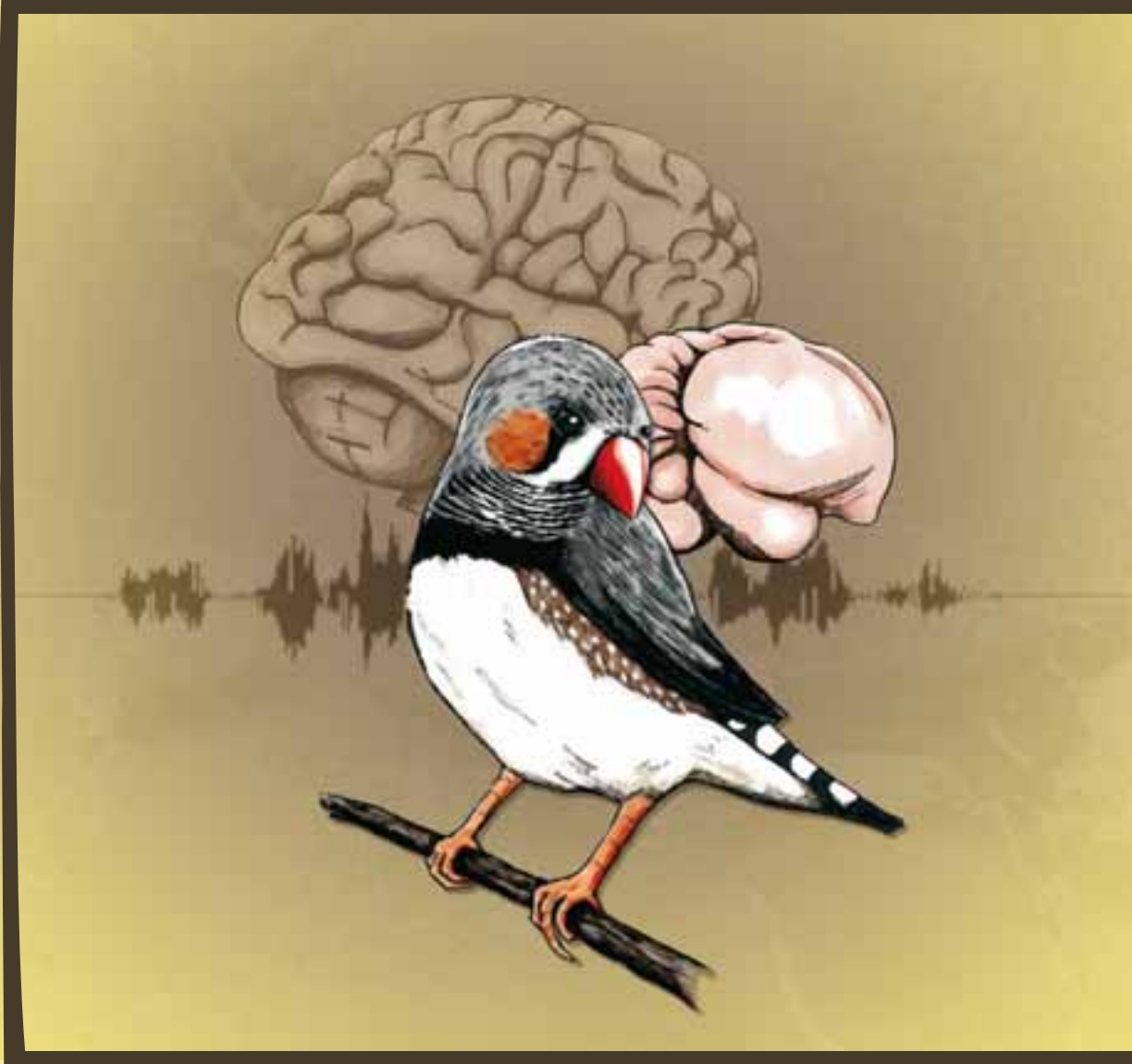
Bird Brains and Songs

neural mechanisms of auditory memory and perception in zebra finches



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Bird brains and songs: Neural mechanisms of auditory memory and perception in zebra finches

Vogelbreinen en liedjes: Neurale mechanismen van auditief geheugen en perceptie bij zebravinken

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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geboren op 28 februari 1981
te 's-Gravenhage

Promotor: Prof. dr. J. J. Bolhuis

aan mijn ouders

Unless we remember we cannot understand.

- E. M. Forster (1879-1970)

Contents

Chapter 1

General Introduction

Introduction

Vocal learning is rare in the animal kingdom. Songbirds (Passeriformes), parrots (Psittaciformes) and hummingbirds (Apodiformes) share with humans and some other mammals (bats, dolphins, whales, seals) the capacity for vocal learning, which is the ability to imitate sounds (Fitch, 2000; Hauser et al., 2002; Jarvis, 2004). The degree to which humans and species of birds learn vocalizations varies. Humans are masters at vocal imitation; we can learn to combine the basic elements of speech ('phonemes') into an almost infinite number of different sounds during our lives. We can even learn new languages at an age past infancy although there are some restrictions to which sounds can be learned if they were not in the initial repertoire (Kuhl, 2000). Parrots are also excellent vocal imitators throughout life. It has been shown that one African grey parrot, Alex, could use English words that it learned to label objects (Pepperberg, 1999).

For successful vocal learning, songbirds have to be exposed to conspecific sounds during a sensitive period when they are young and they learn to produce their own vocalizations by a sensorimotor learning process. Some songbirds (Oscines or 'true' songbirds; a subdivision of the order Passeriformes) are capable of learning new songs throughout life. Canaries (*Serinus canaria*) and European starlings (*Sturnus vulgaris*), for example, have repertoires of songs, to which they add new song phrases beyond the first year of life. These birds are called 'open-ended learners'. In contrast, 'age-limited learners' such as the zebra finch (*Taeniopygia guttata*) learn their vocalizations mainly during a sensitive period early in life from a song 'tutor' which is usually the father. Under specific experimental conditions, when a song tutor is not available or the young bird can not hear itself sing, it has been reported that age-limited learners are also able to change their vocalizations later in life (Eales, 1985, 1987; Slater et al., 1993; Funabiki and Konishi, 2003; but see Mooney, 1999, for review). In zebra finches, only males sing a single song, which is, under normal conditions, produced without change throughout their life. Female zebra finches also learn the characteristics of their father's song but do not sing themselves (Clayton, 1990; Riebel, 2000; Riebel et al., 2002; Riebel and Smallegange, 2003). The zebra finch is an Australian songbird commonly used in laboratory studies (Zann, 1996). In general, the function of birdsong is thought to be attracting females or

defending a territory. Zebra finches are gregarious; song is used for mate attraction and not for territorial defence, as it is in other bird species such as black-capped chickadees (*Poecile atricapillus*). Apart from songs, many songbird species also produce calls. Male and female zebra finches both use 'long calls', which have different acoustic characteristics in the two sexes, for communication. The male long call contains features that are learned from the song tutor whereas the long call produced by females is not learned.

Parallels between song learning and speech acquisition

There are strong behavioural parallels between speech acquisition in humans and song learning in songbirds (Doupe and Kuhl, 1999; Kuhl, 2000; Goldstein et al., 2003; Wilbrecht and Nottebohm, 2003; Bolhuis and Gahr, 2006). In both cases there is a sensitive period for auditory learning, during which the infants or young birds are exposed to and memorize the sounds of adult conspecifics (see Box 1 for a detailed description of the process of song learning). Subsequently, vocal learning proceeds through an intermediate phase that is called 'babbling' in human infants and 'subsong' in songbirds. During this phase, development of song (Eales, 1989) and speech (Kuhl, 2000; Goldstein et al., 2003; Kuhl and Rivera-Gaxiola, 2008) are both affected by social cues. That is, the vocal as well as non-vocal behaviour of adult conspecifics enhances vocal learning. In addition, songbirds as well as humans require auditory feedback for development and maintenance of vocalizations.

In humans, functional Magnetic Resonance Imaging (fMRI) studies have shown that the auditory association cortex in the superior temporal gyrus is the likely neural substrate of speech perception and memory (Hickok and Poeppel, 2000; Hickok et al., 2003; Viceic et al., 2006). Motor representations of speech involve regions in the frontal cortex centred around Broca's area (Hickok and Poeppel, 2000; Hickok et al., 2003; Demonet et al., 2005). With human imaging studies, the neuronal mechanisms of memory cannot be investigated at the cellular level. Thus, it is important to investigate

the neural mechanisms of memory for vocalizations in an animal model because we cannot perform high-resolution experiments, such as single cell recordings, in humans.

Recently, it has become clear that there are analogies and homologies between the brains of birds and mammals (Jarvis et al., 2005). The nomenclature of the avian brain has been completely revised, reflecting these similarities (Reiner et al., 2004b). The new nomenclature emphasises the fact that a large part of the avian telencephalon is homologous to the mammalian cortex (Reiner et al., 2004a; Reiner et al., 2004b; Jarvis et al., 2005). It has recently been suggested that the caudomedial nidopallium (NCM) (Fig. 1.3) is in function the likely avian equivalent of the auditory association cortex in the mammalian temporal lobe (Bolhuis and Gahr, 2006). The NCM and the caudomedial mesopallium (CMM) receive input from Field L1/L3. Field L1/L3 has been suggested to be homologues to layer 2 and 3 of the mammalian auditory cortex; it receives projections from thalamorecipient Field L2 which is likely homologues to layer 4 of the primary auditory cortex in mammals. It has also been suggested that the NCM and CMM are similar to layer 2 and 3 in the primary auditory cortex, because they are reciprocally connected (Jarvis, 2004). However, Bolhuis and Gahr (2006) suggested that the Field L complex could be homologues to the 'core' regions of the mammalian primary auditory cortex, and the NCM / CMM to the belt or parabelt regions of the auditory association cortex because these regions receive projections from the Field L complex.

To summarize, there are homologies and analogies between the mammalian and avian brain, and between birdsong learning and speech acquisition. This makes the acquisition of song in songbirds an excellent model system to study the neural mechanisms of auditory memory and vocal learning.

Box 1 ~ The phases of song learning

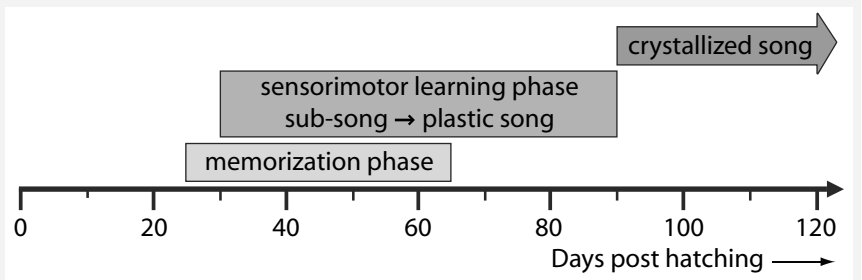

Fig. 1.1

The mechanisms underlying song memorization in birds are often illustrated with the metaphor of the 'template' (Konishi, 1965), which is the internal representation of the tutor song (Konishi, 2004) that is formed in the first phase of song learning. It has been suggested that songbirds are born with a 'crude template', because even in the absence of an adult tutor they develop songs that have some species-specific characteristics (Marler and Sherman, 1985). During the first phase of song learning, the memorization phase (also called sensory acquisition phase), young birds memorize the song of a conspecific tutor, often the father. By hearing conspecific song, the crude template is thought to be refined into a more precise representation of the tutor song. During the second phase of song learning which is called the sensorimotor phase, the young bird starts to vocalize, and it is thought that it matches its song output with the template that was formed in the first phase because the song of the young bird is gradually modified and it will increasingly resemble the tutor song. Eventually the bird will sing a crystallized song that, in the case of age-limited learners such as the zebra finch, does not change during adulthood.

In zebra finches, the memorization stage starts at about 20 days of age and last until about 60 days of age. The sensorimotor phase starts at about 30 days of age with vocalizations that are called 'sub-song' and progresses through 'plastic song' (around day 45) until

song does not change anymore and thus is said to crystallize at about 90 days of age. The memorization phase and sensorimotor phase thus partially overlap in this species. There are also species, such as the white-crowned sparrow (*Zonotrichia leucophrys*), in which the phases of song learning do not overlap (Konishi, 1965). Auditory feedback is required throughout development in order to match the bird's own vocalizations to the template. In adulthood, auditory feedback is necessary for maintenance of song (see Brainard and Doupe, 2000b, for review).

Neuronal mechanisms of memory

The fact that songbirds need to learn their vocalizations by imitation makes it possible to investigate the mechanisms that underlie memory in the context of auditory-vocal learning. The theories on neural mechanisms of learning proposed by Donald Hebb in 'The Organization of Behaviour' (1949) are still important principles underlying memory research. Hebb essentially suggested that when there is simultaneous activity in the pre- and post-synaptic cell, growth of processes or metabolic changes take place at the level of the synapse resulting in changed connectivity between those neurons. A model system to study the neural mechanisms of memory that has provided evidence to support some of Hebb's proposals is filial imprinting in the domestic chick (*Gallus gallus domesticus*) (see Box 2). Imprinting in chicks and song learning in songbirds are both avian model systems for memory and learning, visual and auditory-vocal learning respectively. It is assumed that memory is a general process and that the cellular and molecular mechanisms that underlie memory are thus similar in both systems. In songbirds as well as chicks, there is a memorization phase early in life. It is possible that during auditory learning in songbirds, similar cellular and molecular changes take place as during imprinting in chicks in which there is localization of the neural substrate for the representation of memory (see Box 2). To investigate this, we first need to localize the neural substrate for tutor song memory in zebra finches.

Box 2 ≈ Filial imprinting: Localization of the neural substrate of memory

Domestic chicks learn the visual characteristics of a stimulus to which they are exposed early in life and develop a preference for that stimulus (Horn, 1985; Bolhuis and Honey, 1998). Chicks have a predisposition to approach certain stimuli, such as moving objects. 'Imprinting' is the process in which they develop a preference for a specific object. There is localization of the neural substrate for memory in chicks. In particular, the intermediate and medial mesopallium (IMM) is the likely neural substrate for the representation of the memory of the imprinted stimulus (for review see Horn, 2004). The study of imprinting in chicks has revealed many biochemical changes in the IMM that correlate with learning. For example, the expression of the immediate early gene *c-fos* (the protein product is called 'Fos' and a transcription factor) is related to the strength of learning (McCabe and Horn, 1994), as it is in the songbird brain (Bolhuis et al., 2000, 2001). In addition, the size of post-synaptic densities is increased by imprinting and there is a learning-related increase in other proteins (amyloid precursor protein, APP, and MARCKS , myristoylated alanine-rich protein kinase C) (Solomonia et al., 2003; see Horn, 2004, for an extensive review of all learning-related changes in the chick brain). In electrophysiological studies it was found that the number of neurons selectively responsive to the imprinted stimulus increases during a time frame that is consistent with the reported biochemical changes (Horn et al., 2001; Solomonia et al., 2003).

The song system and song learning

The production of song in birds is controlled by an elaborate network of interconnected brain nuclei known as the 'song system' (Fig. 1.2) (Nottebohm et al., 1976) (see Box 3 for a detailed account of the neuroanatomy of the song system). Some nuclei in the song system are larger in males than in females (Nottebohm and Arnold, 1976) and the song system is unique to birds that learn their song. The song system is subdivided into a posterior pathway,

involved in song production (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Kao et al., 2005) and an anterior forebrain pathway (AFP, see Fig. 1.2), involved in vocal motor learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Kao et al., 2005). Lesions to nuclei in the AFP, including lMAN and area X, do not affect adult song, but cause abnormal song development in young zebra finches (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). Juvenile males with lesions in area X do not develop crystallized songs, while juveniles with lesions in lMAN produce aberrant but stable songs. It has therefore been suggested that the AFP may contain the neural substrate for auditory memory (Basham et al., 1996; Nordeen and Nordeen, 1997). It is difficult to estimate how much birds that received permanent lesions to the AFP have memorized from their tutor because they never develop normal adult song. The impaired strength of song learning found in these birds could thus be the result of deficits in auditory learning and/or sensorimotor learning. To circumvent this problem, the song system nucleus lMAN was temporarily and reversibly inactivated by injections with an NMDA (N-methyl-D-aspartic acid) receptor blocker on days that birds were exposed to tutor song in the sensorimotor phase of song learning, but not in between sessions (Basham et al., 1996). Therefore, normal sensorimotor learning in between session should have been unaltered while only auditory learning during tutoring session should have been affected. Song learning was significantly impaired in birds that received the experimental treatment, consistent with the suggestion that the AFP is involved in memorization of tutor song. However, in zebra finches, there is overlap between the memorization phase and the sensorimotor learning phase. In the study of Basham and colleagues (1996), lMAN was inactivated in the sensorimotor phase, these findings could be due to an effect on sensori-motor integration (Bolhuis and Gahr, 2006) during the tutoring sessions. This could have resulted in reduced song imitation without affecting auditory memory processes. In this interpretation it is assumed that when auditory and/or visual interaction is possible between the young bird and the tutor, this has a positive influence on the sensorimotor integration processes that are essential for vocal learning, independently of the formation of auditory memory (Nordeen and Nordeen, 2004). The exact role of the AFP in song learning and possibly memorization of tutor song thus remains unclear.

Box 3 ~ The songbird 'song system'

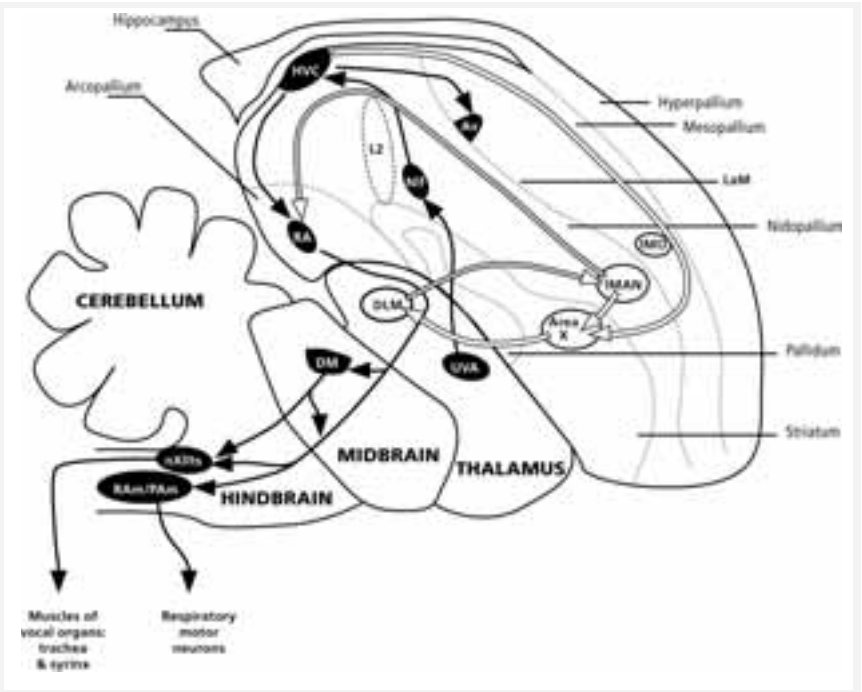

Fig. 1.2 Vocal pathways

Research into the neural mechanisms of song learning and production has traditionally focussed on a network of interconnected nuclei that is called the 'song system'. The song system is divided into a posterior pathway and an anterior forebrain pathway. Recently, regions outside the song system have also been implicated in song perception and memory (see Box 4).

The posterior pathway: The posterior pathway of the song system consists of projections from the premotor nucleus HVC (letter-based proper name) to another premotor nucleus, the robust nucleus of the arcopallium (RA). Projections from RA go to the dorsomedial nucleus (DM) in the midbrain and the hypoglossal nucleus (nXIIts) in the brainstem that innervates the muscles of the syrinx (the avian

vocal organ) (Brenowitz et al., 1997; Wild, 1997). RA also projects to nuclei that innervate the respiratory motor neurons (nucleus parambigualis, PAm, and nucleus retroambigualis, RAm). In addition, HVC projects to nucleus Avalanche (Av) that is activated when the bird is singing (Jarvis and Nottebohm, 1997). Neuronal activity in the premotor nuclei HVC and RA is synchronized with the production of sound and the projection from RA onto the motor neurons in nXIIts is myotopically (in relation to the syringeal muscles) organized (Brenowitz et al., 1997; Margoliash, 1997). Lesions to nuclei in this pathway prevent the bird from producing normal songs, while the bird is still motivated to sing according to its posture and beak movements (Nottebohm et al., 1976). Activity in this pathway might represent a 'vocal motor program' by means of a neural code for the generation of a sequence of action potentials necessary for song production (Hahnloser et al., 2003; Fee et al., 2004). Besides the innervation of the syrinx, the airflow through the syrinx during singing is also controlled by nuclei in the brain. Although nuclei in the midbrain, pons and medulla are not part of the traditional 'motor pathway' of the song system, they receive descending projections from RA and control respiratory movements (see Wild, 1997, for a discussion of this 'respiratory-vocal control pathway').

The anterior forebrain pathway: The anterior forebrain pathway (AFP) includes HVC, area X, the medial part of the dorsolateral nucleus (DLM) in the thalamus, the lateral part of the magnocellular nucleus of the anterior nidopallium (lMAN) and RA. In anesthetized adult zebra finches, neurons in lMAN show auditory responses to the bird's own song (BOS) with temporal (order) and spectral selectivity (Doupe and Konishi, 1991). Thus, these "song selective" neurons respond more strongly to playback of the BOS. HVC neurons also have spectral and temporal selectivity for BOS, and are temporal and harmonic combination sensitive. In Area X, the majority of neurons respond strongly to BOS, but also to simple tone bursts and conspecific songs (see Doupe and Solis, 1997, for review of auditory responses in the AFP). This selectivity for BOS in the AFP is absent in 30-day-old zebra finches, but develops via a stage of less selective responsiveness in 60-day-old birds - including responses to BOS as well as tutor song - to the selectivity that is observed in adult birds (Solis and Doupe, 1999, 2000; Solis et al., 2000).

Beyond the song system

Electrophysiological responsiveness of neurons is commonly measured to investigate the neural response to specific stimuli. Alternatively, the expression of immediate early genes (IEGs) can also be used as a marker for neuronal activation (Sagar et al., 1988). These genes respond rapidly to stimulation by neuronal depolarization (Sagar et al., 1988). The detectable levels of mRNA and protein decrease back to baseline shortly after the stimulus is removed. This technique (as compared to electrophysiology) makes it possible to investigate the neuronal response during a certain time frame of several brain regions simultaneously. Expression of the IEG *ZENK* (an acronym of *zif-268*, *egr-1*, NGF-1A and *krox-24*; 'Zenk' is used to indicate the protein product of this gene) has revealed that nuclei in the song system are activated when the bird is singing (Jarvis and Nottebohm, 1997). The motor act of singing is sufficient to induce *ZENK* in the song system, because neuronal activation in the song system has been demonstrated in deafened birds that do not hear themselves sing (Jarvis and Nottebohm, 1997). In contrast, regions outside the song system, such as the NCM and the CMM (Fig. 1.3) are activated when the bird hears song (Mello et al., 1992; Mello and Clayton, 1994) (see Box 4 for a description of regions involved in auditory perception in songbirds). Neuronal activation in these regions occurs irrespective of the motor act of singing. There is expression of *ZENK* in birds that are exposed to songs played from tapes as well as in birds that hear themselves sing but not in deafened birds (Mello et al., 1992; Mello and Clayton, 1994; Jarvis and Nottebohm, 1997). The expression of *ZENK* decreases after repeated exposure to the same stimulus but can be induced again when the animal is exposed to a novel stimulus (Mello et al., 1995). Habituation of the *ZENK* response - a decline in the expression levels after repeated exposure that is persistent over at least 24 hours - could be an indication that the NCM is involved in processes related to memory (Mello et al., 1995).

In zebra finch males, it was found that expression of Zenk was induced in the NCM and CMM when adult male zebra finches were re-exposed to tutor song (Bolhuis et al., 2000). The expression of Zenk in the NCM was positively correlated with the fidelity of song imitation only after re-exposure to the tutor song (Bolhuis et al., 2000, 2001; Terpstra et al., 2004).

That is, Zenk expression in birds that had copied many elements of their father's song was greater than in birds that had copied few elements. These results led to the suggestion that the NCM contains the neural substrate of the representation for tutor song memory.

This correlation between strength of song learning and Zenk expression was present in birds that were raised by means of tape-tutoring (Bolhuis et al., 2000) as well as in socially reared zebra finches (Bolhuis et al., 2001). In good learners (birds that copied many elements from their song tutor) the BOS resembles the tutor song closely, it was therefore suggested that this correlation could be due to reactivation of BOS memory and not tutor song memory (Marler and Doupe, 2000). Terpstra et al. (2004) addressed this issue, and showed that Zenk expression in the NCM was related to the strength of song learning only in birds that were re-exposed to their tutor's song, but not in birds that were exposed to their own song. In this study, another group of birds was exposed to a novel song to investigate whether this correlation could be due to processes related to attention, i.e. good learners always pay more attention when they listen to songs which results in higher levels of Zenk after song exposure (Marler and Doupe, 2000). Paying more attention to songs might be the reason why they learned the tutor song better during the memorization phase. In the group exposed to novel song, there was no correlation with the strength of song learning in the NCM (Terpstra et al., 2004), which makes it unlikely that the reported correlation in the tutor song group is due to attention.

Alternatively, the Zenk response in the NCM might be dependent on stimulus salience. Stimulus salience refers to the characteristics of the stimulus that make the stimulus stand out from other stimuli and the salience of a stimulus could facilitate attention. Exposure to songs with a greater salience, such as long song bouts in starlings (Gentner et al., 2001) or more complex songs in budgerigars (*Melopsittacus undulatus*) (Eda-Fujiwara et al., 2003), leads to more Zenk induction in the NCM as compared to less salient songs. The songs from the tutors of good learners could have had a greater salience, resulting in the reported correlation. The salience of tutor songs could be dependent on factors other than the number of song elements, because this characteristic of tutor song did not affect the reported correlation (Bolhuis et al., 2001). The novel songs that were used by Terpstra et al. (2004) or the BOS could have had the same salience in

all tested birds and therefore there is no correlation between strength of song learning and Zenk expression in these groups. If the NCM is solely involved in auditory perception, and the magnitude of *ZENK* induction is related to stimulus salience which is determined by a so-far unknown characteristic of song, than the neural substrate for the representation of tutor song memory could in theory be localized outside of the NCM.

A recent electrophysiological study, however, showed that neurons in the NCM of adult zebra finch males showed steeper rates of habituation to novel song than to tutor song (Phan et al., 2006), indicating that neurons in the NCM treat tutor song like other conspecific songs that they are previously familiarized with (Chew et al., 1995). Habituation rates that are different from those of novel song can be interpreted as a neural representation of memory for song. In addition, these authors found that the 'familiarity index' (a measure that indicates how much a song behaves like a familiar song in respect to the habituation rates to novel songs) of NCM neurons correlated significantly and positively with the strength of song learning (Phan et al., 2006). It is unlikely that in the NCM the correlations with the strength of song learning and IEG expression as well as with the familiarity index of habituation rates are both caused only by stimulus salience and independent of a representation of memory. Thus, evidence from IEG as well as electrophysiological studies indicates that the NCM might be (part of) the neural substrate for tutor song memory.

Box 4 ~ The songbird auditory system

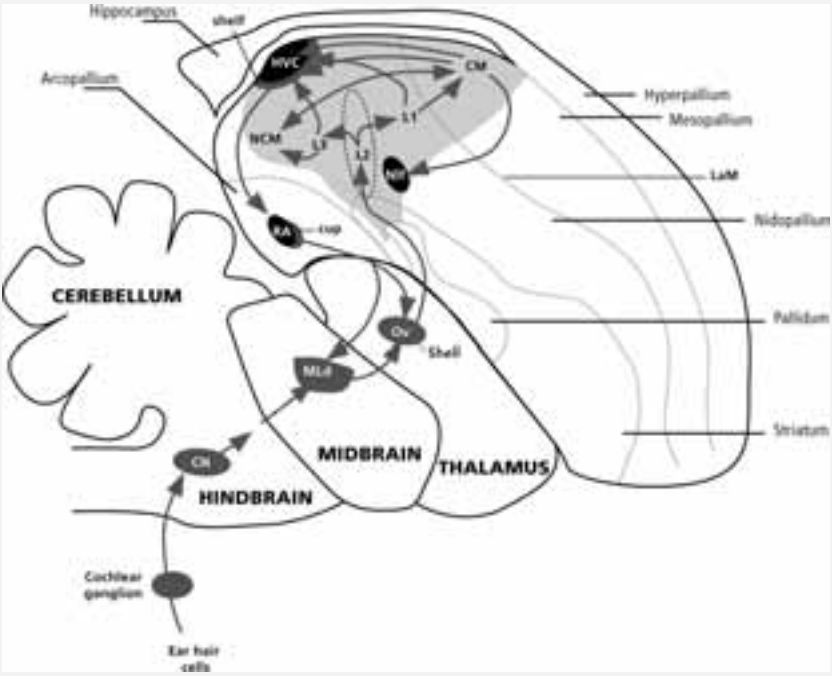

Fig. 1.3 Auditory pathways

Auditory information from the cochlear and lemniscal nuclei travels via nucleus MLd in the midbrain, which is analogous to the inferior colliculus in mammals, to the Nucleus Ovoidalis (Ov) in the thalamus to the primary telencephalic auditory area, Field L2, which is comparable to layer IV of the mammalian primary auditory cortex (Wild, 1997).

Field L is divided in sub-regions (L2, L1 and L3) and has bidirectional connections with secondary auditory areas in the caudomedial nidopallium (NCM) and caudomedial and caudolateral mesopallium (CMM and CLM; which are reciprocally connected and subdivisions of the caudal mesopallium, 'CM') (Vates et al., 1996). These secondary auditory areas were discovered when birds

were exposed to songs, but did not sing themselves. It was shown that the expression of immediate early genes (IEG's) was upregulated in regions outside the song system (Mello and Clayton, 1994). Regions in the brain that are activated when the bird is listening to song, but not singing itself, are the NCM, CMM, the shelf region adjacent to HVC and the cup region adjacent to RA (Mello and Clayton, 1994). These latter two regions also receive projections from sub-regions of Field L and from the CLM (Vates et al., 1996) and the shelf region adjacent to HVC projects to the cup region adjacent to RA (Mello et al., 1998).

Contrary to the selectivity for BOS in the song system, Field L and CM are not selective for BOS (Amin et al., 2004; Amin et al., 2007; Boumans et al., 2008). They show a small preference for BOS over reverse BOS, but this could be explained by a preference for natural spectral-temporal structure of song (Theunissen et al., 2004).

Connections between the auditory system and the song system:
As it has been shown that nuclei in the song system are responsive to auditory stimuli, there must be input from the auditory system to the song system. In addition, auditory input to the song system is necessary for learning and maintaining vocalizations (Brainard and Doupe, 2000b). Currently, no robust projections from a primary auditory region to the song system have been identified (Mello et al., 2004). The best candidate source of auditory input to the song system has long been supposed to be the interfacial nucleus of the nidopallium (NIf) (Margoliash, 1997; Mello et al., 2004) that receives a direct projection from the caudal mesopallium, and projects robustly to HVC (Vates et al., 1996; Mello et al., 2004). Recently, it has become apparent that CM neurons are responsive to song and drive auditory evoked activity in NIf, as well as HVC directly, in anesthetized zebra finches (Bauer et al., 2008). These neurons in CM are also responsive to song playback in freely moving birds, as well as during singing and are thus the likely source of auditory input to the song system.

Bird brains and songs: Neural mechanisms of auditory memory and perception in zebra finches

The aim of this project was to investigate the neural substrate of auditory memory and perception in zebra finches. I investigated the role of the NCM in tutor song recognition memory (Chapter 2) and I analysed neuronal activation after exposure to tutor song and novel song in juveniles (Chapter 3). Because only males learn their songs and females do not sing, it is possible that the auditory association regions process sounds differently in the two sexes. I investigated such potential sex differences in neuronal activation related to auditory processing by using long calls as stimuli (Chapter 4).

If the NCM contains the neural substrate for the representation of tutor song memory, lesions to this structure should impair recognition of the tutor song. In addition, if there are distinct neural representations of tutor song memory and a vocal motor program, then lesions to the NCM should not affect production of the bird's own song (BOS). I investigated this hypothesis in Chapter 2 by placing bilateral neurotoxic lesions in the NCM of adult zebra finch males. I examined the effects of the lesions on tutor song recognition by means of song preference tests and I used spectrogram analysis before and after surgery to evaluate any changes in the bird's own song.

Evidence for a role for the NCM in tutor song memory was obtained in Chapter 2 from experiments with adult zebra finches. However, if the NCM contains the neural substrate for the representation of tutor song memory, then this structure should be activated in juvenile birds that are in the process of learning the tutor song. In Chapter 3 I test this hypothesis by exposing young birds, in the middle of the sensorimotor learning phase to their father's song. To control for non-specific effects of hearing song, I exposed another group of birds to a novel song. I measured the expression of the immediate early gene *ZENK* in the NCM and the CMM and in two nuclei in the song system, RA and HVC.

Male and female zebra finches show different patterns of Zenk expression

in NCM and CMM after exposure to the tutor song. The tutor song is memorized by both male and female zebra finches but only produced by males. If this difference is due to sex differences in the auditory system, resulting in differential auditory processing, there should also be differential Zenk expression after exposure to stimuli that are produced by and relevant to both sexes. In Chapter 4, I investigate the patterns of Zenk expression after exposure to unfamiliar calls, a vocal communication signal used by both sexes.

Chapter 2

Birdsong memory: A neural dissociation between song recognition and production.

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Summary

Songbirds learn their song from an adult conspecific tutor when they are young, much like the acquisition of speech in human infants (Doupe and Kuhl, 1999; Goldstein et al., 2003). When an adult zebra finch is re-exposed to its tutor's song there is increased neuronal activation in the caudomedial nidopallium (NCM), the songbird equivalent of the auditory association cortex. This neuronal activation is related to the fidelity of song imitation (Bolhuis et al., 2000, 2001; Terpstra et al., 2004; Phan et al., 2006), suggesting that the NCM may contain the neural representation of song memory (Bolhuis and Gahr, 2006). We found that bilateral neurotoxic lesions to the NCM of adult male zebra finches impaired tutor song recognition but did not affect the males' song production or their ability to discriminate calls. These findings demonstrate that the NCM performs an essential role in the representation of tutor song memory. In addition, our results show that tutor-song memory and a motor program for the bird's own song have separate neural representations in the songbird brain. Thus, in both humans and songbirds, the cognitive systems of vocal production and auditory recognition memory are subserved by distinct brain regions.

Results and Discussion

There are strong behavioural, cognitive and neural parallels between speech acquisition in humans and song learning in songbirds (Doupe and Kuhl, 1999; Goldstein et al., 2003; Bolhuis and Gahr, 2006). In both cases, there is a sensitive period for auditory learning, and vocal learning proceeds through a practice stage that is called "babbling" in human infants and "subsong" in songbirds. In addition, it has become apparent that there are analogies and homologies between the brains of birds and mammals (Jarvis et al., 2005), prompting a complete overhaul of the nomenclature of the avian brain (Reiner et al., 2004b). Despite these similarities, we know relatively little about the neural substrate of song memory. In the present study, we investigated whether, as in humans, the neural representation of auditory memory and that of vocal production and motor memory are separate in songbirds.

In recent analyses of song-related neuronal activation (Bolhuis et al., 2000, 2001; Terpstra et al., 2004; Phan et al., 2006), the caudomedial nidopallium (NCM) has emerged as a candidate brain region that could contain the neural substrate for tutor song memory. The NCM, which is located outside the conventional song system (Fig. 2.1A), is the likely avian equivalent of the auditory association cortex in the mammalian temporal lobe (Doupe and Kuhl, 1999; Bolhuis and Gahr, 2006). If the NCM contains the neural substrate for the representation of tutor song memory, lesions to this structure should impair recognition of the tutor song. In addition, if there are distinct neural representations of tutor-song memory and a vocal-motor program, then lesions to the NCM should not affect production of the bird's own song (BOS). We investigated this hypothesis by placing bilateral neurotoxic lesions in the NCM of adult zebra finch males (see supplemental Experimental Procedures and Fig. S2.1 in the Supplemental Data). We examined the effects of the lesions on tutor-song recognition by means of song preference tests and used spectrogram analysis before and after surgery to evaluate any changes in the bird's own song.

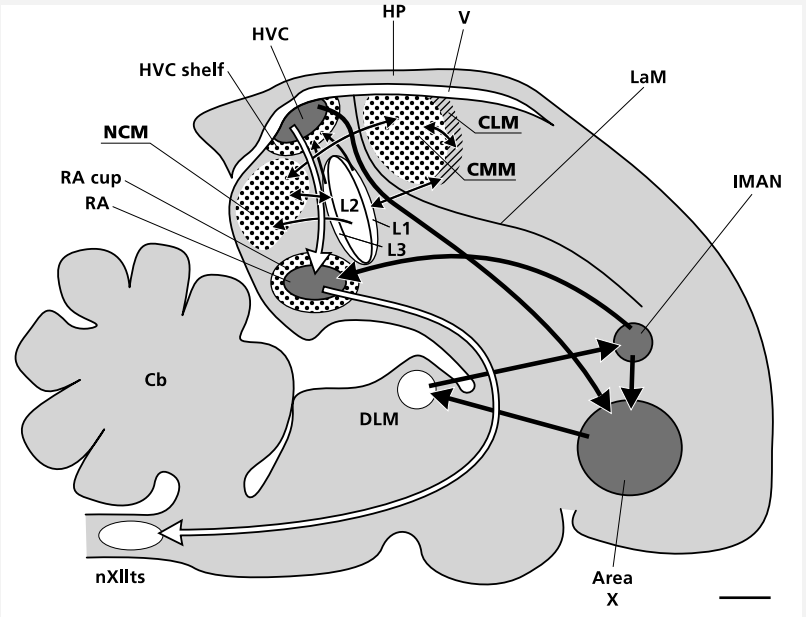

Fig. 2.1 (A) The songbird brain

Schematic diagram of a composite view of parasagittal sections of a songbird brain gives approximate positions of nuclei and brain regions involved in birdsong. The song system is a network of interconnected brain nuclei, consisting of a caudal pathway (white arrows), considered to be involved in the production of song, and a rostral pathway (thick black arrows), thought to have a role in song acquisition (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Thin black arrows indicate known connections between the field L complex, a primary auditory-processing region, and some other forebrain regions. Dark grey nuclei show significantly enhanced expression of immediate early genes (IEGs) when the bird is singing (Jarvis and Nottebohm, 1997). Stippled areas represent brain regions that show increased IEG expression when the bird hears song (Mello et al., 1992; Jarvis and Nottebohm, 1997), including tutor song (Bolhuis et al., 2000, 2001; Terpstra et al., 2004). The scale bar represents 1 mm. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, ref. (Bolhuis and Gahr, 2006), copyright 2006.

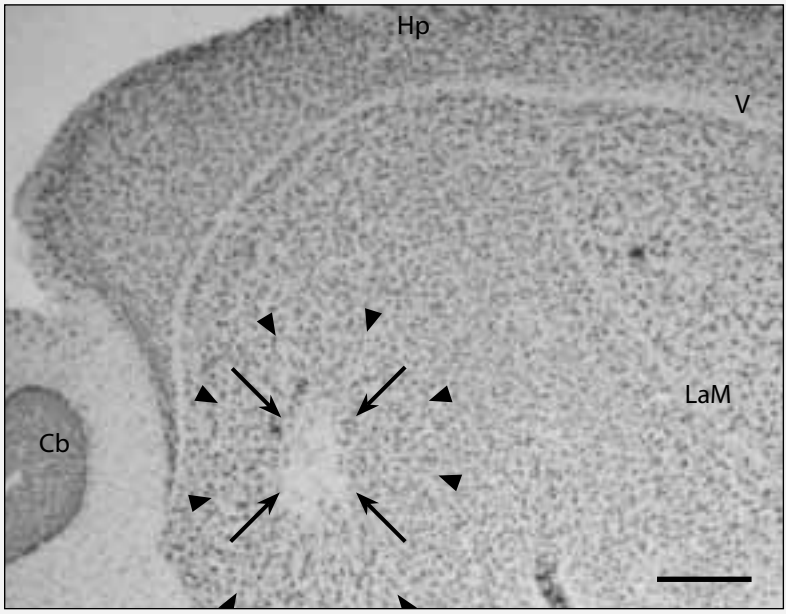

Fig. 2.1 (B) Photomicrographs showing a representative NCM lesions

Parasaggital sections of the brains of an experimental bird, with immunohistochemical staining against neuron-specific nuclear protein (NeuN). Lesion to the NCM 2 weeks after surgery (B) is indicated with arrows. Arrowheads indicate the region that showed structurally changed morphological organization, surrounding the region with dead tissue. All lesions were located within the NCM, overlapping with the area sampled in gene-expression studies (Bolhuis et al., 2000, 2001; Terpstra et al., 2004). The scale bar represents 0.25 mm.

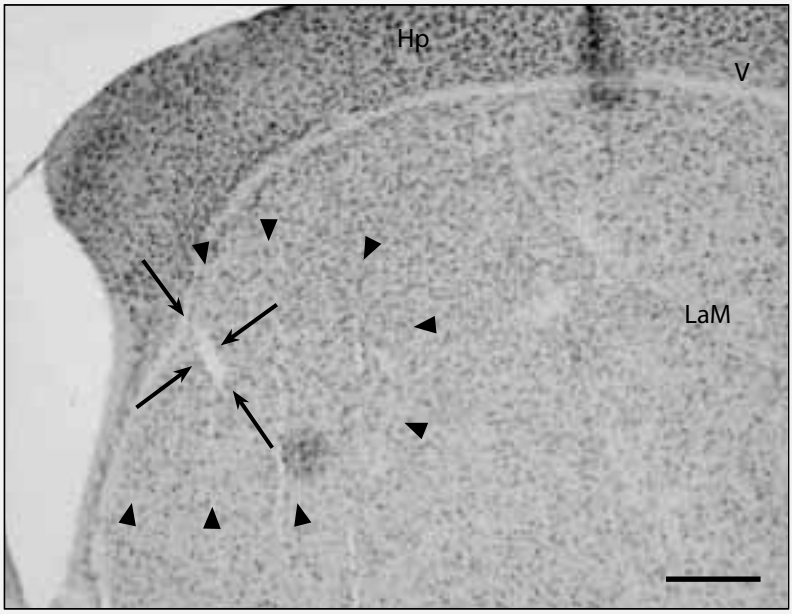

Fig. 2.1 (C) Photomicrographs showing a representative NCM lesions

Parasaggital sections of the brains of an experimental birds, with immunohistochemical staining against neuron-specific nuclear protein (NeuN). Lesion to the NCM 8 weeks after surgery (C) is indicated with arrows. Arrowheads indicate the region that showed structurally changed morphological organization, surrounding the region with dead tissue. All lesions were located within the NCM, overlapping with the area sampled in gene-expression studies (Bolhuis et al., 2000, 2001; Terpstra et al., 2004). The scale bar represents 0.25 mm.

Abbreviations in Fig. 2.1 A, B and C are as follows: Cb, cerebellum; CLM, caudal lateral mesopallium; CMM, caudal medial mesopallium; DLM, nucleus dorsolateralis anterior, pars medialis; Hp, hippocampus; HVC, acronym used as a proper name; L1, L2, L3, subdivisions of field L; LaM, lamina mesopallialis; lMAN, lateral magnocellular nucleus of the anterior nidopallium; NCM, caudal medial nidopallium; nXIIts, tracheosyringeal portion of the nucleus hypoglossus; RA, robust nucleus of the arcopallium; V, ventricle.

Lesions to the caudomedial nidopallium impair song recognition

Zebra finches prefer the song of their tutor to a novel song (Miller, 1979; Riebel et al., 2002; Riebel and Smallegange, 2003), which suggests that they have learned the characteristics of tutor song and have formed an auditory memory of it. We measured the birds' song preferences by calculating the amount of time spent near a speaker that broadcast the song of their tutor compared to a speaker that broadcast a novel song (see supplemental Experimental Procedures). Before surgery, the birds showed a significant mean preference for the tutor song (control group: $t(6) = 15.32$, $p < 0.0001$; experimental group: $t(9) = 17.01$, $p < 0.0001$). There was a significant effect of NCM lesions on preference for the tutor song [$F(1,5) = 22.36$; $p < 0.0001$; see Fig. 2.2A]. These results suggest that tutor-song recognition was impaired after lesions to the NCM.

After surgery, the mean preference for the tutor song in the lesioned group was significantly different from the chance level ($t(9) = 2.84$, $p < 0.05$). It is possible that the remaining preference is subserved by parts of the brain that are outside the lesioned region.

An alternative interpretation of these findings is that birds with lesions to the NCM were less motivated to express a preference. Such a lack of motivation would be reflected in reduced activity directed at the stimulus songs in the preference tests. However, there were no significant differences in the number of transitions made between zones in the preference test, before and after surgery in either group (see Supplemental Data). Thus, an explanation of the effects of NCM lesions in terms of reduced motivation is unlikely.

The more a bird has copied from its tutor, the more its own song will resemble the tutor song. The question thus arises whether the males expressed a preference for the tutor song or for their own song. In operant choice tests, zebra finch males prefer the tutor song to their own song (Adret, 1993), which renders it likely that the preference we measured in the males was indeed a reflection of tutor-song recognition. In addition, there was no significant correlation between song similarity (i.e. the number of elements the bird had copied from its tutor song) and the preference for the tutor song, before or after surgery in either group ($r < 0.14$ in all cases). Thus, tutor song was preferred to novel song regardless of how much of the tutor

song the birds had incorporated into their own song. These results suggest that lesions to the NCM impaired recognition of the tutor song.

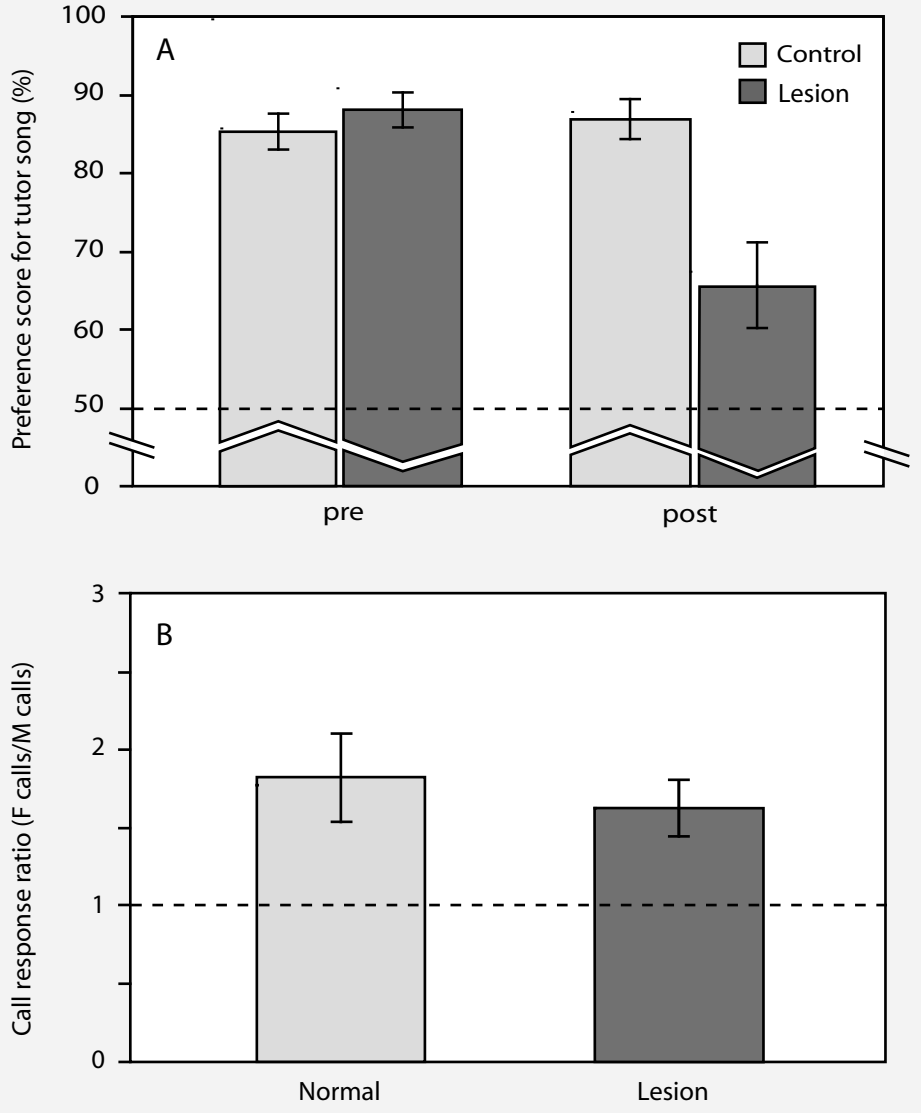

Fig. 2.2

Fig. 2.2 Lesions to the NCM impair song recognition without affecting call discrimination.

(A) Mean preference scores (± SEM) for tutor song in birds in the two groups.
(B) Mean call-response ratios (± SEM) in unoperated control males and males with NCM lesions. The call-response ratio was calculated for each individual by division of the total amount of responses to female calls by the total amount of responses to male calls.

Song production is not affected by lesions to the NCM

Song analysis based on multiple song characteristics (see supplemental Experimental Procedures) revealed that song output was not affected by NCM lesions. That is, within subjects there were no significant differences in songs recorded before surgery and those recorded immediately after surgery [$F(1,12) = 0.38$; group x time interaction: $F(1,12) = 2.84$], or at 11 days [$F(1,15) = 0.29$; interaction: $F(1,15) = 0.24$] or at 56 days [($F(1,6) = 0.26$; interaction: $F(1,6) = 1.00$] after surgery (Fig. 2.3; Table S1). In addition, songs did not change significantly within subjects in the period of 8 weeks after surgery [$F(2,5) = 0.36$; group x time interaction: $F(2,5) = 0.47$]. Thus, within a period of 8 weeks, NCM lesions did not significantly affect the males' vocal production.

In zebra finches, modifications in song production may be protracted, as seen in birds deafened during adulthood (Lombardino and Nottebohm, 2000). In birds that were older than those used in the present study (664 - 2090 days old), effects of deafening were not apparent until 100 weeks after surgery, in contrast to younger birds (< 140 days), of which the songs were affected within 4 weeks after deafening. The authors suggested that every time a bird sings, "a little bit of learning – motor engrainment – occurs" (Lombardino and Nottebohm, 2000, p. 5054). It may be that we would have found significant changes in song production if we had measured at a later time after surgery. Thus, we cannot exclude the possibility that adequate

song production in adults – if it involves learning sensu Lombardino and Nottebohm (2000) – would eventually require an intact NCM. However, our results show that in the short term, song production can proceed unaltered when tutor song recognition is impaired after lesions to the NCM.

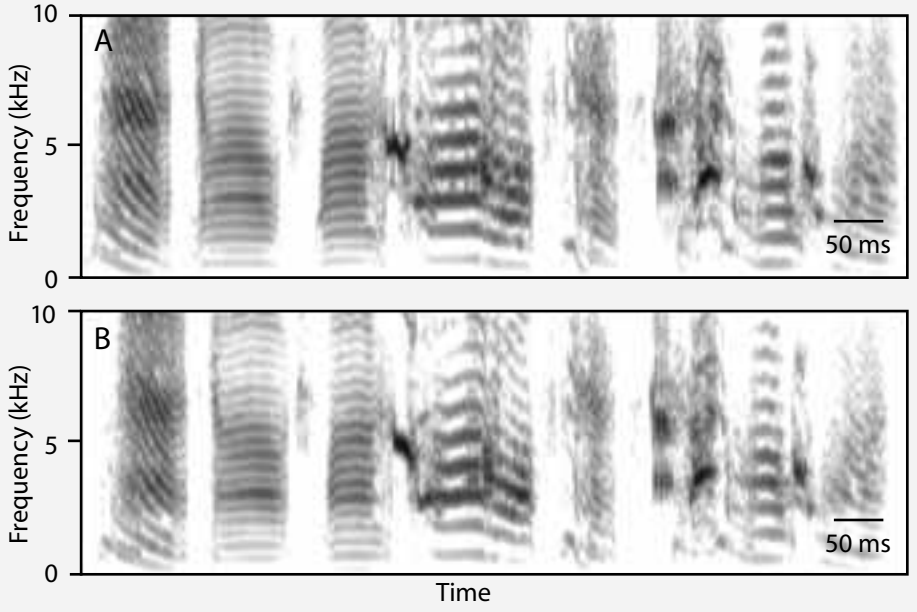

Fig. 2.3 Song production is not affected by NCM lesions.

Spectrograms of one motif of an experimental bird before (A) and eight weeks after (B) NCM lesioning. There were no changes in song production after NCM lesions.

NCM lesions do not affect the ability to discriminate between different calls

In order to recognize the tutor song, birds must be able to discriminate between different complex vocalisations (Gentner et al., 2000). The effect of NCM lesions on tutor-song preference might be a result of an impaired abi-

lity to discriminate between different sounds. To control for this possibility, we subjected a subset of the experimental subjects and a group of unoperated control birds to a call-discrimination test (see supplemental Experimental Procedures). Zebra finch long calls have the same frequency range as songs and consist of several harmonics similar to song syllables. Calls of males and females differ in fundamental frequency, frequency modulation, and duration (Vicario et al., 2001) (see Fig. S2.2A and S2.2B). The calls of males are characterized by a frequency modulation also seen in some types of song syllables. Zebra finch males are able to discriminate calls of the two sexes; they preferentially respond to female calls in playback tests (Vicario et al., 2001).

Figure 2B shows the call-preference scores for birds with NCM lesions and an unoperated control group. Birds with lesions to the NCM exhibited a significant preference for female calls over male calls. In this group, the mean preference for female calls was significantly different from the chance level for both measures used (strength of response, shown in Fig. 2.2B: $t(4) = 3.59$; $p < 0.05$. Probability of responding: mean, 1.48; SEM, 0.15; $t(4) = 3.26$; $p < 0.05$). A similar preference for female calls was found in the control group (strength of response, shown in Fig. 2.2B: $t(9) = 2.98$; $p < 0.05$. Probability of responding: mean, 1.53; SEM, 0.21; $t(9) = 2.54$; $p < 0.05$). There was no significant difference in mean preference score between the two groups [$t(13) = 0.44$ for the strength of the response and $t(13) = 0.18$ for the probability of responding].

The preference for female calls shown by the two groups of birds, was smaller than reported by Vicario et al. (2001) It is important to note that the calls used in the present study (see Table S2) have different characteristics (duration, fundamental frequency) than the calls used by Vicario and coworkers. All these characteristics may influence the males' preference for female calls (Vicario et al., 2001). The fact that in both groups there was a significant preference for female calls demonstrates that discrimination of complex auditory stimuli was not affected by NCM lesions. Thus, it is unlikely that the effect of NCM lesions on tutor-song preference is due to an impaired ability to discriminate sounds.

A neural dissociation – parallels with humans

These results suggest that the NCM is necessary for recognition of the tutor song, but that its integrity is not required for song production or sound discrimination. These findings are consistent with the hypothesis that the NCM is (part of) the neural substrate for the representation of tutor-song memory (Bolhuis et al., 2000). Furthermore, the results suggest a neural dissociation between brain regions (including the NCM) involved in auditory perception and memory and brain regions (including nuclei in the song system) involved in vocal production. This dissociation has a striking parallel in humans, where the neural substrate of speech perception and memory is primarily localized to the auditory association cortex in the superior temporal gyrus (Viceic et al., 2006), whereas motor representations of speech involve frontal-cortex regions centered around Broca's area (Hickok and Poeppel, 2000; Hickok et al., 2003; Demonet et al., 2005). Presently, there is insufficient evidence for one-to-one homologies between the avian and the mammalian brain (Jarvis et al., 2005). Nevertheless, neuroanatomical evidence concerning auditory projections from the thalamus has led to the suggestion that the NCM (and possibly the caudomedial mesopallium) is analogous to the mammalian auditory association cortex (Doupe and Kuhl, 1999; Bolhuis and Gahr, 2006). In human adults, fMRI studies have shown that, in particular, regions in the superior temporal gyrus are involved in speech perception and memory (Hickok and Poeppel, 2000; Hickok et al., 2003; Viceic et al., 2006). In addition, there is evidence from both lesion studies and gene-expression analyses that the auditory association cortex is involved in auditory recognition memory in monkeys and rats (Colombo et al., 1990; Wan et al., 2001; Fritz et al., 2005).

Analyses of expression of immediate early genes (IEGs) have revealed a dissociation between nuclei in the song system, which are activated when the bird is singing (Jarvis and Nottebohm, 1997), and the NCM and the caudomedial mesopallium (CMM, Fig. 2.1A), which are activated when the bird hears song (Mello et al., 1992; Bolhuis et al., 2000, 2001; Terpstra et al., 2004, 2006). There was increased IEG expression in the CMM of female zebra finches when they were exposed to their father's song, compared to novel song (Terpstra et al., 2006). In zebra finch females, lesions to the CMM, but not to the song-system nucleus HVC, impaired a preference for

conspecific over heterospecific song (MacDougall-Shackleton et al., 1998). In contrast, lesions to song-system nuclei in songbirds disrupted song production but not recognition (Nottebohm et al., 1976; Gentner et al., 2000). Taken together with the present findings, showing that lesions to the NCM do not affect song production but impair song recognition, these results reveal a complete double dissociation of the effects of lesions to rostral and caudal brain regions on song in zebra finches.

Recent evidence shows that the functional distinction between the temporal and frontal cortex in human speech is not as strict as was thought previously (Ojemann, 1991; Hickok and Poeppel, 2000; Hickok et al., 2003; Dehaene-Lambertz et al., 2006; Okada and Hickok, 2006). Furthermore, in humans, temporal and frontal regions do not function in isolation but they interact continually, already in juveniles. For instance, an fMRI study in 3-month-old infants revealed neural activation in response to speech not only in the temporal cortex, but also in Broca's area in the frontal cortex (Dehaene-Lambertz et al., 2006). The latter was related to speech repetition, prompting the authors to suggest that in preverbal infants, Broca's area may be involved in memory for speech. This more dynamic view of the involvement of caudal and rostral brain regions in human auditory recognition and vocal production also has a parallel in songbirds. In adult songbirds, some nuclei in the song system are responsive to song, particularly to the bird's own song, with a minority of cells preferentially responding to tutor song (Solis and Doupe, 1999). In the early sensorimotor phase of song acquisition in juvenile zebra finches, neurons in the song-system nucleus HVC show transient preferential responding to the tutor song, whereas in adults there is preferential responsiveness to the bird's own song (Nick and Konishi, 2005). In the present study, the subjects were adults and had crystallized songs. It is possible that song acquisition in the sensorimotor phase requires an intact NCM because early song production involves access to the representation of the tutor song. In that case, lesions to the NCM of juvenile zebra finches are predicted to influence song development. In addition, plastic changes to adult song (Leonardo and Konishi, 1999; Brainard and Doupe, 2000a) may also involve continued interactions between the song system and the NCM (and possibly the CMM; Bolhuis et al., 2000; Terpstra et al., 2006).

In summary, we have demonstrated that lesions to the caudomedial

nidopallium (NCM), a brain region outside the conventional song system, impair song recognition without affecting song production or sound discrimination. These findings suggest that the NCM contains the neural representation of tutor-song memory, and that access to this representation is not necessary for song production. There is a parallel in human speech, where there is a similar dissociation between frontal brain regions mainly involved in speech production and caudal temporal regions mainly involved in speech perception and memory. In both systems, there appears to be continued dynamic interaction between these two brain regions throughout the life of an individual. The parallel between songbirds and humans suggests convergent evolution of the mechanisms underlying vocal learning.

Acknowledgements

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Supplemental Data

Supplemental Experimental Procedures

Subjects and Surgery

The 17 adult male zebra finches used in this study were reared at the central animal facility of Utrecht University. Juveniles stayed with both parents in individual cages (80 x 40 x 40 cm) until 100 days after hatching, after which they were transported to same-sex aviaries. These aviaries were located in a different room from the breeding cages, preventing visual or auditory contact between fathers and sons. Experiments started (day 0; the day of surgery) at mean age 413 (±21, SEM) days after hatching (range: 247–601). Ten subjects received bilateral ibotenic acid lesions in the NCM; 2 subjects received control lesions in the hippocampus, dorsal to the NCM; and 5 subjects underwent a sham procedure, during which vehicle was injected into the NCM.

Two hours prior to surgery, birds received one dose of Meloxicam (Metacam, Boehringer Ingelheim; 0.03 ml, 0.15 mg/ml, given orally in combination with a 0.5 ml 5% sucrose solution), a nonsteroidal anti-inflammatory drug that is thought to have analgesic effects in birds. Birds were anesthetized with Isoflurane (IsoFlo, Abbott Laboratories) and placed in a custom stereotaxic apparatus. Lidocaine (Emla cre` me, AstraZeneca) was applied to the scalp and a small incision was made in the skin and skull overlying the NCM and 110 nl ibotenic acid (1% dissolved in 0.1M NaOH) or 0.1M NaOH (controls) was injected into both hemispheres, targeted at the NCM (1 mm lateral from the bifurcation of the midsaggital sinus; 1.25mm below the dura mater). Ibotenic acid or vehicle was injected through a glass micropipette attached to a Nano-2000 microinjector (World Precision Instruments) in doses of approximately 10 nl at a time with 30 s between injections. Visual inspection of the level of fluid in the pipette during injections confirmed the successful application of ibotenic acid or vehicle. The pipette was left in place for 5 min after the last dose to prevent backflow along the pipette track. Because there were no significant behavioural differences between the birds with control lesions and the sham-operated birds, the results of those two control groups were taken

together. All experimental procedures were in accordance with Dutch law and approved by the Animal Experiments Committee of Utrecht University (DEC 03/160).

Immunohistochemistry and Image Analysis

Four lesioned birds were sacrificed 2 weeks after surgery, immediately after they had been given a preference test for tutor song. For a further six lesioned birds, we recorded songs up to 8 weeks after surgery and sacrificed them subsequently. Birds were anesthetized with 0.06 ml Natriumpentobarbital (intramuscular) (Nembutal, Ceva Sante Animale), and they received intracardial transfusions of 4% paraformaldehyde (PFA). Brains were dissected out and postfixed for 3 hr in PFA. Brains were cryo-protected in a series of 10% (30 min), 20% (5 hr), and 30% (overnight) sucrose solutions. Hemispheres were separated and frozen in OCT compound (Tissue-Tek), and 20 μm parasaggital sections were made on a cryostat and mounted on poly-L-lysine-coated slides. Histological verification of lesion location was carried out with use of an immunocytochemical procedure as described previously (Jongen-Relo and Feldon, 2002), with the primary antibody against neuron-specific nuclear protein, which is expressed in the nucleus and cell body of living neurons.

The region in which no living cells were observed was larger in the birds sacrificed after 3 weeks than after 8 weeks (Fig. 2.1B and 2.1C). An area surrounding the region without living cells was structurally different from normal NCM tissue, further away from the lesion (see arrowheads in Fig. 2.1B and 2.1C). A consequence of ibotenic-acid injections is that after a period of several weeks, the cells damaged by the neurotoxin gradually disappear and the empty space is filled up by other cells. In addition, the NCM is not a discrete nucleus but a more diffuse region. A relative measure, such as the percentage of lesioned tissue, cannot be used to compare the effect of the neurotoxin between animals. Therefore, we developed a method to compare lesion location between animals on the basis of the relative distance between two reference points. First, digital photographs of all sections containing lesioned tissue were taken with a Nikon digital camera DXM 1200 at 25x and 100x magnification on an Axioskop (Carl Zeiss MicroImaging). Image analysis was carried out with a PC-based system

equipped with the KS400 version 3.0 software (Carl Zeiss MicroImaging). In each photomicrograph, the contours of the lesion, the intersection of lamina mesopallialis (LaM) and the lateral ventricle, and the distal tip of the hippocampus overlying the NCM were identified (see Fig. S2.1A). A program written in KS400 calculated the geometric gravity point, or "center" of the lesion and its distance to the two reference points. Thus, a relative grid system made it possible to objectively compare location between animals. Measurements of lesion locations were carried out by two independent observers (mean interobserver-reliability coefficient: 0.92).

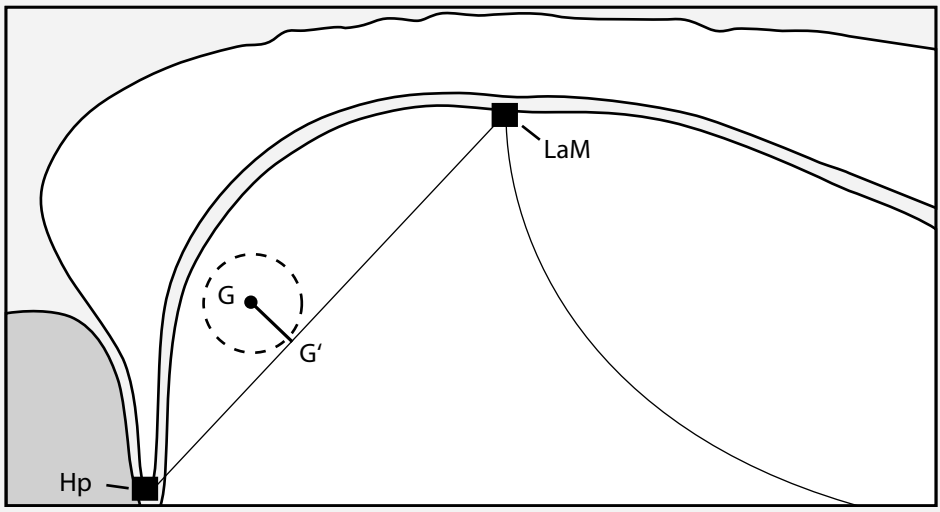

Fig. S2.1 (A) Assessing the Location of Lesions

Method. In each section that contained a lesion, the intersection of the lamina mesopallialis (LaM) with the ventricle and the most distal tip of the hippocampus (Hp) (between cerebellum and caudal NCM in parasaggital sections) were indicated. The borders of the lesioned region were also indicated (dashed lines), from which the gravity point of the lesion (G) was determined. In each section, the distance of G to the intersection (G0) with the reference line between Hp and LaM was calculated and expressed as a percentage of the reference distance between Hp and LaM. The distance from Hp to G0 was also calculated and expressed as a percentage of the reference between Hp and LaM.

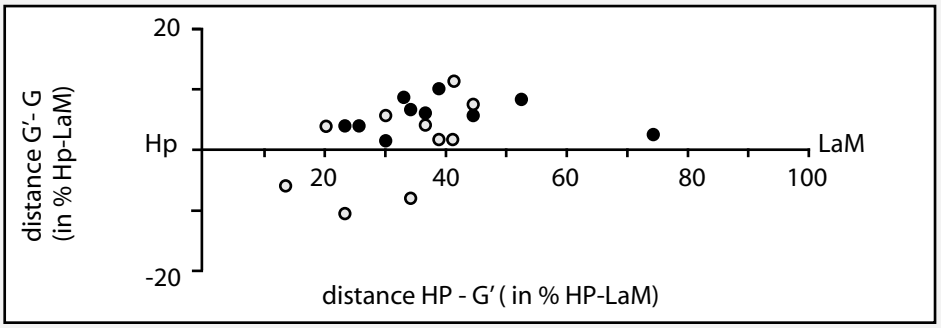

Fig. S2.1 (B) Assessing the Location of Lesions

Results. Graph indicating center of lesions in all experimental birds, plotted relatively to the distance between two reference points, LaM and Hp (for explanation of method, see above). Open circles, left hemisphere; solid circles, right hemisphere.

Song recording and behavioural testing

For all birds, songs were recorded 9 weeks prior to surgery (±1.2 week, SEM), 5 weeks prior to surgery (±1.6, SEM), and at day 11 after surgery. For some birds, song production was monitored continually for the first 72 hr after surgery (experimental, n = 7; control, n = 7), at day 11, and subsequently every 7th day up to 8 weeks after surgery (experimental, n = 6; control, n = 2). For all recording sessions, birds were housed individually in sound-proof chambers (115 x 115 x 205 cm) for about 20 hr, and songs were recorded digitally with Sennheiser MKH 50P48 directional microphones with the recorder of Sound Analysis Pro (SAP (Tchernichovski et al., 2000); http://ofer.sci.ccny.cuny.edu), version 1.056.

Preference tests involving tutor song and novel song were conducted 6 weeks (±1 week, SEM) prior to surgery and 2 weeks after surgery. During a test, birds were placed individually in a cage measuring 120 x 42 x 45 cm (Hernandez and MacDougall-Shackleton, 2004) that was divided into two approach zones (30 cm each), and a central zone (60 cm) in which food and water were available ad libitum. A speaker (vifa MG10SD) with a flat frequency- response curve between 0.1 and 15 kHz was placed at either

side of the cage. A Hypex PC amplifier and Windows Media Player controlled the sound-pressure level (SPL) of songs that were played back. During 14 min, song A (tutor or novel; chosen randomly for each bird) was broadcast for periods of 1 min on the left side alternating with periods of 1 min during which song B (tutor song if song A was novel song and vice versa) was broadcast from the right side. After a silent interval of 15 min, another session of 14 min followed in which the sides from which the two songs were played were reversed. Sound files were constructed with the computer program PRAAT (http://www.praat.org, version 4.2.34 for Windows). The root-mean-square amplitude of all songs was equalized, and stimuli were broadcast at 70 dB SPL (measured at 20 cm from the speaker). Ten songs were chosen from each tutor or novel bird (average duration, 2 s; combined with silence to a 6 s sound file; examples of a novel and a tutor song: Fig. S2.2C and S2.2D) and presented randomly during 1 min. To avoid pseudoreplication, we used songs from 14 birds of our breeding colony that were unfamiliar to the experimental birds. Two novel songs were selected randomly for each subject; one was used in the preference test before surgery and one after.

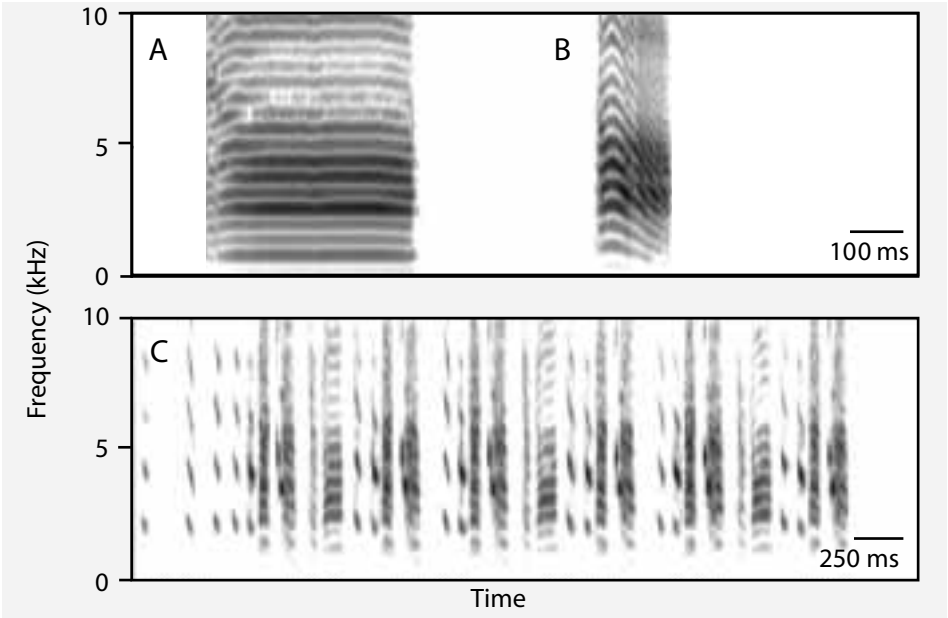

Fig. S2.2 Examples of Vocalisations Used as Auditory Stimuli
(A) Female call, (B) Male call & (C) Novel song

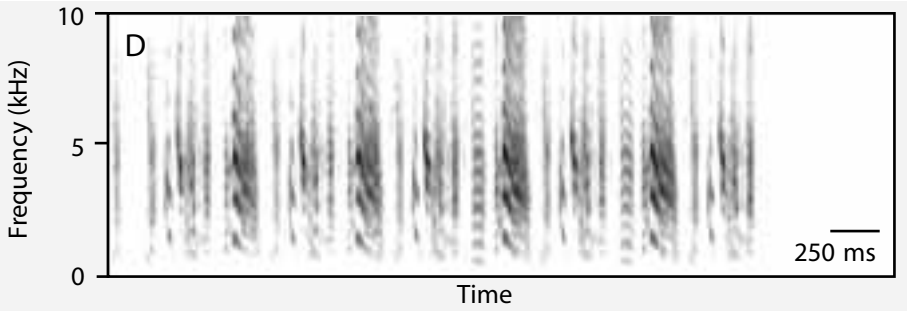

Fig. S2.2 Examples of Vocalisations Used as Auditory Stimuli
(D) Tutor song

Time spent in each zone was scored with a computer by two independent observers who were unaware of the subject's treatment. One observer scored the bird's behaviour in the test "live" from behind a curtain, and the other observer scored from videotape without sound. The interobserver-reliability coefficient between live scores and video scores was 0.99. For each bird, a preference score was calculated by dividing time spent in the approach zone where the tutor song was played back by the total time spent in the two approach zones where song was played. Visits to the approach zones of less than 2 s were not used in calculating the preference score. Birds that failed to reach the following three criteria were excluded from analysis: (1) spend more than 10% of the total test time in one of the approach zones; (2) make at least two choices for the approach zones per session, and (3) show a consistent preference in the two sessions before surgery.

At 28 days after surgery, birds were housed in individual soundproof chambers and the following morning subjected to a call-discrimination procedure, consisting of 240 calls played in random order as described previously (Vicario et al., 2001). Calls of 12 males and 12 females from our breeding colony were selected (see Table S2; examples of a female and a male call: Fig. S2.2A and S2.2B). All stimuli and vocalisations of the experimental subjects were recorded with SAP. Because one of the two animals in the sham group subjected to this test did not respond to more than 10% of the stimulus set, we compared the results of the animals that received lesions to the NCM with those of an unoperated control group (n = 10) from our breeding colony that were reared under the same conditions.

Table S1 ~ Mean song-similarity scores before and after surgery.

	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	C1	C2	C3	C4	C5	C6	C7
0	96	92	95	91	89	91	98	98	99	96	96	96	96	90	96	97	99
1	–	–	–	98	93	96	97	97	98	94	98	95	94	90	94	95	98
11	86	92	95	92	90	96	98	98	97	94	98	94	96	94	94	96	98
56	–	–	–	–	92	97	97	96	97	94	–	–	–	–	–	95	97

Mean song-similarity scores (SAP, % similarity) for ten single motifs compared before surgery (0) with the 1st, 11th and 56th day after surgery for each subject. E1-10 represent experimental birds that received lesions to the NCM; C1-C7 represent birds in the control group.

Analysis of Song and Behavioural Tests

Sound Analysis Pro was used to assess possible changes in vocal production by calculating the similarity index (SI) of ten motifs from before surgery to ten song motifs from after surgery, on day 1, 11, and 56 after surgery. To quantify natural variability in songs within individuals, we also compared songs from two different days before surgery with each other. Based on multiple features (Wiener entropy, spectral continuity, pitch and frequency modulation), this comparison provides an objective quantification of song similarity (Tchernichovski et al., 2000). We evaluated song similarity with repeated-measures ANOVAs with factors Time (SI before surgery, 1, 11, and 56 days after surgery) and Treatment (experimental/control).

Preference scores for tutor song were analysed with a repeated-measures ANOVA with factors Treatment (control/lesion) and Test (before/after). A one-sample t test against the chance level value of 50% was performed for both groups. As a measure of activity during the preference tests and in the baseline periods, we calculated the number of transitions between zones for all stages of the preference test (baseline, session 1, silent interval, session 2). Repeated-measures ANOVAs with factors Time (before/after) and Group showed that the number of transitions between zones did not

change significantly in either group after surgery [baseline: F(1,15) = 0.00; interaction: F(1,15) = 0.04; session 1: F(1,15) = 0.38; interaction: F(1,15) = 0.39; silent interval: F(1,15) = 0.33; interaction: F(1,15) = 0.01; session 2: F(1,15) = 0.43; interaction: F(1,15) = 1.65]. The methods of analysis of vocal responses during the call-discrimination test were described elsewhere (Vicario et al., 2001).

Table S2 ~ Acoustic parameters of Male and Female Call Stimuli

Male	DUR	124	136	152	131	144	110	134	215	142	133	135	246
	FF	312	759	715	520	1481	508	889	477	484	903	478	551
Female	DUR	160	259	287	112	160	248	299	218	238	358	315	348
	FF	478	526	435	403	419	456	538	581	511	677	577	467

Dur denotes duration of the call in ms; FF denotes fundamental frequency in Hz of the part of the call that is not modulated.

Chapter 3

Memory in the making: Localized brain activation related to song learning in young songbirds

With: Matthijs A. Zandbergen and Johan J. Bolhuis

Abstract

Songbird males learn to sing their songs from an adult conspecific (a 'tutor') early in life much like human infants learn how to speak. Similar to humans, in the songbird brain there are separate neural substrates for vocal production and for auditory memory. In adult songbirds, the caudal pallium, the avian equivalent of the auditory association cortex, has been proposed to contain the neural substrate of tutor song memory, while the song system is involved in song production as well as sensorimotor learning. If this hypothesis is correct, then there should be neuronal activation in the caudal pallium, and not in the song system, while the young bird is actually learning the tutor song. We found increased song-induced neuronal activation, measured as the expression of immediate early genes, in the caudal pallium of juvenile zebra finch males that were still learning to sing their songs. No such activation was found in the song system. In addition, in the caudal pallium neuronal activation was greatest in response to tutor song, which was most pronounced relative to novel song in the medial part of the caudomedial nidopallium (NCM). These results suggest that the caudal pallium contains the neural substrate for tutor song memory, which is activated when the young bird is in the process of learning its song. The findings provide insight into the formation of auditory memories that guide vocal production learning, a process fundamental for human speech acquisition.

Introduction

Songbirds learn their songs from an adult conspecific 'tutor' through a process that has parallels with human speech acquisition (Doupe and Kuhl, 1999). In both cases, there is a sensitive period for auditory learning, and vocal learning proceeds through a transitional phase that is called 'babbling' in human infants and 'subsong' in songbirds. Songbirds generally learn to sing in two stages, a memorization phase followed by a sensorimotor phase (Konishi, 1965). During the memorization phase an internal representation of the tutor song is formed that has been called a 'template' (Konishi, 1965). During the sensorimotor phase the young bird starts to vocalize, and it is thought that its song output is matched with the template that was formed in the first phase. Eventually the bird will sing a crystallized song that, in the case of age-limited learners such as the zebra finch, does not change substantially during adulthood. In zebra finches, the memorization phase occurs between approximately 25 to 65 days after hatching, during which 10 days of exposure to a song tutor has been shown to be sufficient to acquire an adult song (Eales, 1985; Eales, 1989; Roper and Zann, 2006). In this species, the sensorimotor learning phase is thought to start around 30 days after hatching, and the song crystallizes when the bird is about 90 days old (Immelmann, 1969; Johnson et al., 2002).

In songbirds, a network of interconnected forebrain nuclei, known as the 'song system' is necessary for vocal learning and song production during the sensorimotor phase and in adulthood (Nottebohm et al., 1976; Bottjer et al., 1984; Scharff and Nottebohm, 1991). In adult songbirds, neurons in the song system preferentially respond to the bird's own song (BOS) (Volman, 1993; Solis et al., 2000). In contrast, brain regions outside the song system, in the nidopallium and mesopallium, are involved in auditory processing (Mello et al., 1992; Mello and Clayton, 1994) and they have been suggested to contain the neural substrate for tutor song memory acquired in the memorization phase (Bolhuis et al., 2000; Bolhuis et al., 2001; Terpstra et al., 2004). The caudomedial nidopallium (NCM) may be the avian equivalent of the auditory association cortex in the mammalian temporal lobe (Bolhuis and Gahr, 2006). In adult zebra finch males, neuronal activation in the NCM in response to tutor song is related to the strength of song learning (Bolhuis et al., 2000; Bolhuis et al., 2001; Terpstra et al., 2004;

Phan et al., 2006). In addition, lesions to the NCM of adult male zebra finches impaired recognition of the tutor song (Gobes and Bolhuis, 2007). The males' own song production was not affected by the lesions suggesting a dissociation between regions in the brain involved in song production and those involved in song perception and memory (Gobes and Bolhuis, 2007). Taken together, these findings suggest that the NCM contains the neural substrate for tutor song memory (Bolhuis and Gahr, 2006). If this hypothesis is correct, neurons in the NCM are expected to be activated in juvenile males that are learning the song of their tutor.

Neuronal activation can be measured as the expression of the immediate early gene *ZENK* (an acronym of *Zif-268*, *Erg-1*, *NKFI-A* and *Krox-24*). Jin and Clayton (1997) found that basal expression of *ZENK* in the NCM of male zebra finches early in the memorization phase (20 - 30 days post hatching), was greater than in adults (Jin and Clayton, 1997; Stripling et al., 2001). At 30 and 45 days post hatching, which is when in the zebra finch the memorization phase and sensorimotor learning phase overlap, there is significantly greater Zenk expression after exposure to conspecific song than in silence (Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2005). Song learning was impaired when an inhibitor of activation of the extracellular signal-regulated kinase (ERK; an enzyme that regulates the transcription of *ZENK*, as well as other genes) was infused into the NCM of juveniles in the sensorimotor learning period (between 40 and 50 days post hatching) when they were first exposed to tutor song (London and Clayton, 2008). These studies show that in juvenile zebra finches, neurons in the NCM are responsive to song, and that this neuronal activation may be important for song learning. To test the hypothesis that the NCM contains the neural substrate of tutor song memory, we studied neuronal activation in this structure in juvenile zebra finches that were in the process of song learning. In particular, we measured Zenk expression of juveniles in response to tutor song, novel song or silence. In addition, we measured neuronal activation in the song system, to determine whether in juveniles there is a similar neural dissociation between song recognition and production as we have found in adults (Gobes and Bolhuis, 2007).

Results

Song learning

The juvenile males had already copied parts of the song of their father, but their songs were still different from those of adults (Fig. 3.1). That is, the songs of the juveniles shared significantly more characteristics with the song of the father than with the songs of novel, unrelated males (similarity: 58.5 ± 4.1 % (SEM) with the tutor and 44.9 ± 2.8 % with unrelated males; $t(11) = 2.5$; $p < 0.05$). In general, the similarity with the tutor's song was representative of birds of that age in our colony (N = 44, similarity: 55.5 ± 2.4 %). In comparison, the mean similarity of songs of adult zebra finch males (90 days post hatching [dph]; N = 39) in our laboratory (separated from their father after the memorization phase at 67 dph), had a mean similarity score of 67.3 ± 3.1%. There was a significant difference between the similarity scores of juvenile and adult males in our colony [N = 44 juveniles and N = 39 adults; $t(81) = 3.02$; $p < 0.01$]. In addition, the juveniles in this study had not copied the songs of their tutors with similar accuracy as adult birds in our laboratory [N = 12 juveniles and N = 39 adults; $t(49) = 3.84$; $p < 0.0001$]. These findings suggest that the juveniles were still in the process of learning their songs.

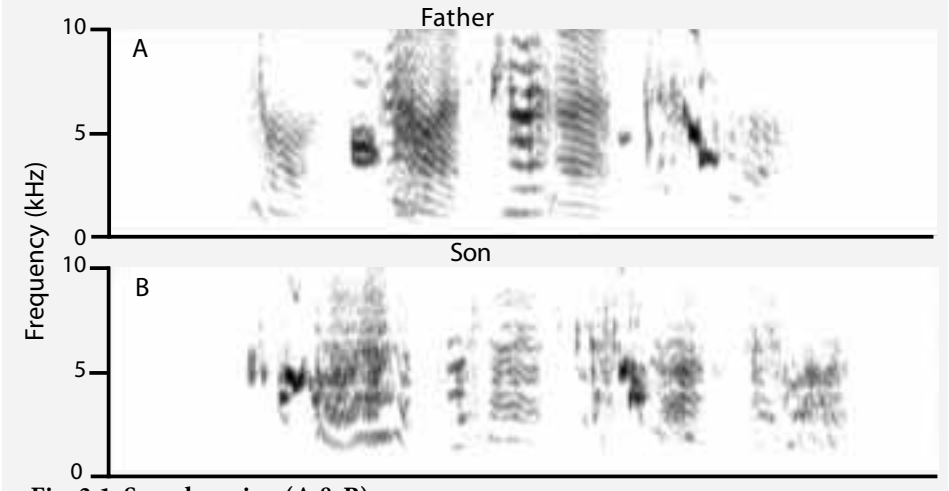

Fig. 3.1 Song learning (A & B)

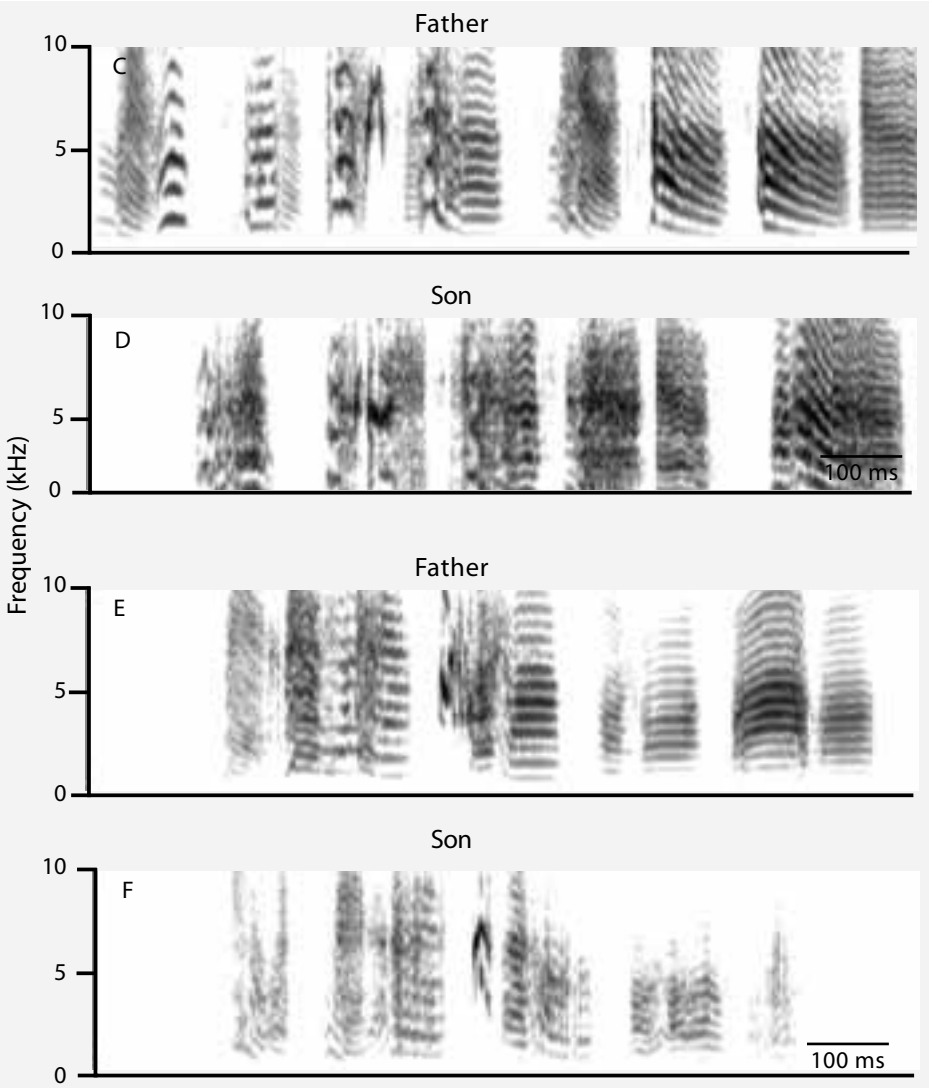

Fig. 3.1 (C,D,E & F) Song learning

Spectrograms of song motifs of some fathers (tutors; A,C,E) and their respective sons (B,D,F). A representative example is shown of one bird in each group.

A neural dissociation in song-induced neuronal activation

Visual inspection of the sections showed that there was song-induced expression of Zenk (the protein product of the IEG *ZENK*) in the two secondary auditory regions (NCM and CMM) but not in the song system (HVC and the robust nucleus of the arcopallium [RA]; see Fig. 3.2). This impression was confirmed by a repeated measures ANOVA with two functional divisions of the sampled forebrain regions as a main factor: the secondary auditory regions (NCM and CMM) and the song system nuclei (HVC and RA). That is, there was a significant interaction between the factors Functional division and Stimulus [$F(2,13) = 4.84$; $p < 0.05$; $N = 16$]. Subsequent ANOVAs revealed a significant effect of Stimulus [$F(2,13) = 8.11$; $p < 0.01$; $N = 16$] in the secondary auditory regions, but not in the song system [$F(2,13) = 0.29$, not significant; $N = 16$].

Localized learning-related neuronal activation in the secondary auditory regions

On the basis of previous findings (Terpstra et al., 2004) we sampled Zenk expression in the secondary auditory regions NCM and CMM at two levels: medial and lateral. A repeated measures ANOVA with factors Region (CMM and NCM), Medial-lateral (as within-region levels) and Stimulus revealed significant main effects of Region [$F(1,13) = 26.55$; $p < 0.0001$; $n = 16$], Lateral-medial ($F(1,13) = 4.95$; $p < 0.05$; $n = 16$] and Stimulus [$F(2,13) = 8.11$; $p < 0.01$; $n = 16$]. There were significant interactions between the factors Region and Stimulus [$F(2,13) = 6.74$; $p < 0.01$; $n = 16$] and Region and Lateral-medial [$F(1,13) = 27.99$; $p < 0.0001$; $n = 16$], and a three-way interaction between Region, Lateral-medial and Stimulus [$F(2,13) = 7.38$; $p < 0.01$; $n = 16$]. Thus, we continued with separate analyses of the different sub-regions.

A one-way ANOVA for the medial NCM revealed a significant effect of Stimulus [$F(2,15) = 10.14$; $p < 0.01$; $n = 16$]. Post-hoc tests showed that there was significantly greater Zenk expression in the NCM of juveniles that had been exposed to tutor song ($p = 0.001$) and novel song ($P = 0.024$)

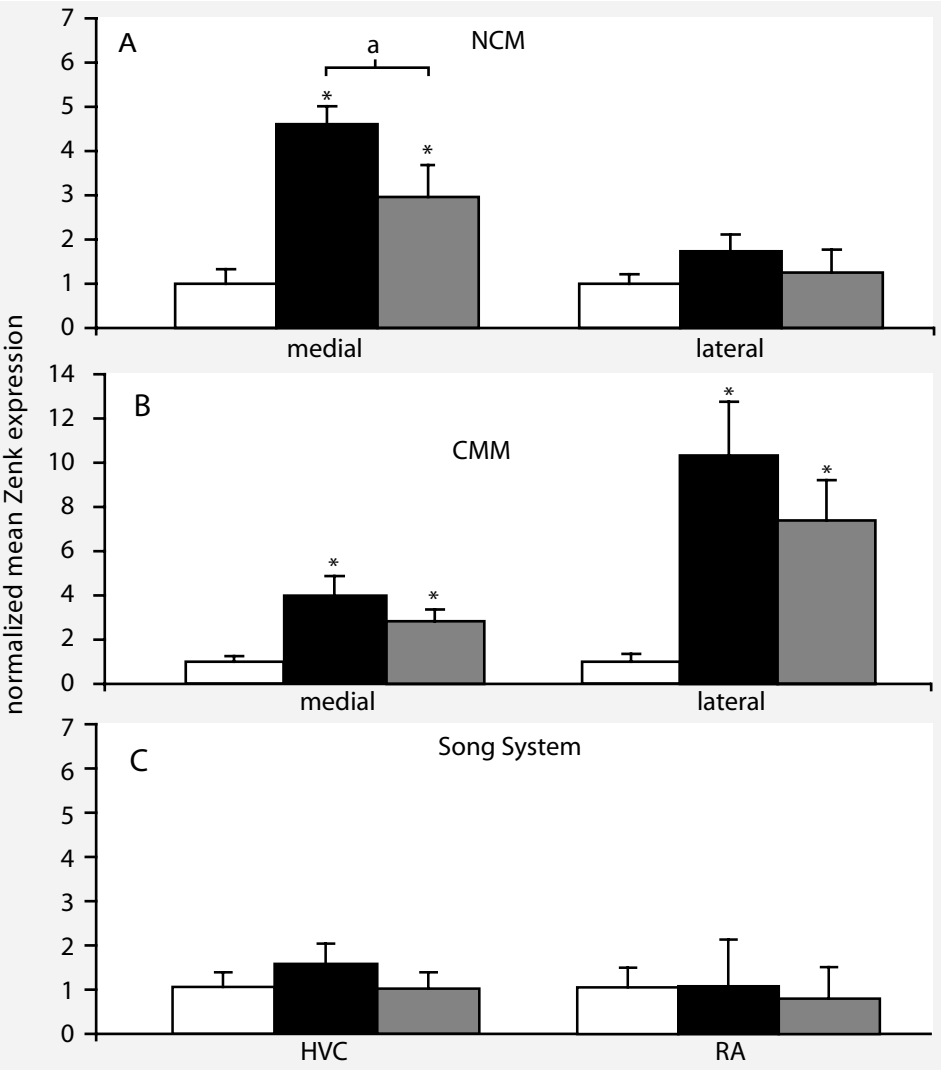

Fig 3.2: Mean Zenk expression normalized to control levels (± SEM) in the NCM (A), the CMM (B) and the Song System (C) for groups of juvenile male zebra finches exposed to tutor song (black bars), novel song (grey bars) or silence (white bars). The number of Zenk-positive nuclei per square millimeter for each subject was divided by the mean number of Zenk-positive nuclei per square millimeter of the Silent control group. Significant effects of exposure to auditory stimuli are indicated with an asterisk; the p-value of the difference indicated with the letter a above the conditions is 0.053.

when compared to the silent control group (see Fig. 3.2A). The difference in Zenk expression in the medial NCM between juveniles that were exposed to tutor or novel song was nearly significant ($p = 0.053$). There were no such effects in the lateral NCM [$F(2,15) = 0.75$, not significant; $n = 16$].

In the CMM, one-way ANOVAs revealed a significant effect of Stimulus [medial CMM: $F(2,15) = 5.94$; $p < 0.05$; lateral CMM: $F(2,15) = 6.71$; $p < 0.01$; $n = 16$]. Post-hoc tests revealed significant differences between the tutor group and the silent group (medial: $p = 0.005$; lateral: $p = 0.003$), and between the novel group and the silence group (medial: $p = 0.046$; lateral: $p = 0.024$; see Fig. 3.2B). There were no significant differences between the tutor and the novel group (medial: $p = 0.199$; lateral: $p = 0.260$).

Singing induced neuronal activation in the song system in juveniles

As shown previously, the act of singing drives Zenk expression in HVC and RA of the developing songbird brain (Jin and Clayton, 1997; Whitney et al., 2000) as it does in adults (Jarvis and Nottebohm, 1997). We exposed a separate group of juvenile males of the same age ($N = 20$) to tutor song, novel song or silence at the same circadian time as the subjects in the main experiment, but not in darkness. Birds that are kept in darkness do not vocalize. Fifteen out of 20 birds that were kept with the lights on sang during the last 2 hours prior to sacrifice. There was no significant effect of Stimulus (tutor song, novel song or silence) on Zenk expression [$F(2,19) = 3.17$; $p > 0.05$; $n = 20$] in the song system of birds that were tested with the lights on. However, Zenk expression in the song system was significantly greater in these subjects than in the birds that were kept in darkness [$F(1,36) = 7.2$; $p < 0.05$; $n = 16$ for darkness and $n = 20$ for lights on), suggesting that the expression of Zenk is induced by the act of singing, and not by hearing song. Furthermore, the amount of singing correlated significantly with the expression of Zenk in the song system nucleus RA (Pearson's correlation: 0.452; $p < 0.05$; $n = 20$).

Discussion

These results show that in juvenile zebra finches that were in the process of learning their songs, there was song-induced neuronal activation in the auditory forebrain (cf. Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2005) that, in the NCM, was greatest in response to tutor song. In contrast, nuclei in the song system showed neuronal activation when the young males were singing (cf. Jin and Clayton, 1997; Johnson and Whitney, 2005), but not when they were exposed to song, including tutor song. These findings suggest that in juvenile songbirds that are in the process of learning to sing a song, the NCM is (part of) the neural substrate for the representation of tutor song, which is activated when the bird is exposed to that song. In addition, in juveniles there is a neural dissociation between the cognitive systems of vocal production and auditory memory, as it is in adults (Gobes and Bolhuis, 2007).

The present results are consistent with the suggestion (Bolhuis and Gahr, 2006) that the NCM contains the neural representation of tutor song memory that is acquired by juveniles, while nuclei in the song system are involved in song production as well as in sensorimotor learning in juveniles and adults (Brainard and Doupe, 2000). Bolhuis & Gahr (2006) suggested that during song learning there is continual interaction between regions in the auditory forebrain and the song system. During the first half of the sensorimotor phase (35 - 69 dph), neurons in the HVC of male zebra finches respond preferentially to the tutor song, whereas during the second half of the sensorimotor phase and in adult zebra finches and white-crowned sparrows there is preferential responding to the bird's own song (Volman, 1993; Nick and Konishi, 2005). Recently, it was found that lesions to the HVC in zebra finch males in the early sensorimotor phase (33 - 44 dph) did not impair the production of subsong (Aronov et al., 2008). Lesions in HVC late in the sensorimotor learning phase (45 - 73 dph) did affect the production of so called 'plastic song' (Aronov et al., 2008). These findings suggest that the HVC is important for song production in the plastic song phase and that during this phase neurons in HVC acquire their preferential responsiveness to the BOS.

A similar dissociation between brain regions involved in auditory perception and memory on the one hand, and vocal production on the other,

exists in the case of human speech. In human adults, the neural substrate of motor representations of speech involves regions in the inferior frontal cortex (Broca's area) (Demonet et al., 2005) whereas perception and memory of speech involves regions in the superior temporal cortex (Wernicke's area and surrounding regions) (Viceic et al., 2006). In human newborns (5 days old) that are exposed to human speech there is increased activity (measured with magnetoencephalography) in the superior temporal lobe, but not in the inferior frontal cortex (Imada et al., 2006). Interestingly, six- and twelve-month-old infants exhibit increased activation in the superior temporal cortex as well as in the inferior frontal cortex when exposed to speech sounds (Imada et al., 2006). In addition, an fMRI study in 3-month old infants (that are in the 'cooing' stage of babbling in which no syllables are produced) showed speech-induced activation in the superior temporal cortex as well as in Broca's area (Dehaene-Lambertz et al., 2006). The activation in Broca's area was specific for speech repetition indicating a role for Broca's area in auditory memory in pre-verbal infants. These studies suggest that the superior temporal cortex is (part of) the neural substrate for speech perception in neonates and that Broca's area becomes active at a later stage, when infants start babbling.

Thus, in humans as well as in songbirds, there is dissociation between the regions involved in auditory perception and memory and the production of vocalizations, in juveniles as well as in adults. Also, in both cases there is evidence for continual interaction between these regions, particularly in juveniles. The present findings suggest that birdsong learning may provide a powerful model for auditory and vocal production learning, processes that are fundamental for human speech acquisition.

Materials & Methods

Subjects. 19 male zebra finches were obtained from the Central Animal facility (GDL) of Utrecht University. Birds were maintained on a 15:9 light:dark cycle, lights on at 6 am. All birds were kept in breeding cages with their parents and siblings until 47 days post hatching (dph). At 47 dph, all sons from a clutch were taken to a different room and kept in communal cages (60 x 40 x 40 cm). Mean age at the day of the experiment was 56 days (range 54 - 59 dph; SD: 1.8). Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University (DEC 06/296).

Experimental procedures. Subjects were divided into three experimental groups: tutor, novel and silence. As auditory stimuli we used the song of the father or the song of a novel zebra finch male. Stimulus presentation started at 11 am and lasted approximately one hour. On the day of the experiment, the lights turned on at 6 am, they were turned off at 8 am and the birds remained in the dark until they were sacrificed 30 min. after the end of the last stimulus presentation.

As stimuli for exposure, we used 10 songs from the same animal that were repeated in random order to a total of 90 stimulus presentations. The playback of the total set lasted an hour. The ten songs that were selected represented the naturally occurring song repertoire with respect to the number of motifs sung in each song, of either the father (tutor) or an unfamiliar male. The root-mean-square amplitude of all songs was equalized and the average duration of the songs used was 2.1 s (SD: 0.4). Sound files were constructed using Praat software (Boersma and Weenink, 2005).

Two days before the start of exposure, a subject bird was placed in a cage measuring 40 x 35 x 35 cm, placed within a soundproof chamber with water and food available *ad libitum*. After the birds were placed in the soundproof chambers, vocalizations were monitored and digitally recorded with Sennheiser MKH 50P48 directional microphones using the recorder of Sound Analysis Pro (SAP), version 1.056 (Tchernichovski et al., 2000; Tchernichovski and Mitra, 2004). Digital video recordings of the subjects were made with infrared video cameras and a NV7000 video card using the program DVR version 5.1.0027 from Avermedia Technologies©. Video and sound recordings were made throughout the experiment.

The results of one subject were excluded from further analyses because video recording on the day of the experiment had failed.

At 11 am on the second day, after the birds had been in the sound-proof chamber for 2 nights and one day, the stimulus songs were broadcast through a speaker (Vifa MG10SD) with a flat frequency-response curve between 0,1 and 15 kHz. A Hypex PC amplifier and Windows Media Player controlled the sound pressure level at 65 dB mean SPL at 30 cm from the speaker.

Immunocytochemistry. At 12.30 (30 min. after the end of exposure to the stimulus set), the experimental subjects were anesthetized with 0.06 ml Natriumpentobarbital (intramuscular) (Nembutal®, Ceva Sante Animale) and subsequently perfused with phosphate buffered saline (PBS), followed by fixation with 4% paraformaldehyde in PBS. Brains were dissected out and post-fixed in 4% paraformaldehyde at 4° C for 6 hours. Brains were cryoprotected in a series of 10% (30 min.), 20% (5 hours) and 30% (overnight) sucrose solutions. Hemispheres were separated, frozen in OCT compound (Tissue-Tek) and parasagittal 20 µm sections were made on a cryostat and mounted on poly-L-lysine coated slides. The brains of two animals were excluded from further analysis because the left hemisphere was damaged. Slides were stored at -18° C until immunocytochemistry. Immunocytochemistry for Zenk was done in a single run, including sections of all animals.

Sections were rinsed in PBS for 5 minutes and incubated in H_2O_2 (0.034%) for 1 minute. Sections were rinsed twice with PBS and once with PBT (0.01% acetylated albumin (BSA-c™, AURION, Wageningen, the Netherlands) and 0.2% triton in PBS) for 5 minutes each, and incubated with 5% normal goat serum (NGS) in 0.3% triton and 0.01% BSA-c for 30 minutes at room temperature. Afterwards, sections were rinsed twice in PBT for 5 minutes and incubated with primary polyclonal rabbit antiserum (Santa Cruz biotechnology, Santa Cruz, CA, USA, catalogue # sc-189, 1:1000) raised against the carboxy-terminus of mouse egr-1 (sequence STGLSDMTATFSPRTIEIC; see Mello and Ribeiro, 1998), in 0.3% triton and 0.01% BSA-c overnight at 4° C. Specificity of this commercially available antiserum for activity-induced Zenk expression has previously been confirmed by Western blots of the zebra finch brain and by preabsorption with anti-Zenk antiserum (Mello and Ribeiro, 1998). The antibody was localized in the nuclei of neurons, which is similar to previous reports of song-induced Zenk expression. In addition, regional localization in the zebra finch brain was similar to previous reports

of protein and mRNA expression (Mello et al., 1992; Mello and Clayton, 1994; Mello and Ribeiro, 1998). Controls were run by omitting the primary antiserum. Sections were rinsed again in PBT three times for 15 minutes, incubated with biotinylated goat-anti-rabbit (IgG, dilution 1:100, Vector Laboratories, Burlingame, CA, USA), in 3% triton and 0.01% BSA-c for one hour, and rinsed three times for 15 minutes in PBS. Afterwards sections were incubated with ABC (avidin-biotinylated enzyme complex, Vector Elite Kit, Vector Laboratories, Burlingame, CA, USA) and rinsed in PBS twice for 5 minutes. Finally, sections were incubated in diaminobenzidine medium with 0.034% H2O2 for 6 minutes. The reaction was stopped in distilled water. Slides were then rinsed in PBS, dehydrated and embedded in DPX.

Image analysis. Quantification of Zenk-immunopositive cells was performed for NCM and CMM on 150 x 200 μm images of sections at both the medial (between 260 and 440 μm from the midline) and the lateral (between 740 and 920 μm from the midline) position at regular intervals (every second section for medial and every fourth section for lateral). For the NCM, a counting frame was placed at the extreme caudal pole of the nidopallium (Terpstra et al., 2004). For the CMM, the frame was placed adjacent to the ventricle and the lamina mesopallialis. Distance from the midline was assessed by calculating the number of serial sections and this location was verified using the atlas of Vates et al. (1996), an unpublished atlas of the zebra finch brain by A. M. den Boer-Visser which was also used in previous studies (Terpstra et al., 2004; Terpstra et al., 2006) and a stereotaxic atlas that is available online (Nixdorf-Bergweiler and Bischof, 2007). For the HVC and the RA, the photomicrographs were taken in the center of the nucleus. For each region, digital photographs of three different sections were taken using a Leica DFC 4206 camera and the program Leica application suite on an Axioskop (Zeiss, Germany) with 20x objective. Image analysis was carried out with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany), A program was developed in KS400 to quantify immunoreactive cells semiautomatically. The circular shape factor, optical density and mean nucleus size were determined for each region to optimize the selection specificity of immunopositive cells. This program uses the circular shape factor to exclude artefacts that are above background-threshold level but are not nuclei, for example, remaining blood cells that are oval in shape. Mean nucleus size was determined by

precisely measuring the circumference of 5 nuclei per picture, in 5 random pictures. This measure is used by the program to exclude artefacts that are much smaller or larger than an average nucleus. We ensured accuracy of our cell counts by visually checking each selection of immunopositive neurons made by the program, and deselecting artefacts manually. To facilitate this process, the selection of what is background staining (based on pixel intensity levels) could be adjusted when necessary for each picture independently. Counts of three sections per region per animal were averaged for further statistical analysis. Image analysis was performed 'blind' as to the experimental history of the subject.

Behavioural analyses. We quantified the total cumulative duration of vocalizations for each bird by estimating the percentage of song that each sound file contained relative to the total duration of the sound file. Sound Analysis Pro (Tchernichovski and Mitra, 2004) was used to assess the fidelity of tutor song imitation of the experimental subjects by calculating the 'percentage similarity' which is a measurement of syllable copying. In addition, 'accuracy' was calculated, which is a measure of how well each syllable is copied. Five songs were randomly selected from the last ten songs that were sung on the day of the experiment just before 8 am; the birds did not sing from the moment the lights were turned off. The songs from the tutors and birds were filtered and equalized using Praat software. Based on multiple features (Wiener entropy, spectral continuity, pitch and frequency modulation), this comparison provides an objective quantification of song similarity. Sound Analysis Pro has a 'floor-effect', because zebra finch songs always resemble each other somewhat on these features. We thus compared these songs also to songs from five unfamiliar non-tutor birds, to investigate whether our juveniles had already learned from their song tutors or if similarity was due to general song characteristics present at that age.

Statistical analysis. All data were first normalised to the mean levels of Zenk expression in the appropriate silent control group because the sampled regions had different basal expression levels and we were interested in Zenk induction ('fold-change') resulting from the exposure. We conducted repeated-measures ANOVAs to examine the effects of playback stimulus on the Zenk response in the different brain regions. When appropriate, brain regions were analysed separately using one-way ANOVAs with Fishers LSD post hoc tests. Data were analysed using SPSS 15.0.0.

Acknowledgements

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Chapter 4

Differential responsiveness in brain and behaviour to sexually dimorphic long calls in male and female zebra finches

Provisionally accepted as:
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Abstract

In zebra finches (*Taeniopygia guttata*), as in most other songbird species, there are robust sex differences in brain morphology and vocal behaviour. First, male zebra finches have larger song system nuclei - involved in sensorimotor learning and production of song - than females. Second, male zebra finches learn their song from a tutor, whereas female zebra finches develop a learned preference for the song of their father but do not sing themselves. Third, female zebra finches produce an unlearned 'long call', while males learn their long call (which is different from that of females) from their song tutor. We investigated behavioural and molecular neuronal responsiveness to this sexually dimorphic communication signal. Behavioural responsiveness was quantified by measuring the number of calls and approaches in response to calls that were broadcast from a speaker. We quantified neuronal activation by measuring the number of neurons expressing Zenk, the protein product of the immediate early gene *ZENK* in a number of different forebrain regions in response to male calls, to female calls or to silence. In both sexes female calls evoked more calls and approaches than male calls. There was significantly greater Zenk expression in response to female calls compared to silence in the caudomedial nidopallium, caudomedial mesopallium and the hippocampus in females, but not in males. Thus, male and female zebra finches both show a behavioural preference for female calls, but differential neuronal activation in response to sexually dimorphic calls.

Introduction

Juvenile zebra finch males do not only learn their songs but also learn another vocal signal – the 'long call' or 'distance call'- from their song tutor, which is typically the bird's father (Zann, 1996). The long call functions as an identity call and is produced when birds are visually but not vocally separated from their flock mates or partners (Marler, 2004). In contrast, female zebra finches do not sing and their long call is not learned. The zebra finch long call is not only sexually dimorphic (Fig. 4.1) but also individually recognizable (Vignal et al., 2004, 2008b) and it has been shown that zebra finches can discriminate female calls from male calls in playback experiments (Vicario et al., 2001).

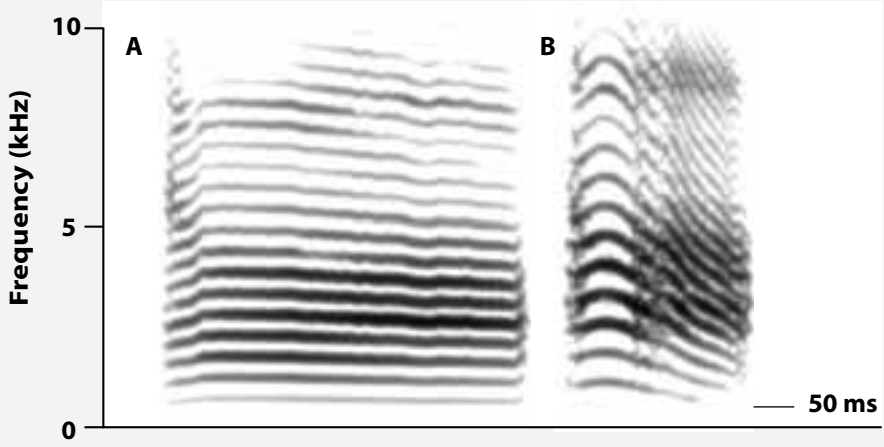


Fig. 4.1: Spectrograms of a female (A) and male (B) long call.

The female long call is usually longer in duration and lower in fundamental frequency than the male long call, which is also characterized by an initial fast frequency modulation.

In addition to sex differences in zebra finch vocal behaviour, there is sexual dimorphism at the level of the brain. The 'song system' (Fig. 4.2A), a set of interconnected brain nuclei involved in song production (Nottebohm et al., 1976) and vocal sensorimotor learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Kao et al., 2005), is larger in males than in females (Nottebohm and Arnold, 1976). Forebrain regions outside the song system, in particular the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), have been shown to be involved in song and call perception (Mello and Clayton, 1994; Bailey et al., 2002; Vignal et al., 2005), and may contain the neural substrate for the memory of conspecific songs and calls (Chew et al., 1995, 1996; Bolhuis et al., 2000, 2001; Terpstra et al., 2004, 2006; Phan et al., 2006; London and Clayton, 2008); for reviews see (Bolhuis and Eda-Fujiwara, 2003; Bolhuis and Gahr, 2006; Bolhuis, 2008). These regions are analogous to the auditory association cortex in mammals (Bolhuis and Gahr, 2006; Bolhuis, 2008). There is some evidence for sexual dimorphism at the level of protein expression in these forebrain regions during development (Bailey and Wade, 2003) and in adult zebra finches (Pinaud et al., 2006), but direct comparisons between males and females investigating differences in the genomic response to auditory stimuli that are produced by both sexes have not been undertaken.

Despite the growing number of studies on auditory perception, little is known of the neural mechanisms of call processing in songbirds. As far as we know, there has been no study in zebra finches that has made a direct comparison between male and female brain activity in response to stimuli produced by and relevant to both sexes. In black-capped chickadees, a songbird species in which both males and females learn their vocalizations, there are sex differences in neuronal responsiveness to songs and calls (Avey et al., 2008). In particular, the genomic response to vocalizations is greater in males than in females (Phillmore et al., 2003; Avey et al., 2008), which according to the authors might be due to the greater salience of male calls in males, because they are territorial (Avey et al., 2008). Exposure to songs with a greater salience, such as long song bouts in starlings (*Sturnus vulgaris*) (Gentner et al., 2001) or more complex songs in budgerigars (*Melopsittacus undulatus*) (Eda-Fujiwara et al., 2003), leads to more Zenk induction in the NCM as compared to less salient songs.

The zebra finch long call is only learned by males and a sexually dimor-

phic communication signal; it is thus a potent auditory stimulus to investigate possible sex differences in the processing of sounds. Here we investigated call processing in two secondary auditory brain regions, the NCM and the CMM. The hippocampus shows selective neuronal activation in response to conspecific song in female zebra finches (Bailey et al., 2002), but this has never been reported for, or extensively studied in males. Therefore we investigated the neuronal response to calls in the hippocampus of both sexes. In this study, we combined a behavioural approach with a detailed investigation of the neural response to calls of both sexes in male as well as female zebra finches.

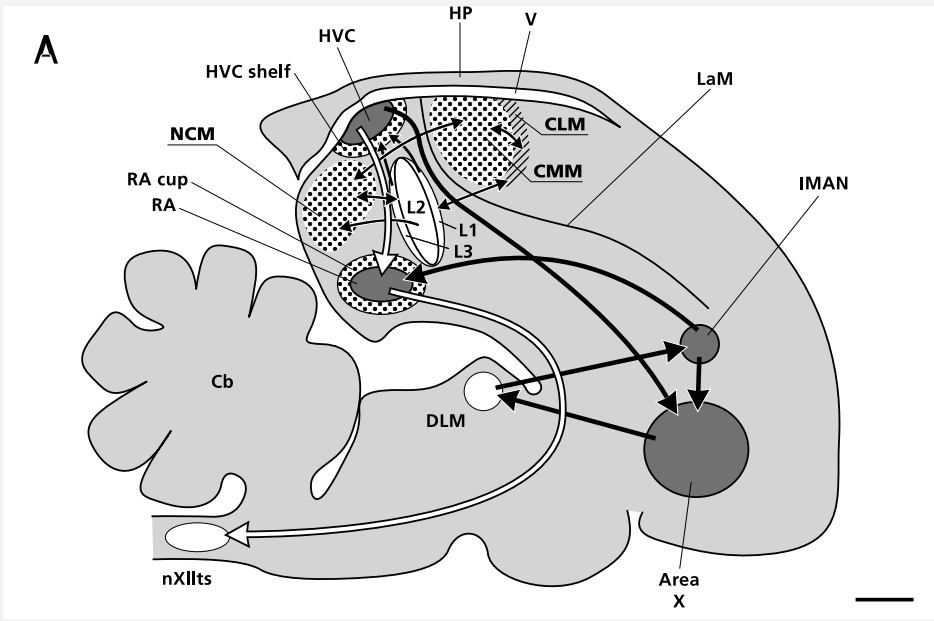
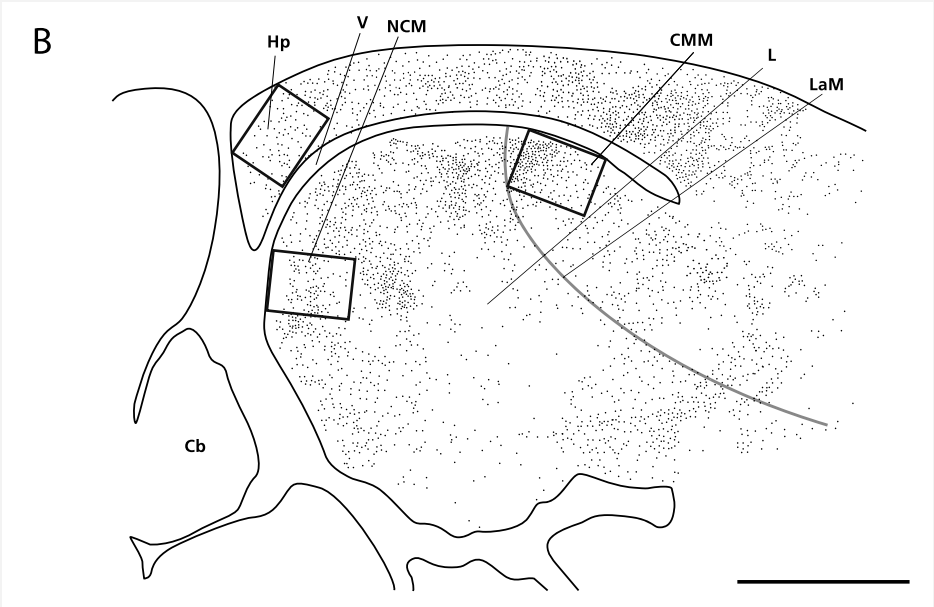


Fig. 4.2: (A) Schematic diagram of a composite view of parasagittal sections of a songbird brain gives approximate positions of nuclei and brain regions involved in birdsong. The song system is a network of interconnected brain nuclei, consisting of a caudal pathway (white arrows), considered to be involved in the production of song, and a rostral pathway (thick black arrows), thought to have a role in song acquisition (Bottjer et al., 1984; Scharff and Nottebohm, 1991). Thin black arrows indicate known connections between

the field L complex, a primary auditory processing region, and some other forebrain regions. Dark grey nuclei show significantly enhanced expression of immediate early genes (IEGs) when the bird is singing (Jarvis and Nottebohm, 1997). Stippled areas represent brain regions that show increased IEG expression when the bird hears song (Mello et al., 1992; Jarvis and Nottebohm, 1997), including tutor song (Bolhuis et al., 2000, 2001; Terpstra et al., 2004). Scale bar represents 1 mm. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, ref. (Bolhuis and Gahr, 2006), copyright 2006.



(B) Schematic representation of a parasaggital section of the brain of an experimental bird. Each dot represents one Zenk-expressing cell. Rectangles indicate the counting area for NCM, CMM and Hippocampus. Scale bar: 1mm. Abbreviations: Cb, cerebellum; CLM, caudolateral mesopallium; CMM, caudomedial mesopallium; DLM, nucleus dorsolateralis anterior, pars medialis; HP, hippocampus; HVC, acronym used as a proper name; L1, L2, L3, subdivisions of field L; LaM, lamina mesopallialis; lMAN, lateral magnocellular nucleus of the anterior nidopallium; NCM, caudomedial nidopallium; nXIIts, tracheosyringeal portion of the nucleus hypoglossus; RA, robust nucleus of the arcopallium; V, ventricle.

Materials and Methods

Subjects. For behavioural testing, we used 6 male and 6 female adult zebra finches (*Taeniopygia guttata*), obtained from the breeding colony at Jean Monnet University. Birds were kept on a 12:12 light:dark cycle with adapted wavelengths (full spectrum with increased blue and red fractions), food and water ad libitum and temperature between 23 and 25°C. Experimental protocols were approved by the Jean Monnet University Animal Care Committee. We measured neuronal activation in 18 male and 18 female adult zebra finches, obtained from the Central Animal facility (GDL) of Utrecht University. Birds were kept on a 12:12 light:dark cycle, lights on at 9.00. Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University (DEC 05/238).

Prior to both experiments, all birds were kept in same sex groups to prevent them from forming pair bonds. The cages of male and female groups were facing each other to enable visual and auditory contact between the sexes. Because birds in the two populations were kept under the same social conditions (i.e. in same sex groups), we assume that the results of the behavioural and the neuronal activation experiment are compatible.

Behavioural testing. We tested responsiveness to sexually dimorphic long calls using a behavioural paradigm in which acoustic stimuli were played back to the subject and the bird's vocal and locomotor activities were measured. One day before the start of the experiment, the experimental subject was placed in a test cage (240 x 50 x 50 cm) in an acoustically isolated room. During isolation and stimulation periods, room temperature and food and water availability were the same as in the aviary. A DAT recorder (Sony DTC-ZE 700) and an amplifier (Yamaha AX-396) were connected to two high fidelity speakers (Triangle Comete 202, France) placed at either end of the experimental cage. During each test only one randomly chosen speaker broadcast the playback stimulus (sound level 60 dB at 1 meter).

Another cage containing one same-sex companion bird was placed near the experimental cage, enabling visual and auditory contact during the day prior to the experiment in which the birds got used to their new environment and during the behavioural experiments, thereby simulating a naturalistic context. Both behavioural (Vignal et al., 2004) and molecular neu-

ronal responsiveness (Vignal et al., 2005) have been shown to be enhanced by the presence of other birds. A different companion bird was used in each trial. All playback experiments took place between 8.00 and 10.00. For each tested bird, playback consisted of one 5 min-set of male calls and one 5 min-set of female calls. To prevent pseudoreplication (Kroodsma, 1989; McGregor et al., 2000), calls from different males and females were used between tests (i.e. calls from 6 females and 6 males). During one set, series of 10 sec call presentations (1 call per second) were broadcast followed by 20 sec silence. Ten different calls from the same individual were played back randomly in each set, so that in each 30 seconds the order in which the 10 calls from the same subject were presented was different. Thus, each tested bird heard a different set of 10 calls from a female and a set of 10 calls from a male, which were randomly presented. The root-mean-square amplitude of all calls was equalized. During the playback test, the vocal and locomotor activity of the experimental bird was recorded with a video recorder (Sony DCR-TRV33). By observing body posture and beak movements, inspection of the video tapes allowed us to distinguish the subject's vocalizations from the vocalizations of the same-sex companion. During each 5 min of stimulus presentation we measured the number of emitted long calls and the number of approaches in the direction of the loudspeaker. One male and one female experimental bird did not vocalize at all during the test. The results of these birds were excluded from the analysis. We analysed male and female responses to male and female calls using a repeated-measures ANOVA with Stimulus (male or female call) as within-subjects factor and Sex (male or female subjects) as between-subjects factor. Because the raw data did not conform to the homogeneity of variances hypothesis (Bartlett's test), the number of approaches was log-transformed. All statistical tests were carried out using Statistica software v. 6 (StatSoft, Tulsa, OK, USA).

Neural analysis. Subjects were divided into six experimental groups: 1. Females exposed to male calls; 2, Females exposed to female calls; 3. Females not exposed to calls (control); 4. Males exposed to male calls; 5. Males exposed to female calls and 6. Males not exposed to calls (control). As stimuli for exposure, we used the same long calls of six males and six females that were used in the behavioural tests. Ten different long calls from each individual were collected and the root-mean-square amplitude of all calls

was equalized. Sound files were constructed using Praat software (Boersma and Weenink, 2005). It has been shown that brief exposure (10 times) to songs evokes a robust immediate early gene (IEG) response in the NCM, comparable to the IEG response after 30 minutes of exposure (Kruse et al., 2000). Accordingly, we chose to use a short exposure time to resemble a natural situation and to match exposure duration with the duration used in the behavioural tests. Sound files with a total duration of 10 minutes were composed, in which each 30 seconds consisted of 10 seconds of randomly ordered calls, 1 call per second, followed by 20 seconds of silence. In each sound file, we used calls of a different individual to prevent pseudoreplication (Kroodsma, 1989; McGregor et al., 2000), resulting in six unique female and six unique male call stimuli.

One day before the start of exposure, the subject bird was placed in a soundproof chamber together with a companion bird of the same sex. The presence of companion birds during exposure has been shown to amplify the effect of auditory stimulation on Zenk-expression (Vignal et al., 2005). In addition, birds are less likely to vocalize in the dark in response to the playback of calls when they are not in social isolation. The subject and companion bird were placed in separate adjacent cages, enabling visual and auditory contact, and with water and food available *ad libitum*. At 9.00 on the day of exposure, the stimulus was broadcast through a speaker (MG10SD, Vifa, Rødovre, Denmark) with a flat frequency-response curve between 0.1 and 15 kHz. A Hypex PC amplifier (Hypex Electronics B.V., Groningen, the Netherlands) and Windows Media Player controlled the sound pressure level at 70 dB mean SPL at 30 cm from the speaker. On testing days the lights were not switched on the morning of the experiment, to avoid visual stimulation and to inhibit vocal responses. Birds in the control groups were also kept in the dark, and no stimulus was presented.

The birds remained in the dark for one hour after stimulus onset and until the experimental bird was sacrificed for further analysis. Starting on the day before exposure and throughout the experiment, vocalizations were monitored and digitally recorded with Sennheiser MKH 50P48 directional microphones (Sennheiser Electronic KG, Wedemark, Germany) using the recorder of Sound Analysis Pro (Tchernichovski et al., 2000; Tchernichovski and Mitra, 2004). Although the experiments were conducted in the dark to prevent the birds from vocalizing, it is likely that the

birds were awake because movement-related sounds (hops, wing-shakes) and low-amplitude vocalizations (tets) were noted on the recordings. The number of vocalizations in response to playback of long calls was counted in the sound files recorded during exposure. We identified long calls and songs of the subject and the audience bird by using the similarity function of Sound Analysis Pro. We excluded cases in which songs were produced or more than 5% of calls heard during stimulus exposure and thereafter were produced by the subject and the audience bird, with a limit of maximally 5 calls (= 2.5%) produced by the subject bird. Using this criterion, the effects of vocalizations produced by the subject and audience bird on Zenk expression should be minor (Kruse et al., 2000). Tets, which are very short vocalizations of low amplitude (Zann, 1996), were not distinctive enough between individuals to reliably separate tets produced by the experimental bird from those produced by the audience bird.

Immunocytochemistry. One hour after stimulus onset, the experimental subjects were anesthetized with 0.06 ml Natriumpentobarbital (intramuscular) (Nembutal®, Ceva Sante Animale, Libourne, France) and subsequently perfused with phosphate buffered saline (PBS) containing 0,2% heparin, followed by fixation with 4% paraformaldehyde in PBS. Brains (in the skull) were stored overnight at 4° C in 4% paraformaldehyde. Brains were dissected out and cryo-protected in a series of 10% (30 min.), 20% (5 hours) and 30% (overnight) sucrose solutions. Hemispheres were separated, frozen in OCT compound (Tissue-Tek®, Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and parasagittal 20 µm sections were made on a cryostat and mounted on poly-L-lysine coated slides. Slides were stored at -18° C until immunocytochemistry, which was done in a single run, including sections of all 32 animals.

Sections were rinsed in PBS for 5 minutes and incubated in H_2O_2 (0.034%) for 1 minute. Sections were rinsed twice with PBS and once with PBT (0.01% acetylated albumin (BSA-c™, AURION, Wageningen, the Netherlands) and 0.2% triton in PBS) for 5 minutes each, and incubated with 5% normal goat serum (NGS) in 0.3% triton and 0.01% BSA-c for 30 minutes at room temperature. Afterwards, sections were rinsed twice in PBT for 5 minutes and incubated with primary polyclonal rabbit antiserum (Santa Cruz biotechnology, Santa Cruz, CA, USA, catalogue # sc-189, 1:1000) raised against the carboxy-terminus of mouse egr-1 (sequence STGLSDMTATFSPRTIEIC;

see (Mello and Ribeiro, 1998), in 0.3% triton and 0.01% BSA-c overnight at 4° C. Specificity of this commercially available antiserum for activity-induced Zenk expression has previously been confirmed by Western blots of the zebra finch brain which revealed a band of the 62.5 kDa protein (Mello and Ribeiro, 1998). Nonspecific and non-induced bands on the Western blots resulting from the detection system alone could successfully be minimized by using Vector's Elite kit and/or the avidin/biotin blocking steps. Preabsorption of brain sections with anti-Zenk antiserum and a 10-fold excess of the preabsorption peptide resulted in a complete loss of specific nuclear staining (Mello and Ribeiro, 1998). The antibody was localized in the nuclei of neurons, which is similar to previous reports of song-induced Zenk expression. In addition, regional localization in the zebra finch brain was similar to previous reports of protein and mRNA expression (Mello et al., 1992; Mello and Clayton, 1994; Mello and Ribeiro, 1998). Controls were run by omitting the primary antiserum. Sections were rinsed again in PBT three times for 15 minutes, incubated with biotinylated goat-anti-rabbit (IgG, dilution 1:100, Vector Laboratories, Burlingame, CA, USA), in 3% triton and 0.01% BSA-c for one hour, and rinsed three times for 15 minutes in PBS. Afterwards sections were incubated with ABC (avidin-biotinylated enzyme complex, Vector Elite Kit, Vector Laboratories, Burlingame, CA, USA) and rinsed in PBS twice for 5 minutes. Finally, sections were incubated in diaminobenzidine medium with 0.034% H_2O_2 for 6 minutes. The reaction was stopped in distilled water. Slides were then rinsed in PBS, dehydrated and embedded in DPX.

Image analysis. We investigated Zenk expression in 2 secondary auditory regions, the NCM and the CMM, that have previously been shown to exhibit a Zenk response after exposure to species-specific songs (see Bolhuis & Gahr, 2006, for review). We also investigated Zenk expression in the hippocampus, because effects of song exposure have been reported for this region in female zebra finches (Bailey et al., 2002), but not in males (Mello et al., 1992). Because differences in neuronal responsiveness between the lateral and medial parts of NCM have been reported in a previous study (Terpstra et al., 2004; these authors only found significant effects related to tutor song memory in the lateral NCM), we sampled this brain region, as well as the CMM and the hippocampus, at both the lateral and the medial position. Quantification of Zenk-immunopositive cells was per-

formed on images covering 300 x 400μm (as determined by reference to photomicrographs of a microscopic scale bar) of sections between 240 and 400μm from the midline for the medial part of NCM, CMM and Hippocampus and between 700 and 1000μm from the midline for the lateral part of the same regions at regular intervals (every second section for medial and every fourth section for lateral). For the NCM, a counting frame was placed at the extreme caudal pole of the nidopallium (Terpstra et al., 2006) (Fig. 4.2B). For the CMM, the frame was placed adjacent to the ventricle and the lamina mesopallialis (Fig. 4.2B). For the hippocampus, the frame was positioned adjacent to the ventricle in the caudal tip of the Hippocampus, where the curve is most pronounced (Fig. 4.2B). Distance from the midline was assessed by calculating the number of serial sections and this location was verified using the atlas of Vates et al. (1996), an unpublished atlas of the zebra finch brain by A. M. den Boer-Visser which was also used in previous studies (Terpstra et al., 2004, 2006) and a stereotaxic atlas that is available online (Nixdorf-Bergweiler and Bischof, 2007). For each region (lateral NCM, medial NCM, lateral CMM, medial CMM, lateral Hippocampus and medial Hippocampus), digital photographs of three different sections of each individual (totaling 18 photomicrographs for each individual) were taken using a Nikon DXM 1200 Digital camera on an Axioskop (Zeiss) with an 20x objective. Image analysis was carried out with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program had previously been developed in KS400 to quantify immunoreactive cells semiautomatically (Terpstra et al., 2004, 2005, 2006). The circular shape factor, optical density and mean nucleus size were determined for each region to optimize the selection specificity of immunopositive cells. This program uses the circular shape factor to exclude artefacts that are above background-threshold level but are not nuclei, for example, remaining blood cells that are oval in shape. Mean nucleus size was determined by precisely measuring the circumference of 5 nuclei per picture, in 5 random pictures. This measure is used by the program to exclude artefacts that are much smaller or larger than an average nucleus. We ensured accuracy of our cells counts by checking each selection of immunopositive neurons made by the program, and deselecting artefacts manually. To facilitate this process, the selection of what is background staining (based on pixel intensity levels) could be adjusted when

necessary for each picture independently. Counts of three sections per region per animal were averaged for further statistical analysis. Image analysis was performed 'blind' as to the experimental history of the subject. An analysis of 49 photomicrographs performed with NIH software (ImageJ) as well as with KS400 software showed that the two programs were highly compatible (Pearson's correlation: 0.9; $p < 0.0001$).

Statistical analysis. All data were natural log transformed before statistical analysis. A repeated measures ANOVA with Brain Region (NCM, CMM, hippocampus) and Medial-lateral as within-subjects factors and Stimulus (male call, female call or silence) and Sex (male or female subjects) as between-subject factors was conducted to examine the effects of playback stimulus on the Zenk response in the different brain regions in both sexes. Males and females were then analysed separately for each brain region using univariate ANOVAs with Fisher's PLSD post-hoc comparisons on play-back stimulus. In addition, we tested the effect of total stimulus duration on the Zenk response. Data were analysed in SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Behavioural response to long calls

Vocal activity. The nature of the stimulus call significantly influenced the vocal activity of the experimental birds (Fig. 4.3A): female calls evoked significantly more long calls than male calls [$F(1,8) = 15.98$; $p = 0.004$]. This effect was significant in both male and female experimental birds (Wilcoxon paired t-test, $p = 0.043$). There was no significant effect of the factor Sex [$F(1,8) = 0.97$] and no significant interaction between Stimulus and Sex [$F(1,8) = 0.90$].

Locomotor activity. The nature of the playback stimulus significantly influenced the locomotion activity: female calls evoked significantly more approaches than male calls [$F(1,8) = 5.77$; $p = 0.043$] (Fig. 4.3B). This effect is not significant in male and female experimental birds taken separately (Wilcoxon paired t-test, $p > 0.05$). There was no significant interaction between Stimulus and Sex [$F(1,8) = 0.17$]. During the test, there was a nearly significant effect of males approaching the loudspeaker more than females [$F(1,8) = 5.14$; $p = 0.053$].

Thus, irrespective of the sex of the experimental birds, female calls evoked more calls and more approaches than male calls.

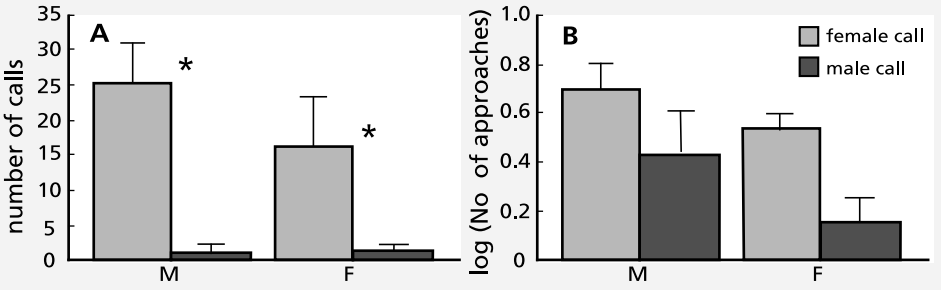


Fig. 4.3: Mean number of calls emitted (A) and approaches towards the speaker (B) by males (M) and females (F) in response to female calls (light bars) and male calls (dark bars). Error bars indicate SEM.

Neuronal response to long calls

Figure 4 shows examples of Zenk immunoreactivity in both sexes. Overall, there were significant effects of Region [$F(2,50) = 8.08$; $p = 0.001$] as well as a significant interaction between Region and Medial-lateral [$F(2,50) = 10.21$; $p < 0.001$]. Finally, there was a significant triple interaction between Region, Sex and Stimulus [$F(4,50) = 3.62$; $p = 0.011$]. Thus, the regions in which we sampled the number of Zenk expressing cells, differed from each other in response magnitude, depending on the nature of the stimulus and the sex of the subject. For this reason, the regions were analysed separately for each sex.

Region-specific analyses of neuronal responsiveness to long calls. We further investigated the effects of the stimuli in both sexes independently by conducting separate ANOVAs for each brain region (NCM, CMM or hippocampus).

In males, no significant effects of Stimulus were found for the NCM, CMM or the hippocampus.

In females, there was a significant effect of Stimulus in the medial NCM [Fig. 4.5B: $F(2,15) = 4.79$; $p = 0.028$]. Post-hoc tests showed that in females' medial NCM there was significantly greater Zenk expression towards female calls in comparison to silence ($p = 0.016$) and in comparison to male calls ($p = 0.027$). In the lateral NCM, there was no significant effect of Stimulus.

In the female CMM, there was a significant effect of Stimulus in the medial [$F(2,15) = 6.36$; $p = 0.012$] as well as lateral CMM [$F(2,15) = 4.32$; $p = 0.036$] (Fig. 4.5 C&D). Post-hoc tests showed that in females' medial and lateral CMM there was significantly greater Zenk expression towards female calls in comparison to silence (medial: $p = 0.004$; lateral: $p = 0.017$). In the lateral CMM, post-hoc tests also revealed a significant difference between females exposed to female calls in comparison to male calls ($p = 0.046$).

In the hippocampus of females, there was a significant effect of Stimulus in the medial [$F(2,15) = 4.46$; $p = 0.034$; see Fig. 4.5 E&F] as well as lateral hippocampus [$F(2,15) = 6.67$; $p = 0.010$]. In females' medial and lateral hippocampus there was significantly greater Zenk expression towards female calls in comparison to silence (medial: $p = 0.013$; lateral: $p = 0.003$).

In lateral hippocampus, post-hoc tests revealed a difference between females exposed to female calls and male calls ($p = 0.045$), whereas in medial hippocampus Zenk expression in females exposed to male calls differed significantly from the silence condition ($p = 0.037$).

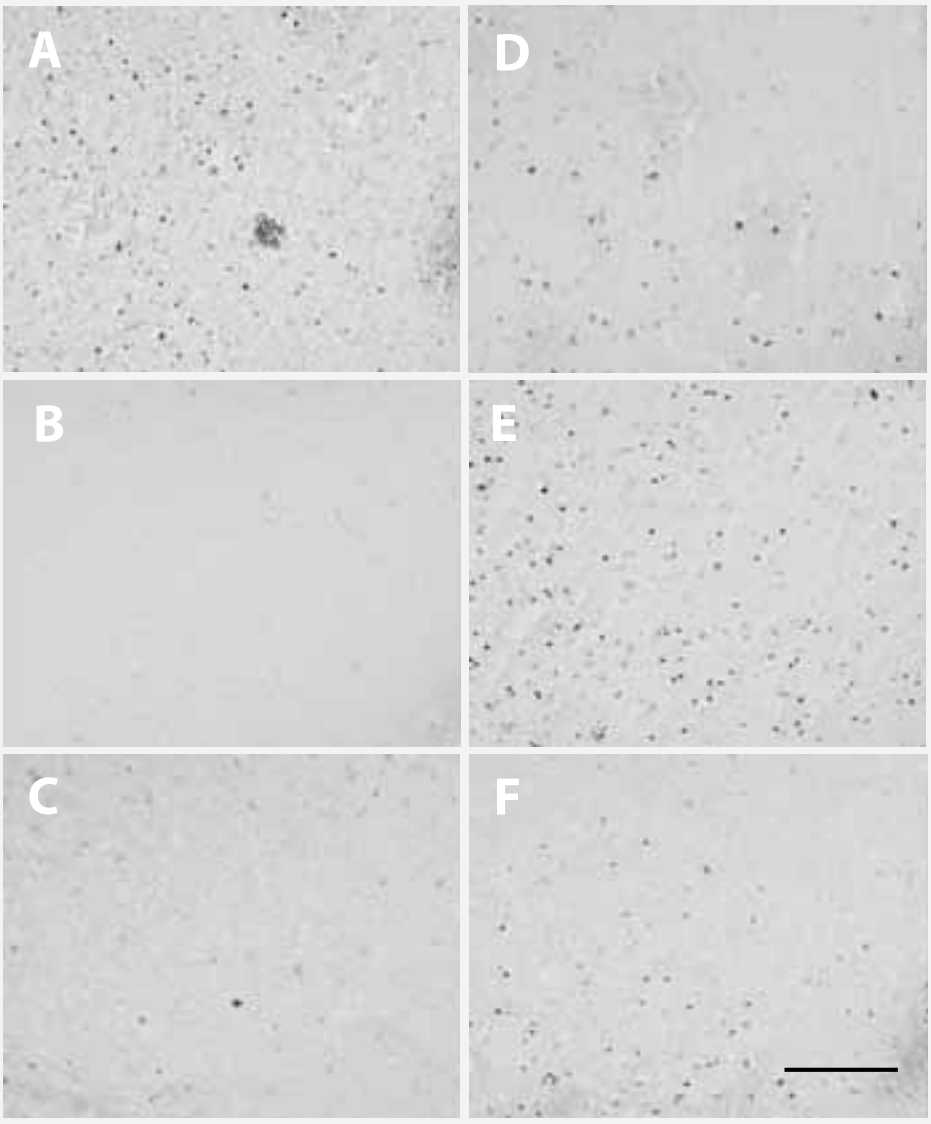


Fig. 4.4

Fig. 4.4: Photomicrographs of parasagittal sections of the zebra finch brain at the level of the NCM at 700 - 1000 µm from the midline (lateral), showing Zenk immunoreactivity. Representative examples of Zenk immunopositive cells in females (A, B, C) and males (D, E, F) that were exposed to female calls (A, D), male calls (B, E) or not exposed to calls (C, F). Scale bar in F represents 0.1 mm

Because the duration of male calls is shorter than that of female calls and all subjects were exposed to a total of 200 calls, subjects in the female-call stimulus groups were subjected to a longer cumulative sound exposure. Pearson's correlation statistic showed no effects of cumulative exposure time on Zenk expression levels in any of the brain regions investigated ($p > 0.05$ in all regions). Only a few birds produced vocalizations themselves during stimulus exposure, and there were no males that produced songs during stimulus exposure. Two females exposed to female calls produced low-amplitude 'tets' (Zann, 1996) and either one or two long calls. One male exposed to male calls produced tets and one long call. All males exposed to female calls produced tets, and three of them also emitted long calls. Of the males that were exposed to female calls, one subject produced more than 2.5% of calls itself, and was therefore excluded from further analysis. Tets, contrary to long calls, are not individually recognizable; therefore, we could not analyse tets emitted by subject and audience bird separately. We could not detect any effects of calls produced by the subject birds on Zenk expression.

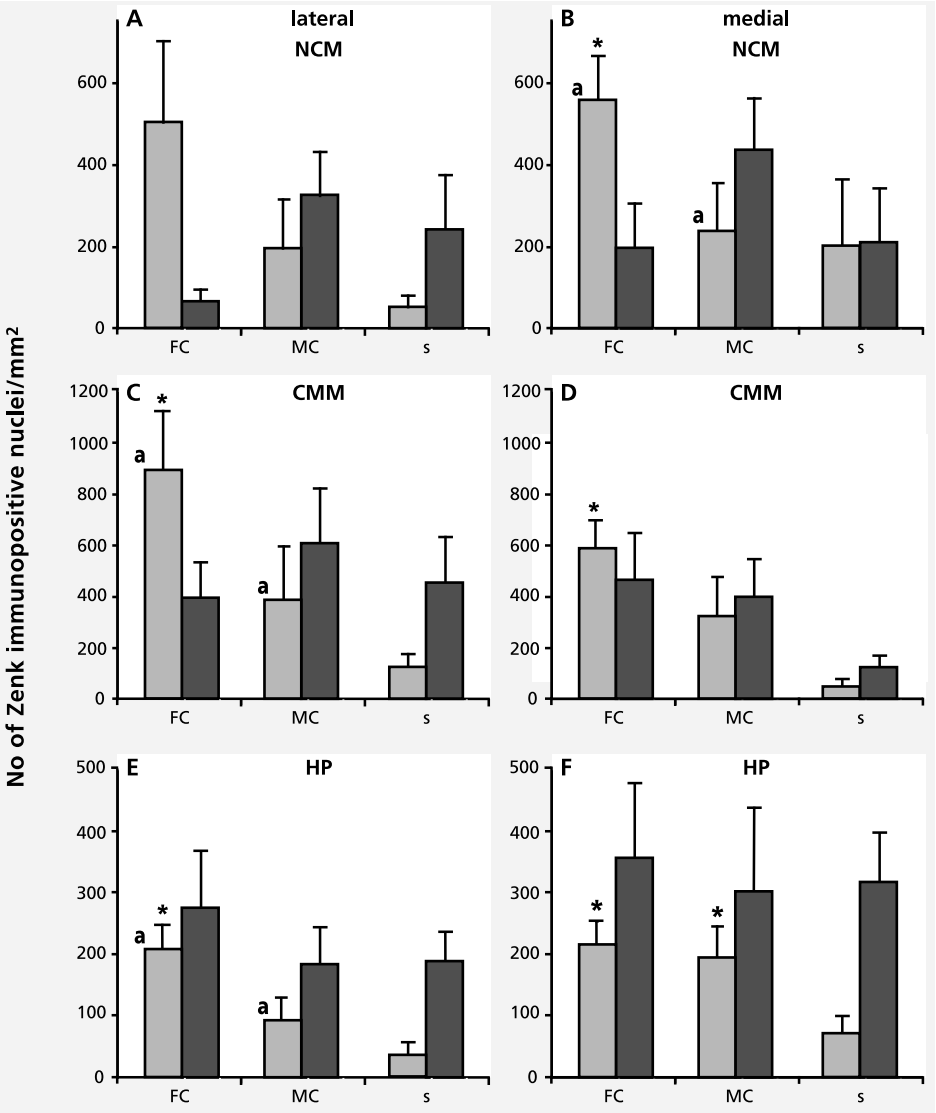


Fig. 4.5: Zenk expression (number of Zenk-positive nuclei per square millimeter ± SEM) in NCM (A, B), CMM (C, D), and hippocampus (E, F) for groups of male (dark bars) and female (light bars) zebra finches exposed to female calls (FC), male calls (MC) or silence (s). Data for the lateral level (A, C, E) were collected at 700 - 1000 µm from the midline and data for the medial level (B, D, F) were collected at 240 - 400 µm from the midline. Significant effects of stimulus (as compared to baseline levels within one sex) are

indicated with an asterisk above the conditions that differ significantly from silence; significant differences between birds of one sex exposed to male calls and female calls are indicated with the letter *a*.

Discussion

Both male and female zebra finches discriminated between calls of the two sexes and preferentially responded to female calls, with increased vocalization as well as more approaches. This behavioural preference for female calls is consistent with previous studies in which only vocal responses were investigated (Vicario et al., 2001; Gobes and Bolhuis, 2007). In contrast, neuronal activation in pallial regions of the forebrain in response to sexually dimorphic long calls differed between males and females. In females only, there was greater neuronal activation in response to female calls than to silence in the NCM, CMM as well as the hippocampus, consistent with their behavioural preference for female calls. In contrast, in males, there was no such effect. Thus, the patterns of neuronal activation in response to calls were not a simple reflection of behavioural preferences.

Sex differences in neural processing of auditory stimuli

The NCM and the CMM may have different functions in the recognition of vocalization in males and females. In the present study we found increased Zenk expression after exposure to female calls in these brain regions in females but not in males. In addition, Zenk expression in the CMM was greater after exposure to the song of the father as compared to a novel song in females but not males (Terpstra et al. 2004, 2006).

In contrast, in male zebra finches we found a significant correlation between the strength of song learning and IEG expression in the NCM, not in the CMM (Bolhuis et al., 2000; Bolhuis et al., 2001; Terpstra et al., 2004). Interestingly, Pinaud et al. (2006) reported neurochemical differences between the male and female NCM of zebra finches. That is, males had more calbindin–positive cells in the NCM than females. These cells are a sub-

population of GABAergic cells that do not express Zenk (Pinaud et al., 2006). These authors suggested that auditory processing circuits in male NCM may be less prone to undergo calcium-dependent plasticity (such as induced gene expression) than in females. This suggestion is consistent with the present finding that call-induced gene expression is greater in females than in males. In contrast to the IEG studies, electrophysiological responsiveness of neurons in the NCM did not reveal sex differences in responsiveness to auditory stimuli in zebra finches (Chew et al., 1996). These responses were equally strong in males and females, and habituated in the same manner after exposure to a number of different conspecific vocalizations, including male song and calls from both sexes (Chew et al., 1996). IEG expression is not a necessary concomitant of neuronal firing (Mello and Jarvis, 2008). For instance, in the song system, electrophysiological analysis revealed preferential responding to the bird's own song, while this was not reflected in increased IEG expression (Jarvis and Nottebohm, 1997; Gobes et al., 2007). Mello & Jarvis (2008) suggested that these discrepancies could be due to the fact that in most electrophysiological studies (e.g. Chew et al., 1996), the animals are anaesthetised, while they are awake in IEG studies.

Familiarity of calls and neuronal activity

Vignal et al. (2005) reported significantly increased Zenk expression in the NCM of males after exposure to familiar female calls. In contrast to the study by Vignal et al. (2005), in which the males were exposed to familiar calls for 30 minutes, the birds in the present study were exposed to unfamiliar calls for only 10 minutes. In addition, in the present study a restricted part of the NCM was sampled while the whole of the NCM was sampled in four sampling areas in the study of Vignal et al. (cf. Fig 1. in Vignal et al., 2005). However, no differences were found in Zenk-response magnitude between the four sampling areas in which NCM was divided. The increased Zenk expression to calls compared to silence in the study by Vignal et al. (2005) could be a consequence of recognition of familiar calls. Thus, a prediction arising from these findings is that males will show greater neuronal activation in the NCM in response to familiar calls, in particular the call of

their father or the call from their mate, than to unfamiliar calls.

Sex differences in auditory processing in other songbird species

While in zebra finches, males learn their songs and calls, and females produce calls that are not learned, both male and female black-capped chickadees learn their calls as well as songs (Avey et al., 2008). In contrast to zebra finches, in chickadees the call is an acoustically complex vocalization, whereas the song is relatively simple. When male and female chickadees were exposed to songs and calls, the expression of Zenk in the NCM was higher in males than in females, and higher for acoustically simple songs than for calls (Phillmore et al., 2003). There was no significant difference in Zenk expression between females that heard the acoustically complex chickadee calls and females in a silent control group. A different study, investigating differences between chickadee males and females to male and female songs and calls separately, confirmed higher Zenk expression in males, but showed the highest response to male calls to be in the CMM of males (Avey et al., 2008). These studies in black-capped chickadees and several studies in other songbirds investigating song (Gentner et al., 2001; Maney et al., 2003; Leitner et al., 2005) provide evidence for the suggestion that vocalizations with the greatest salience (i.e. male songs and calls in territorial male chickadees) evoke the strongest Zenk expression. While male chickadees produce songs in a territorial context, female chickadees produce their song mainly in a nesting context to communicate with their partner. It is thus possible that for female chickadees, their mate's songs and calls would induce higher Zenk expression. In the present study, we report that in zebra finches (that are not territorial), female calls evoked the strongest neuronal response in females (in medial as well as lateral CMM and hippocampus, and medial NCM), while responses in males did not reach levels that were significantly different from controls. Stimulus salience was likely to be similar because both sexes preferred female calls over male calls. While both sexes showed a similar behavioural preference for female calls, the response of females is likely based on call-duration only (with longer female calls receiving more call responses than shorter male calls) whereas males discriminate calls in a more categorical way (Vicario et al., 2001) based on the absence of a frequency modulation in female calls

and independent of call length (see (Vicario, 2004). Interestingly, call discrimination behaviour in males develops during the same developmental time period in which the song and call are learned (Vicario et al., 2002). Thus, although both male and female zebra finches prefer female calls, the neuronal processing of calls might be different resulting in sex differences in neuronal activation as reported in this study.

Differences in activation of lateral and medial subdivisions of the nidopallium

In the NCM, neuronal activation was different between males and females in medial as well as lateral parts of this structure. Only in females and in the medial and not in the lateral subdivision of the NCM was there an increased molecular response to female calls compared to silence. Previously, we have shown differences in molecular responsiveness between lateral and medial subdivisions of the NCM in adult males that were exposed to their tutor's song (Terpstra et al., 2004). In particular, learning-related Zenk expression was only found in the lateral NCM. Interestingly, Velho and Mello (2008) reported that stimulation with conspecific song in female zebra finches induces a significant increase in the expression of synapsins (a class of proteins involved in the regulation of synaptic vesicle availability) only in the lateral NCM and not in the medial NCM. Zenk protein binds to the synapsin promoter in vivo in zebra finches (Velho and Mello, 2008). In addition, the sex differences in the number of calbindin positive cells in the NCM reported by Pinaud et al. (2006), were most pronounced in the medial NCM. Taken together, these findings indicate that the lateral and medial parts of the NCM respond differently to auditory stimuli.

The role of the hippocampus

Overall, there was less expression in the hippocampus than in both NCM and CMM (cf. Terpstra et al., 2004, 2006). The present findings do show that neuronal activation in the hippocampus in response to calls is different in females and males. Similar results regarding the expression of Fos,

the protein product of the immediate early gene *c-fos*, were found in zebra finch females in relation to song perception (Bailey et al., 2002). Bailey and colleagues exposed adult female zebra finches to conspecific and heterospecific songs, tones or silence and quantified Fos immunoreactive cells in a number of forebrain regions. They reported increased Fos expression in response to conspecific song in the NCM, hippocampus and parahippocampus. Thus, immediate early gene responses to songs (Bailey et al., 2002) and calls, as reported here, follow similar patterns in adult female zebra finches, indicating that, in addition to the NCM and the CMM, the hippocampus may also be involved in the processing of sounds relevant for the female zebra finch. In addition, it has recently been shown that Zenk expression is significantly greater after exposure to mate-calls than to the calls of another male in the Hippocampus of female zebra finches, but not in NCM and CMM (Vignal et al., 2008a). Further research is necessary to elucidate the function of the hippocampus in female zebra finches.

This is the first study to analyse neuronal activation in both male and female zebra finches in response to calls. These findings suggest that the neuronal processing of calls is different between males and females. Whether these differences are related to the ability of males to learn their vocalizations is the next question that we need to answer in order to better understand brain mechanisms for communication between the sexes.

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Chapter 5

General discussion

General discussion

Zebra finches, like most other songbird species, learn their songs in two phases; a memorization phase in which a representation of the song of an adult tutor is formed and a sensorimotor learning phase in which the young birds starts singing itself (Fig. 1.1). A network of interconnected brain nuclei, collectively known as the 'song system', is necessary for the production of song and for sensori-motor learning in the second phase of song learning (Fig. 1.2). On the basis of previous research, it has been suggested that regions in the caudal pallium, the songbird equivalent of the auditory association cortex, contain the neural substrate for the representation of tutor song memory that is acquired during the memorization phase (Bolhuis et al., 2000; Bolhuis and Gahr, 2006). In particular, in adult male zebra finch males, song-induced neuronal activation in the caudomedial nidopallium (NCM; Fig. 1.3) is positively correlated with the strength of song learning (Bolhuis et al., 2000, 2001; Terpstra et al., 2004; Phan et al., 2006). In chapter 2, I tested the hypothesis that the NCM contains the neural substrate for the representation of tutor song memory by placing bilateral lesions in this structure in adult male zebra finches. If the NCM contains the neural substrate for the representation of tutor song, lesions to this structure should affect recognition of the tutor song. The results of this experiment confirmed this hypothesis: the preference for the song of the father was significantly impaired, while song production and auditory discrimination were not affected. Another prediction arising from the suggestion that the NCM is the neural substrate for the representation of tutor song is that this structure should be activated in juvenile male zebra finches that are in the process of learning their songs (Chapter 3). I exposed young males to song and found increased neuronal activation in the NCM and caudomedial mesopallium (CMM). Activation in the medial part of the NCM was greatest in response to tutor song. In addition, there was neuronal activation only in the song system of young birds that produced songs themselves but not when they were exposed to songs (Chapter 3).

Taken together, the results described in Chapters 2 and 3 suggest that (part of) the NCM is the likely neural substrate for the representation of tutor song memory. The song-induced neuronal activation in the NCM and the impaired recognition of tutor song after lesions to the NCM are

also consistent with a role for this structure in general auditory processing. For example, the NCM could be the neural substrate for discrimination of sounds based on fine acoustic features or temporal sequences of sounds, such as song syllables within a motif. That is, the NCM could be a 'relay station' for auditory processing, and the neural representation of song memory might be localized outside the NCM (Bolhuis and Gahr, 2006). Neuronal activation in juveniles could be greater after exposure to tutor song than to novel song if the novelty of a stimulus is processed in the NCM by means of making a comparison with a representation of song that is localized outside of the NCM. However, if the NCM would be a 'relay station' for general processing of auditory stimuli, then lesions to the NCM are expected to impair the preference for female calls in comparison to male calls. I have shown that lesions to the NCM did not affect the preference for novel female calls (Chapter 2), making it unlikely that the NCM is only involved in the general processing of auditory stimuli. Taken together, these findings demonstrate that the NCM contains (part of) the neural substrate for representation of tutor song memory in juvenile and adult male zebra finches, and that access to this representation is not necessary for song production in adult zebra finches.

While only male zebra finches produce songs, both males and females produce calls. The 'long call' is produced by both sexes, but only the male long call is learned. We do not know how calls are represented in the brain. Investigating neuronal activation in response to calls might provide clues as to how vocalisations are processed in males and females. In female zebra finches, the CMM is the likely neural substrate for the representation of the song of the father (Terpstra et al., 2006). When female zebra finches are exposed to the song of their father, there is increased neuronal activation in the CMM as compared to a novel song (Terpstra et al., 2006). In contrast, in male zebra finches that are exposed to the song of their father, neuronal activation the NCM (and not the CMM) is related to the strength of song learning (Terpstra et al., 2004). Thus, there are sex differences in the neuronal processing of familiar song. This sexual dimorphism may be related to vocal learning (Bolhuis and Gahr, 2006). It is possible that in general, vocalizations are processed differently in males and females. To address this issue, I exposed male and female zebra finches to long calls of both sexes (Chapter 4). I found that in females, neuronal activation was greatest

after exposure to female calls, in the NCM, the CMM as well as in the hippocampus. In contrast, in male zebra finches there was no such activation. Thus, both male and female zebra finches preferred female calls (cf. Vicario et al., 2001), but in females only, female calls induced expression of the protein product of the immediate early gene *ZENK* in the NCM, CMM and hippocampus. Thus, in male and female zebra finches, there is differential neuronal activation in response to vocalisations. It was suggested by Pinaud et al. (2006) that auditory processing circuits in male NCM may be less prone to undergo calcium-dependent plasticity (such as induced gene expression) than in females, which is consistent with the neuronal activation in response to calls found in the present study (Chapter 4). Possibly, these differences in molecular responsiveness are related to the sex differences in vocal learning ability. Many GABAergic neurons in the NCM and CMM also express *ZENK* in response to auditory stimulation (Pinaud et al., 2004). Males had more calbindin–positive cells in the NCM than females (Pinaud et al., 2006). These cells are a sub-population of GABAergic cells that do not express Zenk (Pinaud et al., 2006). There might be differential inhibitory activity in the auditory system of males and females in response to vocalisations, which could enable males to selectively learn the song that they are most often exposed to, which is the song of the tutor.

A neural dissociation between song memory and production

The results in this thesis show that the NCM is (part of) the neural substrate for the representation of song memory in zebra finches (Chapters 2, 3). There is evidence from both lesion studies and gene expression analyses that the equivalent brain region in monkeys and in rats, the auditory association cortex, is involved in auditory recognition memory (Colombo et al., 1990; Wan et al., 2001; Fritz et al., 2005). In contrast to rats and monkeys, songbirds also learn to imitate the sounds they have memorized. I have shown that the integrity of the NCM in adult zebra finches is not required for song production (Chapter 2). In addition, the song system is not activated when juvenile birds listen to the song of the tutor or to the song of another conspecific male (Chapter 3). Thus, there is a neural dissociation in the regions involved in song memory and production in juvenile and adult songbirds.

In Chapter 2 of this thesis, I reported the results of an experiment in which lesions were made in the NCM of adult zebra finches that had crystallized songs. It is possible that song acquisition in juveniles that are still learning their song requires an intact NCM because the neural representation of tutor song is formed during the memorization phase. In addition, during the sensorimotor phase, when the young bird's song output is matched with the stored representation of tutor song (Konishi, 1965), development of the bird's own song is likely to involve access to this representation. It was predicted (Chapter 2; Bolhuis et al., 2000) that lesions to the NCM of juvenile zebra finches should impair song development. Recently, London and Clayton (2008) infused an inhibitor of the activation of extracellular signal regulated kinase (ERK) in the NCM of juvenile zebra finches during tutoring sessions in which they were exposed to a tutor. The transcription of *ZENK*, as well as other immediate early genes (IEGs) such as *c-fos* and *Arc*, is regulated by ERK (Velho et al., 2005). Juveniles that received this treatment during tutoring sessions, developed poor imitations of the tutor song whereas normal discrimination of songs was unaffected by infusion with this inhibitor (London and Clayton, 2008). Birds that received an inactive compound that is structurally similar to the inhibitor developed songs that

resembled the tutor song. This study shows that a molecular response (regulated by ERK) in the NCM is necessary for some of the processes underlying song learning, but provides no direct evidence that the NCM contains the neural substrate for the memory of tutor song in juvenile zebra finches. It is possible that the reported results are not related to auditory memory formation but to sensorimotor integration (vocal learning) in between or during tutor-song exposure. The experimental treatment was not given to a group of birds in between tutoring sessions. It is possible that the inhibitor of ERK activation affected the young males' own song output and thus the feedback process of sensorimotor learning. In a similar study, an NMDA-receptor (N-methyl-D-aspartate) inhibitor was infused into the song system nucleus lMAN (Fig. 1.2) in juvenile birds during tutoring sessions (Basham et al., 1996). This receptor is thought to play a regulatory role in synaptic plasticity (see for review Rao and Finkbeiner, 2007). A control group received the same treatment on non-tutoring days, i.e in the absence of the tutor. The songs of the experimental birds resembled the tutor song less than the songs of the control birds, but song discrimination was not affected. The studies of London and Clayton (2008) and Basham and colleagues (1996) both investigated song learning in juveniles. These experiments yielded similar results, i.e. impaired song learning, although the studies utilized different pharmacological agents and involved different brain regions. Firm conclusions concerning the localization of tutor song memory in juveniles cannot be drawn from these studies, because the confounding effects on sensori-motor integration and perception cannot be separated from the putative effects on auditory memory formation.

Early in the memorization phase (see Fig. 1.1) of song learning (20 - 30 days post hatching [dph]), basal levels of *ZENK* expression in the NCM are greater than in adults. (Jin and Clayton, 1997; Stripling et al., 2001). Later in the memorization phase and during the early sensorimotor learning phase (30 - 45 dph), there is significantly more *ZENK* expression after exposure to conspecific song than in silence (Jin and Clayton, 1997; Stripling et al., 2001; Bailey and Wade, 2005). The neuronal activation reflected by increased and inducible (after exposure to conspecific song) levels of *ZENK* in the NCM of juvenile zebra finches coincides with the auditory memorization phase of song learning. *ZENK* expression has been suggested to be important for neuronal plasticity and memory consolidation (Jones et al., 2001; Bozon et

al., 2003a, 2003b; Knapska and Kaczmarek, 2004). However, the study by Jin and Clayton (1997) provides no direct evidence for a representation of tutor song in the NCM of juvenile zebra finches, because tutor song was not used as a stimulus. In this thesis, I confirmed the hypothesis that the NCM is (part of) the neural substrate for tutor song memory in juveniles (Chapter 3) as well as adults (Chapter 2). I found that neuronal activation in the NCM en CMM was induced by song, activation in the medial part of the NCM was most strongly induced by the song of the tutor and that the NCM is necessary for recognition of the tutor song. In contrast, the act of singing induced neuronal activation in the song system of adult zebra finches (Jarvis and Nottebohm, 1997) and juveniles (Chapter 3; Jin and Clayton, 1997; Johnson and Whitney, 2005). There was neuronal activation in the NCM but not in the robust nucleus of the arcopallium (RA) or HVC of juveniles that were exposed to the tutor song and production of song was unaltered after lesions to the NCM. Lesions of song system nuclei (Bottjer et al., 1984; Scharff and Nottebohm, 1991) in the anterior forebrain pathway (see Fig. 1.2) affect sensorimotor learning in juveniles but do not affect song production in adults. Juvenile zebra finches with lesions to the song system nucleus Area X never develop stable adult song and lesions to lMAN prematurely stabilize song output. In contrast, lesions to HVC and RA also effect song production in adult songbirds (Nottebohm et al., 1976; Aronov et al., 2008). In combination with the present results, this suggest a dissociation in the neural substrates underlying song memory and song production in juvenile (Chapter 3) and adult (Chapter 2) zebra finches. That is, the NCM (and possibly the CMM) contains the neural substrate for the representation of tutor song memory whereas the song system is involved in the production of song and in sensorimotor learning.

Parallels between birdsong and human speech

In human adults, the auditory association cortex in the superior temporal gyrus is the likely neural substrate for speech perception and memory (Viceic et al., 2006). Regions in the frontal cortex including Broca's area are part of the neural substrate for speech production (Hickok and Poeppel, 2000; Hickok et al., 2003; Demonet et al., 2005). This dissociation in the neural substrates for speech perception and production is strikingly similar to the neural dissociation between song recognition and production in zebra finches, as shown in Chapter 2 and 3 of this thesis.

Recent findings suggest that there is a dynamic interaction between these regions in both adult (Ojemann, 1991; Hickok and Poeppel, 2000; Hickok et al., 2003; Okada and Hickok, 2006) and infant humans (Dehaene-Lambertz et al., 2006; Imada et al., 2006). In pre-verbal human infants, the superior-temporal cortex (or auditory association cortex) and the frontal cortex are both active during speech perception and memory tasks (Dehaene-Lambertz et al., 2006; Imada et al., 2006). In newborn infants (5 days old) there is activation (measured by magnetoencephalography) in the superior temporal cortex (Wernicke's area), but not in the inferior frontal cortex (Broca's area) when they are exposed to human speech (Imada et al., 2006). In older (3-month old) infants that were exposed to sentences, there was activation (measured with fMRI) in the superior-temporal cortex as well as in Broca's area (Dehaene-Lambertz et al., 2006). The activation in Broca's area was specific for speech repetition, indicating a role for Broca's area in memory in pre-verbal infants. Six- and twelve-month-old infants also exhibit activation in the superior-temporal cortex as well as in the inferior-frontal cortex when exposed to speech (Imada et al., 2006). Taken together, these findings suggest that the human superior-temporal cortex is (part of) the neural substrate for speech perception in neonates and that Broca's area in the frontal cortex becomes active later in development, around the age that infants start to produce sounds themselves. The interaction between these areas might be of importance for speech production learning.

There is a parallel in the organisation of the avian brain, where the primary pre-motor nucleus for the production of song, the HVC, exhibits preferential neuronal responsiveness (measured electrophysiologically) to the song of the tutor early in development (35 - 69 dph) (Nick and Konishi,

2005) (cf. activity after exposure to speech in the frontal cortex of human infants when they start babbling). Later in development (in zebra finches of 70 - 90 dph and in adults), neurons in HVC preferentially respond to the bird's own song (BOS) (Margoliash and Konishi, 1985; Margoliash, 1986; Volman, 1993; Nick and Konishi, 2005), indicative of a (motor) representation of the bird's own song in this nucleus. As shown in Chapter 3 of this thesis, in juveniles within the same period (50 - 60 dph) there is neuronal activation, measured as the expression of IEG's, in the NCM in response to the tutor song but nuclei in the song system of juveniles only show neuronal activation when the birds produce song themselves (Chapter 3). In adults, neuronal activation in HVC is also induced by singing (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997). In humans as well as birds, regions that are involved in the perception and production of vocalizations may interact throughout vocal learning; I will elaborate on this in the final sections of the General Discussion.

Bird brains and songs:

A synthesis of previous findings with present results and suggestions for future directions

The 'template' localized?

An important step in understanding how vocalizations are memorized and learned is to understand how the representation of tutor song memory in the NCM is used during song learning. There is learning-related neuronal activation in the NCM of adult birds (Bolhuis et al., 2000, 2001; Terpstra et al., 2004; Phan et al., 2006). In addition, in this thesis I have shown that the NCM is necessary for tutor song recognition in adults. In the NCM of juveniles there is tutor-song specific neuronal activation. However, we do not know if this representation is the 'template', i.e. the memory of the tutor song that is used for learning the bird's own song (Konishi, 1965). It is possible that there are two representations of tutor song memory; one that is used for auditory recognition and one that is used as a 'template' for song learning. On the other hand, the same representation might be used for recognition as well as sensorimotor learning. This problem needs to be investigated in juvenile and adult birds. It could be that regions involved in song memory and production interact more in juveniles that are in the process of learning their song than in adults. Therefore, lesions to the NCM in adult birds do not affect song production (Chapter 2). If the representation of memory in the NCM is a 'template' for learning, lesions to this structure in juveniles should impair song learning, resulting in poor imitations of the tutor song.

Interaction between regions in the brain that contain the neural representation of tutor song memory and a motor program of the BOS might also be necessary when the bird's own song changes due to experimental manipulations such as exposure to white noise or delayed auditory feedback (Leonardo and Konishi, 1999; Brainard and Doupe, 2000b). This hypothesis could be tested in adult birds that have crystallized songs by

destroying those neurons in the song system that are responsible for the pre-motor activity necessary to produce the bird's own song, i.e. the HVC neurons that innervate RA ($HVC_{(RA)}$ neurons). It has been shown that this manipulation immediately leads to highly changed song output, but that there is gradual recovery of the original song (Scharff et al., 2000). If the representation of tutor song memory in NCM is the 'template' for song learning, these adult birds need access to this representation in order to re-learn their songs. Thus, if the NCM is lesioned at the same time as the $HVC_{(RA)}$ neurons are destroyed, these birds will be unable to recover their original songs because they lack the model that is necessary for song modification.

An emerging representation of the bird's own song – a possible role for sleep?

Early in the sensorimotor learning phase (35 - 69 dph), neurons in the song system, in particular in HVC, show selectivity for the tutor's song, whereas later in development (70 - 90 dph) neurons in HVC are selective for BOS (Nick and Konishi, 2005), as they are in adult songbirds (Margoliash and Konishi, 1985; Margoliash, 1986; Volman, 1993). This selectivity for the tutor's song is specific for birds that are awake; HVC neurons in juvenile birds that are asleep show selectivity for BOS (Nick and Konishi, 2005). In birds younger than 45 days, HVC is not necessary to produce sub-song; input from lMAN to RA is sufficient to produce these vocalizations (Aronov et al., 2008). In older juveniles (45 - 67 dph) lMAN is required for inducing song variability, but not necessary for singing per se (Kao et al., 2005; Olveczky et al., 2005; Aronov et al., 2008). In adult zebra finches HVC, and not lMAN, is necessary for the production of song (Bottjer et al., 1984; Aronov et al., 2008).

Thus, early in development input from lMAN to RA is necessary to produce sub-song, which is unstructured and variable (Aronov et al., 2008). In this period, a representation of tutor song memory might be formed in the NCM. A temporary parallel representation of tutor song may then be formed in HVC, likely via the CMM which could be seen as an 'interface' between regions that contain the representation of tutor song (NCM) and the song system (Bauer et al., 2008). It is possible that the representation of

song in HVC functions as a 'comparator' between the representation of tutor song (in NCM) and a pre-motor representation of the BOS in the song system (Mooney, 2004; Nick and Konishi, 2005; Bolhuis and Gahr, 2006; Prather et al., 2008). Recently, the existence of 'mirror neurons', has been demonstrated in HVC in swamp sparrows (*Melospiza georgiana*) (Prather et al., 2008). Mirror neurons respond to auditory stimulation with certain note-types of songs as well as exhibiting motor-activity when similar notes are produced by the bird itself. During the sensorimotor learning phase there could be interaction between auditory regions (NCM, CMM) and regions in the song system (HVC, RA). The representation of tutor song in the HVC perhaps shapes song learning by a Hebbian-like process: every time a sound is produced by the bird (for which lMAN and RA are responsible) with good similarity to the tutor song (for which HVC is the 'comparator'), these pre-motor patterns are consolidated and integrated into a representation of the BOS by changing the connectivity of $HVC_{(RA)}$ neurons. HVC becomes more important late in development in producing song, and neurons engaged in this pre-motor network are less responsive to the song of the tutor. In this way, the representation of tutor song in the HVC could be transformed into a representation of the BOS.

Possibly, the process of consolidating connections between HVC and RA takes place 'off-line', i.e. when the bird is not singing himself or maybe during sleep. There are four indications that sleep might be involved in the process of song learning. First, there is selectivity for the bird's own song in HVC in juvenile birds that are asleep (Nick and Konishi, 2005). Second, in sleeping adult birds, there is 'replay' of pre-motor activity in RA (Dave and Margoliash, 2000) which is driven by HVC and Nif (Hahnloser et al., 2006; Hahnloser and Fee, 2007). Third, sleep might affect the song learning process because there is a positive correlation between post-sleep deterioration of song during development and the strength of song learning (Deregnaucourt et al., 2005). Fourth, nocturnal activity in HVC increases throughout development and is related to overnight changes in song output (Crandall et al., 2007). It has also recently been shown in domestic chicks that sleep affects the stability of neurons involved in a representation of memory of the imprinted stimulus (Jackson et al., 2008). Neuronal activity in the brain of sleeping birds should be studied throughout development in regions involved in production of song, such as HVC and RA, as well as

memory of song, such as the NCM and CMM, to investigate a potential role for sleep in auditory memory and vocal learning.

Molecular and cellular mechanisms underlying song memory

Taken together, the present findings and previously published studies suggest dynamic processes underlying the neural mechanisms of song memory and learning. The NCM is the likely neural substrate for representations of learned vocalizations. The neuronal representation for memory of one song in particular, the tutor song, is probably localized in the NCM and is reactivated after exposure to that song (Chapter 3; Bolhuis et al., 2000, 2001; Terpstra et al., 2004). This representation is used in adulthood to recognize the tutor song (Chapter 2). The NCM may be involved in recognizing many more individual songs and calls (Chapter 4; Chew et al., 1996).

The next level of investigation is that of the cellular and molecular mechanisms underlying memory, in both males that learn their vocalisations and females that only produce unlearned calls. As I have shown in this thesis, neuronal activation in the NCM and CMM in response to unfamiliar calls is different in males and females. We need to investigate whether these differences are related to vocal learning. Are such differences also present after exposure to learned calls, such as the call of the tutor? Males learn their call from their song tutor, but females do not. Perhaps females learn the perceptual characteristics of the call of the tutor. It has been shown that females are able to recognize the call of their mate (Vignal et al., 2008a). As with song, memory-specific effects may be found in the NCM of males and in the CMM of females. Currently, many researchers use female zebra finches to investigate the effects of auditory stimulation on neural processes for practical reasons, namely that they do not sing during the experiments. I have shown in this thesis that there are differences between males and females in the molecular neuronal response to auditory stimuli. Differences in the molecular response might be related to aspects of auditory processing such as the discrimination of stimuli based on acoustic features or the memorization of vocalisations but this remains to be further investigated. If we want to understand the neural mechanisms that underlie auditory memory, we need to investigate this both in males and in females.

The dissociation of brain regions involved in vocal production and memory in the human as well as in the avian brain, suggests convergent evolution of the mechanisms underlying auditory-vocal learning. Thus, by investigating the neural mechanisms underlying birdsong learning, we may gain important insights into the general mechanisms of auditory-vocal learning.

Summary

Songbirds, such as zebra finches, learn their songs from a 'tutor' (usually the father), early in life. There are strong parallels between the behavioural, cognitive and neural processes that underlie vocal learning in humans and songbirds. In both cases there is a sensitive period for auditory learning, during which the young individuals memorize the vocalizations of adult conspecifics to which they are exposed. After this memorization phase, vocal learning proceeds through a sensorimotor learning phase that is called 'babbling' in human infants and 'subsong' in songbirds. There are homologies between the brains of birds and mammals, which makes the songbird a suitable model organism for studying the neural mechanisms of vocal learning and memory. In songbirds, a network of interconnected nuclei, known as the 'song system' is involved in song production and sensorimotor learning. In contrast, it has been suggested that a forebrain region outside the song system, the caudomedial nidopallium (NCM), may contain the neural substrate for the representation of auditory memory acquired in the first phase of song learning. The NCM and the adjacent caudomedial mesopallium (CMM) are thought to be the avian equivalent of the human auditory association cortex. It is essential to investigate the localization of the neural substrate of tutor song memory to be able to unravel the underlying molecular and cellular mechanisms. This can provide insight into how auditory memories are formed that guide vocal production learning, a process fundamental to human speech acquisition. In zebra finches, males learn their vocalisations (calls and songs) while females do not sing, but produce only unlearned calls. Studying the perception of vocalisations in males as well as females may provide clues about how the neural substrate of perception and memory functions in both sexes. This thesis describes three studies investigating the neural mechanisms of auditory memory and perception in zebra finches.

If the NCM contains the neural substrate for the representation of tutor song memory, then lesions to this structure would be expected to impair song recognition. This hypothesis was tested in the experiment described in Chapter 2, where I found that bilateral neurotoxic lesions to the NCM of adult male zebra finches impaired the learned preference for tutor song. Song production was not impaired by NCM lesions. The ability to discrim-

inate calls was unaffected; the subjects exhibited a normal preference for female calls. These findings demonstrate that the NCM performs an essential role in the representation of tutor song memory in adult male zebra finches. In addition, these results show that tutor song memory and a motor program for the bird's own song have separate neural representations in the mature songbird brain. A similar dissociation between regions involved in speech production (such as Broca's area) and speech perception (such as Wernicke's area) exists in the human brain.

Evidence for a role for the NCM in tutor song memory has been obtained from experiments with adult zebra finches. However, if the NCM contains the neural substrate for the representation of tutor song memory, then this structure should be activated in juvenile birds that are in the process of learning the tutor song. This hypothesis was tested in the experiment described in Chapter 3, in which I exposed young zebra finch males that were still learning to produce their own song, to their tutor's song or a novel song. I found increased song-induced neuronal activation, measured as the expression of immediate early genes, in the NCM and CMM of these males. In addition, neuronal activation in the NCM was greatest in response to tutor song relative to novel song. In the song system, there was neuronal activation in birds that had been singing but there was no activation after exposure to tutor song or novel song. These results suggest that the NCM (and possibly also the CMM) contains the neural substrate for tutor song memory, which is activated when the memory is formed.

For vocal communication, zebra finches use not only songs, but also calls such as the 'long call'. Female zebra finches produce an unlearned long call, while males learn their long call (which differs from the female call) from their song tutor. In Chapter 4, I investigated behavioural and molecular neuronal responsiveness to the 'long call' in male and female zebra finches. In both sexes, female calls evoked more calls and approaches than male calls. Neuronal activation in response to female calls was significantly greater in females than in males in the NCM, the CMM and the hippocampus. In addition, there was significantly greater neuronal activation in response to female calls compared to silence in these regions in females, but not in males. Thus, male and female zebra finches both showed a behavioural preference for female calls, but differential neuronal activation when they were exposed to calls of the two sexes.

In the final chapter, I discuss the main findings and their implications for our understanding of the neuronal mechanisms of song learning and memory. The experiments discussed in chapters 2 and 3 confirm that the NCM is the likely neural substrate for the representation of tutor song memory. I have shown for the first time that, in adult zebra finch males, the NCM is necessary for recognition of tutor song, but not for song production. In addition, I demonstrated that in juvenile zebra finches the NCM is activated during auditory memory formation. In juvenile male zebra finches, there is neuronal activation in the song system of birds that are singing themselves but not when they are exposed to songs. Thus, in songbirds the cognitive systems underlying vocal production and auditory recognition memory are subserved by distinct brain regions in juveniles as well as adults. These brain regions may functionally interact continually throughout life. The neuronal processing of auditory signals such as the long call appears to differ between males and females. Unravelling the underlying processes related to the perception and memorization of vocalisations that differ between males and females can increase our understanding of how vocalisations are learned. The localization of the neural substrate of tutor song memory presented in this thesis is a necessary prerequisite for the investigation of the neural mechanisms of auditory memory at the molecular and cellular level. The dissociation of brain regions involved in vocal production and in memory in the human as well as in the avian brain, suggests convergent evolution of the mechanisms underlying auditory-vocal learning. Thus, by investigating the neural mechanisms underlying birdsong learning, we may gain important insights into the general mechanisms of auditory-vocal learning.

Nederlandse samenvatting

Zangvogels, zoals zebravinken, leren hun liedje van een 'tutor' (meestal de vader) wanneer ze jong zijn. Er zijn duidelijke parallellen tussen de cognitieve, neurale en gedragsprocessen die ten grondslag liggen aan vocaal leren bij mensen en bij zangvogels. In beide gevallen is er een gevoelige periode voor auditief leren, waarin de jonge individuen de vocalisaties van volwassenen van de eigen soort waaraan ze zijn blootgesteld opslaan in hun geheugen. Na deze geheugenfase gaat het vocaal leerproces verder via een sensorimotor-leerfase die 'brabbelen' bij kinderen wordt genoemd en 'subsong' bij zangvogels. Er zijn homologiëen tussen de hersenen van vogels en die van zoogdieren die zangvogels tot een geschikt modelorganisme maken voor de bestudering van de neurale mechanismen van vocaal leren en geheugen. In het brein van zangvogels bevindt zich een netwerk van onderling verbonden hersenkernen, het 'song system', dat betrokken is bij de productie van zang en het sensorimotor-leren. Er is echter gesuggereerd dat een hersengebied buiten het *song system*, het caudomediale nidopallium (NCM), het neurale substraat bevat voor de representatie van geheugen voor het tutorliedje dat in de eerste fase van het zangleren wordt gevormd. Het NCM en het naastliggende caudomediale mesopallium (CMM) zijn waarschijnlijk het vogel-equivalent van de humane auditieve associatiecortex. Het is essentieel om de localisatie van het neurale substraat van tutorzanggeheugen te onderzoeken, zodat we de onderliggende moleculaire en cellulaire mechanismen kunnen ontrafelen. Dit kan inzicht verschaffen in het proces van de vorming van auditieve herinneringen, dat ten grondslag ligt aan het leren produceren van vocalisaties, een proces dat fundamenteel is voor het leren van menselijke spraak. Bij zebravinken leren alleen de mannetjes hun vocalisaties (roepen en liedjes), terwijl vrouwtjes niet zingen en slechts niet-geleerde roepen (*calls*) produceren. Het bestuderen van de perceptie van vocalisaties bij zowel mannetjes als vrouwtjes kan ons inzicht geven in hoe dat neurale substraat van perceptie en geheugen functioneert in beide geslachten. Dit proefschrift beschrijft drie onderzoeken waarin de neurale mechanismen van auditief geheugen en perceptie werden onderzocht bij zebravinken.

Als het NCM het neurale substraat bevat voor de representatie van het tutorzanggeheugen, dan zouden lesies in dit gebied een negatieve invloed

moeten hebben op de herkenning van zang. Deze hypothese werd getest in het experiment beschreven in hoofdstuk 2, waar ik aantoon dat neurotoxische lesies in het NCM van volwassen zebravinkmannetjes de geleerde voorkeur voor het liedje van de tutor aantasten. De productie van zang werd niet veranderd door NCM-lesies. Het vermogen om verschillende roepen te kunnen onderscheiden bleef tevens onbeïnvloed; de dieren vertoonden een normale voorkeur voor de roepen van vrouwtjes. Deze bevindingen tonen aan dat het NCM een essentiële rol speelt bij de representatie van tutorzanggeheugen bij volwassen mannelijke zebravinken. Bovendien laten deze resultaten zien dat er gescheiden neurale representaties bestaan in het volwassen zangvogelbrein voor tutorzanggeheugen en een motorprogramma voor het eigen liedje. Een vergelijkbare dissociatie tussen gebieden betrokken bij de productie van spraak (zoals het gebied van Broca) en bij de perceptie van spraak (zoals het gebied van Wernicke) bestaat in het menselijke brein.

Bewijs voor een rol van het NCM bij het geheugen voor tutorzang is verkregen in experimenten met volwassen zebravinken. Indien het NCM inderdaad het neurale substraat voor de representatie van tutorzang zou bevatten, dan zou deze structuur tevens moeten worden geactiveerd in jonge zebravinken die hun liedjes nog aan het leren zijn. Deze hypothese werd getoetst in het experiment beschreven in hoofdstuk 3, waar ik jonge zebravinken blootstelde aan het liedje van hun tutor of aan een onbekend liedje. Ik vond verhoogde zang-geïnduceerde neuronale activiteit, gemeten als de expressie van *immediate early genes*, in het NCM en het CMM van deze mannetjes. Bovendien was de neuronale activatie in het NCM het grootst na blootstelling aan het tutorliedje ten opzichte van het onbekende liedje. Er was neurale activatie in het *song system* van vogels die zelf hadden gezongen, maar niet in dat van vogels die waren blootgesteld aan tutorzang of een onbekend liedje. Deze resultaten suggereren dat het NCM (en mogelijk ook het CMM) het neurale substraat bevat voor tutorzanggeheugen, dat geactiveerd wordt wanneer het geheugen wordt gevormd.

Voor vocale communicatie gebruiken zebravinken niet alleen liedjes, maar ook roepen zoals de 'long call'. Vrouwelijke zebra vinken produceerden een long call die niet aangeleerd is, terwijl mannelijke zebravinken hun *long call* (die verschilt van die van vrouwtjes) aanleren van hun zangtutor. In hoofdstuk 4 onderzoek ik de gedrags- en moleculaire-neuronale respon-

siviteit op de *long call* bij mannelijke en vrouwelijke zebravinken. Bij beide geslachten veroorzaakten vrouwelijke *long calls* meer reacties (roepen, benaderingen) dan mannelijke *long calls*. Neuronale activatie als gevolg van het horen van vrouwelijke roepen was significant groter bij vrouwtjes dan bij mannetjes in het NCM, het CMM en de hippocampus. Bovendien was er significant hogere neuronale activatie in deze gebieden bij vrouwtjes, maar niet bij mannetjes, na het horen van vrouwelijke *long calls* vergeleken met stilte. Mannelijke en vrouwelijke zebravinken vertoonden dus beiden een gedragsmatige voorkeur voor de *long calls* van vrouwtjes, terwijl de neurale activiteit bij hen verschillend was wanneer ze werden blootgesteld aan roepen van beide geslachten.

In het laatste hoofdstuk bespreek ik de belangrijkste bevindingen en hun belang voor het begrip van de neurale mechanismen van het leren van zang en geheugen. De experimenten besproken in de hoofstukken 2 en 3 tonen aan dat het NCM waarschijnlijk het neurale substraat voor de representatie van het geheugen voor tutorzang bevat. Ik heb voor het eerst laten zien dat, bij volwassen mannelijke zebravinken, het NCM noodzakelijk is voor de herkenning van tutorzang, maar niet voor de productie van zang. Bovendien heb ik aangetoond dat bij jonge zebravinken het NCM geactiveerd wordt gedurende de vorming van het auditief geheugen. Bij jonge mannelijke zebravinken is er neuronale activatie van het *song system* in vogels die zelf zingen, maar niet nadat ze slechts zijn blootgesteld aan liedjes. Dus bij zowel jonge als volwassen zangvogels zijn de cognitieve systemen die ten grondslag liggen aan vocale productie en auditieve recognitie geassocieerd met verschillende hersengebieden. Deze hersengebieden vertonen mogelijk continue interactie gedurende het leven van het individu. De neurale verwerking van auditieve signalen, zoals de *long call*, blijkt verschillend te zijn bij mannetjes en vrouwtjes. Het ontrafelen van de onderliggende processen, die gerelateerd zijn aan de perceptie en het geheugen van vocalisaties en die verschillend zijn bij mannetjes en vrouwtjes, kan leiden tot een beter begrip van hoe vocalisaties worden geleerd. Het localiseren van het neurale substraat van tutorzanggeheugen, zoals in dit proefschrift beschreven, is een noodzakelijke voorwaarde om de neurale mechanismen van auditief geheugen te onderzoeken op moleculair en cellulair niveau. De dissociatie tussen hersengebieden betrokken bij vocale productie en bij geheugen in zowel het brein van vogels als dat van mensen duidt op convergente evolu-

tie van de mechanismen die ten grondslag liggen aan auditief-vocaal leren. Door de neurale mechanismen van vogelzang te bestuderen kunnen we dus belangrijke inzichten verwerven over de algemene mechanismen van auditief-vocaal leren.

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Curriculum Vitae

I was born on the 28th of February 1981 in Den Haag, the Netherlands and went to high school at the Gymnasium Haganum in Den Haag where I graduated in 1999. Inspired by the film 'The Double Helix' - about the discovery of DNA -, once shown during biology class in high school, I began my studies in Biology at Leiden University in the same year.

In my first year in Leiden I became also interested in zoology besides my continuing interest in molecular and cellular biology; therefore I combined those study paths in the second year. Motivated because of what I had learned about neuroscience and cognition during a second year course, I started my third year with a research internship investigating circadian rhythms in the laboratory of Prof. J.D. Glass at Kent State University, Ohio. Back in Leiden, I completed courses in the Biomedical Department after which I started my second research internship in the laboratory of Prof. M.K. Richardson concerning the development and evolution of the central nervous system in mammals. I compared brain development of several mammalian species; for data on human embryos I went to the NIH in Washington D.C. In my last year in Leiden, I participated in the Science Based Business program, completed a minor in Philosophy and received my Master degree on the 14th of July 2003, cum laude.

I started my PhD in the Behavioural Biology group of Prof. J.J. Bolhuis at Utrecht University in September 2003 on the neural mechanisms of birdsong memory and perception. In 2008, I spent three months, supported by a fellowship of the Dr. J.L. Dobberke foundation, in the laboratory of Dr. Claudio Mello (OHSU, Portland, OR, USA). In my second year in Utrecht, I finally got to read 'The Double Helix', written by James Watson.

To continue my research as a post-doctoral fellow, the Netherlands Organisation for Scientific Research (NWO) awarded me a Rubicon-fellowship. Following the defense of my thesis, I will join the laboratory of Dr. B.P. Ölveczky at Harvard University (Cambridge, MA, USA) studying the neural mechanisms that underlie birdsong learning.

Publications

Richardson, M.K., Gobes, S.M.H., van Leeuwen, A., Poelman, A., Pieau, C. & Sánchez-Villagra, M.R.; Tetrapod Limb Development: a Case Study in Heterochrony.
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Gobes, S.M.H, ter Haar, S.M., Vignal, C., Vergne, A.L., Mathevon, N. & Bolhuis, J.J. ; Differential responsiveness in brain and behaviour to sexually dimorphic long calls in male and female zebra finches.
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Abbreviations

AFP	anterior forebrain pathway
APP	amyloid precursor protein
Av	nucleus Avalanche
BOS	bird's own song
Cb	cerebellum
CLM	caudolateral mesopallium
CMM	caudomedial mesopallium
DLM	nucleus dorsolateralis anterior, pars medialis
DM	dorsomedial nucleus
dph	days post hatching
ERK	extracellular signal regulated kinase
fMRI	functional Magnetic Resonance Imaging
HP	hippocampus
HVC	acronym used as a proper name
IEG	immediate early gene
IMM	intermediate medial mesopallium
L1, L2, L3	subdivisions of field L
LaM	lamina mesopallialis
lMAN	lateral magnocellular nucleus of the anterior nidopallium
MARCKS	myristoylated alanine-rich protein kinase C
NCM	caudomedial nidopallium
NIf	interfacial nucleus of the nidopallium
NMDA	N-methyl-D-aspartic acid
nXIIts	tracheosyringeal portion of the nucleus hypoglossus
PAm	nucleus parambigualis
PBS	phosphate buffered saline
PFA	paraformaldehyde
RA	robust nucleus of the arcopallium
Ram	nucleus retroambigualis
SAP	sound analysis pro
SI	similarity index
SPL	sound-pressure level
V	ventricle
ZENK	acronym of *zif-268*, *egr-1*, NGF-1A and *krox-24*
Zenk	protein product of the immediate early gene ZENK