expression and the long-term maintenance of song-adaptation yet does affect short-term habituation, indicating separate mechanisms. Blocking the downstream transcription (actinomycin) and translation events (cycloheximide) also results in disruption of long-lasting adaptation.

Chapter 5

Auditory representation of complex sounds and hierarchical selectivity in the zebra finch brain

Introduction

Many animal species use complex acoustic signals to communicate. In order to understand the contribution of sensory/perceptual processing to vocal communication, it is essential to determine how the brain auditory representation of complex sounds is organized. However, in spite of significant progress in understanding the neural basis of sound localization in owls and echolocation in bats, we have just begun to address how complex sounds used in vocal communication are processed by the brain (Ribeiro et al., 1998; Gentner et al., 2001; Ghazanfar and Hauser, 2001).

Songbirds represent one of the most tractable animal models to study the auditory representation of vocal communication signals. The brain circuits controlling song processing and discrimination have been identified and are relatively well characterized. In addition, auditory stimulation with birdsong, a naturally learned behavior widely used for communication, induces the expression of activity dependent genes in auditory processing brain areas (Mello et al., 1992; Bolhuis et al., 2000; Velho et al., 2005). The expression of these genes, especially of the transcription factor *zenk*, can be used to identify neuronal populations responsive to song. The *zenk* gene (a.k.a. *zif268*, *egr1*, *ngfi-a* and *krox24*) encodes a transcription factor whose expression is associated

with neuronal depolarization and has been widely used marker for neuronal activation (see Chaudhuri, 1997 and Mello, 2002 for reviews). Although electrophysiological activity in some areas of the songbird brain is not coupled with the expression activity-dependent genes in (see Velho and Mello, 2007 for a recent discussion), there is no evidence of upregulation of these genes in the absence of neuronal activation.

Mounting evidence from electrophysiology, pharmacology, lesions and gene expression studies indicates that the caudomedial nidopallium (NCM) plays central roles in song perceptual processing, discrimination and perhaps formation and/or storage of song-related auditory memories (Mello et al., 1992; Stripling et al., 1997; Grace et al., 2003; Phan et al., 2006; Gobes and Bolhuis, 2007). NCM receives input from the primary auditory thalamo-recipient area field L and is highly interconnected with other auditory telencephalic areas (Fig 5.1), and as such it can be considered analogous to the supragranular layers of the mammalian auditory cortex (Karten, 1997). NCM has robust electrophysiological responses to song and other auditory stimuli (Chew et al., 1995; Terleph et al., 2006, 2007) (Stripling et al., 1997). NCM is also the area that shows the most pronounced gene expression response to song. This response is rapid and transient, stimulus dependent, and modulated by behavioral context and experience (see Velho and Mello, 2007 for a review). In canaries, expression of the *zenk* gene, in particular, correlates spatially with acoustic features present in the stimulus, with different song syllables leading to expression in different NCM subdomains in a topographically organized manner (Ribeiro et al., 1998).

In the present study, we have used expression analysis of the activity-dependent gene *zenk* to generate global maps of neuronal activation in response to conspecific song compared to other complex sounds of various spectro-temporal structures in the zebra finch brain. Specifically, we compared the neurons responsive to conspecific and heterospecific song, to white noise, tonal sequences, and to decomposed songs, in both NCM and MLd. Our results show that the number and position of song-responsive neurons as measured by *zenk*-expressing cells in NCM are stimulus-dependent. The pattern of spatial distribution of responsive cells differed markedly across different classes of stimuli. More importantly, the density and spatial distribution of responsive neurons were sufficient to discriminate among the NCM maps resulting from the different stimulus types. In contrast, we did not observe differences in the response to complex stimuli in the auditory midbrain. Overall, these results indicate that the auditory pallium has a greater capacity to discriminate among complex sounds than the midbrain, based on the populations of song-responsive neurons recruited, consistent with a hierarchical processing within the songbird auditory system.

Material and Methods

***Modified conspecific song preparation*:** We prepared modified versions of a conspecific song (Song A) using a custom-made algorithm kindly provided by Dr Frederic Theunissen (University of California, Berkeley). The procedure used is essentially as described by Theunissen and Doupe (1998) using two sets of parameters: for the temporal low pass we chose the filter to be 128 Hz and the spectral frequency cutoff to be 0.0005 (or 0.5 cycles/kHz), the upper limit of the temporal frequency was 500 Hz; for the spectral low pass we chose the filter to have 32 Hz bandwidth and the upper frequency for temporal modulations to be 3 Hz. The upper frequency for the spectral modulations was set at 0.016 or 16 cycles/kHz.

***Birds and song stimulation**:* All birds in this study were adult female zebra finches (*Taeniopygia guttata*) from our own breeding colony or obtained from local breeders. For song stimulation, birds were first isolated overnight (16-20 hr) in sound-attenuated chambers (≈76 X 31 X 28 cm) under a 12:12 light:dark cycle (same as the aviary; lights on at 7:00 AM). On the following day, birds were exposed to a playback of a recorded stimulus consisting of a medley of three representative non-familiar conspecific songs (CON), three canary songs (HET),

white noise (WN), or a medley of pure tones (TONE) presented in blocks of 15 seconds followed by a 45-second silent interval for 30 minutes followed by immediate sacrifice. The heterospecific stimulus consisted of a medley of three canary songs arranged to match the duration of the conspecific stimulus. The non-song stimuli consisted of 15 seconds of white noise or of a combination of tones matching the frequency range of the conspecific songs followed by a 45-second silent interval. For the conspecific song and its modified versions comparison, the same procedure was applied except that the animals heard a single song (Song A) or its frequency-preserved (Song Afp) or time-preserved (Song Atp) modified versions also in blocks of 15 seconds followed by 45-second silent interval for 30 min. For catFISH analysis an additional subset of females received a shorter stimulation, 5 minutes, of a single song (Song A), followed by 20 minutes of silent interval, and the presentation of a new song for 5 minutes (Song B). Controls consisted of animals stimulated with a single song for 5 minutes and sacrificed immediately or 30 minutes after stimulus onset, and animals that hear songs A and B in the inverted order. All stimuli were presented at comparable intensities (average 70 dB mean SPL at 35 cm from the speaker). Females were used in all quantitative song stimulation experiments to avoid the confound of singing behavior in males; controls consisted of birds that were not stimulated. All procedures involving birds were approved by OHSU's Institutional Animal Care Use Committee (IACUC) and are in accordance with NIH guidelines.

Tissue preparation: For ISH and Western blots, birds were sacrificed by decapitation. Their brains were quickly dissected from the skull, frozen in Tissue-tek (Sakura Finetek Inc., Torrance, CA, U.S.A) in a dry ice/isopropanol bath, and stored at -80°C. Parasagittal 10 μm brain sections were cut on a cryostat, thaw-mounted onto slides, and stored at -80°C until use. For ChIP assays, sound-stimulated birds (n=3) were killed with an overdose of Nembutal and perfused transcardially with freshly prepared 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were then removed from the skull and the caudomedial telencephalic auditory lobules (including the caudomedial nidopallium and mesopallium - NCM and CMM - and medial field L2a) dissected and frozen.

Probe labeling*:* Riboprobes were used for radioactive and fluorescent *in situ* hybridization (ISH and FISH respectively). Plasmid DNA was isolated using Qiagen Miniprep Kit (Qiagen Inc. Valencia, CA, USA), linearized with the appropriate restriction enzymes, and purified with Qiagen PCR Purification Kit (Qiagen Inc., Valencia, CA, USA). Sense and antisense ^{33}P-labeled riboprobes for all genes analyzed in this study were synthesized as described elsewhere (Mello et al., 1997). For digoxigenin-(DIG)labeled riboprobes, we added 1.0 µl of DIG labeling mix (Roche Diagnostics Corp., Mannheim, Germany) to a 10 µl reaction containing 1 µg of linear plasmid DNA, 10 µg of BSA, 10 mM of DTT, 20 units of RNAse inhibitor, 8-10 units of RNA polymerase, and 2.0 µl of a transcription buffer consisting of 50 mM DTT, 250 mM Tris-HCl pH 7.4, 30 mM MgCl2, 50 mM NaCl, and 10 mM spermidine in DEPC-treated water; otherwise, we followed (Mello et al., 1997). All riboprobes were purified in Sephadex G-50

columns and analyzed using a liquid scintillation counter or by visual inspection of a denaturing formaldehyde-agarose gel.

Radioactive* in situ *hybridization (ISH): ISH was performed using ^{33}P-labelled sense and antisense riboprobes followed by exposure to a KODAK phosphorimager screen or emulsion autoradiography and Nissl counterstaining. The hybridizations and washes for all probes analyzed were performed at 65^{o}C, using essentially the same procedure as previously described (Mello et al., 1997).

Fluorescence ISH (FISH) FISH was performed essentially as described in Velho et all (2005) using DIG-labeled riboprobes. Briefly, after hybridization and washing steps identical to ISH, sections were initially incubated for 10 min in 0.3% H_2O_2 in TNT buffer (0.1 M Tris-HCl pH7.5, 0.15 M NaCl, and 0.3% Triton X-100) to inactivate endogenous peroxidase, rinsed 3X for 5 min in TNT buffer, and incubated for 30 min in blocking buffer (TNT containing 8.3 mg/ml of BSA). The sections were then incubated for 2 hr at room temperature (or overnight at 4^{o}C) with HRP-conjugated anti-DIG (1:200 dil; Roche Diagnostics Corp., Inc) in blocking buffer, washed 3X for 5 min in TNT buffer, followed by a 1-2 hr incubation at room temperature with tyramide coupled with Alexa Fluor 594 or Alexa Fluor 488 (TSA^{TM} Molecular Probes, Inc). Sections were then rinsed 3X for 5 min in TNT buffer, counterstained with Hoechst, and cover slipped with ProLong^{TM} Antifade kit (Molecular Probes, Inc). Control reactions in which the probe or antibody were omitted were included in every experiment.

***Combined ISH and Fluorescent* in situ *hybridization (FISH)*:** We used a double-labeling procedure combining ISH (for the GABAergic marker *gad65*) and FISH (for *zenk*), followed by nuclear counterstaining (Hoechst). We followed the double-labeling procedures described in Jeong et al. (2005) and Velho et al. (2005).

Densitometry: Phosphorimager files were quantified using NIH's Image software. Densitometric measurements taken over specific brain regions were subtracted from the slide background and resulting values averaged for two adjacent sections. For normalization, all densitometric values were divided by the average values from unstimulated controls. To examine the effect of post-stimulation survival time, stimulus duration and drug treatment, we used ANOVA followed by Fisher's PSDL *posthoc* tests for pairwise comparisons, and a probability level of <0.05 for significance.

Mapping of stimulus-responsive neurons: For quantitative analysis of our FISH or combined ISH/FISH experiments, we used Neurolucida software (MicroBrightField Inc., Colchester, VT) and mapped the number of labeled cells in specific brain sections through NCM (~400 μm from the midline), CMM, and MLd of stimulated birds and unstimulated controls (n=3-6 per group). We first outline the area of interest using anatomical landmarks marks as guidelines and mapped all stimulus-responsive neurons by centering a circle (12 μm diameter) over each labelled cell, identified based on *zenk* expression. For the combined ISH/FISH we next counted the number of emulsion autoradiography grains per labeled cell. We note that the typical nuclear diameter of most cells counted was

in the 6-10 μm range and that the diameter of the counting circle was determined to be inclusive of the majority of grains associated with the cells. Cells with grain counts of at least 2 standard deviations above background levels (measured over the glass or over the neuropil between labeled somata) were considered *gad65*-positive. Cell counts were analyzed using ANOVA followed by Fisher's PSDL *posthoc* tests for pair wise comparison, and a probability level of <0.05 for significance.

***Analysis of the spatial distribution of song-responsive cells*:** The cell mapping procedure using Neurolucida Software (MicrobrightField, Inc) results in a set of coordinates for each labeled cell in a particular brain slice. In order to compare such position information across birds, we performed an image registration procedure aligning different brain sections to a common set of landmarks in order to correct for histological distortions and individual variability. As landmarks, we manually chose a set of points common to the anatomy of the zebra finch brain, such as the ventricle wall and the lamina that separates caudal nidopallium and mesopallium, and applied a standard image registration procedure, as provided in Matlab (MathWorks, Inc). Once the sections were aligned, in order to quantify the relative density of activated cells, each individual image was divided using an 8x8 grid and the number of cells in each grid was counted. Using this information in the form of a long vector, we performed Principal Component Analysis in order to reveal the differences in spatial distribution of activated cells across conditions/inputs.

For visualization purposes, individual maps were averaged within group, and a Gaussian spatial filter was applied each aligned image, resulting in a average density map for each stimulus type. This density information was displayed in the form of a heatmap. Individual maps were also generated for visual inspection of intra-stimulus variability.

Results

zenk expression patterns induced by song and other complex sounds

To investigate how song is represented in zebra finch NCM, we used fluorescent ISH for the activity–dependent gene *zenk* (Fig 5.2A; see Suppl. Fig 5.1A for NCM localization), which allows for the generation of global maps at cellular resolution. We analyzed the resulting *zenk* expression maps in birds that heard conspecific song (CON) compared to other sounds that have very different spectro-temporal organizations, including heterospecific song (HET, canary song), white noise (WN), and tonal sequences (TON), and in unstimulated controls (UNSTIM; n=3 adult females per group). We first analyzed the number of *zenk*-expressing cells and found a significant effect of stimulus type (Fig 5.2B; ANOVA, $p<0.001$). Specifically, the number of *zenk*-expressing cells was higher for CON compared to all other stimuli (Fisher's PSLD, $p<0.02$ for HET and $p<0.001$ for all other groups). We also found no difference when comparing tone-stimulated birds to unstimulated controls (Fisher's PSLD, $p=0.21$). Thus, a larger portion of NCM neurons appears to respond to acoustic features present in conspecific songs compared to other stimulus types. Moreover, the lack of significant induction by a sequence of pure tones suggests that NCM preferentially respond to stimuli of more complex spectral characteristics than pure tones. These results parallel those of previous studies using densitometric analysis of *zenk* mRNA levels (Mello et al., 1992; Velho et al., 2005) and strongly suggest that the stimulus differences in the *zenk* response are largely reflective of differences in the number of responsive cells in NCM.

To investigate whether this effect of stimulus type might also occur at lower levels in the auditory ascending pathway, we examined the number of *zenk*-expressing cells in the dorsal part of the lateral mesencephalic nucleus (MLd; see Fig 5.3B for MLd localization), the avian equivalent to the central nucleus of the inferior colliculus. Similar to NCM, auditory stimulation had a significant effect on the number of *zenk*-expressing cells in MLd (Figure 5.2C; ANOVA, $p < 0.05$). However, in contrast to NCM, the number of *zenk*-expressing cells in birds that heard CON was not significantly different from birds hearing HET or WN (Fisher's PSLD, $p=0.67$ and $p=0.21$ respectively). Finally, birds stimulated with tones did not differ from unstimulated controls (Fisher's PSLD, $p=0.93$). These results point to considerable differences in the response properties of NCM compared to the auditory midbrain, also mirroring electrophysiological findings (Woolley and Casseday, 2004, 2005; Woolley et al., 2005; Terleph et al., 2006).

We next generated density maps of *zenk*-expressing cells in NCM, to address the spatial distribution of song-responsive cells and its possible dependence on stimulus type. For this purpose, individual density maps of *zenk* expression were registered and averaged (Fig 5.3 and methods for details). The resulting average density maps revealed overall increases in *zenk*-labeled cells in the NCM of birds hearing CON, HET and WN (Fig 5.4A-C, respectively) compared to unstimulated controls. However, this pattern was not homogeneous and differed significantly among stimuli. In CON-stimulated birds, *zenk*-expressing cells were concentrated mostly in dorsal and ventral NCM, with a

lower density in the more central region; there was also a moderate but significant activation of caudal NCM (Fig 5.4A). In contrast, *zenk*-expressing cells in HET- and WN-stimulated birds were mostly located in central NCM (Fig 5.4B and C respectively); some labeled cells also occurred in dorsal NCM, but at a much lower density compared to CON. Interestingly, *zenk*-expressing cells in caudal NCM were very few or absent for all stimuli except CON, suggesting that this area may be the most selective NCM subregion.

To quantitatively compare *zenk* expression patterns across stimuli, individual maps were divided into smaller bins by an 8X8 grid (Fig 5.4F), and subjected to Principal Component Analysis (PCA) based on the number of labeled cells per bin in each map. We found that the first three components of this analysis were sufficient to separate the *zenk* expression patterns into three clusters (Fig 5.4G). One cluster included unstimulated controls and tones, both of which do not elicit a significant *zenk* response in NCM (Fig 5.4G, red symbols). A second cluster grouped together HET and WN (Fig 5.4G, blue symbols), which both evoke a considerable number of labeled cells in central NCM. Finally, CON-stimulated birds constituted a separate cluster without overlaps with the other stimulus types (Fig 5.4G, green squares). Thus, *zenk* maps in NCM clearly distinguish CON from all other complex sounds tested.

Activation of excitatory and inhibitory neurons

Song stimulation elicits activation of both gabaergic (inhibitory) and non-gabaergic (presumably excitatory) cells (Pinaud et al., 2004). To investigate the separate contributions of these cell types to the ensemble of song-responsive

neurons in NCM, we have identified and mapped cells expressing *zenk* and *gad65*, a gabaergic marker (Fig 5.5A), using double-labeling *in situ* hybridization. In agreement with previous studies, we observed *zenk*+/*gad65*+ (Fig 5.5A, arrowheads) and *zenk*+/*gad65*- cells (Fig 5.5A, green arrows), as well as *gad65*+-only cells (Fig 5.5A, white arrow). Quantitative analysis showed significant effects of stimulation with CON, HET and WN on the number of both *zenk*+/*gad65*- cells (Fig 5.5B; ANOVA, $p<0.001$) and *zenk*+/*gad65*+ cells (Fig 5.5C; $p<0.0001$). Interestingly, we observed an effect of stimulus type, as the number of *zenk*+/*gad65*+ cells was significantly higher for HET than for the other stimuli (Fisher's PSLD $p<0.0001$ for both CON and WN respectively), and CON and WN were not different (Fisher's PSLD $p= 0.06$), although a trend was observed. Indeed, the central region of NCM in the HET group had a high density of *zenk*+/*gad65*+ cells compared to the other stimuli, which had rather low levels of this cell type throughout NCM (Fig 5.5D, lower panels). This contrasted with *zenk*+/*gad65*- cells, which were more numerous for CON and HET than for WN (Fisher's PSLD $p< 0.005$ and $p <0.05$ respectively) and showed a trend of higher values for CON than HET (Fisher's PSLD $p= 0.39$). The distributions of *zenk*+/*gad65*- cells (Fig 5.5D, upper panels) paralleled closely those of the total cell maps (Fig 5.4). When the numbers of both *zenk*+/*gad65*+ and *zenk*+/*gad65*- were plotted together for all groups, the patterns could be separated without overlaps (Fig 5.5E), indicating that information about the neurochemical identity within the ensemble of song-responsive cells in NCM can help separate the responses to CON, HET and WN.

Expression patterns of spectrally and temporally modified songs

The classes of stimuli presented so far differ markedly in their specro-temporal composition. To refine our analysis and isolate the participation of the frequency and time compositions of conspecific song, we compared a normal song (Song A) to the same song after a decomposition that degraded its frequency and time components, resulting in versions that we named time-preserved (Song Atp) and frequency-preserved (Song Afp) songs respectively (Fig 5.6A-C; similar manipulation as in (Theunissen and Doupe, 1998), n= 3 birds per group, further details in Methods). The counts of *zenk*-expressing cells in NCM showed no significant effect of stimulus type (Fig 5.6G; ANOVA, p= 0.38) and quantitative autoradiography from radioactive *in situ* hybridization revealed no group differences in *zenk* expression levels (not shown, ANOVA, p= 0.74), indicating that these stimuli result in comparable levels of *zenk* expression in NCM. In contrast, the expression pattern evoked by song A (Fig 5.6D) differed markedly from those elicited by the modified songs (Fig 5.6D-F): the latter had much higher densities of labeled cells in central NCM and lower ones in dorsal, caudal and ventral regions compared to intact song A. A PCA of these expression patterns clearly separated song A from the 2 modified songs, yet the latter clustered together and could not be distinguished (Fig 5.6H). For an independent quantification, we used linear discrimination analysis (Duda et al. 2000), which readily separated song A patterns from modified songs (Fig 5.6I) but not the two types of modified songs.

We next examined the separate contributions of gabaergic and non-gabaergic cells to these patters using dFISH for *zenk* and *gad65*. The density maps indicated that the spectrally and temporally modified versions of song A resulted in higher densities of *zenk*+/*gad65*- (non-gabaergic) cells in central NCM as compared to the intact song (Fig 5.7A top, compare middle and right to left panel). A similar effect was observed for *zenk*+/*gad65*- (gabaergic) cells (Fig 5.7A top, compare middle and right to left panel). These observations suggest that the maps of modified songs cannot be differentiated based on the identity of the responsive cells. Accordingly, the first 3 components of a PCA based on the distribution and density of both double-labeled excitatory and inhibitory cells (with the same spatial grid as in Fig 5.4F) separated the pattern of song A from the other stimuli, but not between the two modified song stimuli (Fig 5.7B). Thus, the activation patterns triggered by spectrally or temporally degraded songs are quite distinct from that of intact song, mapping preferentially to central NCM, but cannot be distinguished based on *zenk* expression patterns.

Auditory representation of two conspecific songs

We next examined whether two distinct conspecific songs result in distinct *zenk* expression patterns in NCM. Our first approach was to compare *zenk* maps in response to single songs in different birds. We found that density maps resulting from two songs (songs A and B; not shown) were very similar in terms of the density and spatial distribution of *zenk*-expressing cells, and could not be distinguished on that basis. This analysis, however, did not examine whether the

same neuronal populations might respond to the two songs. To address this question, we used a method termed cellular component analysis of temporal activity by fluorescence *in situ* hybridization (catFISH), first developed in rodent to examine the distributions of hippocampal place cells responding to two environmental conditions (Guzowski et al., 1999). This method is based on the nuclear and cytoplasmic localization of the mRNA of inducible genes at short and long survivals after stimulation respectively. We adapted catFISH to examine the auditory representation of birdsong in NCM, specifically to reveal the neuronal populations expressing *zenk* in response to two songs in the same bird. We used a two-epoch song stimulation protocol combined with FISH for *zenk* (se Methods for details). We first determined that *zenk*-expressing cells in the NCM of birds sacrificed immediately after 5 min of stimulation with one conspecific song (Fig 5.8A, top panel) had a nuclear pattern of labeling (Fig 5.8C, SA/5'), consisting in two characteristic transcriptional foci (Fig 5.8C, SA1/5' inset). Quantitative analysis revealed that the great majority (Fig 5.8C'; 96.4 ± 2.6 %; n= 2091 cells in 3 birds) of labeled cells had this nuclear labeling as compared with cells with cytoplasmic (1.7±1%) or double labeling (1.4±0.8) patterns. In contrast, when the same stimulation was followed by a 25-minute survival (Fig 5.8A, bottom panel), the labeling pattern in NCM was predominantly cytoplasmic (Fig 5.8D), consisting of a characteristic doughnut-shaped distribution (Fig 5.8D, inset). The vast majority of neurons showed this pattern (98.5 ± 0.5 %, n= 1065 cells in 3 birds), compared to cells with nuclear (2.8±1.7) or double labeling patterns (1.4±0.3). Thus, the subcellular localization of *zenk* mRNA is an indicative of the precise

time in the cell was activated.

To directly compare the NCM representations of two distinct stimuli, we exposed zebra finches to a two-epoch stimulation protocol (Fig 5.9A) comprising 5 minutes of song A, 20 minutes of silent interval, and 5 minutes of a second song B (both songs are the same used in the single stimulation experiments). As a result, we observed large proportions of NCM cells with nuclear (46±2.2 %; n= 2415 cells from 3 birds) or cytoplasmic (38.3±0.6%) signal, whereas a smaller but considerable proportion (15.54±2 %) had double-labeling pattern (Fig 5.9B and C). Based on comparison with the single-stimulated birds (Fig 5.8), these cell types correspond respectively to the populations that responded to Song B or Song A only, or to both songs. To control for possible effects of the order of presentation, we exposed another 3 birds with the inverse sequence (Song B followed by Song A) and observed similar proportions of the three labeling patterns (i.e., 37.5±2.8 nuclear, 33.7±8.6 cytoplasmic and 28.7±10.5 double-labeled cells), arguing that the order of stimulation has no effect on the resulting patterns (Fig 5.9D). Thus, we have succeeded in identifying NCM cells that respond selectively to two songs, and a population that can have independent *zenk* induction responses to these two songs in the same bird. Finally, we determined the spatial distribution of these cell populations in some representative birds. We observed that the three cell populations are distributed and interspersed over the entire extent of NCM (Fig 5.9D), consistent with a lack of marked differences of the density maps in separate birds.

Discussion

We have presented here evidence that the activation patterns of the zebra finch caudomedial nidopallium (NCM) is stimulus dependent. We found that the number and spatial distribution of activated neurons, as measure by *zenk*-expressing cells, varies with the stimulus presented in the NCM, but not in a preceding auditory station. Importantly, PCA indicates that this information is sufficient to discriminate NCM activation maps based on the type of stimulus presented. Our results also provide evidence for regional specializations of NCM, with increasing selectivity from rostral to caudal and dorsal NCM. In addition, we also directly demonstrated that distinct yet overlapping ensembles of cells respond to two distinct zebra finch songs, further indicating that NCM neurons are tuned to specific features present in conspecific vocal signals. The relevance of these findings to the representation of complex auditory signals is discussed below.

Our understanding of the auditory representation of vocal communication signals in the vertebrate brain is still very rudimentary. The evidence available indicates that, similar to the visual system, neuronal cells in the auditory cortex have more complex response properties than thalamic and midbrain stations, suggesting a hierarchical organization with increasing selectivity along the

ascending auditory pathway. This regional difference can be readily appreciated when one considers the responses to conspecific calls compared to other complex sounds in monkeys, with primary auditory cortex and belt areas exhibiting significantly more selectivity towards conspecific vocalizations (Ghazanfar and Hauser, 2001). In songbirds, auditory areas such as the primary thalamo-recipient area field L and one of its main targets, the caudomedial nidopallium (NCM) may play similar roles. In the present study, we have determined that the pattern of activation, elicited by conspecific song in NCM, as measured by *zenk*-expressing cells, is markedly different from those of other sounds tested. These results are in sharp contrast with what we observed for the avian equivalent of the inferior colliculus, the lateral mesencephalic nucleus (MLd), where no differences were encountered in the number *zenk*-expressing neurons across complex stimuli, providing further evidence of increasing neuronal selectivity as information ascends the auditory pathway. We have also observed noticeable differences in the activation patterns, suggesting the existence of functional subdomains in NCM.

The use of zenk *expression as a mapping tool*

In the present study, we took advantage of activity-dependent gene expression as a mapping tool. This methodology allows for brain activation mapping at cellular resolution in awake behaving animals. Specifically, it allowed us to identify the ensembles of neuronal cells in NCM that were responsive to the various stimuli used in this study. *zenk* induction by song in NCM has been

previously used to map auditory activation in a variety of behavioral conditions, including freely-behaving wild birds (see Mello, 2002 for a review). Furthermore, the expression of this gene reflects the acoustic properties of auditory stimuli. For example, *zenk* expression has been successfully used to reveal a syllabic representation in canary NCM (Ribeiro et al., 1998). In starlings, a different songbird species, the number of *zenk*-expressing cells also correlates with the complexity of the song stimulus.

It should be noted, however, that activity-dependent gene expression cannot be directly equated with electrophysiological activity, since some brain areas do not show gene induction in response to their electrophysiological activation. Thus, although the expression of genes like *zenk* appears to reflect neuronal activation, the absence of gene expression needs to be taken with more caution. Furthermore, this method has low temporal resolution and is terminal, limiting the number of comparisons that can be accomplished for every brain area studied.

Recent advances have increased our understanding of the factors that regulate the expression of *zenk* in responsive neurons. More specifically, the induction of *zenk* and other activity-dependent genes in NCM requires the activation of MAP-kinase signaling, i.e. ERK1/2 phosphorylation (Cheng and Clayton, 2004; Velho et al., 2005), and local noradrenergic transmission (Velho et al, submitted). Thus, attention-related mechanisms that alter the activation of intracellular signaling pathways can affect the levels of *zenk* expression observed in NCM. Consistent with this idea, stimuli of low behavioral relevance and that

are not likely to engage the bird's attention, such as white noise and tones, induce much lower levels of *zenk*, as measured by both autoradiographic densitometry (Mello et al., 1992; Velho et al., 2005) and number of activated cells (current study), compared to conspecific song. In addition, *zenk* induction can be enhanced by increasing the salience of the stimulus, e.g. by pairing song presentation with electric shock (Jarvis et al., 1995). These observations notwithstanding, *zenk* expression mapping is a highly useful tool for comprehensive mapping of brain activation, and has helped us gain significant insights into the auditory representation of birdsong.

zenk-*expression at different brain levels: NCM versus MLd*

Our current results indicate that significant differences exist between the responses of NCM and MLd. While NCM appears to have more cells responding to conspecific song than to heterospecific and the other stimuli, in a graded fashion, such difference across stimuli does not exist in MLd. Although not directly tested, this finding suggests that neuronal cells may be less tuned to complex stimuli in MLd than in NCM. Given the position that these two areas occupy within the ascending auditory pathway and considering that typically there is an increase in complexity and selectivity as sensory information travels away from the periphery, this is not surprising. Indeed, an increase in receptive field complexity and selectivity has been well documented from MLd to the field L complex (Gehr et al., 1999; Sen et al., 2001; Grace et al., 2003; Woolley and Casseday, 2004, 2005; Woolley et al., 2005). The contrasting differences in the

responses to CON, HET and WN in NCM, which is downstream to field L, and lack thereof in the MLd further confirm the increase in selectivity observed in electrophysiological experiments.

Intriguingly, stimulation with tones that are known to evoke electrophysiological activation of both NCM and MLd neurons (Woolley and Casseday, 2004; Terleph et al., 2007) does not trigger significant *zenk*-expression in either area. A possible explanation is that the amount and/or pattern of neuronal activation triggered by tones are not sufficient to induce gene expression. For instance, the majority of the cells in MLd have onset only responses (Woolley and Casseday, 2004) whereas NCM responses to tones are relatively smaller compared to conspecific song (Chew et al 1996). Alternatively, the absence of gene expression may reflect a lack of neuromodulatory signals required for transcriptional activation. For example, it could reflect a lack of engagement of the noradrenergic system by pure tones.

Regional differences of zenk*-expressing cells in NCM*

The distribution of *zenk*-expressing cells in the NCM of stimulated birds was far from homogeneous. This lack of homogeneity, however, was not random: the spatial distributions in response to a given stimulus were consistent across individuals. Indeed, the PCA results often showed clustering of the patterns according to the stimulus type. For example, stimulation with conspecific songs characteristically induced more *zenk* cells in dorsal and ventral NCM, even when different songs were presented (both a medley of songs and song A). This

observation suggests that *zenk* expression patterns reflect specific acoustic properties present in each stimulus type. The specific patterns evoked by different stimuli also indicate that NCM has the capability of discriminating the different stimuli tested.

Our results also point to regional variations in the NCM responses to complex sounds. For example, caudal NCM showed the fewest overall number of *zenk*-expressing cells. In fact, the only stimulus that induced significant expression in that region was conspecific song. In contrast, the central region of the NCM showed higher density of *zenk*-expressing cells in response to canary songs and white noise compared to conspecific songs. Because these stimuli have very different spectral and temporal structures, these results indicate central NCM probably responds to a broad spectrum of features, rather than representing particular spectro-temporal features. Moreover, we observed a general inverse correlation between the activation of central NCM and of its caudal and dorsal domains. This suggests that central NCM may represent or contribute to a filter function that controls the activation of other NCM domains.

As the degraded stimuli contain virtually the same envelope as the song from which it was generated but lack its fine temporal or spectral organizations, the differences observed in the *zenk* patterns in comparison to normal song are particularly informative. We observed two main differences, the disengagement of caudal and dorsal NCM and the recruitment of central NCM. The disengagement of caudal and ventral regions for both song manipulations indicates that the activation of these areas requires both normal temporal and

spectral features of conspecific song. Conversely, the recruitment of central NCM by both degraded stimuli further suggests that this region is less selective, responding to as much broader range of auditory stimuli. We do not presently know however, whether the same cells in central NCM respond to both kinds of degraded song. In fact, based on the results of the catFISH analysis for intact songs, it is likely that the response to the degraded songs is separated at the single cell level of representation. Such result would imply that cells selectively tuned to spectral or temporal features of intact song are interspersed in central NCM. Based on these observations we suggest that caudal and dorsal NCM are primarily activated by stimuli that meet both spectral and temporal requirements of normal conspecific songs, i.e. intact song.

Thus, distinct subdomains in zebra finch NCM appear to respond differently to different stimuli, even when these stimuli share the same spectral or temporal components (e.g., intact vs temporally or spectrally degraded songs). Interestingly, emerging evidence indicates that NCM may in fact be composed of distinct subdomains. The expression of the calcium binding protein calbindin and of the estrogen generating enzyme aromatase is restricted to caudal NCM (Pinaud et al., 2006). We have obtained preliminary evidence that the neuronal activity regulated pentraxin 1 (*narp*) is also enriched in large neuronal cells of this region (Velho & Mello personal observation). In addition to these neurochemical differences, caudal and rostral NCM also exhibit different response properties. Specifically, the reduction of response amplitude (song adaptation) in NCM after repeated presentations of the same stimulus occurs most robustly in caudal and

less frequently in rostral sites (Chew et al., 1995). Previous and recent tract-tracing evidence also suggest that rostral and caudal NCM differ in their connectivity [i.e., the input from field L appears to be primarily to rostral NCM (Vates et al., 1996) and rostral and caudal NCM may not have the same projections (Mello, unpublished)].

Cell types and the response to complex sounds

One of the most intriguing results generated by this set of experiments is that canary songs recruited a larger number of gabaergic cells than the other stimuli. A possible explanation likely lies on the different acoustic structures of zebra finch and canary song. The former is characterized by syllables containing modulated frequency stacks (harmonics), whereas canary song typically contains several narrowband trills and whistles. We suggest that the zebra finch may have evolved mechanisms for filtering out the response to complex acoustic patterns built predominantly on whistle-like notes such as those of canary song. Such filtering would involve the recruitment of inhibition in NCM, predominantly in the rostral portion. Stimuli that are less complex temporally and spectrally, such as pure tones, or less structure, such as white noise, would fail to evoke a significant activation. It will thus be interesting to investigate the extent to which the removal of narrow-band components from canary song alters the recruitment of gabaergic neurons.

The representation of two zebra finch songs

While *zenk* mapping was extremely useful to reveal global patterns of activation triggered by very different stimuli, the analysis of more subtle differences, such as two conspecific songs, require resolving particular populations of cells occupying similar spatial domains. To advance further our ability to map song-induced gene expression triggered by two songs, we adapted the catFISH method, first developed for mapping place cells in the hippocampus under different environmental conditions (Guzowski et al., 1999), to zebra finches. Here, we have effectively used this technique to identify and map NCM cells that respond to two distinct zebra finch songs. Our data shows that distinct yet overlapping ensembles of cells responded to song A or B. It is important to note, however, that similar numbers and spatial distribution were engaged by song A or B and thus a traditional method of gene expression mapping would not distinguish these two populations. Instead we have not only managed to determine the population of neurons specifically responding to a given song, but also managed to identify neurons responding to both songs. The latter likely representing cells responding to features shared by the songs. This also represents a direct demonstration that NCM neurons can respond to more than one song independently. It also provides an explanation for the fact that after repeated presentations leading to the induction of gene expression adaptation (each song has been estimated to recruit up to 40% of NCM cells), NCM cells promptly respond with gene expression when a novel song is presented.

Altogether, the results presented here suggest that the auditory pallium is in a better position to discriminate complex sounds than the midbrain, consistent

with a hierarchical processing within auditory system. They also indicate the existence regional specializations in the processing of complex sounds in zebra finches, suggesting different mechanisms for categorization of complex sounds. These differential representations may have evolved to facilitate conspecific communication. Alternatively, they may represent experience dependent processes in young zebra finches.

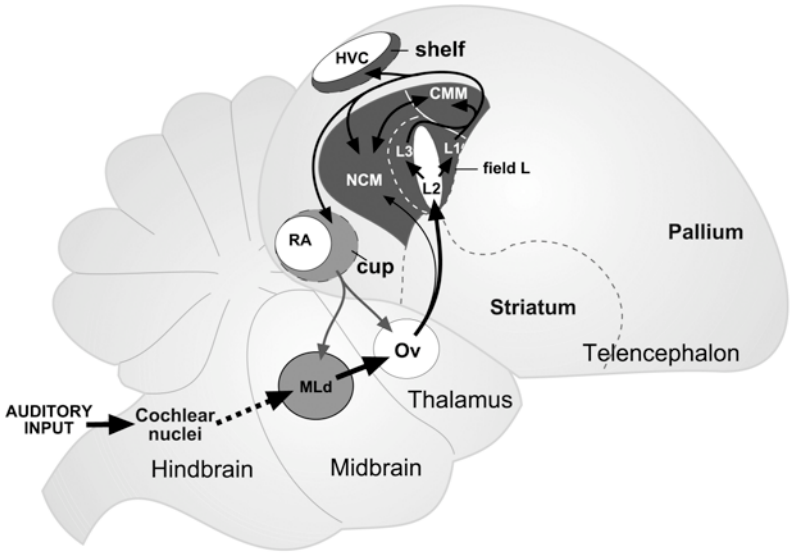


Figure 5.1. Schematic representation of the auditory pathway in zebra finches and associated genomic responses to song in songbirds. Dark grey indicates areas that show activity-dependent transcription in songbirds upon song-stimulation. White indicates areas that lack this response. CMM, caudomedial mesopallium; L1, L2 and L3, fields L1, L2, and L3; MLd, dorsal lateral nucleus of the anterior mesencephalon; NCM, caudal medial nidopallium; Ov, nucleus ovoidalis; RA, robustus nucleus of the arcopallium.

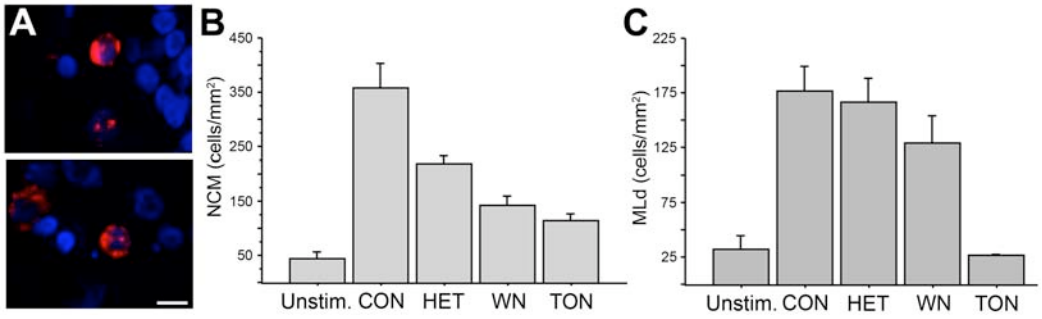


Figure 5.2. The number of *zenk* expressing cells in NCM is stimulus dependent. A) High magnification confocal images of fluorescent *in situ* hybridization for *zenk* (red fluorescence) in NCM sections counterstained for Hoechst (blue). B-C) Number of *zenk*-expressing cells in NCM and MLd, respectively. CON, conspefic song; HET, heterospecific, WN, white noise; TON, pure tones. Plotted are mean± S.E.; Scale bar, 10 μm.

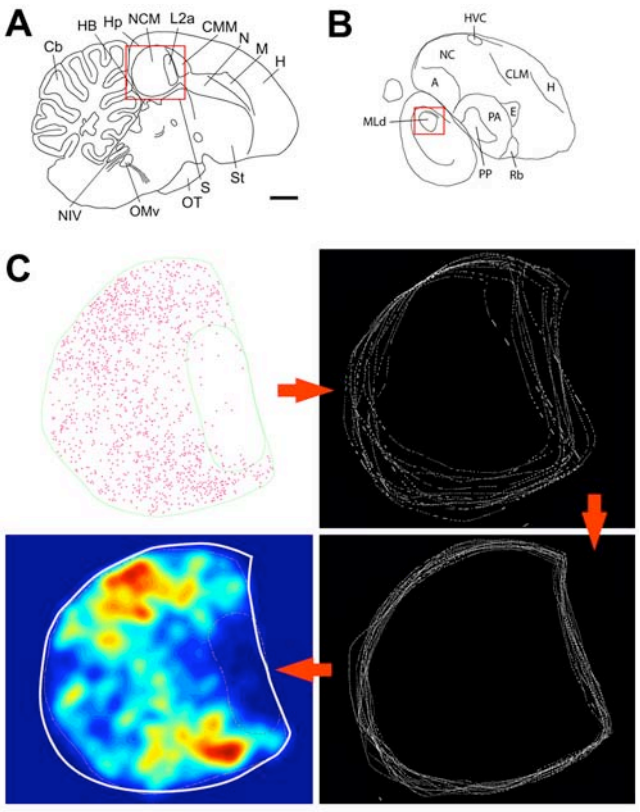

A
HB
Hp
NCM
L2a
CMM
N
M
H
Cb
St
S
OT
OMv
NIV
B
HVC
NC
A
CLM
H
MLd
PA
E
PP
Rb
C

Figure 5.3. Mapping and comparing the distribution of *zenk*-expressing cells in NCM and MLd. A-B) Camera lucida drawings of parasagittal sections of zebra finch brain at the planes studied; the areas enclosed by the rectangle indicates NCM and MLd as shown in all *zenk* expression maps. C) Alignment, registration and density maps of *zenk* expression. Top left, camera lucida mapping of *zenk* cells (red dots) in NCM; right panels, superposition of several NCM outlines before (top) and after (bottom) registration; density map of a representative NCM of a female zebra finch that heard conspecific song. A, arcopallium; Bas, basorostral pallial nucleus; Cb, cerebellum; CLM, caudomedial lateral mesopallium; CMM, caudomedial mesopallium; E, entopallium; H, hyperpallium; HB, habenular nuclei; Hp, hippocampus; HVC, nucleus HVC of the nidopallium; L2a, subfield L2a of field L; M, mesopallium; MLd dorsal part of the lateral mesencephalic nucleus; N, nidopallium; NCM, caudomedial nidopallium; NIV, nucleus of the trochear nerve; OMv, ventral part of the oculomotor nucleus; OT, optic tract; GP, globus pallidus; S, septum; St, striatum; LSt, lateral striatum. Scale bar, 1 mm.

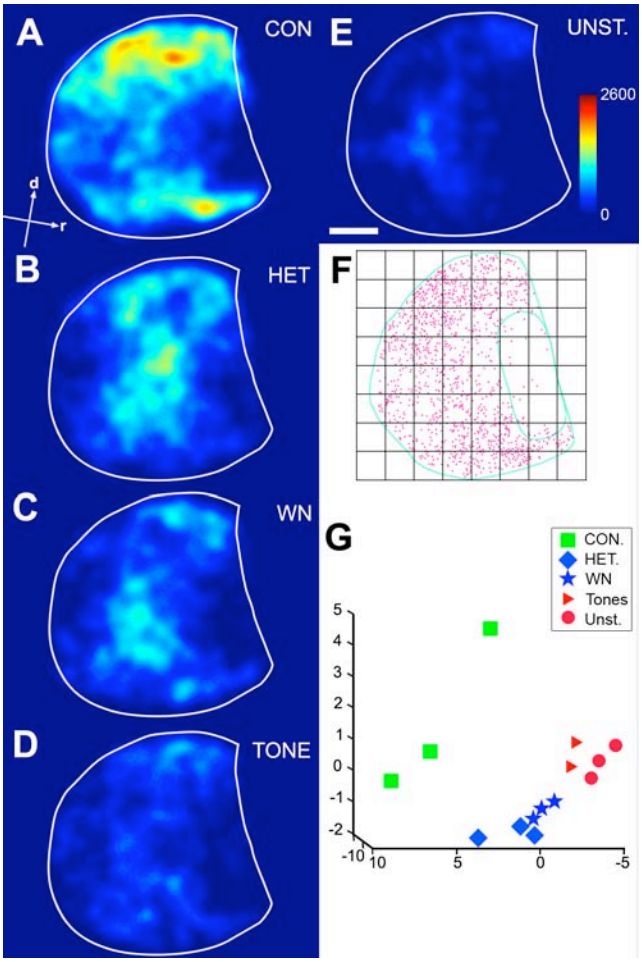


Figure 5.4. Auditory representation of zebra finch song and other complex sounds in NCM. A to E) Average maps of *zenk* expression resulting from presentation of conspecific songs, canary songs, white noise, and pure tones; F) Camera lucida map of a representative individual divided into smaller bins by an 8 x 8 grid; G) Principal component analysis (PCA) of *zenk* expression patterns of individual maps resulting from different stimuli. Shown is a perspective of the first three components. CON, conspefic song; HET, heterospecific, WN, white noise; TON, pure tones.

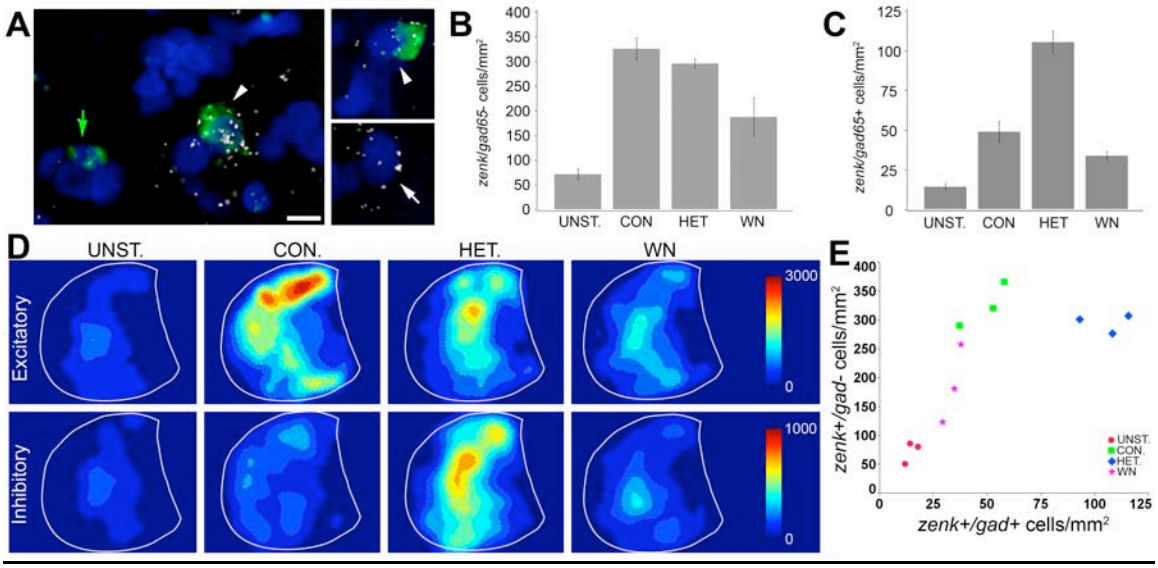


Figure 5.5. Patterns of activation for excitatory and inhibitory cells. A) High magnification images of combined fluorescent and radioactive *in situ* hybridization for *zenk* (green fluorescence) and *gad65* (white silver grains) in NCM sections counterstained for Hoechst (blue); arrowheads indicate *zenk+/gad65+*; green arrow indicates a *zenk+/gad65-* cell; and white arrow indicates a *zenk-/gad65+* cell. B and C) Number of stimulus responsive excitatory (*gad65* negative) and inhibitory (*gad65* positive) cells in NCM, respectively. A to E) Average maps of excitatory cells (*zenk+/gad65-*) cells resulting from presentation of conspecific songs, canary songs, white noise, and pure tones; Average maps of inhibitory (*zenk+/gad65+*) cells resulting from presentation of the same stimulus; Color scale represents cells/mm^2. E) Bivariate plot of *zenk+/gad65-* and *zenk+/gad65+* cell density of individual maps. Scale bar, 10 µm.

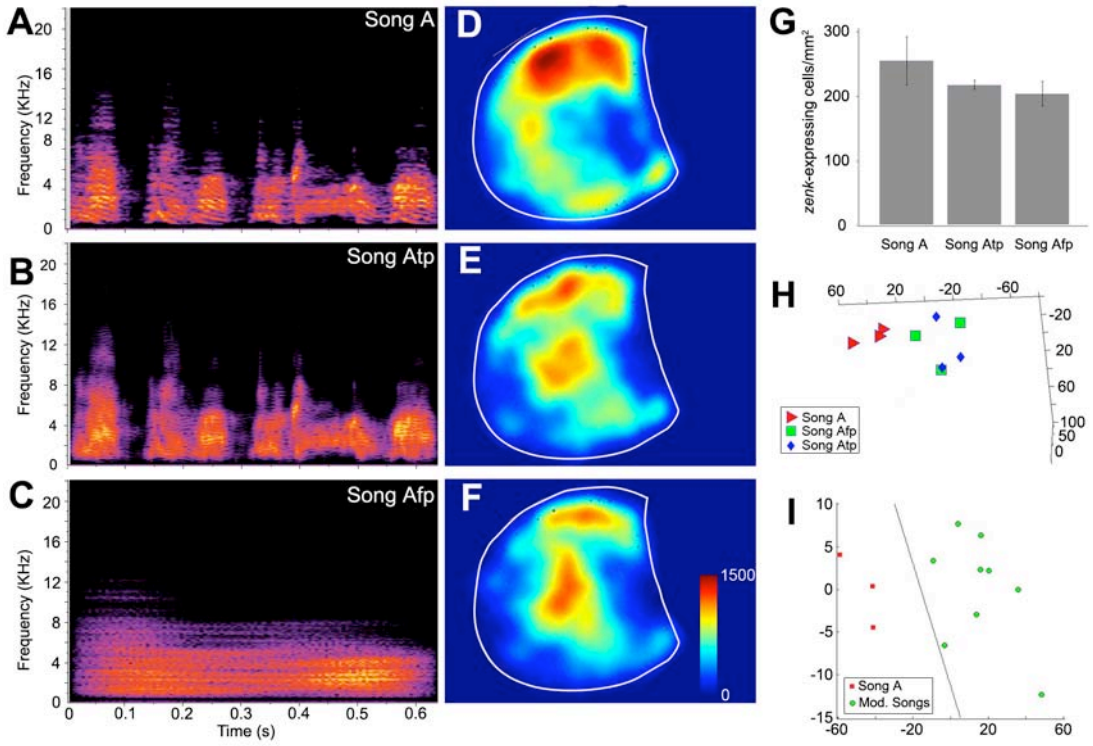


Figure 5.6. Auditory representation of spectrally and temporally modified songs. A to C) Spectrogram of a normal zebra finch song (Song A) and its frequency- (Song Atp) and time-decomposed (Song Afp) versions respectively. D to F) Average density maps of *zenk*-expressing cells in NCM of birds hearing Song A, Song Atp and Song Afp respectively; Color scale represents cells/mm^2. G) Number of *zenk*-expressing neurons in the NCM of the same birds. H) Principal component analysis for individual NCM maps. I) Linear discrimination analysis of the same density maps.

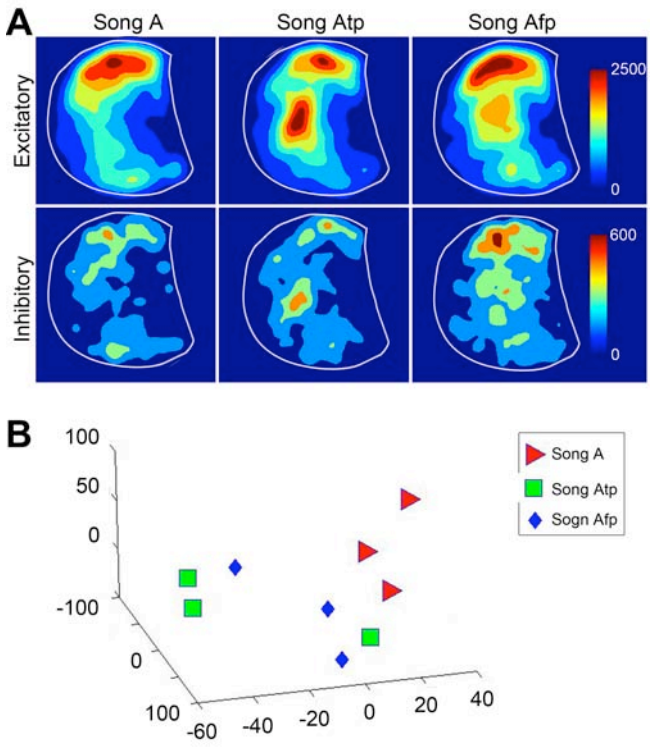


Figure 5.7. Patterns of activation for excitatory and inhibitory cells in spectrally and temporally modified songs. A) Average density maps of excitatory (*zenk+/gad65-*) and inhibitory (*zenk+/gad65+*) cells resulting from presentation of Song A, Song Afp and Song Atp; Color scale represents cells/mm^2. B) Principal component analysis for individual NCM maps.

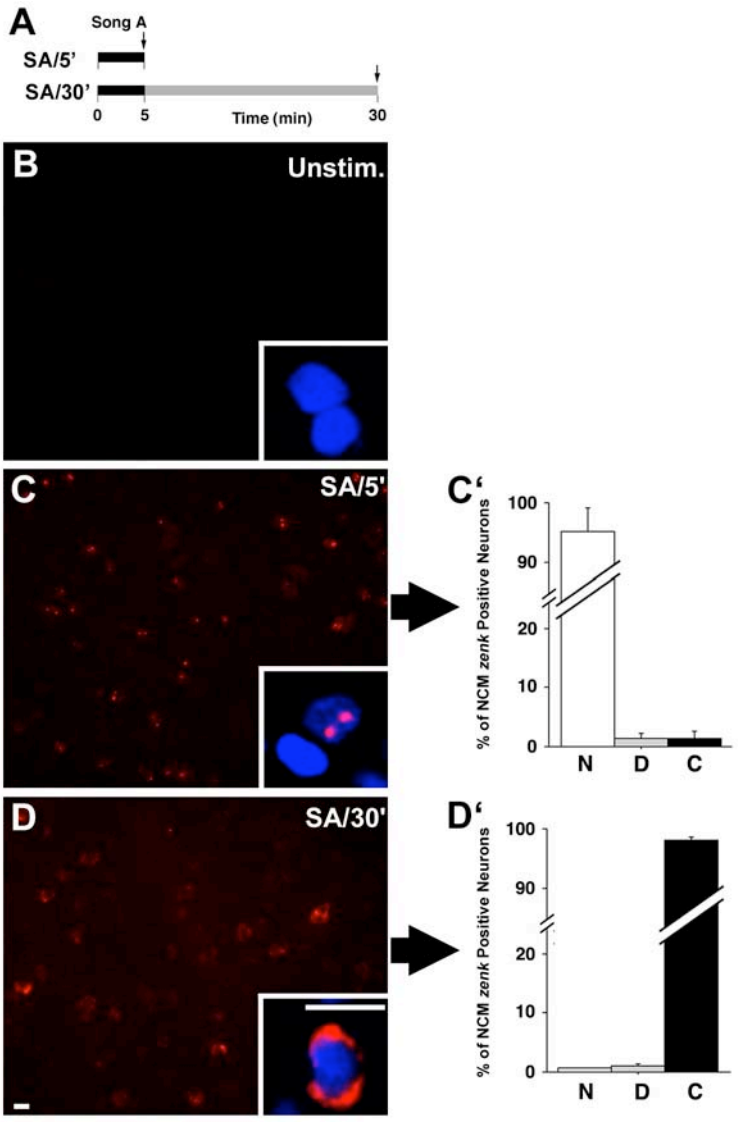

A
Song A
SA/5'
SA/30'
0
5
Time (min)
30
B
Unstim.
C
SA/5'
C'
% of NCM zenk Positive Neurons
100
90
20
10
0
N
D
C
D
SA/30'
D'
% of NCM zenk Positive Neurons
100
90
20
10
0
N
D
C

Figure 5.8. Time-dependent *zenk* mRNA localization reveals the neuronal populations responding to two distinct songs in the NCM. A) Schematic representation of the stimulation protocol. SA/5', birds stimulated for 5' and immediately sacrificed; SA/30', birds stimulated for 5' and sacrificed at 30'. B to D) Representative images of *zenk* FISH of the NCM of control (unstimulated), SA/5' and SA/30' birds respectively; insets, representative high magnification images of the typical subcellular distribution of the *zenk* mRNA for each group. C' and D') Quantification of *zenk* expressing neurons showing nuclear, cytoplasmic, or both patterns of mRNA localization (illustrated in the left column insets) of SA/5' and SA/50' birds. N, nuclear; D, double; and C, cytoplasmic. Scale bar, 10 μm.

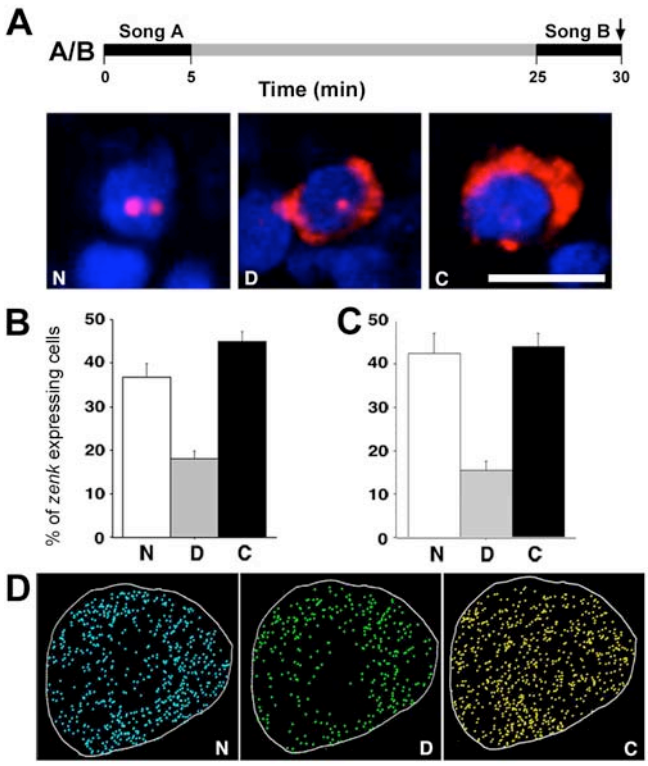


Figure 5.9. catFISH reveals the populations of neurons responding to two conspecific songs in the same bird. A) Schematic representation of the stimulation protocol. SA/B, birds stimulated with Song A for 5', 20' of silent interval, Song B for 5', and immediately sacrificed. B) Quantification of *zenk* expressing neurons showing nuclear (N), cytoplasmic (C), or both patterns (D) of mRNA localization in SA/B birds. C) Quantification of *zenk* expressing showing nuclear, cytoplasmic, or both patterns of mRNA localization in birds hearing first Song B and then Song A. D) Representative individual map of *zenk* cells subdivided into the same categories as above from a SA/B bird.

Chapter 6

DISCUSSION

The main goals of this work were to further characterize the gene expression program triggered by song and to examine the neuronal representation of songs in NCM. Using molecular, cellular, pharmacological and electrophysiological approaches I have identified and characterized integral components of the early (0-2 hr) and late (2-8 hr) genomic response to song, established a link between early and late genes, unraveled some basic regulatory mechanisms controlling gene expression, and gained novel insights into the organization of song representation in comparison with other complex acoustic stimuli in the zebra finch NCM.

Considering that a detailed discussion of each particular set of experiments was presented for individual chapters, I will summarize the main conclusions of my work as a cellular model for the transcriptional response to song in NCM auditory neurons. Hopefully, this model will serve as a framework for future studies investigating the details of the transcriptional response triggered not only by song, but also by sensory stimulation in general. In addition, I will further discuss the potential implications of activation patterns triggered by song and other complex auditory stimuli for the understanding of sensory processing and for long-lasting synaptic changes.

The main findings presented in the previous chapters can be summarized as follows:

-auditory stimulation with birdsong induces a well-orchestrated cascade of gene expression in NCM neurons that includes genes representing distinct

genomic and cellular programs, i.e. transcription factors and effector proteins;

-this gene expression program appears to be regulated by the canonical MAP-Kinase signaling pathway;

-the early transcription factor ZENK interacts *in vivo* with the promoter of at least one late song-induced gene (*syn3*);

-noradrenaline acting on α-adrenergic receptors regulates the genomic response to song and long-lasting changes in song-responsive neurons.

-the patterns of activation in zebra finch NCM, as measured by *zenk* expressing cells are stimulus dependent, with a large portion of cells particularly in caudal and dorsal domains tuned to acoustic elements present in the conspecific song.

Song stimulation thus triggers the activation of a gene expression program in auditory neurons that ultimately leads to long-lasting electrophysiological changes (Fig. 6.1). The program is regulated by attention-related (noradrenergic) and auditory inputs (likely glutamatergic). These converging inputs likely elevate intracellular calcium levels and lead to the activation of MAP kinase kinase (MEK1/2), which in turn activates the extracellular-signal regulated kinase (ERK). Once activated, ERK translocates into the nucleus where it triggers the activation

of regulatory transcription factors (RTFs). RTFs will drive the induction of immediately early genes (i.e. *arc*, *zenk*, *c-fos* and *c-jun*). Early effector proteins such as Arc can directly alter neuronal physiology independent of new rounds of RNA and protein synthesis. In contrast, inducible transcription factors (ITFs) exert a more protracted effect through the regulation of downstream target genes (for example *syn2* and *3*). ITF targets likely encode late effector proteins that can also alter the properties of song-responsive cells leading to long-lasting physiological changes such as long-term adaptation.

The implications and assumptions of this model are discussed below.

Song-induced gene expression: functional consequences

As stated in the Introduction, activity-dependent gene expression has been suggested to represent a link between neuronal activation and long-lasting changes underlying neuronal plasticity, learning and memory (Goelet et al., 1986; Clayton, 2000). To fully understand the contribution of such genomic events to neuronal change, however, it is fundamental to characterize the components involved in this response, its induction kinetics, and possible functional interactions among induced proteins.

The fact that the maintenance of long-term adaptation of NCM responses to song depends on local RNA and protein synthesis after song stimulation, suggests that song-induced genes might be involved in its consolidation. Indeed, song-induced genes like *Arc* and *zenk* have been linked to the long-term maintenance of synaptic plasticity in the mammalian hippocampus (Guzowski et

al., 2000; Jones et al., 2001). Although the contributions of specific genes to long-term adaptation to song have yet to be determined, the existence of two distinct periods or epochs of *de novo* RNA and protein synthesis likely reflects a well-orchestrated cascade. For example, the two *epochs* could reflect sequential waves of gene expression in which the initially synthesized proteins influence and regulate late targets. More specifically, during the first wave, synthesis inhibitors likely prevent the induction of immediate-early genes such as *zenk*, *c-fos*, *c-jun* and *Arc*. During the second wave, synthesis inhibitors likely block the expression of slowly rising genes and of targets of regulatory transcription factors induced during the first wave of gene expression. The identified late song-regulated transcripts *syn2* and *3* fit well within this second category. The second wave could also reflect new activation of early genes induced by re-activation (reverberation) of song-responsive circuits. In favor of the latter hypothesis is the fact that the *Arc* response to song contains a second period of mRNA accumulation around 4 hours after stimulation onset (Velho et al., 2005), a phenomenon that had been previously described for this gene during some forms of learning in rodents (Montag-Sallaz et al., 1999; Montag-Sallaz and Montag, 2003).

It is intriguing that while activity-dependent transcription in mammals has been linked to the maintenance of a potentiated state of activated synapses (hippocampal LTP), in songbird NCM it appears to be associated with a depression (adaptation) of song-induced responses (Chew et al., 1995). One possibility is that the mechanisms involved in long-lasting synaptic changes, and

the functions of activity-dependent genes like *zenk*, *c-fos, c-jun*, *bdnf* and *Arc* are not conserved across birds and mammals. This possibility seems unlikely, given the high degree of conservation in sequence (e.g., the DNA-binding motif of ZENK is 100% identical between birds and mammals; Mello, unpublished observation), kinetics of activity-dependent regulation and cellular distribution patterns of activity-dependent gene expression between birds and mammals. A more likely possibility is that activity-dependent transcriptional regulation is associated with synaptic plasticity independently of the direction of the synaptic change. In this case, even though the early genomic responses to neuronal activation share several components, the end result is a circuit-specific activation of distinct target molecules leading to different synaptic modifications in both birds and mammals. It is also possible that this species difference reflects different brain regions: it remains to be determined whether stimulus-specific changes akin to adaptation in NCM are also seen in comparable portions of the mammalian auditory cortex. Conversely, it remains to be determined whether different stimulation protocols could also lead to potentiation in NCM.

A better understanding of the significance of the transcriptional response to song also requires an analysis of the function of individual song-induced genes. The *zenk* gene (also known as *zif-268*, *egr-1*, *NGFI-A* and *Krox-24*), encodes a zinc-finger DNA-binding protein that can regulate the expression of downstream target genes with the appropriate binding motif in their promoters (Milbrandt, 1987; Christy and Nathans, 1989; Knapska and Kaczmarek, 2004). Two other known song-induced genes, *c-fos* and *c-jun*, together comprise the

activator protein-1 (AP-1) complex (Sonnenberg et al., 1989). Disruption of expression of one member of the AP-1 complex, *c-fos*, using antisense oligodeoxynucleotides prevents the long-term maintenance of some forms of hippocampal-dependent memory (Grimm et al., 1997; Guzowski and McGaugh, 1997). Since transcription factors such as *zenk* and *c-fos* exert their effects by regulating downstream targets, understanding the mechanisms of action of these genes in long-term plasticity and memory requires the identification of such targets. In the present work, I have searched and identified putative ZENK targets through GenBank screenings for genes that have the conserved ZENK binding domain in their promoters (Mello and Velho, unpublished observations). Because an avian genome was not yet available, I relied on searches of mammalian genomic sequences. This work resulted in the identification of synapsins as integral components of the genomic response to song, consistent with the fact that ZENK (Egr1) regulates *synI* and *II* in cultured cells (Thiel et al., 1994; Petersohn et al., 1995; James et al., 2006).

The interaction between the several components of the genomic response to song includes constitutive regulatory transcription factors (RTFs; e.g. Elk, CREB) acting on inducible transcription factors (ITFs; zenk, ap-1), which in turn up or downregulate target genes (e.g. synapsins). However, such interactions are not simply positive ones. In fact, the results using the protein synthesis inhibitor cycloheximide to prevent protein expression during the early phase of the response to song indicate that synapsin genes are under positive regulation by RTFs activated by song, but also are under suppressive action by early song-

induced proteins. With the recent sequencing of the zebra finch genome, I have identified ZENK binding sites in the *syn3* promoter and examined and demonstrated through chromatin immunoprecipitation (ChIP) that ZENK interacts with this promoter. Further experiments specifically manipulating the ZENK protein are needed to further clarify its role on the expression of synapsins and other late genes.

In contrast to transcription factors, other song-induced genes such as *Arc* and *bdnf* encode effectors proteins thought to act by altering cellular properties independently of further transcription (Lanahan and Worley, 1998). Arc subcellular localization indicates that it acts specifically at recently activated synapses (Link et al., 1995; Steward et al., 1998; Steward and Worley, 2001b) through interactions with cytoskeletal (F-actin) and signal transduction (NMDA receptor complex and possibly calcium-dependent calmodulin kinase II) elements associated with the post-synaptic density (Husi et al., 2000; Steward and Worley, 2001b; Guzowski, 2002). Indeed, recent studies suggest that Arc regulates different aspects of synaptic physiology, including AMPA receptor trafficking and endocytosis (Chowdhury et al., 2006; Shepherd et al., 2006). Moreover, by downregulating the surface expression of AMPA receptors, Arc also alters synaptic strength, ultimately leading to long-term depression of hippocampal neurons (Rial Verde et al., 2006). Finally and more importantly, Arc knockout mice show significant memory retention deficits (Plath et al., 2006) in agreement with this protein playing a significant role on synaptic physiology.

In songbirds, the cellular distribution we observed for *Arc* mRNA is consistent with a dendritic localization of this transcript in song-responsive neurons (Velho et al., 2005). Interestingly, the properties of song adaptation, i.e. simultaneous and independent adaptation to multiple songs, suggest that different songs activate the same NCM neurons differently, likely involving distinct portions of the dendritic arborization. Thus, Arc and associated molecules could serve as molecular tags that help differentiate activated vs. non-activated synapses, thus contributing to long-term song-specific adaptation at the post-synaptic level in NCM. This possibility is consistent with Arc exerting a key regulatory role on synaptic depression (Plath et al., 2006; Rial Verde et al., 2006). Another song-induced direct effector, *bdnf*, is also thought to be involved in different aspects of synaptic plasticity, such as regulating dendritic and axonal morphology, synaptic efficacy and synaptogenesis (Lohof et al., 1993; Cabelli et al., 1995; Kang and Schuman, 1995; Levine et al., 1995; McAllister et al., 1995; Patterson et al., 1996; Causing et al., 1997; Horch et al., 1999). BDNF has also been associated with the consolidation of contextual fear memories in rats (Lee et al., 2004), and could play a similar role in the formation of song-related memories in songbirds.

Towards an understanding of the norepinephrine's role in learning and memory

One of the most interesting findings of this whole set of experiments is the regulation of gene expression and long-term adaptation by norepinephrine (NE).

As discussed earlier in this dissertation, noradrenergic transmission has been widely linked to the formation of long-term memories in the vertebrate brain (see McGaugh, 2000; McGaugh and Izquierdo, 2000; Gibbs and Summers, 2002 for reviews). Pharmacological blockade of noradrenergic transmission disrupts the consolidation of memories without affecting the acquisition (short-term memory) of the specific task. Conversely, low doses of NE can improve learning (see Gibbs and Summers, 2002 for a review). However, the cellular mechanisms underlying these effects are not understood. Interestingly, the effects of NE antagonism are similar to those observed for protein and RNA synthesis inhibitors. Our current findings may provide a mechanistic explanation for NE effects and its similarities with *de novo* RNA/protein synthesis requirements for memory consolidation.

In the zebra finch NCM, the blockade of α-adrenergic transmission not only prevents the induction of gene expression by song, but also disrupts the long-term maintenance of a neural correlate of learning and memory, i.e. song adaptation. Importantly, the pharmacological blockade does not affect the acquisition phase or the short-term adaptation, much like the effects of RNA and protein and RNA synthesis blockers (Chew et al., 1995). Therefore, NE action in learning and memory may largely reflect the activation or amplification of intracellular signaling cascades that lead to the expression of plasticity related genes.

As discussed in the introduction and shown in Chapter 5, song-induced gene expression depends on the stimulus, the context and is largely associated

with novelty. The modulation of song-induced gene expression by noradrenergic system may help explaining some of these characteristics. Specifically, the increase in gene expression observed when pairing electric-shocks (Jarvis et al., 1995) or visual cues (Kruse et al., 2004; Avey et al., 2005) with song stimulation may represent further engagement of the noradrenergic system by cross-modal stimuli (i.e. somatosensory or visual). Conversely, the absence of induction by tones, for example, may represent the lack of engagement of the noradrenergic system by this behaviorally irrelevant stimulus. In addition, a disengagement of the noradrenergic system could also explain why young zebra finches learn best when they are adequately engaged during sensory exposure to tutor song either by the presence of a live tutor or by actively triggering the playback (Tchernichovski et al., 1999) and less when social interaction is limited (Price, 1979). More specifically, by modulating activity-dependent gene expression norepinephrine could also have a significant effect on the consolidation of sensory memories essential for normal song development.

Taken together, I believe the current results represent a significant step towards the understanding of the role of NE in learning and memory. Our cellular model (Fig. 6.1) proposes that a convergence of attention-related mechanisms (NE transmission) and activation-dependent signaling (inputs from the ascending auditory pathway) results in the activation of intracellular signaling cascade that modulate long-lasting changes without necessarily causing immediate effects on the electrophysiological responses to stimulation. Perhaps it can also help

explaining why we need to pay attention in order to learn and why emotionally charged experiences in humans are so memorable.

Are there functional subdomains in NCM?

Growing evidence suggests that NCM is not a homogeneous area. Recent gene expression studies show marked neurochemical differences between caudal and rostral NCM. For instance, calbindin and aromatase expressing cells are concentrated in caudal NCM (Pinaud et al., 2006). Similarly, I found that the activity-dependent neuronal pentraxin 1 (*narp*) is also enriched in large neuronal cells in this same region. Intriguingly, electrophysiological studies also indicate the existence of fundamental differences between caudal and rostral regions, i.e. long-term adaptation occurs primarily in units located within the caudal NCM (Chew et al., 1995).

Recent and previous evidence from our lab indicates that rostral and caudal NCM may also differ in their connectivity. Field L input to NCM appears to arrive primarily into rostral NCM (Vates et al., 1996). Injections of retrograde anatomical tracers into caudal NCM result in labeled cells in rostral NCM, whereas small biocitin injections into dorso-caudal NCM revealed fibers traveling downwards towards ventro-caudal region but not into rostral NCM (C.M. personal observation). Although these results await further confirmation, they further suggest that NCM contains at least two anatomically distinct subdomains.

Our gene mapping experiments (Chapter 5) demonstrated the existence of regional variations in the density of *zenk*-expressing cells. For example, the

caudal most region of NCM does not show *zenk* induction for any stimuli used other than conspecific song, suggesting a higher degree of selectivity. In contrast, *zenk*-expressing cells are highly represented in central and parts of the dorsal NCM of all stimulated birds (except birds stimulated with tones that do not show a significant induction throughout NCM), indicating lower selectivity.

Is there a relationship between the regional variations in gene expression and adaptation? I believe this is quite possible. All stimuli tested by Chew and colleagues led to short-term electrophysiological adaptation, from human voice to white noise (Chew et al., 1995; Chew et al., 1996). This adaptation however, was quickly lost (~3 hr) for the canary song and human voice, while it was maintained for zebra finch vocalizations (calls and songs). It is thus tempting to speculate that the lack of long-term adaptation relates to the reduced levels of activity-dependent gene expression triggered by non-zebra finch stimuli such as the canary song and white noise in caudal NCM.

Summary and future directions

The fascinating general question of how learning occurs represents an enormous quest that not even more than 100 years of active research and countless brilliant minds have managed to conquer. We have just scratched the surface and many questions are yet to be answered. Nonetheless, what I have learned from the set of experiments that compose this dissertation represent some firm steps towards a general understanding of how auditory stimulation influences gene expression and the formation of long-lasting changes in neuronal

circuits. I would like to close this dissertation with some less ambitious and much more specific questions raised by our current results. Although the list of future experiments is certainly more extensive than the ones listed below, these would be the most immediate questions.

In the first set of experiments, I have demonstrated that activity-dependent early genes linked to synaptic plasticity and learning in mammals are co-activated in NCM auditory neurons. This represents one of the first demonstrations that neuronal activation leads to the co-activation of multiple genes representing distinct cellular programs, an assumption that has been largely untested. Nonetheless, the number of genes that are regulated in concert with neuronal activation is likely much larger than we have tested. With the fast development of new resources such as a zebra finch microarray (containing up to 15,000 unique annotated transcripts), we can now examine the full extent of the genomic response to song in these birds. Moreover, with new technologies such as laser-capture and the ability to extract and work with minute amounts of RNA, one can also systematically investigate the extent to which the genomic response differs in excitatory vs. inhibitory song-responsive neurons.

In the second set of experiments, I have shown that synapsins also represent an integral part of the genomic response to song. Moreover, I have demonstrated that *zenk* interacts with the *syn3* promoter *in vivo*, and that the regulation of *syn3* by song is altered (de-repressed) by protein synthesis inhibitors. Although these results provided evidence of suppression by early song-induced proteins, it would be interesting to directly investigate the

contribution of ZENK to *syn3* regulation. In addition, even though we know that song-induced gene expression is required for long-lasting synaptic changes in the NCM, the precise contribution of individual component genes remains unknown. With the recent development of new genetic tools for birds, i.e transgenic animals (Scott and Lois, 2005) and effective viral vectors (Haesler et al., 2007), we may soon be able to answer these questions in zebra finches.

In the third set of experiments I have shown that song-induced gene expression depends on local noradrenergic transmission. I have also shown that noradrenergic transmission is required for the consolidation of long-term (not short-term) adaptation that follows repeated song presentations. These results suggest a fundamental role for the noradrenergic system in the formation of long-lasting synaptic changes. Presumably the locus coeruleus (LoC), the main source of noradrenergic inputs to the brain, must be directly activated by song or by a secondary process triggered by song stimulation. It would be interesting to directly investigate the contribution of the LoC to NCM auditory responses using simultaneous recordings of both areas. Although I have attempted to probe LoC activation using gene expression, I could not confidently determine differences between unstimulated control and song-stimulates birds.

The nature of the auditory input onto NCM neurons has not been studied yet. Although this input is likely to be glutamatergic, this hypothesis needs to be directly tested. Once the nature of the auditory input is determined, it would be extremely appealing to determine the precise contribution of noradrenergic and

glutamatergic (?) inputs to sensory induced gene expression and to short and long-term adaptation.

In the fourth set of experiments, I have shown that the patterns of *zenk*-expression in NCM are stimulus dependent. Moreover, these patterns indicate the existence of subdomains in the NCM. One particularly interesting observation regards the stronger recruitment of gabaergic cells by heterospecific songs compared to all other stimuli used. Because canary song contains narrow-band syllables that are mostly absent in zebra finch songs (and white noise), it would be interesting to investigate the extent to which these syllables contribute to the recruitment of gabaergic cells.

Temporal and spectral decomposition of songs also revealed some intriguing converging patterns. These patterns tend to resemble more of patterns evoked by white noise and canary songs. It would be interesting to investigate the representation of songs with a more intermediate level of degradation (we originally used 128 and 32 Hz as low pass filters). Such intermediate levels may be more informative given the current converging patterns we have obtained. In addition, one can also use the more refined method of catFISH and examine two modified songs in a single bird to determine the levels of overlap for different levels of song degradation.

A full understanding of the sensory-induced gene expression is fundamental for understanding the formation of sensory memories. Songbirds represent one of the most tractable animal models for such studies. I hope the work I have developed with the help of so many colleagues in the last 5 years will

help direct future investigations aiming to unravel this more than a century old question of memory consolidation. I also hope my brain spent enough energy consolidating some of the great experiences I have lived at OHSU.

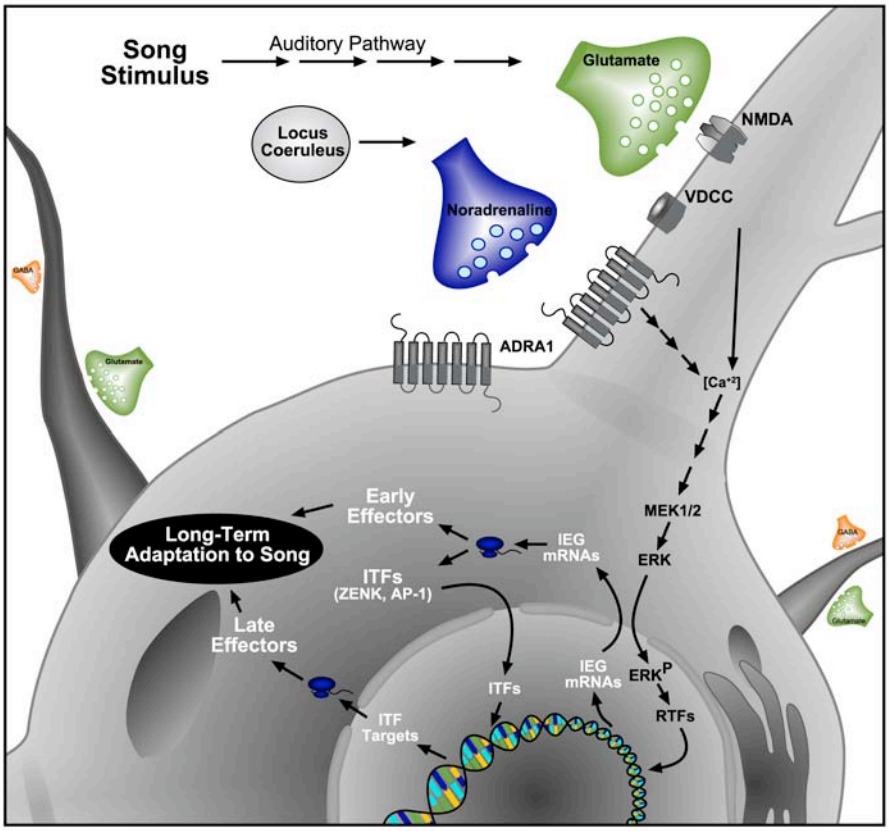


Figure 6.1. Song-induced gene expression program in auditory neurons.

7 REFERENCES

Alfonso J, Frick LR, Silberman DM, Palumbo ML, Genaro AM, Frasch AC (2006) Regulation of hippocampal gene expression is conserved in two species subjected to different stressors and antidepressant treatments. Biol Psychiatry 59:244-251.

Angel P, Allegretto EA, Okino ST, Hattori K, Boyle WJ, Hunter T, Karin M (1988) Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. Nature 332:166-171.

Arnold AP (1980) Effects of androgens on volumes of sexually dimorphic brain regions in the zebra finch. Brain Research 185:441-444.

Arnold AP, Bottjer SW, Brenowitz EA, Nordeen EJ, Nordeen KW (1986) Sexual dimorphisms in the neural vocal control system in song birds: ontogeny and phylogeny. Brain, Behavior and Evolution 28:22-31.

Aston-Jones G, Bloom FE (1981a) Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 1:876-886.

Aston-Jones G, Bloom FE (1981b) Nonrepinephrine-containing locus coeruleus neurons in behaving rats exhibit pronounced responses to non-noxious environmental stimuli. J Neurosci 1:887-900.

Aston-Jones G, Cohen JD (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. Annu Rev Neurosci 28:403-450.

Avey MT, Phillmore LS, MacDougall-Shackleton SA (2005) Immediate early gene expression following exposure to acoustic and visual components of courtship in zebra finches. Behav Brain Res 165:247-253.

Bailey CH, Bartsch D, Kandel ER (1996) Toward a molecular definition of long-term memory storage. Proceedings of the National Academy of Sciences U S A 93:13445-13452.

Bailey DJ, Wade J (2003) Differential expression of the immediate early genes FOS and ZENK following auditory stimulation in the juvenile male and female zebra finch. Brain Res Mol Brain Res 116:147-154.

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13-26.

Bolhuis JJ, Zijlstra GG, den Boer-Visser AM, Van Der Zee EA (2000) Localized neuronal activation in the zebra finch brain is related to the strength of song learning. Proc Natl Acad Sci U S A 97:2282-2285.
Bonke BA, Bonke D, Scheich H (1979) Connectivity of the auditory forebrain nuclei in the guinea fowl (*Numida meleagris*). Cell and Tissue Research 200:101-121.
Bottjer SW (1993) The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. Journal of Neurobiology 24:51-69.
Bottjer SW, Miesner EA, Arnold AP (1984) Forebrain lesions disrupt development but not maintenance of song in passerine birds. Science 224:901-903.
Bottjer SW, Halsema KA, Brown SA, Miesner EA (1989) Axonal connections of a forebrain nucleus involved with vocal learning in zebra finches. Journal of Comparative Neurology 279:312-326.
Bramham CR, Bacher-Svendsen K, Sarvey JM (1997) LTP in the lateral perforant path is beta-adrenergic receptor-dependent. Neuroreport 8:719-724.
Brauth SE, McHale CM (1988) Auditory pathways in the budgerigar. II. Intratelencephalic pathways. Brain, Behavior and Evolution 32:193-207.
Brauth SE, McHale CM, Brasher CA, Dooling RJ (1987) Auditory pathways in the budgerigar. I. Thalamo-telencephalic projections. Brain, Behavior and Evolution 30:174-199.
Brenowitz EA (1997) Comparative approaches to the avian song system. Journal of Neurobiology 33:517-531.
Brenowitz EA (2004) Plasticity of the adult avian song control system. Ann N Y Acad Sci 1016:560-585.
Brenowitz EA, Arnold AP (1990) The effects of systemic androgen treatment on androgen accumulation in song control regions of the adult female canary brain. Journal of Neurobiology 21:837-843.
Brenowitz EA, Margoliash D, Nordeen KW (1997a) The neurobiology of birdsong. Journal of Neurobiology 33.
Brenowitz EA, Margoliash D, Nordeen KW (1997b) An introduction to birdsong and the avian song system. Journal of Neurobiology 33:495-500.
Butler AB, Hodos W (1996) Comparative Vertebrate Neuroanatomy: Evolution and Adaptation. New York, NY: Wiley-Liss.
Cabelli RJ, Hohn A, Shatz CJ (1995) Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. Science 267:1662-1666.
Cardin JA, Schmidt MF (2003) Song system auditory responses are stable and highly tuned during sedation, rapidly modulated and unselective during wakefulness, and suppressed by arousal. J Neurophysiol 90:2884-2899.
Castelino CB, Ball GF (2005) A role for norepinephrine in the regulation of context-dependent ZENK expression in male zebra finches (Taeniopygia guttata). Eur J Neurosci 21:1962-1972.
Casto JM, Ball GF (1996) Early administration of 17beta-estradiol partially masculinizes song control regions and alpha2-adrenergic receptor

distribution in European starlings (Sturnus vulgaris). Hormones and Behavior 30:387-406.
Catchpole CK, Slater PJB (1995) Bird song: biological themes and variations. U.K.: Cambridge University Press.
Causing CG, Gloster A, Aloyz R, Bamji SX, Chang E, Fawcett J, Kuchel G, Miller FD (1997) Synaptic innervation density is regulated by neuron-derived BDNF. Neuron 18:257-267.
Cavalleri GL, Weale ME, Shianna KV, Singh R, Lynch JM, Grinton B, Szoeke C, Murphy K, Kinirons P, O'Rourke D, Ge D, Depondt C, Claeys KG, Pandolfo M, Gumbs C, Walley N, McNamara J, Mulley JC, Linney KN, Sheffield LJ, Radtke RA, Tate SK, Chissoe SL, Gibson RA, Hosford D, Stanton A, Graves TD, Hanna MG, Eriksson K, Kantanen AM, Kalviainen R, O'Brien TJ, Sander JW, Duncan JS, Scheffer IE, Berkovic SF, Wood NW, Doherty CP, Delanty N, Sisodiya SM, Goldstein DB (2007) Multicentre search for genetic susceptibility loci in sporadic epilepsy syndrome and seizure types: a case-control study. Lancet Neurol 6:970-980.
Cha-Molstad H, Keller DM, Yochum GS, Impey S, Goodman RH (2004) Cell-type-specific binding of the transcription factor CREB to the cAMP-response element. Proc Natl Acad Sci U S A 101:13572-13577.
Chaudhuri A (1997) Neural activity mapping with inducible transcription factors. Neuroreport 8:v-ix.
Chen Q, He G, Qin W, Chen QY, Zhao XZ, Duan SW, Liu XM, Feng GY, Xu YF, St Clair D, Li M, Wang JH, Xing YL, Shi JG, He L (2004) Family-based association study of synapsin II and schizophrenia. Am J Hum Genet 75:873-877.
Cheng HY, Clayton DF (2004) Activation and habituation of extracellular signal-regulated kinase phosphorylation in zebra finch auditory forebrain during song presentation. J Neurosci 24:7503-7513.
Chew SJ, Vicario DS, Nottebohm F (1996) A large-capacity memory system that recognizes the calls and songs of individual birds. Proceedings of the National Academy of Sciences U S A 93:1950-1955.
Chew SJ, Mello C, Nottebohm F, Jarvis E, Vicario DS (1995) Decrements in auditory responses to a repeated conspecific song are long-lasting and require two periods of protein synthesis in the songbird forebrain. Proc Natl Acad Sci U S A 92:3406-3410.
Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Kuhl D, Huganir RL, Worley PF (2006) Arc/Arg3.1 interacts with the endocytic machinery to regulate AMPA receptor trafficking. Neuron 52:445-459.
Christy B, Nathans D (1989) DNA binding site of the growth factor-inducible protein Zif268. Proceedings of the National Academy of Sciences U S A 86:8737-8741.
Christy BA, Lau LF, Nathans D (1988) A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc Natl Acad Sci U S A 85:7857-7861.

Cirelli C, Tononi G (2000) Differential expression of plasticity-related genes in waking and sleep and their regulation by the noradrenergic system. Journal of Neuroscience 20:9187-9194.
Cirelli C, Pompeiano M, Tononi G (1996) Neuronal gene expression in the waking state: a role for the locus coeruleus. Science 274:1211-1215.
Clayton DF (2000) The genomic action potential. Neurobiol Learn Mem 74:185-216.
Cole AJ, Saffen DW, Baraban JM, Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature 340:474-476.
Curran T, Morgan JI (1985) Superinduction of c-fos by nerve growth factor in the presence of peripherally active benzodiazepines. Science 229:1265-1268.
Curran T, Miller AD, Zokas L, Verma IM (1984) Viral and cellular fos proteins: a comparative analysis. Cell 36:259-268.
Dave AS, Yu AC, Margoliash D (1998) Behavioral state modulation of auditory activity in a vocal motor system. Science 282:2250-2254.
Davis S, Bozon B, Laroche S (2003) How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? Behav Brain Res 142:17-30.
Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S (2000) The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to control long-term potentiation-dependent gene expression in the dentate gyrus in vivo. J Neurosci 20:4563-4572.
Denisenko-Nehrbass NI, Jarvis E, Scharff C, Nottebohm F, Mello CV (2000) Site-specific retinoic acid production in the brain of adult songbirds. Neuron 27:359-370.
Ding L, Perkel DJ, Farries MA (2003) Presynaptic depression of glutamatergic synaptic transmission by D1-like dopamine receptor activation in the avian basal ganglia. J Neurosci 23:6086-6095.
Doupe AJ, Konishi M (1991) Song-selective auditory circuits in the vocal control system of the zebra finch. Proc Natl Acad Sci U S A 88:11339-11343.
Doupe AJ, Kuhl PK (1999) Birdsong and human speech: common themes and mechanisms. Annual Review of Neuroscience 22:567-631.
Duda RO, Hart PE, Stork DG Pattern classification, 2nd ed. Edition: New York : John Wiley, 2000-.
Finkbeiner S, Greenberg ME (1998) Ca2+ channel-regulated neuronal gene expression. J Neurobiol 37:171-189.
Foote SL, Aston-Jones G, Bloom FE (1980) Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. Proc Natl Acad Sci U S A 77:3033-3037.
Gaffield MA, Betz WJ (2007) Synaptic vesicle mobility in mouse motor nerve terminals with and without synapsin. J Neurosci 27:13691-13700.
Gehr DD, Capsius B, Grabner P, Gahr M, Leppelsack HJ (1999) Functional organisation of the field-L-complex of adult male zebra finches. Neuroreport 10:375-380.

Gentner TQ, Hulse SH (2000) Female european starling preference and choice for variation in conspecific male song. Animal Behavior 59:443-458.
Gentner TQ, Hulse SH, Duffy D, Ball GF (2001) Response biases in auditory forebrain regions of female songbirds following exposure to sexually relevant variation in male song. Journal of Neurobiology 46:48-58.
George JM, Clayton DF (1992) Differential regulation in the avian song control circuit of an mRNA predicting a highly conserved protein related to protein kinase C and the bcr oncogene. Molecular Brain Research 12:323-329.
Ghazanfar AA, Hauser MD (2001) The auditory behaviour of primates: a neuroethological perspective. Curr Opin Neurobiol 11:712-720.
Ghosh A, Ginty DD, Bading H, Greenberg ME (1994) Calcium regulation of gene expression in neuronal cells. Journal of Neurobiology 25:294-303.
Gibbs ME, Summers RJ (2002) Role of adrenoceptor subtypes in memory consolidation. Prog Neurobiol 67:345-391.
Gitler D, Takagishi Y, Feng J, Ren Y, Rodriguiz RM, Wetsel WC, Greengard P, Augustine GJ (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. J Neurosci 24:11368-11380.
Gobes SM, Bolhuis JJ (2007) Birdsong memory: a neural dissociation between song recognition and production. Curr Biol 17:789-793.
Goelet P, Castellucci VF, Schacher S, Kandel ER (1986) The long and the short of long-term memory--a molecular framework. Nature 322:419-422.
Goldman SA, Nottebohm F (1983) Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proceedings of the National Academy of Sciences U S A 80:2390-2394.
Gomez-Pinilla F, So V, Kesslak JP (2001) Spatial learning induces neurotrophin receptor and synapsin I in the hippocampus. Brain Res 904:13-19.
Grace JA, Amin N, Singh NC, Theunissen FE (2003) Selectivity for conspecific song in the zebra finch auditory forebrain. J Neurophysiol 89:472-487.
Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780-785.
Grewal SS, York RD, Stork PJS (1999) Extracellular-signal-regulated kinase signallin in neurons. Curr Opin Neurobiol 9:544-553.
Grimm R, Schicknick H, Riede I, Gundelfinger ED, Herdegen T, Zuschratter W, Tischmeyer W (1997) Suppression of c-fos induction in rat brain impairs retention of a brightness discrimination reaction. Learn Mem 3:402-413.
Guzowski JF (2002) Insights into immediate-early gene function in hippocampal memory consolidation using antisense oligonucleotide and fluorescent imaging approaches. Hippocampus 12:86-104.
Guzowski JF, McGaugh JL (1997) Antisense oligodeoxynucleotide-mediated disruption of hippocampal cAMP response element binding protein levels impairs consolidation of memory for water maze training. Proc Natl Acad Sci U S A 94:2693-2698.

Guzowski JF, McNaughton BL, Barnes CA, Worley PF (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci 2:1120-1124.
Guzowski JF, Setlow B, Wagner EK, McGaugh JL (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. J Neurosci 21:5089-5098.
Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA (2000) Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci 20:3993-4001.
Hackett JT, Cochran SL, Greenfield LJ, Jr., Brosius DC, Ueda T (1990) Synapsin I injected presynaptically into goldfish mauthner axons reduces quantal synaptic transmission. J Neurophysiol 63:701-706.
Haesler S, Rochefort C, Georgi B, Licznerski P, Osten P, Scharff C (2007) Incomplete and inaccurate vocal imitation after knockdown of FoxP2 in songbird basal ganglia nucleus Area X. PLoS Biol 5:e321.
Han HQ, Nichols RA, Rubin MR, Bahler M, Greengard P (1991) Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. Nature 349:697-700.
Hessler NA, Doupe AJ (1999a) Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. Journal of Neuroscience 19:10461-10481.
Hessler NA, Doupe AJ (1999b) Social context modulates singing-related neural activity in the songbird forebrain. Nature Neuroscience 2:209-211.
Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354:269-279.
Holzenberger M, Jarvis ED, Chong C, Grossman M, Nottebohm F, Scharff C (1997) Selective expression of insulin-like growth factor II in the songbird brain. Journal of Neuroscience 17:6974-6987.
Horch HW, Kruttgen A, Portbury SD, Katz LC (1999) Destabilization of cortical dendrites and spines by BDNF. Neuron 23:353-364.
Huang YY, Kandel ER (1996) Modulation of both the early and the late phase of mossy fiber LTP by the activation of beta-adrenergic receptors. Neuron 16:611-617.
Huang YY, Martin KC, Kandel ER (2000) Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. Journal of Neuroscience 20:6317-6325.
Humeau Y, Doussau F, Vitiello F, Greengard P, Benfenati F, Poulain B (2001) Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in Aplysia. J Neurosci 21:4195-4206.

Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG (2000) Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. Nat Neurosci 3:661-669.

Impey S, McCorkle SR, Cha-Molstad H, Dwyer JM, Yochum GS, Boss JM, McWeeney S, Dunn JJ, Mandel G, Goodman RH (2004) Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119:1041-1054.

Iwata M, Shirayama Y, Ishida H, Kawahara R (2006) Hippocampal synapsin I, growth-associated protein-43, and microtubule-associated protein-2 immunoreactivity in learned helplessness rats and antidepressant-treated rats. Neuroscience 141:1301-1313.

James AB, Conway AM, Morris BJ (2006) Regulation of the neuronal proteasome by Zif268 (Egr1). J Neurosci 26:1624-1634.

Jarvis ED, Nottebohm F (1997) Motor-driven gene expression. Proc Natl Acad Sci U S A 94:4097-4102.

Jarvis ED, Mello CV, Nottebohm F (1995) Associative learning and stimulus novelty influence the song-induced expression of an immediate early gene in the canary forebrain. Learn Mem 2:62-80.

Jarvis ED, Scharff C, Grossman MR, Ramos JA, Nottebohm F (1998) For whom the bird sings: context-dependent gene expression. Neuron 21:775-788.

Jarvis ED, Gunturkun O, Bruce L, Csillag A, Karten H, Kuenzel W, Medina L, Paxinos G, Perkel DJ, Shimizu T, Striedter G, Wild JM, Ball GF, Dugas-Ford J, Durand SE, Hough GE, Husband S, Kubikova L, Lee DW, Mello CV, Powers A, Siang C, Smulders TV, Wada K, White SA, Yamamoto K, Yu J, Reiner A, Butler AB (2005) Avian brains and a new understanding of vertebrate brain evolution. Nat Rev Neurosci 6:151-159.

Jeong JK, Velho TA, Mello CV (2005) Cloning and expression analysis of retinoic acid receptors in the zebra finch brain. J Comp Neurol 489:23-41.

Johnson F, Sablan MM, Bottjer SW (1995) Topographic organization of a forebrain pathway involved with vocal learning in zebra finches. Journal of Comparative Neurology 358:260-278.

Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S, Davis S (2001) A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. Nat Neurosci 4:289-296.

Kang H, Schuman EM (1995) Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. Science 267:1658-1662.

Kao HT, Porton B, Hilfiker S, Stefani G, Pieribone VA, DeSalle R, Greengard P (1999) Molecular evolution of the synapsin gene family. J Exp Zool 285:360-377.

Karten HJ (1967) The organization of the ascending auditory pathway in the pigeon (Columba livia). I. Diencephalic projections of the inferior colliculus (nucleus mesencephali lateralis, pars dorsalis). Brain Res 6:409-427.

Karten HJ (1968) The ascending auditory pathway in the pigeon (*Columba livia*). II. Telencephalic projections of the nucleus ovoidalis thalami. Brain Research 11:134-153.
Karten HJ (1997) Evolutionary developmental biology meets the brain: the origins of mammalian cortex. Proc Natl Acad Sci U S A 94:2800-2804.
Karten HJ, Hodos W (1967) A stereotaxic atlas of the brain of the pigeon (*Columba livia*). Baltimore, MD: Johns Hopkins Press.
Kelley DB, Nottebohm F (1979) Projections of a telencephalic auditory nucleus-field L-in the canary. J Comp Neurol 183:455-469.
Kimpo RR, Doupe AJ (1997) FOS is induced by singing in distinct neuronal populations in a motor network. Neuron 18:315-325.
Knapska E, Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? Prog Neurobiol 74:183-211.
Konishi M (1965) Effects of deafening on song development in American robins and black- headed grosbeaks. Z Tierpsychol 22:584-599.
Kroodsma DE, Miller EH (1996) Ecology and Evolution of Acoustic Communication in Birds. Ithaca, NY: Cornell University Press.
Kruse AA, Stripling R, Clayton DF (2000) Minimal experience required for immediate-early gene induction in zebra finch neostriatum. Neurobiology of Learning and Memory 74:179-184.
Kruse AA, Stripling R, Clayton DF (2004) Context-specific habituation of the zenk gene response to song in adult zebra finches. Neurobiol Learn Mem 82:99-108.
Lachman HM, Stopkova P, Rafael MA, Saito T (2005) Association of schizophrenia in African Americans to polymorphism in synapsin III gene. Psychiatr Genet 15:127-132.
Lanahan A, Worley P (1998) Immediate-early genes and synaptic function. Neurobiol Learn Mem 70:37-43.
Lee JL, Everitt BJ, Thomas KL (2004) Independent cellular processes for hippocampal memory consolidation and reconsolidation. Science 304:839-843.
Leitner S, Voigt C, Metzdorf R, Catchpole CK (2005) Immediate early gene (ZENK, Arc) expression in the auditory forebrain of female canaries varies in response to male song quality. J Neurobiol 64:275-284.
Leonardo A, Konishi M (1999) Decrystallization of adult birdsong by perturbation of auditory feedback. Nature 399:466-470.
Levine ES, Dreyfus CF, Black IB, Plummer MR (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. Proc Natl Acad Sci U S A 92:8074-8077.
Lewicki MS (1996) Intracellular characterization of song-specific neurons in the zebra finch auditory forebrain. J Neurosci 16:5855-5863.

Lewis JW, Ryan SM, Arnold AP, Butcher LL (1981) Evidence for a catecholaminergic projection to area X in the zebra finch. Journal of Comparative Neurology 196:347-354.

Li L, Chin LS, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P, et al. (1995) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9235-9239.

Lindquist JM, Fredriksson JM, Rehnmark S, Cannon B, Nedergaard J (2000) Beta 3- and alpha1-adrenergic Erk1/2 activation is Src- but not Gi-mediated in Brown adipocytes. J Biol Chem 275:22670-22677.

Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U, Kuhl D (1995) Somatodendritic expression of an immediate early gene is regulated by synaptic activity. Proc Natl Acad Sci U S A 92:5734-5738.

Lohof AM, Ip NY, Poo MM (1993) Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363:350-353.

London SE, Clayton DF (2008) Functional identification of sensory mechanisms required for developmental song learning. Nat Neurosci 11:579-586.

Luo M, Ding L, Perkel DJ (2001) An avian basal ganglia pathway essential for vocal learning forms a closed topographic loop. J Neurosci 21:6836-6845.

Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, Gilbert DJ, Jenkins NA, Lanahan AA, Worley PF (1995) Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 14:433-445.

Margoliash D (1986) Preference for autogenous song by auditory neurons in a song system nucleus of the white-crowned sparrow. J Neurosci 6:1643-1661.

Margoliash D, Fortune ES (1992) Temporal and harmonic combination-sensitive neurons in the zebra finch's HVc. J Neurosci 12:4309-4326.

Marler P, Peters S (1977) Selective vocal learning in a sparrow. Science 198:519-521.

McAllister AK, Lo DC, Katz LC (1995) Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15:791-803.

McGaugh JL (2000) Memory--a century of consolidation. Science 287:248-251.

McGaugh JL, Izquierdo I (2000) The contribution of pharmacology to research on the mechanisms of memory formation. Trends Pharmacol Sci 21:208-210.

McGaugh JL, Roozendaal B (2002) Role of adrenal stress hormones in forming lasting memories in the brain. Curr Opin Neurobiol 12:205-210.

McKenzie TL, Hernandez AM, Macdougall-Shackleton SA (2006) Experience with songs in adulthood reduces song-induced gene expression in songbird auditory forebrain. Neurobiol Learn Mem.

Mello C, Nottebohm F, Clayton D (1995) Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. J Neurosci 15:6919-6925.

Mello CV (1998) Auditory experience, gene regulation and auditory memories in songbirds. Journal of the Brazilian Association for the Advancement of Science 50:189-196.
Mello CV (2002) Mapping vocal communication pathways in birds with inducible gene expression. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 188:943-959.
Mello CV, Clayton DF (1994) Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. J Neurosci 14:6652-6666.
Mello CV, Clayton DF (1995) Differential induction of the ZENK gene in the avian forebrain and song control circuit after metrazole-induced depolarization. J Neurobiol 26:145-161.
Mello CV, Ribeiro S (1998) ZENK protein regulation by song in the brain of songbirds. Journal of Comparative Neurology 393:426-438.
Mello CV, Vicario DS, Clayton DF (1992) Song presentation induces gene expression in the songbird forebrain. Proc Natl Acad Sci U S A 89:6818-6822.
Mello CV, Pinaud R, Ribeiro S (1998a) Noradrenergic system of the zebra finch brain: immunocytochemical study of dopamine-beta-hydroxylase. Journal of Comparative Neurology 400:207-228.
Mello CV, Velho TA, Pinaud R (2004) Song-induced gene expression: a window on song auditory processing and perception. Ann N Y Acad Sci 1016:263-281.
Mello CV, Jarvis ED, Denisenko N, Rivas M (1997) Isolation of song-regulated genes in the brain of songbirds. Methods Mol Biol 85:205-217.
Mello CV, Vates GE, Okuhata S, Nottebohm F (1998b) Descending auditory pathways in the adult male zebra finch (*Taeniopygia guttata*). Journal of Comparative Neurology 395:137-160.
Metzger M, Jiang S, Braun K (1998) Organization of the dorsocaudal neostriatal complex: a retrograde and anterograde tracing study in the domestic chick with special emphasis on pathways relevant to imprinting. Journal of Comparative Neurology 395:380-404.
Milbrandt J (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797-799.
Montag-Sallaz M, Montag D (2003) Learning-induced arg 3.1/arc mRNA expression in the mouse brain. Learn Mem 10:99-107.
Montag-Sallaz M, Welzl H, Kuhl D, Montag D, Schachner M (1999) Novelty-induced increased expression of immediate-early genes c-fos and arg 3.1 in the mouse brain. J Neurobiol 38:234-246.
Moore RY, Bloom FE (1979) Central catecholamine neuron systems: anatomy and physiology of the norepinephrine and epinephrine systems. Annu Rev Neurosci 2:113-168.
Morgan JI, Cohen DR, Hempstead JL, Curran T (1987) Mapping patterns of c-fos expression in the central nervous system after seizure. Science 237:192-197.

Morimoto K, Sato K, Sato S, Yamada N, Hayabara T (1998a) Time-dependent changes in rat hippocampal synapsin I mRNA expression during long-term potentiation. Brain Res 783:57-62.
Morimoto K, Sato K, Sato S, Suemaru S, Sato T, Yamada N, Hayabara T (1998b) Increases in mRNA levels for synapsin I but not synapsin II in the hippocampus of the rat kindling model of epilepsy. Seizure 7:229-235.
Morrow BA, Elsworth JD, Inglis FM, Roth RH (1999) An antisense oligonucleotide reverses the footshock-induced expression of fos in the rat medial prefrontal cortex and the subsequent expression of conditioned fear-induced immobility. J Neurosci 19:5666-5673.
Muller CM, Leppelsack HJ (1985) Feature extraction and tonotopic organization in the avian auditory forebrain. Exp Brain Res 59:587-599.
Murchison CF, Zhang XY, Zhang WP, Ouyang M, Lee A, Thomas SA (2004) A distinct role for norepinephrine in memory retrieval. Cell 117:131-143.
Nastiuk KL, Clayton DF (1995) The canary androgen receptor mRNA is localized in the song control nuclei of the brain and is rapidly regulated by testosterone. Journal of Neurobiology 26:213-224.
Nastiuk KL, Mello CV, George JM, Clayton DF (1994) Immediate-early gene responses in the avian song control system: cloning and expression analysis of the canary c-jun cDNA. Brain Res Mol Brain Res 27:299-309.
Nedivi E, Hevroni D, Naot D, Israeli D, Citri Y (1993) Numerous candidate plasticity-related genes revealed by differential cDNA cloning. Nature 363:718-722.
Nordeen KW, Nordeen EJ (1992) Auditory feedback is necessary for the maintenance of stereotyped song in adult zebra finches. Behavioral and Neural Biology 57:58-66.
Nottebohm F (1972) The origins of vocal learning. American Naturalist 106:116-140.
Nottebohm F (1981) A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. Science 214:1368-1370.
Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. Science 194:211-213.
Nottebohm F, Stokes TM, Leonard CM (1976) Central control of song in the canary, Serinus canarius. Journal of Comparative Neurology 165:457-486.
Nottebohm F, Kelley DB, Paton JA (1982) Connections of vocal control nuclei in the canary telencephalon. Journal of Comparative Neurology 207:344-357.
Nottebohm F, Nottebohm ME, Crane L (1986) Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song-control nuclei. Behavioral and Neural Biology 46:445-471.
Olveczky BP, Andalman AS, Fee MS (2005) Vocal experimentation in the juvenile songbird requires a basal ganglia circuit. PLoS Biol 3:e153.
Park KH, Clayton DF (2002) Influence of restraint and acute isolation on the selectivity of the adult zebra finch zenk gene response to acoustic stimuli. Behav Brain Res 136:185-191.

Paton JA, Nottebohm FN (1984) Neurons generated in the adult brain are recruited into functional circuits. Science 225:1046-1048.
Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. Neuron 16:1137-1145.
Petersohn D, Schoch S, Brinkmann DR, Thiel G (1995) The human synapsin II gene promoter. Possible role for the transcription factor zif268/egr-1, polyoma enhancer activator 3, and AP2. J Biol Chem 270:24361-24369.
Phan ML, Pytte CL, Vicario DS (2006) Early auditory experience generates long-lasting memories that may subserve vocal learning in songbirds. Proc Natl Acad Sci U S A 103:1088-1093.
Pinaud R, Fortes AF, Lovell P, Mello CV (2006) Calbindin-positive neurons reveal a sexual dimorphism within the songbird analogue of the mammalian auditory cortex. J Neurobiol 66:182-195.
Pinaud R, Osorio C, Alzate O, Jarvis ED (2008a) Profiling of experience-regulated proteins in the songbird auditory forebrain using quantitative proteomics. Eur J Neurosci 27:1409-1422.
Pinaud R, Tremere LA, Penner MR, Hess FF, Barnes S, Robertson HA, Currie RW (2002) Plasticity-driven gene expression in the rat retina. Brain Res Mol Brain Res 98:93-101.
Pinaud R, Velho TA, Jeong JK, Tremere LA, Leao RM, von Gersdorff H, Mello CV (2004) GABAergic neurons participate in the brain's response to birdsong auditory stimulation. Eur J Neurosci 20:1318-1330.
Pinaud R, Terleph TA, Tremere LA, Phan ML, Dagostin AA, Leao RM, Mello CV, Vicario DS (2008b) Inhibitory Network Interactions Shape the Auditory Processing of Natural Communication Signals in the Songbird Auditory Forebrain. J Neurophysiol.
Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke SF, Blanquet V, Wurst W, Salmen B, Bosl MR, Lipp HP, Grant SG, Bliss TV, Wolfer DP, Kuhl D (2006) Arc/Arg3.1 is essential for the consolidation of synaptic plasticity and memories. Neuron 52:437-444.
Pospelov VA, Pospelova TV, Julien JP (1994) AP-1 and Krox-24 transcription factors activate the neurofilament light gene promoter in P19 embryonal carcinoma cells. Cell Growth Differ 5:187-196.
Price P (1979) Developmenteal determinants of structure in zebra finch song. Journal of Comparative Physiology and Psychology 93:260-277.
Ramos BP, Arnsten AF (2007) Adrenergic pharmacology and cognition: focus on the prefrontal cortex. Pharmacol Ther 113:523-536.
Reiner A, Perkel DJ, Bruce LL, Butler AB, Csillag A, Kuenzel W, Medina L, Paxinos G, Shimizu T, Striedter G, Wild M, Ball GF, Durand S, Gunturkun O, Lee DW, Mello CV, Powers A, White SA, Hough G, Kubikova L, Smulders TV, Wada K, Dugas-Ford J, Husband S, Yamamoto K, Yu J,

Siang C, Jarvis ED (2004) Revised nomenclature for avian telencephalon and some related brainstem nuclei. J Comp Neurol 473:377-414.
Rial Verde EM, Lee-Osbourne J, Worley PF, Malinow R, Cline HT (2006) Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. Neuron 52:461-474.
Ribeiro S, Mello CV (2000) Gene expression and synaptic plasticity in the auditory forebrain of songbirds. Learn Mem 7:235-243.
Ribeiro S, Cecchi GA, Magnasco MO, Mello CV (1998) Toward a song code: evidence for a syllabic representation in the canary brain. Neuron 21:359-371.
Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, Wolff JR, Hammer RE, Malenka RC, Sudhof TC (1995) Essential functions of synapsins I and II in synaptic vesicle regulation. Nature 375:488-493.
Sambucetti LC, Curran T (1986) The Fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. Science 234:1417-1419.
Sasaki A, Sotnikova TD, Gainetdinov RR, Jarvis ED (2006) Social context-dependent singing-regulated dopamine. J Neurosci 26:9010-9014.
Scharff C, Nottebohm F (1991) A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. Journal of Neuroscience 11:2896-2913.
Scheiderer CL, Dobrunz LE, McMahon LL (2004) Novel form of long-term synaptic depression in rat hippocampus induced by activation of alpha 1 adrenergic receptors. J Neurophysiol 91:1071-1077.
Schmidt MF, Konishi M (1998) Gating of auditory responses in the vocal control system of awake songbirds. Nat Neurosci 1:513-518.
Scott BB, Lois C (2005) Generation of tissue-specific transgenic birds with lentiviral vectors. Proc Natl Acad Sci U S A 102:16443-16447.
Sen K, Theunissen FE, Doupe AJ (2001) Feature analysis of natural sounds in the songbird auditory forebrain. J Neurophysiol 86:1445-1458.
Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821-861.
Sheng M, Greenberg ME (1990) The regulation and function of c-fos and other immediate early genes in the nervous system. Neuron 4:477-485.
Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF (2006) Arc/Arg3.1 mediates homeostatic synaptic scaling of AMPA receptors. Neuron 52:475-484.
Sockman KW, Gentner TQ, Ball GF (2002) Recent experience modulates forebrain gene-expression in response to mate-choice cues in European starlings. Proc R Soc Lond B Biol Sci 269:2479-2485.
Sockman KW, Gentner TQ, Ball GF (2004) Complementary neural systems for the experience-dependent integration of mate-choice cues in European starlings. J Neurobiol 62:72-81.

Sohrabji F, Nordeen EJ, Nordeen KW (1990) Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. Behavioral and Neural Biology 53:51-63.
Sonnenberg JL, Rauscher FJ, Morgan JI, Curran T (1989) Regulation of proenkephalin by Fos and Jun. Science 246:1622-1625.
Stanton PK, Sarvey JM (1985) Depletion of norepinephrine, but not serotonin, reduces long-term potentiation in the dentate gyrus of rat hippocampal slices. J Neurosci 5:2169-2176.
Steward O, Worley PF (2001a) A cellular mechanism for targeting newly synthesized mRNAs to synaptic sites on dendrites. Proc Natl Acad Sci U S A 98:7062-7068.
Steward O, Worley PF (2001b) Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation. Neuron 30:227-240.
Steward O, Worley P (2002) Local synthesis of proteins at synaptic sites on dendrites: role in synaptic plasticity and memory consolidation? Neurobiol Learn Mem 78:508-527.
Steward O, Wallace CS, Lyford GL, Worley PF (1998) Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. Neuron 21:741-751.
Stripling R, Volman SF, Clayton DF (1997) Response modulation in the zebra finch neostriatum: relationship to nuclear gene regulation. J Neurosci 17:3883-3893.
Strosberg AD (1993) Structure, function, and regulation of adrenergic receptors. Protein Sci 2:1198-1209.
Tchernichovski O, Lints T, Mitra PP, Nottebohm F (1999) Vocal imitation in zebra finches is inversely related to model abundance. Proceedings of the National Academy of Sciences U S A 96:12901-12904.
Terleph TA, Mello CV, Vicario DS (2006) Auditory topography and temporal response dynamics of canary caudal telencephalon. J Neurobiol 66:281-292.
Terleph TA, Mello CV, Vicario DS (2007) Species differences in auditory processing dynamics in songbird auditory telencephalon. Dev Neurobiol 67:1498-1510.
Terpstra NJ, Bolhuis JJ, den Boer-Visser AM (2004) An analysis of the neural representation of birdsong memory. J Neurosci 24:4971-4977.
Terpstra NJ, Bolhuis JJ, Riebel K, van der Burg JM, den Boer-Visser AM (2006) Localized brain activation specific to auditory memory in a female songbird. J Comp Neurol 494:784-791.
Theunissen FE, Doupe AJ (1998) Temporal and spectral sensitivity of complex auditory neurons in the nucleus HVc of male zebra finches. Journal of Neuroscience 18:3786-3802.
Theunissen FE, Sen K, Doupe AJ (2000) Spectral-temporal receptive fields of nonlinear auditory neurons obtained using natural sounds. J Neurosci 20:2315-2331.

Thiel G, Schoch S, Petersohn D (1994) Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. J Biol Chem 269:15294-15301.
Vallet E, Beme II, Kreutzer M (1998) Two-note syllables in canary songs elicit high levels of sexual display. Anim Behav 55:291-297.
Vates GE, Vicario DS, Nottebohm F (1997) Reafferent thalamo- "cortical" loops in the song system of oscine songbirds. Journal of Comparative Neurology 380:275-290.
Vates GE, Broome BM, Mello CV, Nottebohm F (1996) Auditory pathways of caudal telencephalon and their relation to the song system of adult male zebra finches. J Comp Neurol 366:613-642.
Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF (2002) Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. J Neurosci 22:10067-10071.
Velho TA, Lovell P, Mello CV (2007) Enriched expression and developmental regulation of the middle-weight neurofilament (NF-M) gene in song control nuclei of the zebra finch. J Comp Neurol 500:477-497.
Velho TA, Pinaud R, Rodrigues PV, Mello CV (2005) Co-induction of activity-dependent genes in songbirds. Eur J Neurosci 22:1667-1678.
Velho TAF, Mello CV (2007) Transcriptional regulation of activity-dependent genes by birdsong. In: Transcriptional Regulation by Neuronal Activity, 1st Edition (Dudek S, ed), pp 3-26: Springer.
Vicario DS, Yohay KH (1993) Song-selective auditory input to a forebrain vocal control nucleus in the zebra finch. J Neurobiol 24:488-505.
Wada K, Sakaguchi H, Jarvis ED, Hagiwara M (2004) Differential expression of glutamate receptors in avian neural pathways for learned vocalization. J Comp Neurol 476:44-64.
Wade J, Arnold AP (2004) Sexual differentiation of the zebra finch song system. Ann N Y Acad Sci 1016:540-559.
Walling SG, Harley CW (2004) Locus ceruleus activation initiates delayed synaptic potentiation of perforant path input to the dentate gyrus in awake rats: a novel beta-adrenergic- and protein synthesis-dependent mammalian plasticity mechanism. J Neurosci 24:598-604.
Waltereit R, Dammermann B, Wulff P, Scafidi J, Staubli U, Kauselmann G, Bundman M, Kuhl D (2001) Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. J Neurosci 21:5484-5493.
Wang JQ, Tang Q, Parelkar NK, Liu Z, Samdani S, Choe ES, Yang LM, L. (2004) Glutamate signaling to Ras-MAPK in striatal neurons. Molecular Neurobiology 29:1-14.
Weaver IC, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M, Meaney MJ (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7:847-854.

Wild JM (1993) Descending projections of the songbird nucleus robustus archistriatalis. Journal of Comparative Neurology 338:225-241.
Woolley SC, Doupe AJ (2008) Social context-induced song variation affects female behavior and gene expression. PLoS Biol 6:e62.
Woolley SM, Rubel EW (2002) Vocal memory and learning in adult Bengalese Finches with regenerated hair cells. J Neurosci 22:7774-7787.
Woolley SM, Casseday JH (2004) Response properties of single neurons in the zebra finch auditory midbrain: response patterns, frequency coding, intensity coding, and spike latencies. J Neurophysiol 91:136-151.
Woolley SM, Casseday JH (2005) Processing of modulated sounds in the zebra finch auditory midbrain: responses to noise, frequency sweeps, and sinusoidal amplitude modulations. J Neurophysiol 94:1143-1157.
Woolley SM, Fremouw TE, Hsu A, Theunissen FE (2005) Tuning for spectro-temporal modulations as a mechanism for auditory discrimination of natural sounds. Nat Neurosci 8:1371-1379.
Worley PF, Christy BA, Nakabeppu Y, Bhat RV, Cole AJ, Baraban JM (1991) Constitutive expression of zif268 in neocortex is regulated by synaptic activity. Proc Natl Acad Sci U S A 88:5106-5110.
Worley PF, Bhat RV, Baraban JM, Erickson CA, McNaughton BL, Barnes CA (1993) Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. J Neurosci 13:4776-4786.
Yu AC, Margoliash D (1996) Temporal hierarchical control of singing in birds. Science 273:1871-1875.
Zeigler HP, Marler P (2004) Behavioral Neurology of birdsong, 1st Edition. New York: The New York Academy of Sciences.
Zhong ZG, Noda M, Takahashi H, Higashida H (1999) Overexpression of rat synapsins in NG108-15 neuronal cells enhances functional synapse formation with myotubes. Neurosci Lett 260:93-96.