

Neural mechanisms of song memory formation in juvenile zebra finches

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Neural mechanisms of song memory formation in juvenile zebra finches

Neurale mechanismen van de vorming van zanggeheugen in jonge zebrafinches

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Introduction

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Introduction to studying neural mechanisms of song memory formation in juvenile zebra finches

Charles Darwin already noticed almost a century and a half ago that “The sounds uttered by birds offer in several respects the nearest analogy to language [...]” (Darwin 1871, p.55). Indeed, there are many parallels between the acquisition of spoken language in human infants and song learning in songbirds, at the behavioural, neural, genetic and cognitive levels (Doupe & Kuhl 1999; Bolhuis et al. 2010). Both human infants and juvenile songbirds are vocal learners, meaning that they are able to imitate sounds that adult conspecifics (often their parents) make. Vocal learning is a relatively rare ability in the animal kingdom. Until now, only a few mammalian taxa have been identified as vocal learners, namely humans, certain marine mammals and bats, while vocal learning appears to be absent in our closest relatives, non-human primates (Hauser et al. 2002). In contrast, almost 5,000 avian species are vocal learners, in three avian taxa: songbirds (Oscine Passeriformes), parrots (Psittaciformes) and hummingbirds (Apodiformes). Most research is conducted in songbirds: in the zebra finch (*Taeniopygia guttata*), Bengalese finch (*Lonchura striata var. domestica*), canary (*Serinus canaria*), several species of sparrows (song sparrow *Melospiza melodia*, white-crowned sparrow *Zonotrichia leucophrys*, and swamp sparrow *Melospiza georgiana*), European starling (*Sturnus vulgaris*) and the brown-headed cowbird (*Molothrus ater*; Williams 2008). The zebra finch, the species studied in this thesis, is originally an Australian songbird that lives in large flocks (Zann 1996). Zebra finches are very social animals, and although both sexes vocalise frequently, only males sing. Zebra finches are a good model species because they are easily maintained in the lab and are opportunistic breeders, meaning that they will reproduce year-round under the right circumstances (Doupe & Kuhl 1999).

Birdsong functions and characteristics

Birdsongs consist of ordered strings of sounds (visualized in a sonogram in figure 1.1). The separate sound elements are called syllables, and one complete string is a motif. Songbirds often sing multiple motifs that are together called a song bout. Some bird species, such as the zebra finch, have a highly stereotyped song, where only one motif is repeated with just minor variability. Other species, such as Bengalese finches or European starlings, sing several different motifs and thus they have more variable songs (figure 1.2).

The main function of song is hypothesized to be defence of territory and mate attraction (Collins 2004). The importance of song in territory defence was shown in wild-living great tits (*Parus major*). Researchers caught and removed great tits from their territories, and placed speakers that broadcast songs in half of the empty territories. They found that empty territories were occupied faster in comparison to the territories where male songs were played (Krebs 1977). However, gregarious species such as the zebra finch do not have a territory and consequently, it is unlikely that their songs would have the function of

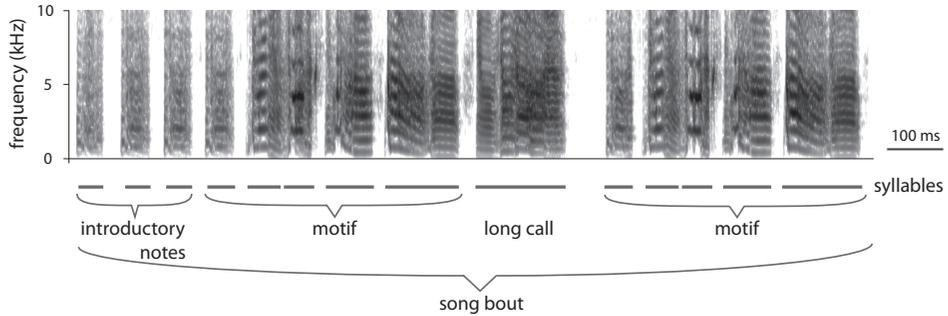


Figure 1.1. Song of a zebra finch. Sonograms are figures that show sound frequencies over time. The X-axis represents time in seconds; the Y-axis represents frequency in kilohertz. One bout of a zebra finch song is depicted. It consists of 2 motifs, which are made up of 7 syllables each (illustrated by the lines underneath the sonogram).

territory defence. Indeed, singing males do not behave aggressively and their songs do not evoke aggressive behaviour in other males; either (Zann 1996).

Learning by auditory-vocal imitation

Young songbirds copy many elements of the songs of their tutors, as can be observed by comparing sonograms of tutors and their tutees (figure 1.3). Since the middle of the last century, research has demonstrated that songbirds indeed have to learn their songs. When young birds are reared in isolation, they will produce a highly abnormal song, the so-called isolate song (Marler 1970; Fehér et al. 2009; Fehér & Tchernichovski 2013). Isolate song does have some recognizable species-specific features, but it has a relatively simple structure and does not sound like the songs of socially raised birds. There is a predisposition to preferentially learn songs of conspecifics: when songbirds are exposed both to songs from their own and from another species, they will mainly imitate the conspecific song (Marler & Peters 1977). However, if their preferred input is lacking, they will copy from songs that are available to them. If young songbirds are raised by parents of another species, they will imitate sounds of their foster parents (Clayton 1988). Of course there are physical constraints to what sounds can be heard, remembered and imitated. Physical constraints are formed on the one hand by neural mechanisms involved in the analysis, processing and storage of auditory information, and on the other hand by the functioning of the ear and the sound production organs (the vocal tract and the larynx or syrinx in humans or birds, respectively; Bolhuis et al. 2010).

By manipulating auditory experience of songbirds early in life it was shown that there is a sensitive period in which songs are learned best (reviewed in Doupe & Kuhl 1999). Some species, such as the zebra finch, sing only one song during their life, while others are able to learn multiple songs or adjust their songs every breeding season. Birds that learn one song that stays stable over the years are called 'age-limited learners'; an example is the zebra

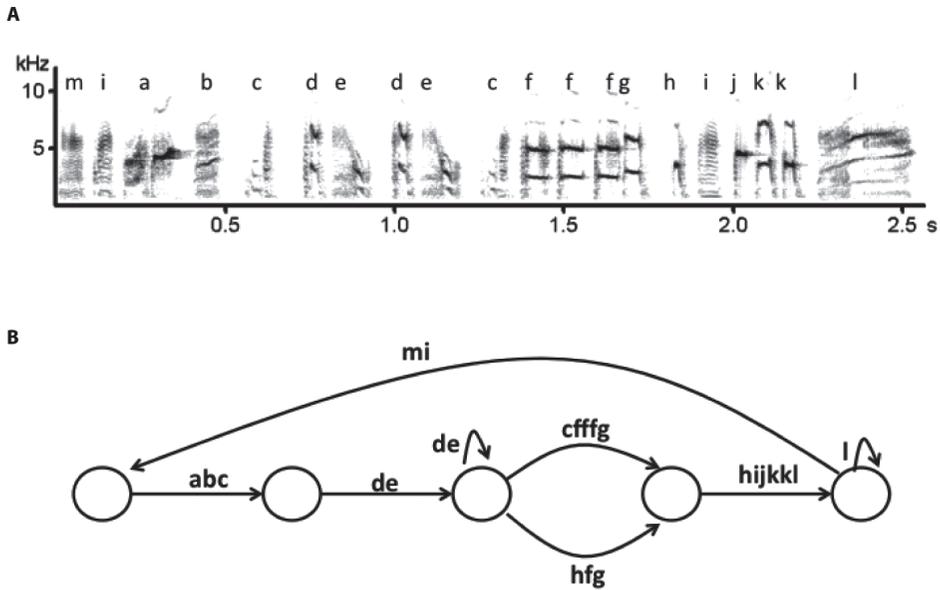


Figure 1.2. Song of a Bengalese finch. Bengalese finches sing longer and more variable songs than zebra finches. **A:** The sonogram in the top panel depicts one example song motif. Bengalese finches sing several different motifs in which the order of the syllables can vary according to specific rules. **B:** In the lower panel, a scheme depicts the possible orders of syllables that this specific bird sings.

finch. Birds that can learn songs throughout their lives, such as starlings, are called ‘open-ended learners’ (Doupe & Kuhl 1999; Bolhuis & Gahr 2006).

In the songbird species that have been studied, song learning has two phases: a memorization phase, during which a memory of the song of the tutor is formed, and a sensorimotor phase, in which the young bird learns to sing itself (figure 1.4). The memorization phase can precede the sensorimotor phase by months (figure 1.4b). This is the case for many ‘seasonal breeders’ such as the white-crowned sparrow. In other species, such as the zebra finch, the two phases overlap (figure 1.4a). Konishi first introduced the concept of the ‘template’, which is essentially a central representation of the species-specific tutor song that is used for vocal learning (Konishi 1985). In this framework, songbirds are thought to be born with an elementary representation of their species’ song, called a crude template. During the memorization phase, birds modify this crude template towards a more precise representation of the song of their tutor, resulting in a more exact template. During the sensorimotor phase, the young bird starts to produce sounds, just like human infants’ first vocalisations, which are called ‘babbling’ (Doupe & Kuhl 1999). This is called subsong (see figure 1.3 for a zebra finch example). Through auditory feedback, the bird matches its song to the template to improve its imitation of the parent’s song, and through a plastic song phase in which song is highly variable, the bird develops a more or less accurate copy of the tutor song when it reaches adulthood. The latter is known as the crystallized song (Konishi 1965; Nottebohm 1968; Marler 1970).

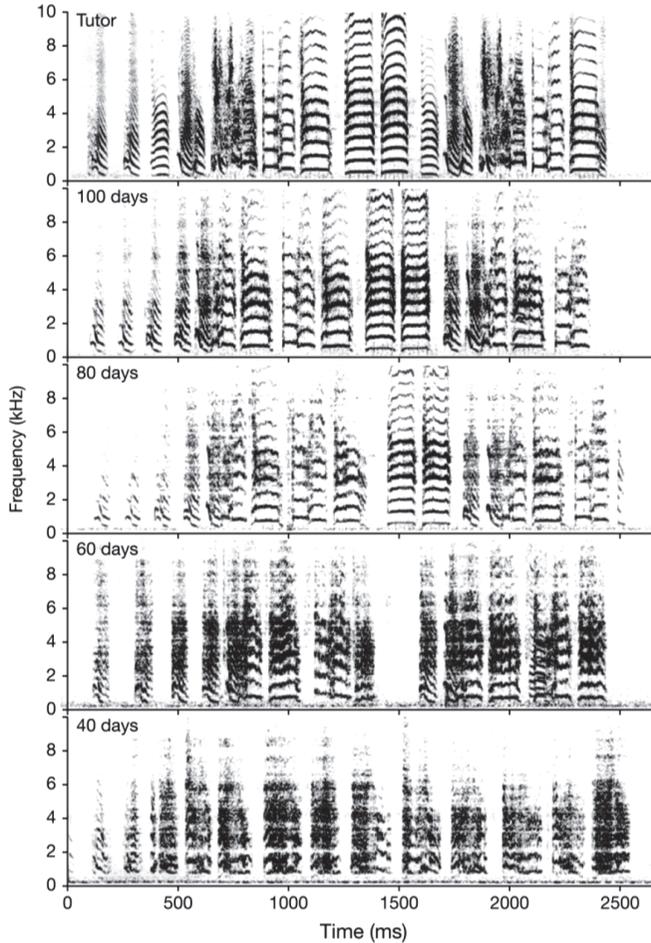


Figure 1.3. Song imitation by zebra finches. Sonograms of two zebra finch males are shown. The top panel is the sonogram of a tutor song. Below are sonograms of the song of its son through development. When the son is 40 days old (bottom), he produces subsong, which sounds unstructured and does not resemble the tutor song yet. At 60 days he sings plastic song, which looks more like normal zebra finch songs already. Zebra finches develop their song until they are approximately 90 days old; from that age on their song is very stable. Here in this figure, when the son is 100 days old, his song is crystallized and it is a proper imitation of the tutor song. Reproduced, with permission, from Bolhuis and Gahr (2006).

There is a remarkable developmental parallel between birdsong learning and speech acquisition in human children. As early as in the uterus (DeCasper & Spence 1986), but also in early stages after birth (Kuhl & Rivera-Gaxiola 2008; Dehaene-Lambertz et al. 2010), human babies may learn characteristics of their parents' speech. From six or seven months of age, babies start babbling, when they produce sounds that are still very different from adult speech. Then, after practice, the sounds develop into proper human speech from about the age of three (Doupe & Kuhl 1999; Kuhl & Rivera-Gaxiola 2008).

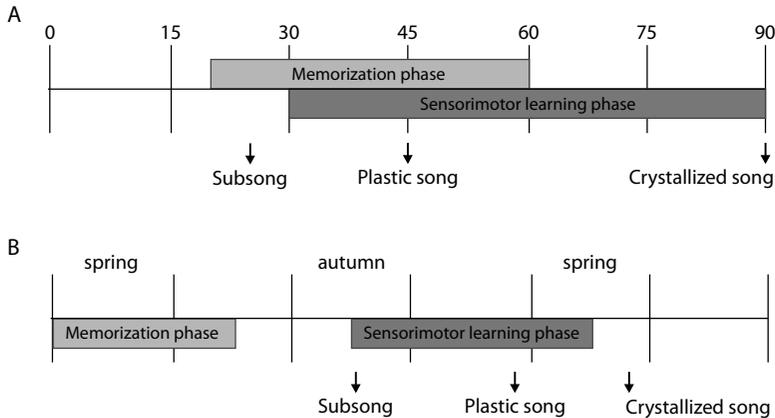


Figure 1.4. Sensitive phases for song learning. Timelines showing the memorization and sensorimotor learning phase of different species of songbirds. **A:** The top timeline shows that zebra finches have overlapping memorization and production phases in which they learn their song. After 90 days post hatching, their song does not change any more. **B:** The bottom time line shows the learning phases of song sparrows, and covers a longer period than **A**. Song sparrows first form a memory representation of the song of their tutor when they are young and do not sing themselves yet. Later in autumn, and also in absence of their tutor, they start singing themselves and imitate their tutor's song.

Human infants are able to learn the language(s) to which they are exposed early in life, which could be any one of more than 6,000 languages. They can do this with relative ease and without formal instruction (Kuhl & Rivera-Gaxiola 2008; Bolhuis et al. 2010). There are some anecdotal accounts of children that grew up separated from human contact, by themselves in the wild or in the presence of animals. Although most of these stories are probably fiction, there are some feral children that were studied scientifically. One famous example is “Genie”, a girl that grew up alone in her bedroom. Her father brought her food, but he never spoke to her, so she was never exposed to language. At the age of twelve, she was freed and people tried to socialize her and teach her English. Although she made some progress, she never learned to speak properly (Krashen 1973).

Feral children's failure of learning to speak suggests there is a sensitive period early in life, in which language learning is much easier than at a later time. More important evidence is that, in contrast to young children that can easily learn more than one language fluently, human adults have more trouble with learning new languages, and they will never learn to speak them fluently (Lenneberg 1967; Doupe & Kuhl 1999; Bolhuis & Eda-Fujiwara 2010).

Female songbirds

Female zebra finches are able to recognize songs of male conspecifics. They form a memory representation of the song of their father when they are juveniles, and it has been suggested that they might use this as a reference for mate selection later in life (e.g., Collins 2004;

Riebel 2009). For example, zebra finch females that were fostered by another songbird species preferred mates that sung the fostered songs rather than their own species' songs (Clayton 1990). Indeed, females have sexual preferences for certain songs over others (reviewed in Riebel 2009), and they may perform copulation solicitation displays in response to playbacks of male songs (Searcy 1992). Are females selecting for high-quality males through their songs? How would male quality be expressed in song?

One possibility is that male song quality might be related to tutor song imitation. Indeed, female zebra finches preferred the songs of males that imitated a tutor song over the songs of males raised in auditory isolation (Williams et al. 1993), and female song sparrows prefer songs of 'good-learners' over those of 'poor-learners' (Nowicki et al. 2002).

In addition, it has been shown that female songbirds prefer more complex songs (Okanoya 2004; Leitão et al. 2006). In female budgerigars, neuronal activation in a brain region that is important for song perception and song memory correlated with complexity of the stimulus song (Eda-Fujiwara et al. 2003). In the canary and swamp sparrow, females prefer syllables with a high trill rate and large frequency bandwidth, the so-called 'sexy syllables' (Vallet & Kreuzer 1995; Draganoiu et al. 2002). Furthermore, females prefer songs with a large number of song motifs, even in species whose songs would naturally have only one motif – such as the common grackle (*Quiscalus quiscula*; Searcy 1992) or the zebra finch (Collins 1999). The preference for a large number of song motifs is not caused by a longer song duration *per se* (Riebel 2009). In addition, females prefer a high variety in syllables (Holveck & Riebel 2007; Woodgate et al. 2011) and a high song rate (reviewed in Collins 2004; Riebel 2009).

However, song preferences were found to be dependent on the quality of the female as well. In an experiment where zebra finch quality was manipulated through brood size – birds from small broods have higher growth rates, a better condition and a stronger immune response when they are young than individuals from large broods – it was shown that low-quality female zebra finches preferred low-quality males, and high-quality females preferred high-quality males (Holveck & Riebel 2010).

In some species, such as the European starling, females also sing. The functions of female song are probably similar to those of males: territory defence and mate attraction (Collins 2004). Zebra finch females do not sing, but it was demonstrated that zebra finch partners do communicate with each other through soft calls (Elie et al. 2010). Vocal duets between males and females may enhance pair bonds (Collins 2004; Elie et al. 2010).

Neural mechanisms of human speech

Speech production and perception in humans involve multiple functional brain circuits, in which Broca's and Wernicke's areas are well-known and important brain regions (figure 1.5). Both brain regions were discovered in the 19th century, in patients who had suffered damage in these regions and suffered from language disorders. Paul Broca discovered that damage to

the left inferior frontal gyrus caused severe difficulties with speech production, while speech perception was intact (Broca 1861; Pearce 2009). Carl Wernicke discovered that damage to the left temporal lobe caused an opposite syndrome, where the patient could still speak, but without any meaning in its utterances, and had largely lost the ability to understand others (Wernicke 1874; Specht 2014). Later, more regions were found to be involved in language processing, and more functions were ascribed to Broca's and Wernicke's areas. The brain regions involved in speech and language are not domain-specific, but implicated in different tasks (Friederici & Alter 2004; Poeppel & Hickok 2004; Specht 2014). Their specific functions are caused by the association with functional networks, rather than being intrinsic to the brain regions (Friederici & Alter 2004).

Furthermore, it was found that the left anterior insula, located in the sulcus under the frontal operculum (figure 1.5), was involved in the articulation of speech, while Broca's area is more important for more complex speech production (Ackermann & Riecker 2004; Price 2012). The cerebellum (see figure 1.6c) and basal ganglia (a subcortical brain region, not shown in the figure) are also involved in language processing (Ackermann 2008; Price 2012). In the temporal lobe, in addition to the posterior superior temporal gyrus that is part of Wernicke's area, the anterior superior temporal gyrus and superior temporal sulcus are involved in speech perception, and

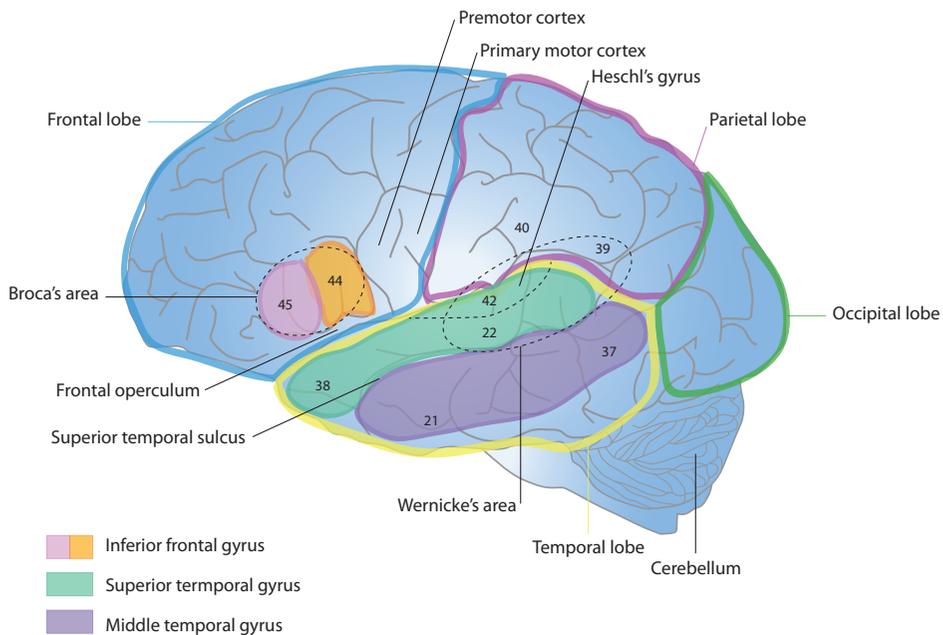


Figure 1.5. Human brain regions involved in language processing. This schematic illustration of the human brain depicts the brain regions that are involved in language processing. The numbers represent language-relevant Brodmann Areas. The four different lobes of the human brain are indicated by coloured outlines, and the most important language-related brain regions are colour-filled. The names of language-related brain regions are indicated in the figure. This figure is adapted with permission from Friederici (2011).

the right temporal lobe has a role in it as well (Poeppel & Hickok 2004; Friederici 2011; Price 2012; Specht 2014). Even though there is an extended linguistic brain network, Broca's and Wernicke's area are two major regions within the network. Among other functions, Broca's area is involved in speech production and generation and processing of language syntax, while Wernicke's area plays a major role in speech perception and comprehension (Friederici & Alter 2004; Friederici & Alter 2004; Scott et al. 2009; Friederici 2011).

Brain regions involved in song learning

The neural mechanisms of song production and song learning and memory in songbirds have been investigated in considerable detail (Brainard & Doupe 2002; Bolhuis & Gahr 2006; Mooney 2009; Bolhuis & Eda-Fujiwara 2010). One remarkable characteristic of song-related brain activation is a neural dissociation that is similar to the dissociation between speech production and perception in humans. Songbird brains have functional networks that are mainly associated with song production, and a connected but different network of brain regions that is involved in song perception, recognition and memory (Bolhuis & Gahr 2006; Pinaud & Terleph 2008; Bolhuis et al. 2010; Hahnloser & Kotowicz 2010; Woolley 2012; Roberts & Mooney 2013). Thus in a functional sense, these networks can be considered to be similar to Broca's and Wernicke's areas in humans, respectively (Bolhuis et al. 2010).

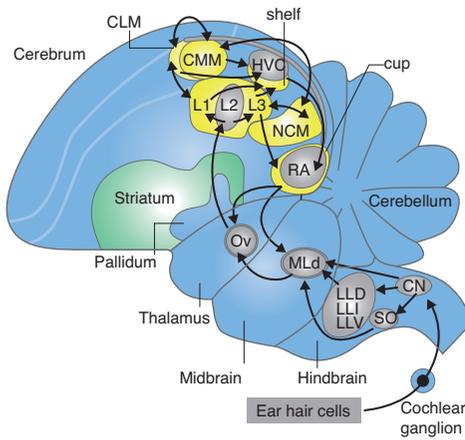
The brain circuitry involved in singing consists of two networks of interconnected structures (figure 1.6). The song motor pathway (SMP; see figure 1.6b; Mooney 2009) originates in HVC (used as a proper name; Reiner et al. 2004; Jarvis 2005) and passes via a caudal route through the robust nucleus of the arcopallium (RA) to vocal-motor and respiratory centers in the midbrain and hindbrain (Vicario 1991; Mooney 2009). Second, the anterior forebrain pathway (AFP; figure 1.6b) consists of the projection from striatal nucleus Area X through a thalamic relay to the lateral magnocellular nucleus of the anterior nidopallium (LMAN), which projects back to Area X forming a loop reminiscent of human cortico-striatal-thalamic-cortical loops (Bottjer et al. 1989; Vates et al. 1997; Luo et al. 2001). The AFP and SMP are connected through HVC-to-X and LMAN-to-RA projections. An intact SMP is required for song production (Nottebohm et al. 1976), whereas the AFP plays important roles in sensorimotor learning (Bottjer et al. 1984; Scharff & Nottebohm 1991) and adult song plasticity (Brainard & Doupe 2000b; Olveczky et al. 2005). Together, the SMP and AFP are known as the song system, which is only present in birds that learn their vocalizations (Brenowitz 1997; Doupe et al. 2005; Bolhuis & Gahr 2006; Roberts et al. 2008; Mooney 2009). For comprehensive reviews, see (Zeigler 2004; Bolhuis 2008; Zeigler & Marler 2008).

In addition to the song system, a number of regions in the caudal pallium are involved in auditory processing and perception (Mello 2004b; Mello et al. 2004; figure 1.6a). Initially, the role of these brain regions was discovered using expression analysis of immediate early genes (IEGs). As the name implies, these genes are rapidly induced following the activation of a neuron and as such they are useful molecular markers of neuronal activation (Sagar

et al. 1988; Farivar et al. 2004). They include both effectors that can exert direct actions on cellular processes, and transcription factors that act by regulating the expression of downstream target genes. In all cases their transcription is regulated very quickly after cellular activation (Mello & Clayton 1994; Velho et al. 2005). IEGs have been extremely helpful for mapping brain activation in the context of vocal communication in birds (Mello 2002) and for identifying neural substrates underlying birdsong perceptual processing (Ribeiro et al. 1998) and memory (Bolhuis & Gahr 2006).

The brain regions that are involved in auditory processing and perception include field L, the primary termination site of the ascending auditory pathway in the nidopallium, and adjacent higher-order auditory cortex-like areas, including the caudomedial nidopallium (NCM), the

A) Auditory pathways



B) Vocal pathways

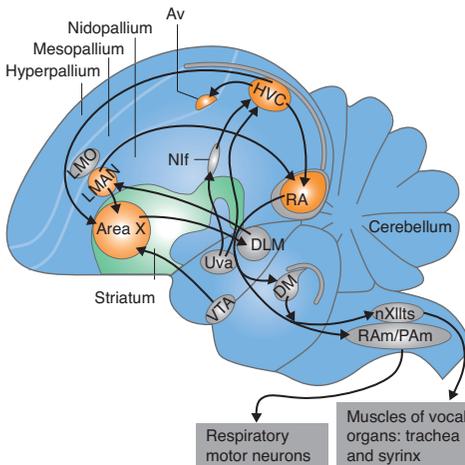


Figure 1.6. Schematic side views of the songbird (a,b) and human (c) brain. **A:** Regions depicted in a light shade show increased neuronal activation when the bird hears song. The NCM and CMM regions are assumed to contain the neural substrate for tutor song memory. **B:** Nuclei HVC, Av, RA, LMAN and Area X show increased neuronal activation when the bird is singing. **C:** In the human brain, Broca's area is most importantly involved in speech production, while Wernicke's area is mainly involved in speech perception and understanding. **Abbreviations:** Area X, Area X of the striatum; Av, avalanche; CLM, caudolateral mesopallium; CMM, caudomedial meso-pallium; CN, cochlear nucleus; DLM, medial subdivision of the dorsolateral nucleus of the anterior thalamus; DM, dorsomedial subdivision of nucleus intercollicularis of the mesencephalon; HVC, a letter-based name; L1, L2 and L3 are subdivisions of Field L; LLD, lateral lemniscus, dorsal nucleus; LLI, lateral lemniscus, intermediate nucleus; LLV, lateral lemniscus, ventral nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LMO, lateral oval nucleus of the mesopallium; MLd, dorsal part of the lateral nucleus of the mesencephalon; NCM, caudomedial nidopallium; Nif, interfacial nucleus of the nidopallium; nXIIts, tracheo-syringeal portion of the nucleus hypoglossus (nucleus XII); Ov, nucleus ovoidalis; PAm, nucleus para-ambiguous medullaris; RA, robust nucleus of the arcopallium; RAm, nucleus retroambiguus medullaris; SO, superior olive; Uva, nucleus uvae-formis; VTA, ventral tegmental area. Modified, with permission, from Bolhuis et al. (2010).

caudomedial mesopallium (CMM), the shelf region adjacent to HVC, and the cup region adjacent to RA (Fortune & Margoliash 1992; Vates et al. 1996; Mello et al. 1998). These regions are activated when songbirds hear conspecific songs, based on the robust induction of activity-dependent IEGs (Mello et al. 1992; Mello & Clayton 1994; Mello et al. 1995; Velho et al. 2005) and/or electrophysiological responses (Chew et al. 1995; Chew et al. 1996; Stripling et al. 1997; Gentner & Margoliash 2003; Phan et al. 2006; Terleph et al. 2006). Since these or similar areas seem to be present in all birds studied to date (Wild et al. 1993), regardless of the occurrence of vocal learning, they are likely to play general roles in auditory processing. Although the focus of this thesis is on memory aspects, it is important to point out that the NCM and CMM also play a broader role in auditory and perceptual processing of birdsong.

IEG expression has also been used to measure neuronal activation in relation to the strength of learning in the context of filial imprinting (McCabe & Nicol 1999; Horn 2004). In songbirds, several immediate early genes are induced by song in NCM neurons (Nastiuk et al. 1994; Bolhuis et al. 2000b; Velho et al. 2005). By far the most robust example of a song-inducible gene is *Zenk* (an acronym of *zif-268*, *egr-1*, *ngfi-a* and *krox-24*; Mello et al. 1992; Mello & Clayton 1994; Jin & Clayton 1997; Mello & Ribeiro 1998; Leitner et al. 2005). Due to very low basal expression levels, high signal to background ratio and rapid induction and degradation kinetics, the *Zenk* gene response has been highly useful for mapping neuronal pathways activated by hearing song and for mapping the auditory representation of acoustic features of song (Mello & Ribeiro 1998; Ribeiro et al. 1998; Mello 2002; Mello 2004a).

A possible neural substrate of birdsong memory

Evidence from both electrophysiological and molecular studies point to the NCM as a site that undergoes plastic changes in response to song perception, thus representing a potential neural substrate for birdsong memory. Electrophysiological studies in the NCM revealed that evoked auditory responses habituate to repeated presentation of the same stimulus, a phenomenon that has been postulated as a contributing mechanism for the auditory memorization of songs and calls (Chew et al. 1995; Mello et al. 1995; Chew et al. 1996; Stripling et al. 1997; Thompson & Gentner 2010). This habituation is specific for the learned stimulus and its maintenance depends on gene expression induced locally in the NCM by song stimulation, based on the inhibitory effect of RNA or protein synthesis blockers within specific time windows after song presentation (Chew et al. 1995). The association between activity-induced gene expression, neuronal plasticity and memory formation is highly reminiscent of what occurs in simpler organisms such as *Aplysia* or in the rodent hippocampus in the context of induction of long-term potentiation (Pittenger & Kandel 2003). In all these cases the long-term maintenance of neuronal plasticity requires gene expression events activated at defined time windows following neuronal stimulation. In songbirds, the evidence indicates that song-induced gene expression networks activated in higher-order auditory regions such as the NCM may play an essential role in birdsong auditory memory (Mello 2004b; Dong et al. 2009).

On the basis of a series of studies, it has been postulated that the NCM also contains the neural substrate of tutor song memory (Bolhuis et al. 2000b; Terpstra et al. 2004; Bolhuis & Gahr 2006; Gobes & Bolhuis 2007; Bolhuis & Eda-Fujiwara 2010; Bolhuis et al. 2010; Hahnloser & Kotowicz 2010). Using Zenk immunocytochemistry, it was shown that the NCM is activated in response to tutor song, and higher activation levels were correlated with a better imitation of the tutor song (Bolhuis et al. 2000b; Bolhuis et al. 2001; Terpstra et al. 2004). In addition, a correlation was found between the neuronal preference for tutor song in the NCM of juvenile zebra finches (measured as relative neuronal habituation rates using electrophysiology) with the degree to which the bird has copied its tutor song (Phan et al. 2006). Confirming the role of the NCM in tutor song memory, when the NCM was lesioned in adult male zebra finches, tutor song recognition was significantly impaired (Gobes & Bolhuis 2007). Furthermore, Zenk is directly regulated by song in the NCM at times when the auditory memory of tutor song is being acquired (Jin & Clayton 1997; Stripling et al. 2001; Gobes et al. 2010). Upon cellular depolarization, several intracellular signaling pathways are activated that could result in Zenk induction. For example, the mitogen-activated protein kinase kinase (MEK)/ extracellular-signal-regulated kinase (ERK) is activated in the caudal pallium when birds are exposed to song playbacks (Cheng & Clayton 2004). This pathway is required for Zenk induction, since local injections of an inhibitor that blocks both MEK and (downstream) mitogen activated protein kinases (MAPKs) activation can downregulate the responses of Zenk to song (Cheng & Clayton 2004; Velho et al. 2005). When ERK was inhibited in the NCM in juvenile birds, tutor song imitation was impaired (London & Clayton 2008). In a training paradigm where noise is played when the bird sings, birds alter their song in order to avoid the interference. When after the training period the negative reinforcement was withheld, birds quickly change their song back to the original song (e.g., Charlesworth et al. 2011). Using this paradigm, the role of the NCM in song restoration in adult zebra finches was recently tested. In adult zebra finches that had altered their song under auditory feedback manipulations, lesions to the NCM impaired song recovery after the manipulations stopped (Canopoli et al. 2014), suggesting that the tutor song representation in the NCM is a reference model for accurate song production in adults. In adult female zebra finches, which do not learn how to sing but do form a memory of their father's song when they are young (Riebel et al. 2002), IEG expression in the CMM was significantly greater when they were re-exposed to their father's song than to novel song (Terpstra et al. 2006). Taken together, these findings point to a central role of the caudomedial pallium in tutor song memorization (Bolhuis & Gahr 2006; Hahnloser & Kotowicz 2010).

A role for sleep in memory formation

Many studies conducted in a whole range of different species indicate that task performance increases when the subjects are allowed to sleep after learning the task. In humans, this was true for example in motor learning tasks (Van Der Werf et al. 2009; Määttä et al. 2010) or language learning tasks (Gómez et al. 2006). Furthermore, a beneficial role of sleep

on memory performance was demonstrated in domestic chicks (Jackson et al. 2008) and in European starlings (Brawn et al. 2010; Brawn et al. 2013). Also, juvenile zebra finches nap more often during the day in early stages of song learning (unpublished observations discussed in Margoliash & Schmidt 2010). In juvenile zebra finches in the sensorimotor phase, song structure deteriorated after night-time sleep, and improved during the morning. After improvement, the song was a better imitation of the tutor song than before sleep. Moreover, the juveniles that showed most deterioration after sleep achieved the best tutor song imitation as adults (Derégnaucourt et al. 2005).

Why does sleep have a positive effect on performance of a learned task? Sleep is widely believed to be important for learning and memory, particularly in the process of consolidation (Walker & Stickgold 2004; Diekelmann & Born 2010; Dudai 2012; Stickgold 2013). Consolidation is a process that transforms short-term memories into more stable and long-term stored memory representations (McGaugh 2000; Dudai 2004; Diekelmann & Born 2010). It is not clear why memory consolidation would benefit from sleep, but it has been suggested that sleep prevents the possible interference of daily activities on memory consolidation (Diekelmann & Born 2010).

Human sleep consists of cycles in which superficial and deep sleep stages alternate, with more slow-wave sleep (SWS) stages in the first half of the night and more rapid-eye movement sleep (REM sleep) stages in the last part of the night (Schulz 2008; Diekelmann & Born 2010; Rattenborg et al. 2011). Songbirds, including zebra finches, have a sleep pattern that is remarkably similar to that of humans, with a similar distribution of SWS and REM sleep (Low et al. 2008; Rattenborg et al. 2009). Therefore, zebra finches are a good model to study the role of sleep in auditory-vocal memory formation.

It was shown that songbirds have brain activation during sleep, for example in the RA, a brain region involved in song production. Remarkably, spontaneous neuronal activation during sleep in RA resembled motor activation patterns during singing, which is called neuronal replay (Dave & Margoliash 2000). Furthermore, after the first day of tutor exposure in juvenile male zebra finches, patterns of neuronal replay were found in RA during sleep that resembled motor patterns for song production (Shank & Margoliash 2009). In addition, Gobes and colleagues (2010) demonstrated memory-related neuronal activation during sleep in the NCM. Spontaneous neuronal activation during sleep in juvenile male zebra finches was correlated to the quality of tutor song imitation (Gobes et al. 2010). In conclusion, there is strong evidence that sleep plays a role in memory formation in mammals, and investigations in songbirds suggest that sleep may also be involved in song learning and memory (Gobes & Bolhuis 2008).

Neural similarity between brain regions involved in human language and in birdsong

Lesions to the NCM impaired tutor song recognition but did not affect song production or call discrimination (Gobes & Bolhuis 2007). Combined with earlier evidence from molecular and electrophysiological studies, this observation has led to the suggestion that the neural mechanisms related to song production and recognition are largely dissociated in songbirds, similar to the functional organization of speech- and language related brain regions in humans (Bolhuis et al. 2010). Functionally, on the one hand, the caudomedial pallium resembles Wernicke's area, a human brain region in the auditory association cortex that is involved in speech perception and understanding (Bolhuis & Gahr 2006; Bolhuis et al. 2010). On the other hand, nuclei in the avian song system have been compared to Broca's area in the human frontal lobe and to human basal ganglia (Jarvis 2005; Bolhuis et al. 2010). The identification of auditory pallial areas such as the NCM as central to the memorization of song, including the tutor song during the vocal learning period, opens the door for mechanistic investigations of the cellular and molecular basis of auditory memory, since learning and memory mechanisms of speech and language cannot be studied at the cellular and molecular level in human infants.

Language-related brain lateralisation

Paul Broca examined many patients that suffered from language disorders, and most of them had damage restricted to the left hemisphere. He concluded: "*nous parlons avec l'hémisphère gauche*", or translated in English: "we speak with the left hemisphere" (Broca 1865; Pearce 2009). Indeed, using imaging techniques such as fMRI, it was subsequently found that both speaking and perception of speech sounds predominantly activates the left hemisphere (Tervaniemi & Hugdahl 2003; Price 2012). However, there is evidence for a role of the right hemisphere in speech perception as well. Recent experiments showed that the right temporal lobe was activated during speech perception in addition to activation in the left hemisphere (Hickok & Poeppel 2007; Cogan et al. 2014). Speech contains perceptual information on many different levels: e.g., temporal, spectral, phonetic and semantic, and therefore the specific tasks that are used greatly influence the outcome of the experiment (McGettigan & Scott 2012). Generally it is suggested that the left hemisphere is mainly involved in semantic and syntactic processes, while the right hemisphere is implicated in prosodic processing (Friederici 2002; Friederici & Alter 2004; Specht 2013) and voice processing, acoustic information that is related to the identity of the speaker (Belin et al. 2000; Grossmann et al. 2010; McGettigan & Scott 2012). Language-related lateralisation was first demonstrated in adults, but can also be found in infants and even babies (table 6.3 in chapter 6). An adult-like activation network was found in response to speech perception in three-month-old babies (Dehaene-Lambertz et al. 2006). Broca's area is not responsive to speech in neonates, but activation was found in 6- or 12-months old babies (Imada et

al. 2006). Interestingly, Broca's area was also activated during speech perception in three-month-old babies, although they do not yet babble or speak; this suggests that Broca's area might have a role in perceptually driven speech production learning (Dehaene-Lambertz et al. 2006). The temporal lobe is activated in response to speech perception in neonates, and the activation pattern is left dominant (Peña et al. 2003). Two-and-a-half-month-old infants showed left-sided dominance in neural activation in temporal lobe regions for speech compared to music. Moreover, there was stronger activation in the left posterior temporal lobe (including Wernicke's area) in response to mother's speech than to speech produced by a stranger (Dehaene-Lambertz et al. 2010). Interestingly, voice recognition is right dominant, and has not developed yet in four-month-old infants (Grossmann et al. 2010). Therefore, it is likely that mother's speech induces some other type of processing rather than voice recognition, perhaps memory-related language processing involved in language acquisition. Indeed, another study showed that mother's voice induced language-related processing, while strangers' voice induced voice-related processing (Beauchemin et al. 2011). Another indication that the left temporal lobe in infants is involved in memory is that varied speech stimuli greatly activated the left temporal lobe, while repetition of speech sounds evoked less activation. This effect was not found in the right hemisphere (Dehaene-Lambertz et al. 2010). In approximately ten-year-old children, Broca's and Wernicke's areas are activated in response to speech. The activation pattern is similar to language-related activation in adults. However, there are more regions involved in children, and the right hemisphere is more active in children than in adults (Gaillard et al. 2000; Berl et al. 2010; Berl et al. 2014; table 6.3 in chapter 6).

There is growing evidence for lateralisation of neural activation in relation to song production and perception in songbirds, similar to humans, although it is not yet clear what specific roles the hemispheres play (reviewed by Ocklenburg et al. 2013).

Scope of this thesis

There are many behavioural and neural parallels between birdsong learning and human speech acquisition, making songbirds a good model to study the neural underpinnings of auditory-vocal learning and memory. The brain regions involved in song learning are functionally similar to the brain regions in the human language network. Here, I investigated the neural underpinnings of early song memory formation. I aimed to find mechanisms of memory formation in juvenile zebra finches by studying juveniles that were at different stages in the song learning process and measuring neuronal activation in the NCM, an important brain region for tutor song memory, in different experimental conditions.

I demonstrated lateralisation in juvenile zebra finches in response to tutor song exposure in **chapter 2**. These juveniles were raised with their parents and had ample exposure to the tutors' songs. When they were still in the sensorimotor phase, auditory stimuli were presented to the birds (silence, an unfamiliar song or the song of their tutor). I examined neuronal

activation in both hemispheres using immediate early gene expression, and showed that the NCM of juveniles was activated left-dominantly after tutor song re-exposure specifically. In the next experiment (**chapter 3**), I asked whether neuronal activation during sleep is lateralised as during the day. In juveniles that were experimentally treated similarly as in the previous study (chapter 2), there were high levels of neuronal activation during sleep, which was lateralised as well. Birds that had imitated their father well had left-dominant neuronal activation, while poor imitators had right-dominant neuronal activation in the NCM. Then in **chapter 4**, I studied juvenile male zebra finches that were of the same age as the birds in the previous experiments, and were therefore at the same stage in physical development. However, I exposed them to tutor song for brief periods only (two groups, receiving two or ten days of tutor song training), and therefore they were at an earlier stage in the song learning process. Both groups of juveniles significantly imitated tutor song. Again I found that neuronal activation was lateralised, but in this stage activation in the NCM was generally left-dominant, and not for tutor song perception specifically. Furthermore, I studied brain activation in juvenile females compared to males (**chapter 5**). Similar to males, juvenile females also memorize tutor song, although they do not imitate it. Comparing sexes might therefore give insight in which brain mechanisms are involved memory only, and which play a role in sensorimotor integration or song production. In contrast to males, I found equal levels of activation in the two hemispheres in females, and no evidence for tutor song memory. Because brain lateralisation related to auditory-vocal memory is an important finding in my experiments, I summarize all existing evidence of birdsong-related lateralisation in songbirds in **chapter 6**, and compare it to another example of avian memory-related lateralisation: visual imprinting in domestic chicks (*Gallus gallus domesticus*). This chapter emphasises a role for lateralisation in memory formation. In **chapter 7** I discuss the characteristics of early birdsong memory formation, the role of sleep and the results regarding birdsong memory in female songbirds.

2

Human-like brain hemispheric dominance in birdsong learning

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Abstract

Unlike non-human primates, songbirds learn to vocalize very much like human infants acquire spoken language. In humans, Broca's area in the frontal lobe and Wernicke's area in the temporal lobe are crucially involved in speech production and perception, respectively. Songbirds have analogous brain regions that show a similar neural dissociation between vocal production and auditory perception and memory. In both humans and songbirds there is evidence for lateralisation of neural responsiveness in these brain regions. Human infants already show left-sided dominance in their brain activation when exposed to speech. Moreover, a memory-specific left-sided dominance in Wernicke's area for speech perception has been demonstrated in 2.5 month-old babies. It is possible that auditory-vocal learning is associated with hemispheric dominance, and that this association arose in songbirds and humans through convergent evolution. Therefore, we investigated whether there is similar song memory-related lateralisation in the songbird brain. We exposed male zebra finches to tutor or unfamiliar song. We found left-sided dominance of neuronal activation in a Broca-like brain region (HVC, a letter-based name) of juvenile and adult zebra finch males, independent of the song-stimulus presented. In addition, juvenile males showed left-sided dominance for tutor song, but not for unfamiliar song, in a Wernicke-like brain region (the caudomedial nidopallium, NCM). Thus, left-sided dominance in the NCM was specific for the song-learning phase and memory-related. These findings demonstrate a remarkable neural parallel between birdsong and human spoken language, and have important consequences for our understanding of the evolution of auditory-vocal learning and its neural mechanisms.

Introduction

There are remarkable similarities between the acquisition of human speech and avian song learning. Like human infants, songbirds learn their vocalizations from an adult tutor during a sensitive period early in life, and in both cases there is a transitional, ‘babbling’ phase that precedes adult vocalizations (Doupe & Kuhl 1999; Tchernichovski et al. 2001; Bolhuis et al. 2010). Such vocal imitation has been demonstrated in humans, certain marine mammals, bats, and three avian taxa: songbirds, parrots, and hummingbirds, but seems to be absent in our closest relatives, apes (Hauser et al. 2002; Bolhuis et al. 2010). Furthermore, the regions of the songbird brain involved in vocal production and auditory perception are analogous to the brain regions that are important for producing and understanding speech in humans (Doupe & Kuhl 1999; Jarvis 2007; Bolhuis et al. 2010).

The caudomedial nidopallium (NCM) of songbirds (figure 1.6a in the introduction of this thesis, chapter 1) is thought to be the avian equivalent of the human auditory association cortex in the temporal lobe, including Wernicke’s region (Bolhuis & Gahr 2006; Bolhuis et al. 2010; figure 1.5 and 1.6c in chapter 1). In addition to a general role in auditory perception (Mello et al. 1992), the NCM is also involved in auditory memory (Chew et al. 1996). Specifically, the NCM is thought to contain (part of) the neural substrate of the memory of the tutor song (Mello et al. 1992; Chew et al. 1996; Bolhuis et al. 2000b; Bolhuis & Gahr 2006; Phan et al. 2006; Gobes & Bolhuis 2007; London & Clayton 2008; Bolhuis et al. 2010; Gobes et al. 2010). In contrast, the premotor nucleus HVC (a letter-based name, figure 1.6b) plays an important role in song production and sensorimotor learning (Hahnloser et al. 2002; Kozhevnikov & Fee 2007; Andalman & Fee 2009; Day et al. 2009), and may thus be functionally analogous to Broca’s area in the human frontal lobe (Doupe & Kuhl 1999; Bolhuis & Gahr 2006; Bolhuis et al. 2010; figure 1.5 and 1.6c in chapter 1). Thus, similar to the functional dissociation between Broca’s and Wernicke’s areas in humans, vocal production and auditory perception and recognition are subserved by distinct regions in the songbird brain (Gobes & Bolhuis 2007). It is well documented that human speech- and language-related neural activity occurs predominantly in the left hemisphere. Left-sided dominance of temporal lobe activation (including Wernicke’s area) could already be demonstrated in neonates who were exposed to speech (Peña et al. 2003). In addition, in older babies that are in the early babbling phase, exposure to speech evokes a left-dominant activation pattern of Broca’s area (Dehaene-Lambertz et al. 2006).

To investigate whether there is lateralisation in the NCM and HVC in songbirds, we measured brain activation in response to tutor song exposure in both juvenile and adult zebra finches (*Taeniopygia guttata*). First, we exposed juvenile male zebra finches that were in the middle of their sensorimotor song-learning (‘babbling’) phase (mean age 56 days old, range 54-59 days) to either songs of their father, songs of an unfamiliar conspecific, or to silence. After stimulus exposure, the birds were sacrificed and the brains were processed with immunocytochemistry to label Zenk, the protein product of the immediate early gene *ZENK* (an acronym of *zif-268*, *egr-1*, *ngf-1a* and *krox-24*; Moorman et al. 2011). The degree of expression of Zenk is a marker

for neuronal activation (Mello et al. 1992; Moorman et al. 2011). We quantified the number of Zenk-immunopositive neurons bilaterally in the NCM, HVC and the hippocampus, a brain region that has not previously been implicated in birdsong.

Materials and methods

Animals. The 22 juveniles in the present experiment had been used in a previous study (Gobes et al. 2010), which was only concerned with the left side of the brain. In addition, 15 adult male zebra finches were used that were bred at the Central Animal Facility of Utrecht University and raised by both parents until 72 days post hatching (dph). The adult males were separated from their tutors at 78 dph, and kept in aviaries until the start of the experiment. Preceding the experiment, all birds were housed individually in soundproof chambers for 48 hours. Mean age at the day of the experiment was 56 days (range 54-59 dph) for the juveniles and 37 months for the adults. Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University.

Stimuli. As stimuli, we used ten songs from the same animal. These songs were repeated in random order to a total of 90 song presentations. The playback of the total stimulus lasted an hour. For tutor stimuli, songs of the fathers of the experimental males were used. Novel stimuli were songs of conspecific males that were not present in the aviary during the life of the subjects. The root-mean-square amplitude of all songs was equalized and the average duration of songs used was 2.1 seconds (sound files were constructed using Praat software).

Experimental design. On the day of the experiment, the lights turned on at 6 am. For most juvenile birds ($n=19$) and all adult birds, the lights turned off at 8 am to prevent them from singing. The other juvenile birds ($n=3$) were kept with lights on, but did not sing. At that time, the songs of the young males were recorded to analyse its similarity with the tutor song. Between 11 and 12 am, the juvenile birds were exposed to tutor song, novel conspecific song or silence. The adult birds were exposed to tutor song or silence at 1.30 pm. For a more detailed protocol, see Gobes et al. (2010).

Immunocytochemistry. 30 minutes after the end of exposure to the stimulus set, the experimental subjects were anesthetized with 0.06 ml Natriumpentobarbital (intramuscular) 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. Parasagittal 20 μ m sections were made on a cryostat and mounted on poly-L-lysine coated slides. The brains were stained immunocytochemically for *egr-1* (Zenk). For a more detailed protocol, see Gobes et al. (2009).

Image analysis. Quantification of Zenk-immunopositive cells was performed for the NCM, HVC and hippocampus as described before (cf. Terpstra et al. 2006; Gobes et al. 2009; Gobes et al. 2010). Digital photographs were taken using a Leica DFC 4206 camera and the Leica Application Suite program on an Axioskop (Zeiss, Germany) with 20x objective. The images

were taken at the extreme caudal pole of the medial NCM, the centre of the HVC nucleus and for the hippocampus at the point at which the curve is most pronounced (cf. Terpstra et al. 2006). Image analysis was performed with a personal computer-based system using KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A program was developed in KS400 to quantify the number of immunoreactive cells semi-automatically. Counts of 3 sections for each region in both hemispheres per animal were averaged for further statistical analysis. Image analysis was performed blind as to the experimental history of the subject.

Song analysis. For the juveniles, 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. We recorded the songs of the adult experimental birds between 1-12 months before the experiment. The songs from the tutors and birds were filtered and equalized using Praat software (Boersma & Weenink 2005). The computer program Sound Analysis Pro 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. Based on multiple features (Wiener entropy, spectral continuity, pitch and frequency modulation), this comparison provides an objective quantification of song similarity (<http://soundanalysispro.com>; Tchernichovski et al. 2000). Sound Analysis Pro has a 'floor-effect', because zebra finch songs always resemble each other somewhat on these features. We thus compared songs from birds in the main experiment also to songs from five unfamiliar non-tutor birds, to investigate whether our zebra finches had learned from their song tutors specifically or if similarity was due to general song characteristics.

Statistical analyses. We log-transformed the data, because the values in the left-sided NCM were not normally distributed (z -values for Skewness and Kurtosis >1.96) and the variances were not equal (Levene's test, $p=0.013$). We conducted repeated measures ANOVA to compare the effect of stimulus exposure on the Zenk response in the left and right NCM, HVC and the hippocampus. To test for lateralisation effects within the stimuli groups, post-hoc paired t -tests with Bonferroni corrections for multiple testing were performed. A lateralisation ratio was calculated by dividing the difference in IEG expression levels between the two hemispheres in a brain region by the total amount of IEG expression of the two hemispheres: $(L-R)/(L+R)$. This lateralisation ratio enabled us to look at true lateralisation levels, not influenced by differences in absolute neuronal activation. We tested for a correlation between the lateralisation ratio and song similarity percentage using Spearman's rho correlation test. Data were analyzed using SPSS 20.0.0.

Results

The juveniles had already learned parts of their father's song, as measured in the morning prior to stimulus exposure (similarity score: $58.5 \pm 4.1\%$ (SEM) with the tutor, which was significantly greater than similarity with an unfamiliar conspecific song: $44.9 \pm 2.8\%$ (SEM); $t(11)=2.5$, $p=0.029$). There was no significant difference between the mean similarity score

in the Tutor, Novel and Silence groups ($F(2,11)=0.074$, n.s.). Figure 2.1 has representative photomicrographs of Zenk expression, while the mean number of Zenk-immunopositive neurons for the different groups is shown in figure 2.2. As the Zenk expression values were not normally distributed, we first log-transformed the data. An overall repeated-measures ANOVA revealed a significant effect of Brain Region ($F(2,11)=4.592$, $p=0.035$) and a significant interaction between Brain Region and Hemisphere ($F(2,11)=22.617$, $p<0.001$). We conducted subsequent analyses on the results for each of the three brain regions.

There was a significant effect of Hemisphere in the NCM in juveniles ($F(1,20)=13.284$, $p=0.002$). Moreover, there was a significant interaction between Stimulus and Hemisphere ($F(2,20)=4.400$, $p=0.026$). In particular, responsiveness to the father's song was greater in the left NCM than in the right NCM (paired t-test, $t(7)=3.314$, $p=0.013$. Bonferroni-corrected $\alpha=0.01667$), but there was no such left-sided dominance in response to novel song or in silence. Thus, lateralised neuronal activation in the NCM of juveniles was memory-specific. In view of this result, we subsequently investigated whether the degree of lateralisation was related to the quality of song imitation. We only had song recordings from the pre-experimental day for 5 of the juveniles in each of the Tutor and Silence groups and 6 juveniles in the Novel group, so the results shown in figure 2.3 are preliminary. Nevertheless, we found that the lateralisation ratio ($[L-R]/[L+R]$; see Methods) was positively correlated with the degree of song similarity between tutor and tutee in the juvenile zebra finches that were exposed to tutor song ($r=0.900$, $p=0.037$, $n=5$) (figure 2.3). There was no significant correlation in the Novel and Silence groups. In addition, there was a significant difference between the correlations in the three experimental groups (Fisher r to z transformation, Q -value 6.689 > Chi-square 5.99). The absolute level of activation in the left NCM in the Tutor group was not significantly correlated with song similarity, suggesting that the strength of song learning was related to lateralisation *per se*, and not to absolute left-sided neuronal activation.

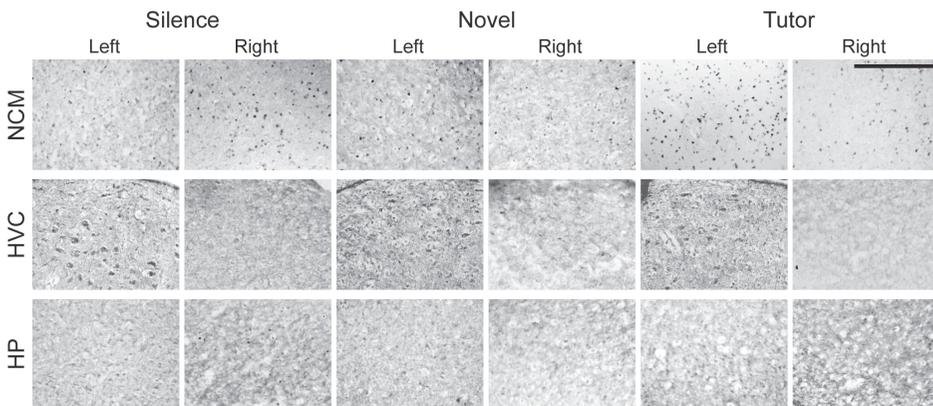


Figure 2.1. Photomicrographs of juvenile zebra finch brains showing Zenk-immunostaining. Representative images at the level of the NCM, HVC and hippocampus are shown for the Silence, Novel and Tutor stimulus groups. Scale bar represents 0.2 mm.

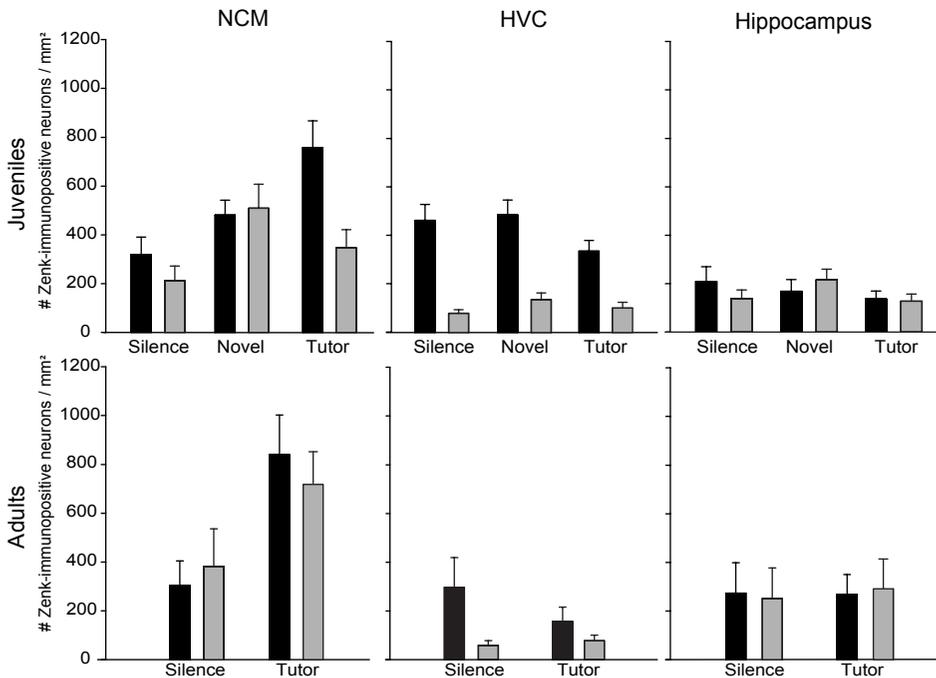


Figure 2.2. Zenk expression in the brains of juvenile and adult male zebra finches. From left to right, the figure presents the mean number of Zenk-immunopositive neurons per mm² for the different brain regions. The top half of the figure shows the results for juvenile male zebra finches. There is left-sided dominance in the NCM of juveniles in response to tutor song, but not to novel song or silence. In HVC, there is a left-sided dominance irrespective of the stimulus presented. The levels of neuronal activation in the hippocampus did not differ between stimulus groups or hemispheres. The bottom half of the figure shows the results for adult male zebra finches. The mean activation level is higher in the NCM of adult zebra finches that were exposed to tutor song than to silence, but there is no difference between the hemispheres. There is left-sided dominance in HVC, irrespective of the stimulus presented, similar to the juveniles. The levels of neuronal activation in the hippocampus did not differ between stimulus groups or hemispheres. Black bars represent the left hemisphere, while the grey bars represent the right hemisphere. Error bars represent SEM.

In HVC of the juvenile zebra finch males, we found that neuronal activation was significantly greater in the left hemisphere than in the right ($F(1,14)=46.061, p<0.001$). However, there was no significant effect of Stimulus in HVC ($F(2,15)=1.114$). This means that the left HVC was activated spontaneously, irrespective of the stimulus presented, and even though these birds had not sung for at least 4 hours before neuronal activation was measured. The levels of neuronal activation in the hippocampus did not differ between stimulus groups or hemispheres.

In a separate experiment, we investigated lateralisation of neuronal activation in response to song in adult songbirds. These birds showed significant imitation of the song of their fathers (song similarity with the tutor song: 69.2 ± 3.44 SEM; which was significantly greater than similarity with an unfamiliar conspecific song: 45.5 ± 3.57 SEM, $t(14)=5.809, p<0.001$).

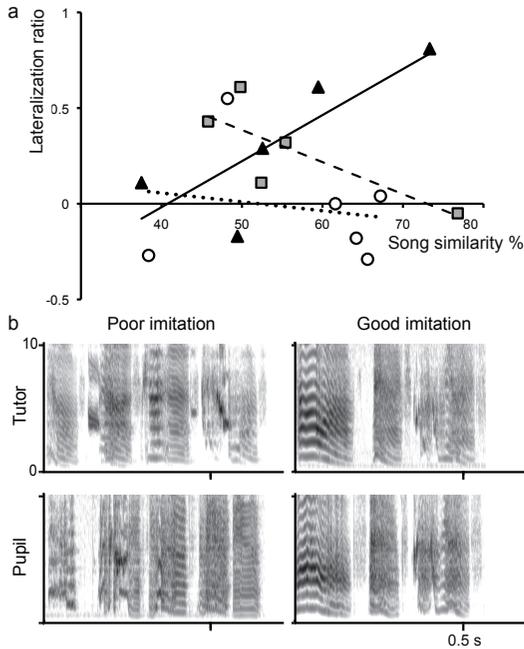


Figure 2.3. Correlation between lateralisation ratio and degree of song similarity. **A:** Lateralisation ratios (L-R) / (L+R) were calculated for each subject from the number of Zenk-immunopositive cells per mm² in the NCM of juvenile zebra finches, and correlated with song similarity scores. In the birds that were exposed to tutor song (black triangles) the correlation was significant (solid line, plotted by linear regression, $R^2=0.67$). There were no significant correlations in the Novel (open circles, dotted line) or Silence groups (grey squares, dashed line). **B:** Spectrograms of juveniles that produced poor or good imitations of their tutor's song. The juvenile in the bottom left panel had a song similarity of 49.4% with its tutor, while the juvenile in the bottom right panel had a song similarity of 73.3%.

There was no significant difference between the mean similarity score in the Tutor and in the Silent group ($t(13)=0.302$, n.s.). The mean number of Zenk-immunopositive neurons for the two groups is shown in figure 2.2, while figure 2.4 has representative photomicrographs of Zenk expression patterns. Although there was a significant difference between the response to father's song and to silence in the NCM ($F(1,30)=5.191$, $p=0.040$), there was no significant interaction between Stimulus and Hemisphere, and there was no significant difference between neuronal activation in the left and right NCM. Similar to the results in juveniles, in the adult zebra finches there was left-sided dominance in HVC irrespective of the stimulus presented ($F(1,30)=5.789$, $p=0.032$). In the hippocampus, we found basal levels of neuronal activation that did not differ between stimulus groups or hemispheres.

Discussion

By comparing neuronal activation patterns in response to song playback, we revealed left-hemispheric dominance in both a Wernicke-like region (the NCM) and a Broca-like region (HVC) in zebra finches that is similar to the brain lateralisation associated with human speech and language. We found left-sided dominance in the NCM of young zebra finches that were exposed to their father's song, reminiscent of early left-sided dominance of temporal brain regions that is found in human infants (Dehaene-Lambertz et al. 2002; Friederici & Alter 2004). Moreover, we demonstrated that the lateralised response in the NCM is memory-specific, since lateralisation only occurred when the juveniles were

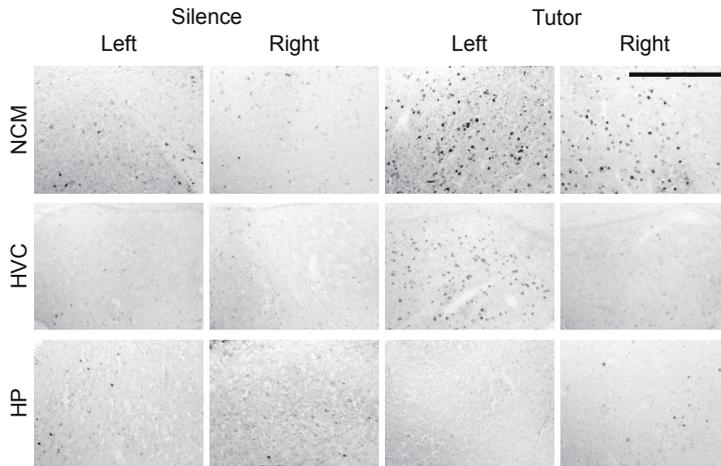


Figure 2.4. Photo-micrographs of adult zebra finch brains showing Zenk-immunostaining. Representative images at the level of the NCM, HVC and hippocampus are shown for the Silence and Tutor stimulus groups. The scale bar represents 0.2 mm.

exposed to their father's song, and not when they were exposed to unfamiliar conspecific songs or to silence. Similarly, in an fMRI study in two-and-a-half-months old infants, exposure to the mother's voice was shown to elicit greater neural activation than exposure to an unfamiliar voice in the left temporal lobe, but not in the right (Dehaene-Lambertz et al. 2010). Repetition of speech stimuli decreased the initial strong response in the left temporal lobe, but did not affect activation in the right hemisphere (Dehaene-Lambertz et al. 2010), showing memory-specific activation in the left temporal lobe. Thus, the present results in songbirds are similar to memory-related left-sided dominance of Wernicke's area in human infants. In human adults that are exposed to speech, Wernicke's area in the left hemisphere is more active than the corresponding area in the right (Friederici & Alter 2004). Furthermore, the left superior temporal sulcus (STS) shows a suppressed response when the same sentence is repeated (Dehaene-Lambertz et al. 2006), similar to repetition suppression in young infants (Dehaene-Lambertz et al. 2010). In contrast, in our experiment in adult zebra finches, we found no significant difference in neuronal activation between the left and the right NCM. In other studies, lateralisation of auditory processing in the NCM was found in adult zebra finches (Avey et al. 2005; Voss et al. 2007; Phan & Vicario 2010; Remage-Healey et al. 2010; Yang & Vicario 2011), although the direction of lateralisation that was found differs between studies. Apart from the different methodology that they used (electrophysiology, aromatase inhibition or fMRI), in contrast to the present study, these authors did not investigate memory-related lateralisation.

The left-sided dominance of HVC activation in adult and juvenile male zebra finches in the present study was not caused by exposure to the stimulus songs or by the subjects singing themselves. The unexpected activation of the left-sided HVC in the Silence groups might

reflect off-line song processing. Most birds were kept in the dark until the stimulus was presented, and may have slept. Electrophysiological studies have demonstrated that HVC shows spontaneous neuronal activity during sleep, similar to the activity recorded during singing (Nick & Konishi 2001; Hahnloser et al. 2006; Margoliash & Schmidt 2010). Thus, in the present study a similar process may have occurred, which would then be limited to the left hemisphere. To further investigate what causes spontaneous left-sided activation in HVC, our experiment could be repeated in, for example, canaries (*Serinus canarius*), where – less ambiguously than in the zebra finch – neuronal activation related to song production is found to be lateralised to left HVC (Nottebohm et al. 1976; Halle et al. 2003). Although there are several reports of HVC-lateralisation in zebra finches, most of them were concerned with neural activity during song production, or only perception (and not memory-related activation) was studied.

In human infants of three months old (Dehaene-Lambertz et al. 2006) or 4-12 years old (Berl et al. 2010) that were exposed to speech, left-sided dominance of Broca's area was found. In human adults, Broca's area in the left hemisphere was activated during syntactic processing of speech (Friederici & Alter 2004). Also, in adults who learned a new language, Broca's area in the left hemisphere was activated (Raboyeau et al. 2010). The left-sided dominance of HVC in juvenile and adult zebra finches is in this respect similar to lateralisation of Broca's area in humans. However, fMRI studies did not show spontaneous lateralised responsiveness in Broca's area in either infant or adult humans (Damoiseaux et al. 2006; Fransson et al. 2007; Fransson et al. 2009; Doria et al. 2010).

There are conflicting reports on lateralisation in non-human primates, which do not show vocal learning. In macaques, left-sided dominance of the superior temporal gyrus was found for species-specific sounds (Heffner & Heffner 1984; Poremba et al. 2004), but others found no lateralisation, in neither the Broca- nor Wernicke-homologue (Gil-Da-Costa et al. 2006). In contrast, right-sided dominance of the superior temporal gyrus for species-specific sounds was reported for chimpanzees (Tagliabue et al. 2009). In the visual domain, memory-related left-hemispheric dominance has been reported for filial imprinting in domestic chicks (Horn 2004), similar to the present results in juvenile zebra finches. In an extensive series of studies, Horn and collaborators found that the left intermediate and medial mesopallium (IMM) shows greater memory-related activation (measured in a number of ways, including the size of the postsynaptic densities and NMDA receptor binding) than the right. Horn has suggested that the left IMM acts as a permanent store, while the right IMM relays to an additional storage system dubbed S', which lies outside the IMM and is important for temporary memory storage between 4-6 and at least 26 hours after imprinting training (Horn 2004).

Minagawa-Kawai et al. (2011) suggested that human language lateralisation might arise as a result of both pre-existing left-right biases for aspects of generic auditory processing and left-sided language-learning mechanisms (Minagawa-Kawai et al. 2011). In the present study, the left NCM showed greater activation for memory-specific auditory stimuli in juveniles

specifically. An interesting possibility is that there is a temporal left-sided dominance associated with song memory formation early in development, which disappears once the memory is formed. That we do not see any lateralisation in adult zebra finches could indicate that there is no lateralisation bias for general auditory perception, or at least it is not manifest as cellular activation. To test whether it is the case that lateralisation is dependent on song learning phase, this experiment should be repeated with different developmental groups included in the experimental design. Additionally, it would be interesting to investigate the effect of lesions to the left NCM on song memory compared to right-sided lesions, an experiment which ideally should also be conducted in several age groups.

In conclusion, in addition to behavioural, genetic and neural parallels that were found between adult songbirds and adult humans (Bolhuis et al. 2010), our findings suggest that perception of human speech and birdsong show similar patterns of lateralised brain activation. Brain lateralisation may be a corollary of auditory-vocal learning that arose in humans and songbirds as a result of convergent evolution (Bolhuis & Wynne 2009).

3

Learning-related brain hemispheric dominance in sleeping songbirds

A different version of this manuscript has been submitted.

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Abstract

There are striking behavioral and neural parallels between the acquisition of speech in humans and song learning in songbirds. In humans, language-related brain activation is lateralised to the left hemisphere. During language acquisition in humans, lateralisation develops as language proficiency increases. Here we show that during sleep in juvenile zebra finches, there is lateralisation of spontaneous neuronal activation in a secondary auditory brain region, and the direction of lateralisation is related to the degree of song imitation. Specifically, birds that imitated their tutors well were left dominant, while poor imitators were right dominant, similar to language-proficiency related lateralisation in humans. Given the avian-human parallels, lateralised neural activation during sleep may also be important for speech and language acquisition in human infants.

Introduction

Humans and songbirds share the ability of vocal production learning by imitation – a trait that is absent in our closest relatives, the apes (Bolhuis et al. 2010; Bolhuis & Everaert 2013). There are remarkable behavioral, neural and genetic similarities between speech acquisition in human infants and birdsong learning (Doupe & Kuhl 1999; Jarvis 2004; Bolhuis et al. 2010; Bolhuis & Everaert 2013; Moorman & Bolhuis 2013). In humans, there is a language-related brain network involving multiple functional circuits. It is well established that Broca's area in the left frontal lobe and Wernicke's area in the left temporal lobe are central brain regions in this network (Friederici 2011; Friederici 2012; Berwick et al. 2013; figure 1.5 in chapter 1 and 3.1a). Among other functions, Broca's area is involved in speech production and generation and processing of language syntax, while Wernicke's area plays a major role in speech perception and comprehension (Price 2010; Friederici 2011; Friederici 2012; Berwick et al. 2013). In the songbird brain, there is a somewhat similar neural dissociation, with a neural network for song production (the 'song system', including the song nucleus HVC, used as a proper noun; Hahnloser et al. 2006; Roberts et al. 2008; Roberts & Mooney 2013) and brain regions that are involved in song perception and recognition (including the caudomedial nidopallium, NCM; figure 1.6a, b in chapter 1 and 3.1a; Mello et al. 1992; Gobes & Bolhuis 2007; London & Clayton 2008). Thus in a functional sense, these networks are considered to be analogous to Broca's and Wernicke's areas in humans, respectively (Bolhuis & Gahr 2006; Bolhuis et al. 2010). Neural activation in relation to speech and language in Broca's and Wernicke's areas occurs predominantly in the left hemisphere in human babies, infants and adults (table 6.3 in chapter 6, Peña et al. 2003; Dehaene-Lambertz et al. 2006; Imada et al. 2006; Friederici et al. 2007; Dehaene-Lambertz et al. 2010; Grossmann et al. 2010; Friederici 2011). There is growing evidence for lateralisation of neural activation in relation to song production and perception in songbirds as well; although it is not yet clear which specific roles the hemispheres play (table 6.1 in chapter 6; reviewed by Ocklenburg et al. 2013).

Sleep plays an important role in learning and memory in mammals, particularly in the process of consolidation (Walker & Stickgold 2004; Diekelmann & Born 2010; Dudai 2012; Stickgold 2013) and insects (Donlea et al. 2011; Beyaert et al. 2012). Consolidation refers to a process in which short-term memories are transformed into more stable and long-term stored memory representations (McGaugh 2000; Dudai 2004; Diekelmann & Born 2010). It has been suggested that memory consolidation may also occur during sleep, to prevent interference between daily activities and memory consolidation (Diekelmann & Born 2010).

Human sleep consists of cycles in which superficial and deep sleep stages alternate, with more slow-wave sleep (SWS) stages in the first half of the night and more rapid-eye movement sleep (REM sleep) stages in the last part of the night (Schulz 2008; Diekelmann & Born 2010; Rattenborg et al. 2011). Songbirds, including zebra finches, have a sleep pattern that is remarkably similar to that of humans, with a similar distribution of SWS and REM sleep (Low et al. 2008; Rattenborg et al. 2009). Sleep is important for memory consolidation

in birds (Gobes & Bolhuis 2008; Rattenborg et al. 2009). Jackson and colleagues (Jackson et al. 2008) showed that a period of sleep after imprinting training with visual stimuli is necessary for consolidation to occur in domestic chicks. Similarly, it was shown that sleep enhances consolidation of auditory memory in European starlings (Brawn et al. 2010; Brawn et al. 2013). Furthermore, sleep in juvenile zebra finches affects tutor song imitation (Derégnaucourt et al. 2005), and learning-related changes in neuronal activation during sleep have been observed in a song system nucleus (the robust nucleus of the arcopallium) after a single day of song tutoring in juvenile zebra finches (Shank & Margoliash 2009).

Here, we investigated brain lateralisation during sleep in songbirds. Previously, we found that there is memory-related neuronal activation in the NCM of juvenile zebra finches during sleep (Gobes et al. 2010). More recently, we demonstrated memory-specific left-hemispheric dominance of neuronal activation in the NCM and general left-hemispheric dominance in HVC in awake juvenile zebra finches (chapter 2 of this thesis). In human infants, brain lateralisation during language processing is dependent on language proficiency. We here show that brain lateralisation in songbirds is similar to language-related lateralisation in humans.

Materials and methods

Animals. The 22 juvenile zebra finch males were bred at the central animal facility of Utrecht University. The subjects have been used in a previous study (Gobes et al. 2010) for analysis of the left hemisphere. Here, we took new photomicrographs of the left NCM and HVC of the same subjects, and added photomicrographs of the right NCM and right HVC and left and right hippocampus of the same birds. Thus, we conducted entirely new measurements of neuronal activation in the left and right hemispheres of these subjects, and calculated relative activation ratios. Birds were kept on a 15:9 hr light:dark cycle (lights were turned on at 6 AM and off at 9 PM) and food and water were provided ad libitum. Birds were kept in breeding cages with their parents and siblings until 47 days post hatching (dph). At 47 dph, all males from a clutch were removed from the breeding cages and kept in communal cages. The experiments were performed at a mean age of 56 dph (range: 54-59 dph). Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University.

Experimental design. Subjects were exposed to their tutor's song, novel conspecific song, or silence on the day of the experiment. The stimuli were constructed using Praat software (Boersma & Weenink 2005) from 10 different song renditions of each animal (tutor, the fathers of the subjects; novel, conspecific males that were unfamiliar to the subjects). The root mean square amplitude of all songs was equalized and the 10 songs were repeated in random order for a total of 90 song presentations in one hour of playback. Before stimulus exposure, the birds had been placed in a soundproof chamber for two nights. On the day of the experiment, the stimuli were presented between 11 AM and 12 PM. After that, the birds were not disturbed until the lights were turned off at 9 PM. Birds were sacrificed at midnight. Video and sound recordings were made throughout the experimental day, to

record the birds' singing activity, the total daytime napping duration and to measure how long the birds had been asleep before being sacrificed.

Tissue collection and immunocytochemistry. At midnight, the birds were deeply anesthetized with an overdose of Natriumpentobarbital (Nembutal, Ceva Sante Animale, Libourne, France, 0.06 mL i.m.) and perfused with phosphate-buffered saline (PBS), followed by fixation with fresh, ice-cold 4% paraformaldehyde in PBS. Brains were dissected out, postfixed at 4°C, cryoprotected in 10%, 20% and 30% sucrose in PBS at 4°C, frozen and kept at -20°C until cut. Parasagittal 20- μ m sections were made on a cryostat and mounted on poly-L-lysine coated slides. The brains were incubated with primary polyclonal rabbit antiserum for *egr-1* (Zenk; Santa Cruz Biotechnology; dilution 1:1000), overnight at 4°C; biotinylated goat antirabbit (IgG, Vector Laboratories, Burlingame, CA; dilution 1:100), for 1 hour at room temperature; ABC (avidin-biotinylated enzyme complex, Vector Elite Kit, Vector Laboratories), for 1 hour at room temperature; and diaminobenzidine (DAB) medium containing 0.034% H₂O₂ for 6 minutes at room temperature.

Image analysis. Quantification of Zenk-immunopositive cells was performed for the NCM, HVC and hippocampus as described before (Terpstra et al. 2004). Brain areas were identified using a stereotaxic atlas that is available online (Nixdorf-Bergweiler & Bischof 2007). Digital photographs were taken using a Leica DFC 4206 camera and the Leica Application Suite program on an Axioskop (Zeiss, Germany) with 20x objective. The NCM photomicrographs were taken at the extreme caudal pole of the lateral neostriatum, immediately adjacent to the ventricle separating the NCM from the hippocampus. The HVC photomicrographs were taken in the centre of the HVC nucleus, and the hippocampus images at the point at which the curve is most pronounced (cf. Terpstra et al. 2004). Image analysis was performed with a personal computer-based system using KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A program had been previously developed in KS400 to quantify the number of immunoreactive cells semi-automatically (Terpstra et al. 2004). Counts of 3 sections for each region in both hemispheres per animal were averaged for further statistical analysis. All image analysis was performed blind as to the experimental history of the subject.

Song analysis. Sound recordings were analyzed using Sound Analysis Pro to calculate song similarities between the subjects and their tutors. To do so, five representative motifs of the juveniles were randomly selected from the afternoon on the day of the experiment, between 12 and 4 PM. Tutor songs were recorded between 1-12 months before the experiment. All songs were filtered and the root-mean-square amplitude was equalized using Praat software (Boersma & Weenink 2005). We calculated the 'percentage similarity', which is a measurement of syllable copying, with the computer program Sound Analysis Pro [<http://soundanalysispro.com/>]. 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). The mean song similarity percentage of each of the juveniles' and tutors' motifs was calculated. We then separated the birds into two learning proficiency groups: 'poor learners' and 'good learners'. We set the boundary between poor

learners and good learners at the middle of the song similarity range, at 55%. The significant difference in song similarity between the poor and good learners could be caused by more variation or exploration from song to song in the poor learners, thus a less stable song than the good learners, or by an actual qualitative difference in their vocal learning abilities. There was no significant difference between the poor and good learners in the variation between song similarities calculated with different juveniles' and tutors' motifs (individual birds' standard deviations; One-way ANOVA: $F_{1,16}=0.000$, $p=0.984$), suggesting that the difference between poor and good learners was not due to differences in song stability.

Video analysis. We analyzed video data to quantify the time a bird had been asleep during the day and subsequent night prior to sacrifice. We used the behavioral indicators of slow-wave sleep as our criterion to quantify sleep. Birds sitting with the head under the wing or sitting without moving, with deep slow breathing and closed eyes for more than 2 minutes were considered to be sleeping (Dave & Margoliash 2000; Derégnaucourt et al. 2005; Hahnloser et al. 2006; Low et al. 2008; Gobes et al. 2010). A waking-up or REM-event was scored when the bird made a sudden movement from this posture, accompanied by opening the eyes. Because head movements can occur during both wakefulness and REM sleep, we could not visually distinguish those.

Statistical analyses. We conducted repeated measures ANOVA to compare the effect of stimulus exposure on the Zenk response in the left and right NCM, HVC, and hippocampus. We further analyzed the brain regions separately using repeated measures ANOVA. We separated the birds into two song learning proficiency groups: 'poor learners' and 'good learners' (see above). To test for lateralisation effects between the learning proficiency groups, a repeated-measures ANOVA was performed. To test for lateralisation effects within the learning proficiency groups, post hoc paired t-tests were performed. A lateralisation ratio was calculated by dividing the difference in Zenk expression levels between the two hemispheres in a brain region by the total amount of Zenk expression of the two hemispheres: $(\text{left} - \text{right}) / (\text{left} + \text{right})$. This lateralisation ratio enabled us to look at true lateralisation levels not influenced by differences in absolute neuronal activation (chapter 2 of this thesis). Furthermore, we analyzed the total time that the birds spent napping on the day of the experiment, the total time that the birds spent sleeping during the night of the experiment, and the total amount of singing during the day of the experiment. We tested for correlations between those measurements and levels of neuronal activation or lateralisation ratios using Pearson's correlation tests. Data were analyzed using SPSS 20.0.0 (IBM Corporation).

Results

Figure 3.1b depicts the experimental design. After tutoring, we separated the juveniles from their parents, so the juveniles could not hear their tutor's song anymore. When the juveniles were approximately 56 days old and in the sensorimotor learning phase, we exposed the juveniles to either the song of their father, which they had started to imitate, to an unfamiliar conspecific

song or to silence. We hypothesized that playbacks of the target song (tutor song) during the day would trigger memory consolidation, which might occur during sleep. Thus, instead of measuring neuronal activation caused by stimulus exposure directly, we measured neuronal activation during sleep. We expected to find differential neuronal activation between the tutor song-exposed juveniles, and the ones that heard novel song or remained in silence during the day. Apart from exposure to the stimulus, we left the juvenile males undisturbed. During the subsequent night, when the birds slept, the birds were sacrificed (figure 3.1b). The mean time that the juveniles had slept before perfusion was 2 hours and 32 minutes (range: 1 hour and 56 minutes – 2 hours and 51 minutes). The brains were processed with immunocytochemistry to label Zenk (the protein product of the immediate early gene *ZENK*, which is an acronym of *zif-268*, *egr-1*, *ngf-1a* and *krox-24*; Figure 3.2e), a marker for neuronal activation that is expressed upon postsynaptic membrane depolarization (Mello & Ribeiro 1998).

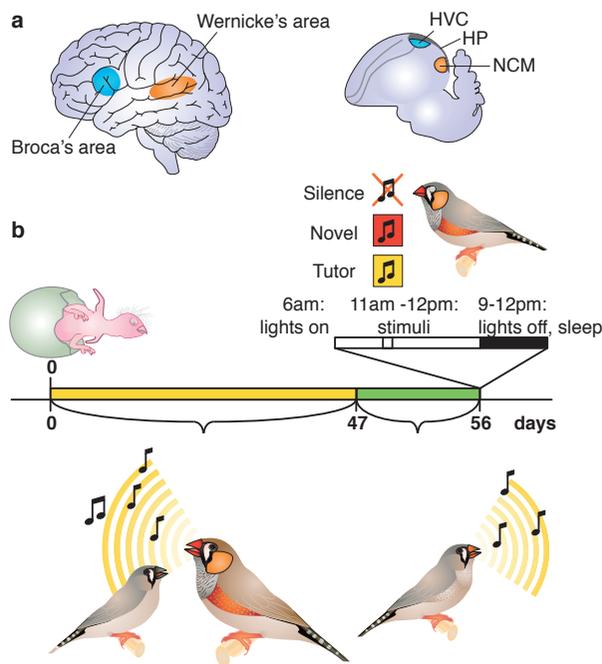


Figure 3.1. Schematic of experimental design. Immediate Early Gene (IEG) expression was measured bilaterally in juvenile male zebra finches during sleep. **A:** We measured IEG expression in brain areas NCM and HVC. NCM is part of an auditory pathway that could be considered analogous to Wernicke's area in the human brain. HVC is part of a song production pathway that could be considered analogous to Broca's area in the human brain. **B:** Birds were reared with their parents until 47 days post hatching. During this time, they could memorize and imitate their fathers' songs. Then, they were moved to siblings-only groups to prevent them from hearing their fathers' songs. Two nights before the experiment, juveniles were moved to sound-isolation chambers. On the day of the experiment, one group of experimental subjects did not receive auditory stimulation ('silence'), a second group was exposed to unfamiliar conspecific song ('novel'), and a third to their fathers' song ('tutor'). All groups consisted of 6 animals. They were undisturbed during the rest of the day. During the subsequent night, when the birds were asleep, they were sacrificed.

We quantified the number of Zenk-immunopositive neurons bilaterally in the NCM, HVC and hippocampus (figure 3.1a) to investigate lateralisation of neuronal activation during sleep (figure 3.2a,b,c). An overall repeated measures ANOVA revealed significant effects of Brain Region ($F_{2,9}=17.501$, $p=0.001$) and Hemisphere ($F_{1,10}=5.485$, $p=0.041$), and a significant interaction between these two factors ($F_{2,9}=4.411$, $p=0.046$). Therefore, we conducted subsequent analyses on the three brain regions separately. In the hippocampus, the level of activation was low and did not differ between stimulus groups ($F_{2,14}=0.439$, $p=0.653$, n.s.) or hemispheres ($F_{1,14}=0.107$, $p=0.748$, n.s.; figure 3.2a).

Differential lateralisation of neuronal activation in the NCM. There was no significant difference in Zenk expression between the stimulus groups (silence, novel and tutor) or between the left and right NCM (figure 3.2b). However, when we analyzed the results of “poor learners” (juveniles with a song similarity of 55% or less – 55% was the mid-range value, see materials and methods) and “good learners” (juveniles with a song similarity of more than 55%; figure 3.3c) separately, there was differential lateralisation. Repeated measures ANOVA revealed a significant interaction between Hemisphere (Left or Right NCM) and Learning proficiency (Poor or Good) ($F_{1,15}=11.615$, $p=0.004$). Poor learners were right dominant ($t=-3.299$, $p=0.011$, $n=9$; mean song similarity=43.44%), while good learners showed left dominance in the NCM ($t=4.219$, $p=0.004$, $n=8$; mean song similarity=65.83%; figure 3.3a).

In addition, we calculated a lateralisation ratio (calculated as: neuronal activation (left – right) / (left + right); chapter 2 of this thesis). We found a significant positive correlation between song similarity of the juveniles and the lateralisation ratio in the NCM during sleep (Pearson’s $r=0.630$, $p=0.007$, $n=17$; figure 3.3b). In contrast to lateralisation during the day, where we previously found a correlation in the tutor stimulus-group only (chapter 2 of this thesis), here we found the correlation in all stimulus groups. These results suggest that there is left hemisphere dominance regarding neuronal activation in the NCM, proportional to the strength of song learning that has occurred.

Neuronal activation in HVC is right dominant during sleep. A repeated-measures ANOVA revealed right-dominance of neuronal activation in HVC during sleep in all stimulus groups ($F_{2,11}=12.209$, $p=0.005$; figure 3.2c). In contrast, in an earlier study with awake juvenile zebra finch males we found left-dominance in HVC (chapter 2 of this thesis). There are two important differences between these two studies. First, in the previous study neuronal activation was determined during daytime right after stimulus exposure (the lights were off for four hours to prevent the birds from singing, before and during stimulus exposure), while here, we assessed neuronal activation when the birds were asleep at nighttime (the lights were off for three hours prior to sacrifice). Second, the birds in the previous study sang in the first two hours of the day only, and did not sing in the 4 hours before they were sacrificed, while in the current study, the birds could sing during the whole day, before the lights were turned off in the evening. To investigate which of these factors may have affected the difference in HVC lateralisation between day and night, we conducted a study with a separate group of juvenile male zebra finches (reared

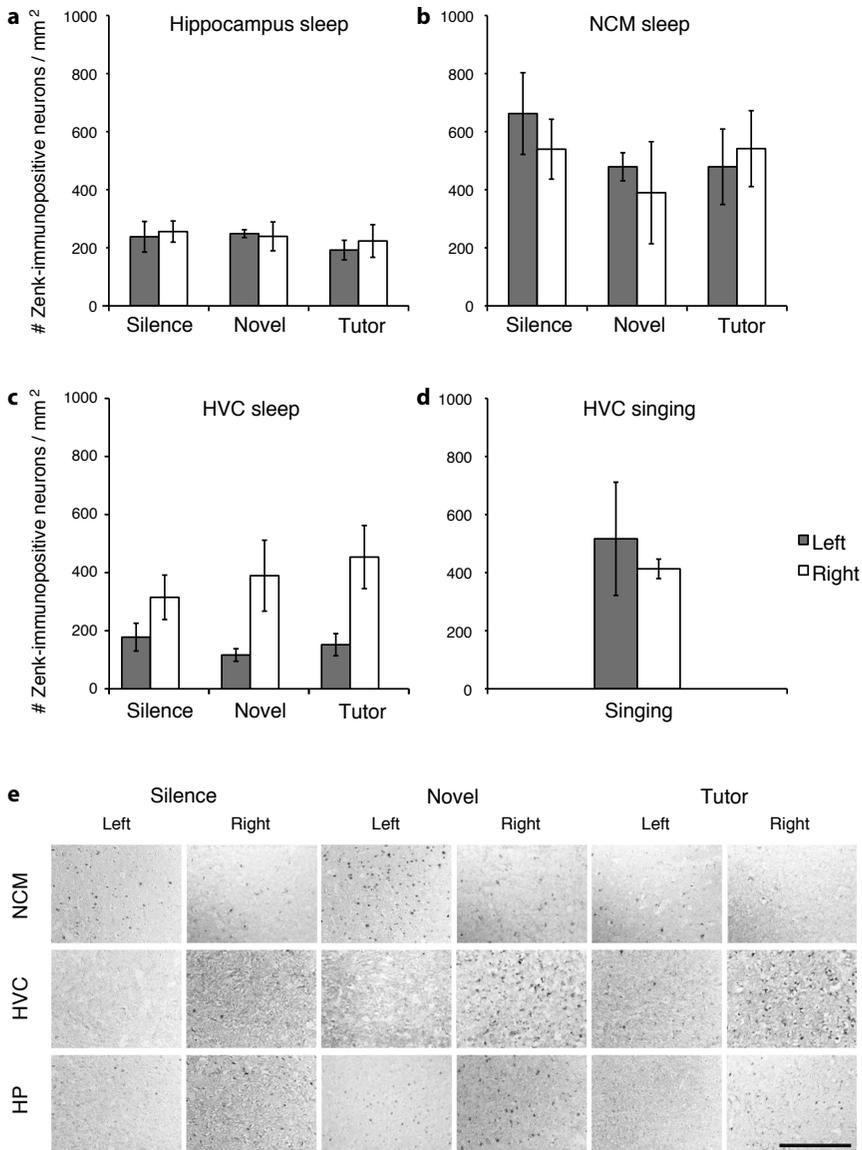


Figure 3.2. Neuronal activation in HVC is lateralised during sleep. Mean number of Zenk-immunopositive neurons per square millimeter is shown for the different brain regions. Grey bars represent the left hemisphere, and white bars represent the right hemisphere. Error bars represent \pm SEM. **A:** In the hippocampus, a control brain region that is not implicated in song learning, Zenk expression levels are low and not lateralised. **B:** In NCM during sleep, there were high levels of Zenk expression but no differences between groups or hemispheres. **C:** We found high activation levels in the right HVC in all conditions, while Zenk expression levels were low in the left HVC. **D:** In a separate group of juvenile males in which IEG expression was measured immediately after they had been singing, there were high levels of Zenk expression in both left and right HVC. ($n=4$) **E:** Photomicrographs of juvenile zebra finch brains showing Zenk-immunostaining. Representative images at the level of the NCM, HVC, and hippocampus (HP) are shown for the silence, novel, and tutor stimulus groups, in the left and right hemispheres. Scale bar represents 0.2 mm.

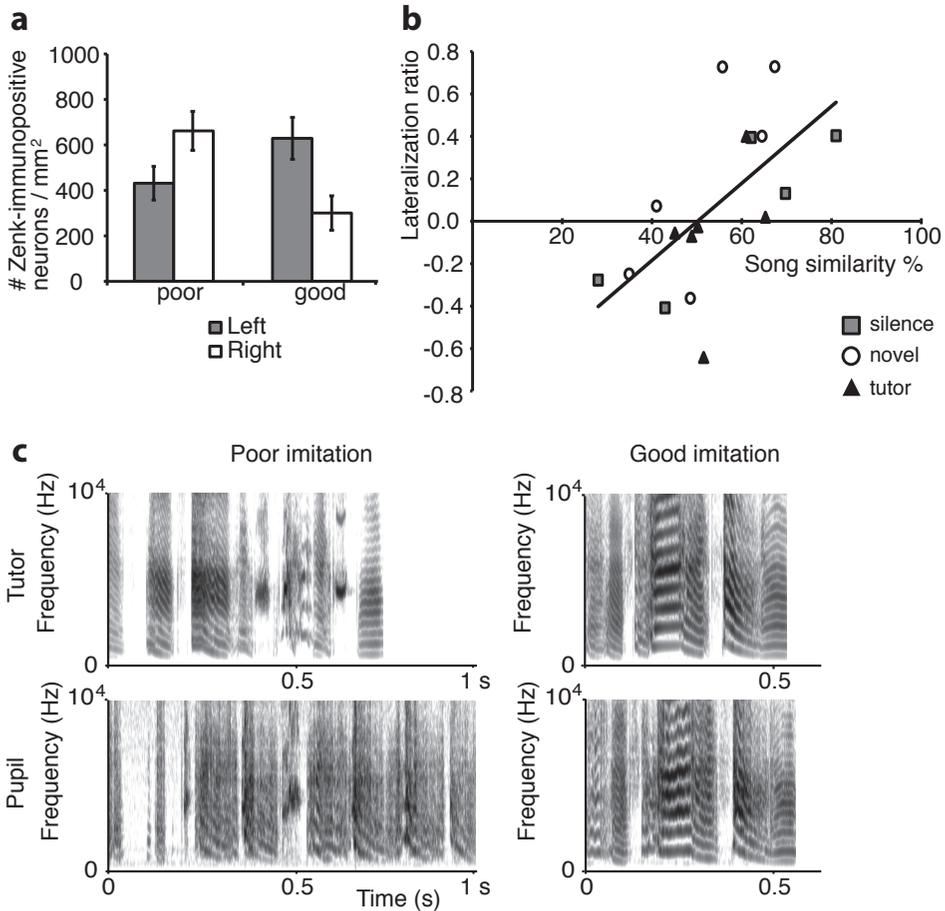


Figure 3.3. Neuronal activation in NCM is lateralised during sleep: poor learners show right-dominant activation in NCM, while good learners have a left-dominant expression pattern. **A:** Mean number of Zenk-immunopositive neurons per square millimeter in NCM for all experimental groups grouped together (silence, novel, tutor), but individuals are divided into two groups based on learning proficiency. 'Poor': song similarity <55% (=mid-range); 'good': song similarity >55%. Grey bars represent the left hemisphere, and white bars represent the right hemisphere. Poor, $n=9$, good, $n=8$. Error bars represent \pm s.e.m. **B:** Lateralisation ratios $(L-R)/(L+R)$ were calculated for each subject from the number of Zenk-immunopositive cells per square millimeter in the NCM. Song similarity scores on the x-axis indicate to which degree the juveniles had imitated their fathers' songs. Black triangles represent birds that were exposed to tutor song during the day; open circles, novel song; grey squares, silence. The regression line shown is for the three stimulus groups together, and the correlation is significant (Pearson's $r=0.630$, $p=0.007$). **C:** Spectrograms of juveniles that produced a poor or good imitation of their tutor's song. Left: Juvenile had a song similarity of 28% with its tutor. Right: Juvenile had a song similarity of 81% with its tutor.

in the same way as the experimental males in the main experiment) that sang during the day, and were also sacrificed during the day ($n=4$). In accordance with the literature (see table 6.1), we found that the left and right HVC were activated approximately equally in these birds ($t=0.545$, $p=0.623$, $n=4$; figure 3.2d). Therefore, it seems that during singing, HVC is activated bilaterally.

In summary, neuronal activation occurs predominantly in the left HVC in non-singing juvenile zebra finches during the day (chapter 2 of this thesis); during singing, HVC is activated bilaterally (see table 6.1 in chapter 6), our singing data; and during sleep, the right HVC is dominant (present results).

Discussion

We demonstrated differential lateralisation of neuronal activation in a ‘Wernicke-like’ brain region (the NCM) and a ‘Broca-like’ brain region (HVC) during sleep in juvenile male zebra finches. The strength of song learning was correlated with the lateralisation ratio in the NCM during sleep, with greater song similarity related to stronger neuronal activation in the left hemisphere. When we divided the experimental subjects in “poor learners” and “good learners”, we found that for the NCM, the right hemisphere was predominantly activated during sleep only in poor learners, while the left hemisphere was dominant in good learners. HVC of sleeping juveniles was right dominant independent of the strength of song learning. Previously we demonstrated that in awake juvenile zebra finches, neuronal activation in the NCM and HVC is also lateralised, but in a different manner: the left NCM of awake juveniles is dominant when the bird is exposed to tutor song, while HVC of awake, non-singing zebra finches is left dominant irrespective of the stimulus to which they are exposed (chapter 2 of this thesis). Taken together these results suggest differential lateralisation of neuronal activation depending on the behavioral state of the animal (sleeping or awake; figure 3.4).

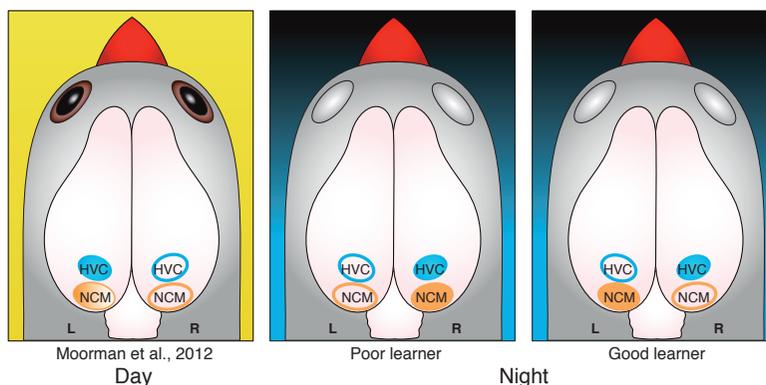


Figure 3.4. Results overview. Left panel: Previously in a similar experiment, we found that HVC showed a left-dominant activation in awake and silent juvenile zebra finch males, while NCM was left-dominantly activated during tutor song playback only (and not during silence or novel, demonstrating memory-specific lateralisation; Moorman et al. 2012). Middle and right panel: Here we have demonstrated that during sleep at night, HVC was activated right-dominantly in all birds, while NCM showed differential lateralisation between learning proficiency groups. In poor learners, NCM activation was right dominant (middle panel), while in good learners NCM activation was left dominant (right panel).

The finding that poor learners exhibited neuronal activation predominantly in the right NCM, while neuronal activation in good learners was left lateralised, is reminiscent of language-related lateralisation in humans that are acquiring a new language. Language-related neuronal activation in babies and infants is already left-dominant (i.e., Dehaene-Lambertz et al. 2002; Holowka & Petitto 2002; Peña et al. 2003; Dehaene-Lambertz et al. 2010; Mahmoudzadeh et al. 2013), which could be related to extensive prenatal or early-life auditory experience. However, there are some papers that report that in children who are developing their language skills, brain regions activated during speech perception were right dominant or bilateral. Activity shifted to the left side of the brain with increased language proficiency, with adult-like left-dominance only occurring at older ages and more developed language abilities (Gaillard et al. 2000; Conboy & Mills 2006; Holland et al. 2007; Spironelli & Angrilli 2010). Furthermore, the degree of left-dominance in infants correlated positively with proficiency (measured as word-fluency in a verb generation task) and age (Holland et al. 2001). Similarly, language-related lateralisation in adults who learn a new language is also correlated to proficiency. Lateralisation of neural activation during speaking and speech perception was correlated to proficiency of the newly-learned language, in which proficient learners were more left dominant than less proficient individuals (Perani et al. 1996; Perani et al. 1998). Two studies found that, when proficiency of a newly-learned language was poor, there was a largely bilateral activation pattern (Dehaene et al. 1997; Minagawa-Kawai et al. 2011). However, in another study it was found that less proficient second language speakers showed more left dominance than more proficient speakers (Hull & Vaid 2007), or linguistic proficiency was found to be not related to the direction nor the degree of lateralisation (Knecht et al. 2001). What seems to be true for all these studies is that less proficient speakers engage a neural network for the second language that encompasses more brain regions than the network for their first language, and the extent of the network is reduced when higher proficiency is reached (Tatsuno & Sakai 2005; Raboyeau et al. 2010). Except for one case report (Wilke et al. 2003), as far as we are aware, no studies in humans have investigated language-related lateralisation during sleep.

What is the function of brain lateralisation? In humans, different brain regions are involved in different aspects of language processing, for example neural activation during processing of prosody is found predominantly in the right temporal lobe (Friederici 2011). There may be a functional division of the two hemispheres in songbirds as well (George et al. 2005b), which could perhaps explain the difference in lateralisation between the learning groups in our current study. For example, the difference between poor and good learning zebra finches might be due to song perception characteristics (e.g. attentional processes involved, or perceived song complexity). In addition, there are clinical examples of lateralisation abnormalities associated with language impairments (table 6.3 in chapter 6). In dyslexic children, auditory perception is significantly less lateralised to the left hemisphere than it is in healthy controls (Johnson et al. 2013). Furthermore, the temporal lobe is right dominant for language perception in both autistic children and autistic adults (Pierce 2011). An important hallmark of autism is language impairment, although language skills can vary

from very poor to outstandingly excellent (Bigler et al. 2007). Some schizophrenic patients suffer from auditory hallucinations (hearing voices). It was shown that those patients have increased neural activation levels in the right hemisphere (Sommer et al. 2001). Lateralisation and hallucinating were correlated: patients with bilateral neural activation had more severe hallucinations than patients with less activation in the right hemisphere.

Thus, although there are conflicting reports on a direct correlation between lateralisation ratio and language abilities in human adults (Dehaene et al. 1997; Hull & Vaid 2007; Minagawa-Kawai et al. 2011), infants show increased left-dominance with increasing linguistic proficiency (Holland et al. 2001; Holland et al. 2007), and abnormal lateralisation in humans seems to be linked to language impairments.

We found right-hemispheric dominance in HVC during sleep. In contrast, awake juveniles showed left-sided dominance of neuronal activation in HVC (chapter 2 of this thesis; figure 3.4). In a separate singing experiment, we found that both the left and right HVC were activated approximately equally in birds that were sacrificed immediately after they had sung (present study). Other studies have reported rapid hemispheric switching (bilateral contributions that alternate on a millisecond time scale) or bilateral activation of HVC during singing (see table 6.1 in chapter 6). We compared all published results on lateralisation in songbirds (table 6.1). We found that results differ between experiments, probably caused by the type of stimulus used, the methods employed and whether the birds were awake, anesthetized or asleep. Generally, both the left and right HVC contribute to singing in zebra finches, perhaps in an alternating way (“hemispheric switching”), while in canaries the left HVC is crucial for singing. During song perception, in HVC and the NCM usually one hemisphere is selective for some song types over others (e.g. bird’s own song vs conspecific song or conspecific vs heterospecific song), and the other hemisphere responds more to birdsong in general, although the direction of lateralisation is not consistent. We suggest that this directional ambiguity could be due to the behavioral state of the animal (awake, sleeping or under anesthesia). It has been argued that anesthesia induces “artificial sleep”, because (1) some anesthetics can reduce the homeostatic need for sleep (Nelson et al. 2010), or (2) induce neuronal activation in a brain region that promotes sleep (the ventrolateral preoptic nucleus; Moore et al. 2012). Furthermore, (3) the oscillations measured during anesthesia closely resemble the oscillations measured during slow-wave sleep (SWS; Steriade 2006). We sacrificed birds at 3 hours after the lights were turned off, and the mean time that the birds had slept before perfusion was 2.5 hours. In the beginning of the night, birds have high SWS density and very low REM sleep density, just like humans. The maximum SWS density is at 2 hours after sleep onset (Low et al. 2008). Therefore, the birds in our experiment experienced a relatively high proportion of SWS episodes at the time that we measured Zenk expression (Zenk-levels are present in cells from 15 minutes after neuronal activation and peak at 1-2 hours; Mello & Ribeiro 1998). Table 6.1 (in chapter 6) shows that most studies measuring neural activity during anesthesia or sleep show a right-dominant pattern, while studies in awake animals mostly show left-sided dominance, in both the NCM and HVC. Our findings

concerning behavioral state-dependent neuronal activation in HVC fit this pattern of results. The lateralisation in the NCM of sleeping songbirds is the first report concerning such effects during sleep and relating them to measures of song learning.

In addition to the NCM, HVC might play a role in tutor song memory (Roberts et al. 2012; Roberts & Mooney 2013). Song memory acquisition might involve dynamic interaction between the NCM and HVC, perhaps to provide a tutor song template with which to compare the birds' own (Gobes & Bolhuis 2007; Bolhuis et al. 2010; Gobes et al. 2013). Lateralisation in the NCM is more complex than the pattern we found in HVC. During sleep, we did not find absolute hemispheric differences, but lateralisation ratios differed between birds and were correlated to the strength of song learning. This might be related to the dynamics of information processing, where the left hemisphere could be involved in tutor song memory processing, while the right hemisphere may subserve reciprocal interaction between song recognition memory and motor learning systems. Although a direct connection between the NCM and HVC is not known, it was shown that HVC receives auditory input from the CM (caudal mesopallium; Bauer et al. 2008). The NCM projects to the CM (Vates et al. 1996), so the NCM is connected to HVC via the CM.

Such hemispheric specialization is reminiscent of filial imprinting in domestic chicks, where the left IMM (intermediate and medial mesopallium) has been shown to be a permanent memory store, while the right IMM is implicated in the formation of a memory store elsewhere in the brain (the hypothetical S'; Cipolla-Neto et al. 1982; Horn 2004). In juvenile zebra finches, the right NCM could have a similar role to the right IMM of chicks, being involved in the formation of a representation of bird's own song in the song system. In that case, the more the birds already learned from their tutor's song, the less neural interaction would be needed and the less activation we would find in the right NCM. An alternative explanation could be that the left NCM inhibits activation in the right NCM in good learners. In humans, it is hypothesized that the left temporal lobe inhibits activation in its right counterpart and when this does not happen very efficiently, for example in schizophrenic patients, right temporal lobe activation can cause the illusion of hearing voices (Sommer et al. 2001). Interestingly, most of the IMM neurons of chicks activated after imprinting contain GABA (Horn 2004), and similarly, 42% of the neurons that are Zenk-immunopositive after song stimulation in the NCM are GABA-ergic cells (Pinaud et al. 2004), indicating that inhibition does play an important role in the NCM. Since we do not know of any direct projections between the left and right NCM, if the left NCM would indeed inhibit activation in the right NCM, it should project via a longer route, for example via L3 (a primary auditory region) to RA cup (the surrounding region of the robust nucleus of the arcopallium) to Ov (nucleus ovoidalis), and cross hemispheres at the level of the midbrain or the thalamus (Vates et al. 1996; Wild et al. 1997; Jarvis 2004). In accordance with both hypotheses, the most extreme left-dominant birds (which were the best learners) had very low levels of activation in the right NCM in comparison to right-dominant birds – rather than higher levels in the left NCM.

In conclusion, we found lateralised brain activation during sleep in juvenile songbirds that were in the process of learning their song. A 'Broca-like' brain region (HVC) showed differential activation of the two hemispheres depending on behavioral state: left dominant during the day (chapter 2 of this thesis), bilateral during singing, and right dominant during sleep. Spontaneous neuronal activation during sleep in a 'Wernicke-like' brain region (the NCM) was left lateralised in good learners, and right lateralised in poor learners. This is similar to language lateralisation in awake humans, in which left-hemispheric dominance develops with increasing language proficiency. Given the parallels that exist between birdsong and human speech, lateralised neuronal activation during sleep may also be important for speech and language acquisition in human infants, and lateralisation might be a fundamental principle for auditory-vocal learning.

4

Neural mechanisms of early birdsong memory formation in male zebra finches

A different version of this chapter is being prepared for publication.
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Abstract

There are striking similarities between the neural mechanisms of human speech acquisition and birdsong learning. In humans, a language-related brain network including Broca's and Wernicke's area is involved in speech production and perception, respectively. In songbirds, similar brain regions are crucially involved in song learning: the song system including HVC (used as a proper name) is important for song sensorimotor learning and production, and the caudomedial nidopallium (NCM) is a higher-order auditory brain region that is important for song perception and tutor song memory. Research into the neural mechanisms of birdsong perception and memory has mainly involved adult zebra finches, but we know relatively little about these mechanisms in juveniles that are in the early stages of memory formation. Previously, we studied tutor song memory in juveniles that had been raised with their father, and found that tutor-song specific neuronal activation was lateralised. Here, memory-related brain activation was studied in juvenile male zebra finches that had minimal tutoring experience. Surprisingly, juvenile male zebra finches already showed significant song learning after just two days of experience with a live tutor. Neuronal activation in the NCM in response to tutor song re-exposure was greatest in the group of juveniles that had been housed with a tutor for two days. Overall, neuronal activation in the NCM was left brain dominant.

Introduction

Auditory-vocal learning in songbirds shares many characteristics with human speech acquisition, at the behavioural, neural and cognitive level. Therefore, investigating the neural mechanisms of birdsong may help to elucidate the neurobiology of human speech (Bolhuis et al. 2010). Previously, it was shown that a higher-order auditory brain region, the caudomedial nidopallium (NCM, figure 1.6a in chapter 1, and figures 4.2a, b), is important for song perception and tutor song memory (Mello et al. 1992; Mello et al. 1995; Bolhuis et al. 2000b; Terpstra et al. 2004; Gobes & Bolhuis 2007; London & Clayton 2008). We found that the NCM in juvenile male zebra finches showed human-like brain hemispheric dominance during song learning (chapter 2 of this thesis). Furthermore, there was neuronal activation in the NCM during sleep (Gobes et al. 2010). The sleep-related activation was lateralised and correlated with the level of song learning: left-dominant in juveniles that had imitated tutor song very well, and right-dominant in birds that sung a poor imitation of the tutor song (chapter 3 of this thesis). Here, we studied neuronal mechanisms of song memory consolidation in juveniles after a relatively brief period of tutor song learning, thus at an early stage in the song learning process.

In a filial imprinting experiment in domestic chicks (*Gallus gallus domesticus*), subjects were trained with a visual stimulus for several different durations (Bateson et al. 1973). Day-time learning resulted in memory consolidation during sleep, with the strongest neuronal activation after brief training periods, showing that memory consolidation mainly occurred after initial learning (Bateson et al. 1973). Similarly, in juvenile songbirds, it may be that tutor song memory consolidation occurs most strongly during initial tutor song learning, and would occur less with increasing learning proficiency. If this were true, then we would expect more memory-related neuronal activation when birds had not fully memorized tutor song yet. Thus, we raised two groups of birds: one group in which juveniles had very brief experience with the tutor song (two days), and one in which birds received longer tutoring experience (ten days; see figure 4.1). The durations of tutor song experience that we chose were based on earlier behavioural results in zebra finch males. Just eight days of exposure to several songs during the memorization phase (which is from approximately 20 until 60 days post hatching) was sufficient to recognize those songs in an operant task (Braaten 2010), and five or ten days of tutor song experience early in life was sufficient for significant song imitation in adulthood (Tchernichovski et al. 2001; Roper & Zann 2006).

There is ample evidence to show that sleep is important for memory consolidation (Diekelmann & Born 2010). In zebra finches it was shown that neuronal activation during sleep in brain regions important for song production, the robust nucleus of the arcopallium and HVC, resembled motor activation patterns as recorded during day-time singing (Hahnloser et al. 2006; Shank & Margoliash 2009). Furthermore, spontaneous neuronal activation during sleep in the NCM correlated with the strength of song learning (Gobes et al. 2010; chapter 3 of this thesis). Therefore, we investigated neuronal activation during night-time sleep as well as immediately after stimulus exposure during daytime (figure 4.1).

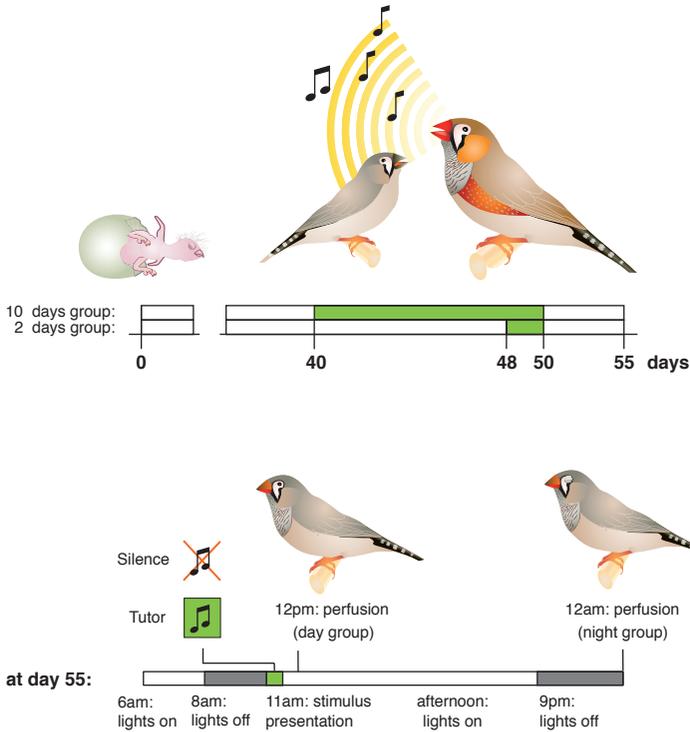


Figure 4.1. Schematic of experimental design. Top: Birds were raised in isolation until 40 or 48 days post hatching. Then, an adult male was moved to their cage for a period of either two or ten days, so that the juveniles could memorize and imitate their songs. Bottom: At day 55 half of the experimental subjects did not receive auditory stimulation ('silence'), while the other half was re-exposed to their tutors' song. Half of the animals were sacrificed after stimulus presentations, the other half during the subsequent night, when the birds were asleep. Therefore, there were 8 experimental groups: 10SD, 10TD, 10SN, 10TN, 2SD, 2TD, 2SN and 2TN - housed with the tutor for a period of 10 or 2 days, stimulus is silence (S) or tutor (T), sacrifice during the day (D) or night (N).

We analysed neuronal activation in the NCM, HVC, and the hippocampus (figure 4.2). Although the hippocampus is involved in many mammalian memory tasks, it is not involved in song memory or song production in males (Bailey et al. 2009). Since we previously found brain lateralisation of tutor song memory (chapter 2 of this thesis), we analysed neuronal activation in both hemispheres.

Materials and methods

Animals. The 57 juvenile zebra finch males were bred at the Central Animal Facility of Utrecht University. We used the hatching date of the oldest bird as a generalized hatching date for the whole clutch. These dates were usually accurate within a few days. The fathers were removed when the oldest hatchlings were 7 days old, and the cage was moved from the

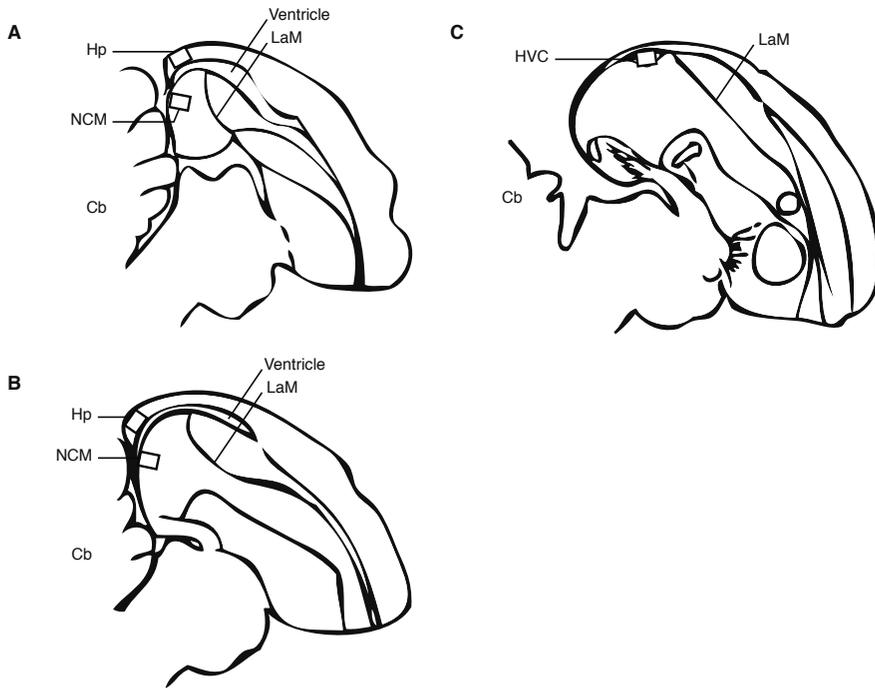


Figure 4.2. Schematic sagittal brain slices showing neural anatomy. Immediate Early Gene (IEG) expression was measured bilaterally in the brain regions NCM, HVC and hippocampus in juvenile male zebra finches. The NCM and hippocampus were measured (A) medially and (B) laterally. C: HVC is located more laterally. The squares indicate the locations where photomicrographs were made. Abbreviations: Cb, cerebellum; Hp, hippo-campus; HVC, used as a proper name; LaM, lamina mesopallialis; NCM, caudomedial nidopallium. Adapted with permission from Terpstra et al. (2006).

animal breeding room to a sound attenuation chamber where the birds could not hear any adult males sing. The young birds were reared with their mothers until 30 days post hatching, after which they were housed separately in similar sound attenuation chambers (chamber size: 115 x 115 x 205 cm; cage size: 40 x 35 x 35 cm). An adult male was moved to each juvenile's cage from day 40 until 50 for the juveniles that received ten days of tutoring experience, or from day 48 until 50 for the juveniles that received two days of tutoring experience (figure 4.1). We chose to not make the tutoring period shorter than 2 days, because we wanted the juvenile to recognize the adult bird as his tutor. We wanted to investigate initial stages of tutor song memory, and not the recognition of a bird that they had heard singing before. From day 50 until day 55, the juveniles remained in sound isolation. Mean age at the day of the experiment was 54.97 days (range 53-56 days). The birds were kept at a 15:9 light regime, with the lights turning on at 6 AM and off at 9 PM, and at a constant temperature of 21°C. Food and water were provided ad libitum, and each bird received millet, egg food or a water bath daily. Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University.

Stimuli preparation. We selected 18 adult males that readily sang when they were moved to the recording chambers as tutors for the experimental birds. The songs of these tutors were recorded prior to the experiment. Recordings were made digitally with Sennheiser MKH 50P48 directional microphones with the recorder of Sound Analysis Pro (Tchernichovski et al. 2000). We calculated how many motifs birds sang in each song bout across a large number of recordings that we made. The songs that were selected for the tutor song re-exposure stimuli consisted of a similar number of motifs. Therefore, the stimuli were a reflection of the natural song pattern of the birds. Ten different song bouts were selected for making the tutor song stimuli. These songs were high-pass filtered (0-400 Hz) and the root-mean-square amplitude of all songs was equalized using Praat software (Boersma & Weenink 2005). Each song was played twice, in random order, so that a total of 20 song bouts was presented to the juveniles. Presenting juveniles with 20 song playbacks per day is sufficient for them to successfully imitate the song (Tchernichovski et al. 1999), and therefore we chose to present 20 playbacks as tutor song re-exposure stimulus. During a 30-minute stimulus exposure session, we used 5 cycles of song playbacks with 7 minutes of silence in between each rendition. Each playback lasted 24 seconds and contained 4 song bouts.

Experimental design. After 2 or 10 days of tutoring experience, birds remained in isolation until day 55 (figure 4.1). On day 55, the lights turned on at 6 AM as usual. However, at 8 AM we turned off the lights, to prevent the birds from singing. For half of the birds, tutor song stimuli were presented from 11 AM until 11.30 AM. The other half of the birds remained in silence. Half of the birds were perfused at 12 PM (noon), the other half at 12 AM (midnight). For the latter birds, we turned the lights on at 12 PM. The birds remained undisturbed during the afternoon, and lights were turned off at 9 PM as usual (figure 4.1). Video and sound recordings were made throughout the experimental day.

Tissue collection and immunocytochemistry. At 12 PM (noon) or 12 AM (midnight), the birds were deeply anesthetized with an overdose of Natriumpentobarbital (Nembutal, Ceva Sante Animale, Libourne, France, 0.06 mL i.m.) and perfused with phosphate-buffered saline (PBS), followed by fixation with fresh, ice-cold 4% paraformaldehyde in PBS. Brains were dissected out, postfixed at 4°C, cryoprotected in 10%, 20% and 30% sucrose in PBS at 4°C, frozen and kept at -20°C until cut. Parasagittal 20- μ m sections were made on a cryostat and mounted on poly-L-lysine coated slides or commercially available StarFrost yellow slides. The brains were incubated with primary polyclonal rabbit antiserum for egr-1 (Zenk; Santa Cruz Biotechnology; dilution 1:1000), overnight at 4°C; biotinylated goat antirabbit (IgG, Vector Laboratories, Burlingame, CA; dilution 1:100), for 1 hour at room temperature; ABC (avidin-biotinylated enzyme complex, Vector Elite Kit, Vector Laboratories), for 1 hour at room temperature; and diaminobenzidine (DAB) medium containing 0.034% H₂O₂ for 6 minutes at room temperature. Left and right sections of the same bird were processed at the same time.

Image analyses. Quantification of Zenk-immunopositive cells was performed for the NCM, HVC and hippocampus as described previously (Terpstra et al. 2004; Terpstra et al.

2006; chapter 2 of this thesis). Brain areas were identified using a stereotaxic atlas that is available online (Nixdorf-Bergweiler & Bischof 2007). Digital photographs were taken using a Leica DFC 4206 camera and the Leica Application Suite program on an Axioskop (Zeiss, Germany) with 20x objective. The NCM photomicrographs were taken at the extreme caudal pole of the neostriatum, immediately adjacent to the ventricle separating the NCM from the hippocampus, at two distances from the midline: medial and lateral. We determined medial and lateral regions based on anatomical characteristics according to previous studies (figure 4.2a, b; c.f. Terpstra et al. 2006). The HVC photomicrographs were taken in the centre of the HVC nucleus (figure 4.2c), and the hippocampus images at the point at which the curve is most pronounced (figure 4.2a, b; cf. Terpstra et al. 2004; Terpstra et al. 2006). Image analysis was performed with a personal computer-based system using KS400 version 3.0 software (Zeiss, Oberkochen, Germany). A program had been previously developed in KS400 to quantify the number of immunoreactive cells semi-automatically (Terpstra et al. 2004; Gobes et al. 2010; chapter 2 of this thesis). Counts of 3 sections for each region in both hemispheres per animal were averaged for further statistical analysis. All image analysis was performed blind as to the experimental history of the subject and brain hemisphere.

Song similarity analyses. At least 10 songs of each juvenile were randomly selected that were sung between 12 PM (noon) and 4 PM on day 54 (the day before perfusion). It was shown that juvenile song has more variance in the morning, while it is more stable in the afternoon (Derégnaucourt et al. 2005). Sound Analysis Pro was used to assess the quality of tutor song imitation of the experimental subjects by calculating the ‘percentage similarity’, which is a measurement of syllable copying. 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). Sound Analysis Pro has a ‘floor-effect’, because zebra finch songs always resemble each other somewhat on these features. We hypothesized that this effect might even be stronger for juvenile song, since it has more noisy features than adult song. To assess the extent of this floor-effect, we compared songs from all juveniles to the songs of 17 unfamiliar birds (“novels”, birds that were used as tutor for the other juveniles). Thereby, we investigated whether our zebra finches had learned from their song tutors specifically or that the similarity was due to general song characteristics. Also, we compared the juveniles’ song similarities to their tutor and novels to an adult example from a previous experiment (chapter 2 of this thesis).

Statistical analyses. *Song similarity data.* We conducted a repeated measures ANOVA to test the differences between similarity to the tutor and the similarity to novels in the two different tutoring experience groups. Then, we did post-hoc paired t-tests with Bonferroni corrections for multiple testing between tutor and novel similarities within each tutoring experience group. We did a one-way ANOVA to compare the tutor song similarities of the juveniles of the current experiment to tutor song similarities of example adults (from a previous study from our lab, chapter 2 of this thesis), to their tutors with post-hoc tests and Bonferroni correction for multiple comparisons. Also, a one-way ANOVA was conducted

to compare the tutor song similarities between the two- and ten-day tutor song experience groups. In addition, we calculated a “learning score” as (mean song similarity percentage to the tutor – mean song similarity percentage to novels). We did this in order to correct for general song similarities caused by noisy syllables of the juveniles, which might differ between the two and ten days tutoring experienced males. The learning score indicates how much of the tutor song similarity is tutor-song specific. We compared learning scores between the two- and ten-day tutor song experience groups and adults using a one-way ANOVA with post-hoc tests and Bonferroni correction for multiple comparisons. *Neural data.* Raw data were square-root transformed, because the data were not normally distributed (z -values for Skewness and Kurtosis >1.96). Repeated measures ANOVA revealed significant differences between brain regions. Then, we conducted 4 separate repeated measures ANOVAs to compare the effect of tutoring experience, stimulus exposure and day or night perfusion on the Zenk response in the left and right lateral NCM, medial NCM, HVC and hippocampus. Post-hoc tests with Bonferroni correction for multiple comparisons were conducted to compare neuronal activation between experimental groups. A lateralisation ratio was calculated by dividing the difference in IEG expression levels between the two hemispheres in the lateral NCM by the total amount of IEG expression of the two hemispheres: $(L-R)/(L+R)$, as was done previously and found to correlate with tutor song similarity (chapters 2 and 3 of this thesis). Data were analyzed using SPSS 20.0.0.

Results

Song learning. Figure 4.3 shows an example tutor song and the songs of two of its tutees, one that was tutored for two days and the other for ten days. We analyzed the similarity between juveniles’ and tutor songs and the similarity between juveniles’ songs and songs of unfamiliar adults (“novels”). Overall, there was a significantly greater percentage similarity (as calculated using Sound Analysis Pro) with the tutor song than with novel songs (figure 4.4; $F_{1,45}=83.238$, $p=0.000$). This was also true for the two-day group separately (paired samples t -test: $t(18)=6.211$, $p=0.000$) and the ten-day group separately (paired samples t -test: $t(27)=7.615$, $p=0.000$). The mean tutor song similarity of the ten-day group (60.97%) was significantly greater than that of the two-day group (54.12%; $F_{1,46}=4.289$, $p=0.044$). We compared the song similarity percentages of the juveniles with adults from an earlier study (chapter 2 of this thesis). The adults had a significantly greater song similarity with the tutor song (69.21%), than with a novel song (45.52%; figure 4.4; paired samples t -test: $t(14)=7.094$, $p=0.000$). The adults had a higher similarity to the tutor song than the juveniles with two days of tutoring experience (Post-hoc test, $p=0.000$, $\alpha=0.017$). The adults also had a higher song similarity to the tutor than juveniles with ten days of tutoring experience, but this was not significant after correction for multiple testing (Post-hoc test, $p=0.030$, $\alpha=0.017$). There was no difference in the similarity to the novels between the groups with two or ten days of tutoring experience or the adults ($F_{2,61}=0.689$, $p=0.502$).

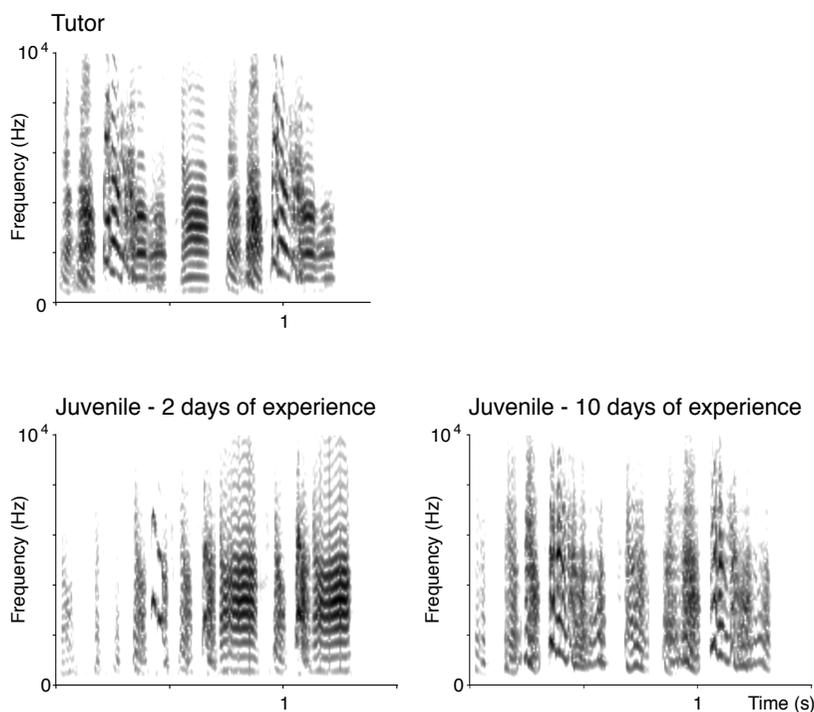


Figure 4.3. Spectrograms of a tutor and two of its tutees. Top: one of the adult males that was a tutor to several of the experimental birds. Bottom: A two-days tutoring experienced juvenile that had a song similarity of 41.02% with its tutor. (left), and a juvenile with ten days of tutoring experience, that had a song similarity of 64.12% with its tutor.

Furthermore, learning scores were calculated for each bird (mean song similarity percentage to the tutor – mean song similarity percentage to novels). The learning scores of both tutoring experience groups were significantly different from zero (One-sample t-tests: 2 days, mean learning score=9.00, $t(17)=7.409$, $p=0.000$; 10 days, mean learning score=12.10, $t(25)=6.838$, $p=0.000$), thus again showing that birds in both tutoring experience groups significantly imitated the tutor song. A one-way ANOVA revealed a trend towards a significant difference between the learning scores of two- and ten-days tutored birds ($F_{1,46}=3.384$, $p=0.072$), suggesting that the ten-days tutored group might have been more proficient already than the two-days tutored group. We also analysed the imitation accuracy of each syllable that was imitated from the tutor song, and the order of the juvenile's syllables in comparison to the tutor song (as calculated using Sound Analysis Pro), to assess other aspects of tutor song imitation in addition to song similarity percentage. The two tutoring experience groups had a similar imitation accuracy ($F_{1,46}=0.160$, $p=0.691$) and sequential match ($F_{1,46}=0.028$, $p=0.868$) with the tutors' songs. In conclusion, even though the juveniles were in the sensorimotor phase and still practising their song, we were able to show significant tutor song imitation in both groups.

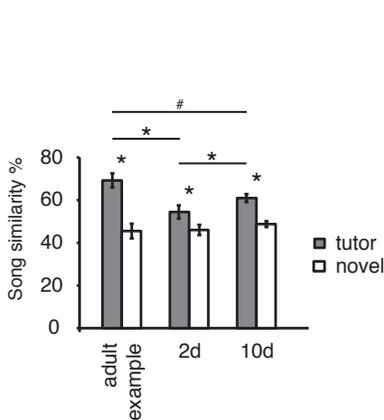


Figure 4.4. Song similarities. Mean song similarity percentages per tutor experience group, as calculated using Sound Analysis Pro (SAP 2011). Filled bars represent the song similarity of the experimental birds to its tutor; open bars the song similarity of the experimental bird to 17 novels – adult birds that were tutors to other juveniles, but were unfamiliar to the experimental bird. The two- and ten-days tutored groups are shown separately, and in addition a group of example adult birds is presented in the graph. These birds were not used for the current experiment, but are shown here to illustrate the level of tutor song imitation in adult birds that were raised with their father. Error bars represent the SEM. An asterisk (*) indicates a significant difference ($p < 0.05$), while the hash sign (#) indicates a trend towards a significant effect that was not significant after correction of multiple testing ($p = 0.030$, $\alpha = 0.017$).

Brain activation. Repeated measures ANOVA revealed a significant effect of Brain Regions ($F_{3,41} = 23.132$, $p = 0.000$; figure 5). Visual inspection of neuronal activation shows great neuronal activation in the NCM, and low levels of activation in HVC and the hippocampus. Furthermore, there was little neuronal activation during sleep (darkly coloured bars in figure 4.5a, b). Generally, neuronal activation in the medial NCM was very similar to the lateral NCM, but neuronal activation in the lateral part of the NCM was slightly lower than in the medial part (figure 4.5a, b). Indeed there was no significant difference between the lateral and medial part of the NCM ($F_{1,45} = 0.003$, $p = 0.959$), but ANOVA revealed a significant interaction between the NCM-region (the lateral and medial part of the NCM) and Hemisphere ($F_{1,45} = 7.075$, $p = 0.011$). For this reason, we analysed the two subregions separately. The lateral NCM might be more important for tutor song memory, since previous studies demonstrated a correlation between amount of Zenk-expression in the lateral NCM and the strength of tutor song imitation (Bolhuis et al. 2000b; Bolhuis et al. 2001; Gobes et al. 2010; Van Der Kant et al. 2013; chapter 3 of this thesis). Furthermore, the lateral NCM is necessary to imitate tutor song during song learning (London & Clayton 2008) and to recognise tutor song when adult (Gobes & Bolhuis 2007). *Neuronal activation in the lateral NCM was left-lateralised.* As the interaction between NCM-region and Hemisphere suggests, there was a significant effect of Hemisphere in the lateral part of the NCM ($F_{1,45} = 7.741$, $p = 0.008$), with the left hemisphere being more activated than the right consistently (figure 4.5a). Neuronal activation in the medial NCM was not lateralised ($F_{1,45} = 0.016$, $p = 0.900$). The left-sided dominance in the lateral NCM was significant for all experimental groups together, but not within groups separately. When the two tutoring experience groups were analysed separately, we found that lateralisation was significant in the birds that had been housed with the tutor for ten days ($F_{1,21} = 4.505$, $p = 0.046$), and a trend towards significance was found in the two day group ($F_{1,24} = 3.103$, $p = 0.091$). Unlike previous findings in adults and more tutor song-experienced juveniles (Bolhuis et al. 2000b; Terpstra et al. 2004; Gobes et al. 2010; chapters 2 and 3 of this thesis), the juveniles' song

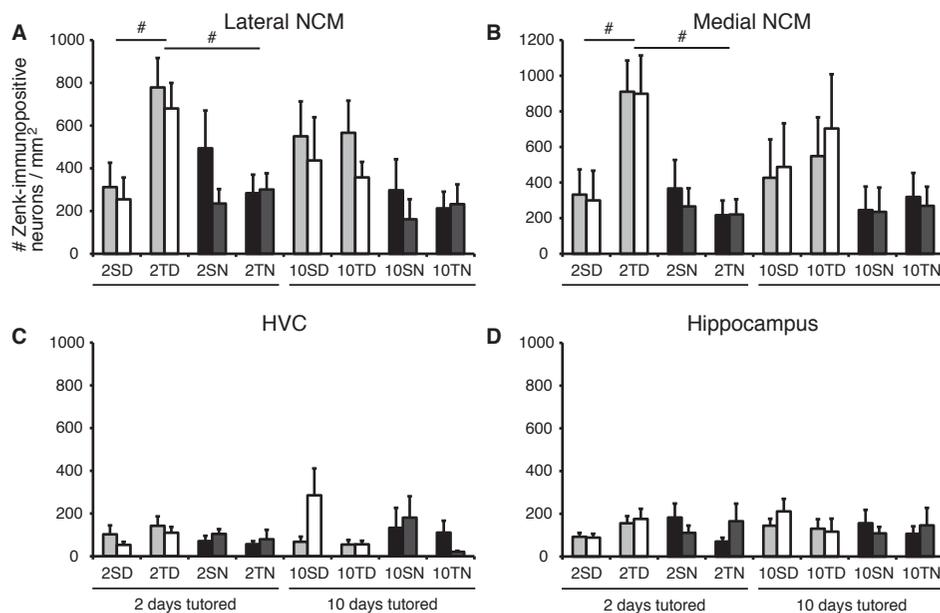


Figure 4.5. Mean Zenk expression levels. The mean number of Zenk-immunopositive neurons per square millimeter is shown for the different brain regions. Results are shown for all eight experimental groups, and both the left and right hemispheres. **Abbreviations:** 2SD, 2TD, 2SN, 2TN, 10SD, 10TD, 10SN and 10TN: housed with the tutor for a period of 2 or 10 days, stimulus is silence (S) or tutor (T), sacrifice during the day (D) or night (N). The light bar-combinations indicate groups that were sacrificed during the day (grey, left hemisphere; white, right hemisphere) and the dark bar-combination indicate groups that were sacrificed during the night (black, left hemisphere; dark grey, right hemisphere). Error bars represent the SEM. A hash sign (#) indicates a difference between experimental groups, with $p < 0.05$, but uncorrected for multiple testing. **A:** Neuronal activation was left-dominant in the lateral NCM of juveniles in all stimulus groups together, and 2TD had the highest level of neuronal activation. **B:** Neuronal activation in the medial part of the NCM was slightly higher than in the lateral part (note the different y-axis scales), and 2TD had the highest level of neuronal activation. There was greater tutoring-induced neuronal activation than spontaneous activation during the day, but not during sleep. **C:** Low levels of neuronal activation in HVC. **D:** Low levels of neuronal activation in the hippocampus. Levels of neuronal activation did not differ between experimental groups or hemispheres.

similarity percentage with the tutor song was not correlated to neuronal activation in the NCM nor to the lateralisation ratio (see materials and methods).

Birds with two days of tutoring experience had the highest neuronal responses to tutor song in the NCM. There was no significant effect of tutoring experience in the NCM ($F_{1,45} = 0.510$, $p = 0.479$). Perhaps this could be related to the fact that juveniles in both experience groups had already started imitating the tutor song, and thus had already formed a tutor song memory representation. Although there was no significant effect of Tutoring Experience, there were some differences between the experimental groups. During the day, there was a significant effect of Stimulus in the medial NCM, with greater neuronal activation after tutor song re-exposure than silence ($F_{1,22} = 5.191$, $p = 0.033$), and we found

a strong trend towards a significant effect of Stimulus during the day in the lateral NCM ($F_{1,22}=4.085$, $p=0.056$). The group that had been housed with the tutor song for two days, received the tutor song as stimulus on day 55 and was sacrificed during the day (2TD) had the highest levels of neuronal activation (figure 4.5a, b). Post-hoc tests revealed that there was a difference between the tutor and silence stimulus groups (2TD versus 2SD: lateral $p=0.014$, medial $p=0.017$; although not significant after correction for multiple testing: $\alpha=0.05/28=0.0018$) and between day and night (2TD versus 2TN; lateral $p=0.028$, medial $p=0.008$; although not significant after correction for multiple testing: $\alpha=0.05/28=0.0018$). These differences were not demonstrated in birds that had been housed with the tutor for ten days (10TD versus 10SD; 10TD versus 10TN; figure 4.5a, b). Visual inspection showed greater neuronal activation after two days than ten days of tutoring experience in birds that had been re-exposed to the tutor song and were sacrificed during the day (2TD versus 10TD; figure 4.5a, b), but this effect was not significant (lateral: $p=0.228$, medial: $p=0.287$). Furthermore, birds that received ten days of tutoring experience and remained in silence on the experimental day (10SD) showed greater spontaneous neuronal activation in the lateral part of the NCM than birds that had been housed with the tutor for two days (2SD; figure 4.5a), also non-significantly (lateral: $p=0.260$, medial: $p=0.690$).

Low levels of neuronal activation during sleep. There was significantly greater neuronal activation during the day than during the night in the NCM (lightly versus darkly coloured bars in figure 4.5a, b; lateral: $F_{1,45}=7.875$, $p=0.007$; medial: $F_{1,45}=7.260$, $p=0.010$), indicating that contradictory to our expectations, there was little neuronal activation during night-time sleep. This might indicate that tutor song memory consolidation does not occur when birds are sleeping in these early stages of memory formation. There was not much Zenk expression during night-time sleep, but we found a significant interaction between Hemisphere and Stimulus in the lateral NCM during sleep ($F_{1,23}=6.846$, $p=0.015$). This was caused by stronger left-dominance in the silence groups, while the birds that had been re-exposed to tutor song showed more bilateral activation patterns. This effect was strongest in the birds that had experienced two days of tutoring (figure 4.5a, darkly coloured bars).

Low levels of neuronal activation in HVC and the hippocampus. We found relatively little neuronal activation in the hippocampus (mean was approximately 135 Zenk-immunopositive cells per mm^2), and there were no significant effects or interactions (figure 4.5d). HVC also showed a generally low level of neuronal activation (figure 4.5c). The mean activation was approximately 100 Zenk-immunopositive neurons per mm^2 , which was even lower than in the hippocampus. In one bird that sang in the hour before sacrifice (which was during the day; we discovered this on audio recordings that we made), HVC was highly activated, and we excluded this bird from analyses. There were some other juveniles that had higher levels of HVC activation than others: 9 out of 56 birds distributed over different experimental groups had more than 200 Zenk-immunopositive neurons per mm^2 in the left, right or bilateral HVC, including values as high as 900 Zenk-immunopositive neurons per mm^2 . These birds did not sing during the experiment, and they were distributed over different

experimental groups. Thus, we do not know why neuronal activation in HVC of these birds was higher than in other birds. There was a significant interaction between Hemisphere and Stimulus ($F_{1,47}=5.694$, $p=0.021$), caused by a difference between tutor and silence in the right HVC ($F_{1,55}=5.125$, $p=0.028$). In the birds that had ten days of tutoring experience, remained in silence on the experimental day and were sacrificed during daytime (10SD), the highest level of neuronal activation was found in the right HVC (figure 4.5c). However, neuronal activation in the right HVC was not significantly greater than the left within this experimental group (paired samples t-test, $t(5)=-1.487$, $p=0.197$). Two out of six birds in this experimental group had high levels of activation in the right HVC, but since these birds did not sing during the experiment, and we did not notice any differences between them and the rest of the birds, we do not know why their right-sided HVC was activated.

Discussion

In this study we explored the neural mechanisms underlying the early stages of tutor song memory formation in juvenile male zebra finches. We investigated whether birds with relatively brief tutoring experience showed differential brain activation patterns from birds that received ample tutor song exposure. We explored patterns of brain activation during the day and during night-time sleep, spontaneous activation versus activation induced by tutor song re-exposure, and compared activation in both cerebral hemispheres.

Significant song learning after only two days of tutoring experience. By comparing the juveniles' songs to songs of their tutor and to unfamiliar songs, we showed that the juveniles exhibited significant song learning. Interestingly, this was also true for the birds that had just two days of tutoring experience, which is even shorter than what was reported previously (Tchernichovski et al. 2001; Roper & Zann 2006; Braaten 2010). Similar to our results, it was recently shown that juvenile male zebra finches that had heard only 75 seconds of tutor song (which equals 100 motif representations; playbacks spread out over 2 hours on one day, triggered by the juveniles pulling a string) were able to imitate the song later in life. Song imitation was measured when the birds had reached adulthood, but changes in the juveniles' songs were detected on the day of tutoring and the day after tutoring already, suggesting very rapid song learning (Deshpande et al. 2014). Here we show that significant tutor song imitation indeed happens very rapidly and can already be quantified in juveniles.

Low levels of neuronal activation in the NCM during the night. Since sleep is important for memory consolidation (Diekelmann & Born 2010), and high levels of neuronal activation during sleep were previously shown in songbirds (Hahnloser et al. 2006; Shank & Margoliash 2009; Gobes et al. 2010; chapter 3 of this thesis). Consequently, we expected to find great neuronal activation in the NCM during sleep. In contrast, we found relatively little neuronal activation in the NCM or HVC during night-time sleep in both tutor-experience groups. It could be argued that we did not measure neuronal activation at the right time point (due to our method, Zenk immunocytochemistry, we had to kill each animal in order to measure

neuronal activation, and thus we had only one time point during the night). However, previous studies using similar methods demonstrated high levels of spontaneous activation at this time point during sleep (Gobes et al. 2010; chapter 3 of this thesis). The birds used in those studies received longer periods of tutoring and were kept in silence during the day. However, when they were re-exposed to the tutor song, neuronal activation levels during sleep were low. The authors suggested this might have been the result of neuronal habituation (Gobes et al. 2010). It may be that we induced habituation to the tutor song as well, even though we used brief tutoring periods. Indeed, habituation occurs very quickly, already after a few song presentations (the neuronal response to a song stimulus is already much lower to the second than to the first stimulus playback, and it is about half of the initial response after about 50 presentations; Chew et al. 1995; Stripling et al. 1997). Moreover, it was recently shown that neuronal activation in response to tutor song habituated faster in juvenile zebra finch males than adults (Miller-Sims & Bottjer 2012). The birds in our experiment had not heard the tutor song for four days; however, song habituation is a long-term effect that can last 48 hours at least (Chew et al. 1995) and even up to two months (Woolley & Doupe 2008). Alternatively, memory consolidation-related neuronal activation might not occur during sleep, but mainly during the day in these initial stages of song learning. This is a hypothesis that was also recently suggested by others (Deshpande et al. 2014). In an operant song tutoring experiment in juvenile male zebra finches in which birds received only one day of tutoring, it was shown that tutoring experience-related song changes were found on the day of tutoring already, so before the juveniles had had the chance to sleep during the night (Deshpande et al. 2014). Day-time consolidation could either happen when the birds are awake, or while they are taking day-time naps. It has been described that juvenile songbirds frequently take day-time naps (Margoliash & Schmidt 2010), including directly after their first tutoring session in the lab (T. Lints and O. Tchernichovski, unpublished results, as cited in Margoliash & Schmidt 2010), which might promote song imitation (Whitaker et al. 2012).

The NCM was highly activated in two-days tutored, awake birds that were re-exposed to the tutor song. Both two and ten days-tutored birds significantly imitated the tutor song, and similarly, there was no significant effect of tutoring experience (two versus ten days) on neuronal activation. However, during the day, the birds that had two days of tutoring experience and were exposed to the tutor stimulus on the day of the experiment (2TD) showed higher levels of neuronal activation in the lateral NCM than ten-days experienced birds (10TD). This suggests that the NCM is involved in the early memorization process of tutor song. Furthermore, birds that received ten days of tutoring experience and remained in silence on the experimental day (10SD) showed non-significant greater spontaneous neuronal activation in the NCM than birds that had been housed with the tutor for two days (2SD). Perhaps this indicates a difference in spontaneous memory consolidation, which might occur more readily after longer training durations.

Left-sided dominance in the NCM. The left lateral NCM was consistently more activated than the right in all groups taken together. That means that also in the birds that remained in silence

before sacrifice, spontaneous neuronal activation was slightly left-dominant. In contrast, in a previous study in juveniles that had received longer tutoring experience, it was shown that lateralisation in the NCM was tutor-stimulus specific (chapter 2 of this thesis). When birds completely lacked auditory experiences, there was no lateralisation (Phan & Vicario 2010; Chirathivat et al. 2013). The initial phase of learning that the birds in our current experiment were in might have caused lateralised spontaneous NCM activation during the day and the night. Since lateralisation is implicated in human speech and speech acquisition, but its exact role is unknown, songbird lateralisation provides an opportunity to study the function of brain lateralisation in auditory-vocal learning. Understanding the temporal and spatial dynamics of lateralisation in juvenile songbirds that are learning to sing is crucial in this respect.

Low levels of neuronal activation in HVC. There were very low levels of activation in HVC. The behavioural state of the animals might have caused lateralised spontaneous activation in some individuals (as in chapter 3 of this thesis). Because these birds were in the sensorimotor phase, HVC might have been spontaneously activated as in previous experiments (chapters 2 and 3 of this thesis). In those studies, juveniles of a similar age but with more tutoring experience showed left-dominance of spontaneous HVC activation during the day, and right-dominance during night-time sleep. However, the direction of lateralisation differed between birds and lateralisation was not a consistent effect within groups.

Conclusions. Here we showed that juvenile male zebra finches already started to imitate their tutor's song after just two or ten days of tutoring experience. Neuronal responsiveness to the tutor song in the NCM was highest in the juveniles that had two days of tutoring experience. In both two and ten days tutoring experienced juveniles, neuronal activation in the NCM was left-dominant. Even though sleep is considered to be important for song acquisition and memory consolidation, there was not much neuronal activation during night-time sleep. Thus, memory consolidation might take place primarily during the day in these initial stages of song learning.

5

Early formation of neural representations of song memory in juvenile female zebra finches

A different version of this chapter is being prepared for publication.

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Abstract

Birdsong memory formation is often studied in male songbirds, because they vocally imitate tutor song. Consequently, the neural mechanisms of tutor song memory in male songbirds are relatively well understood. However, song memory in males also guides song imitation, and neuronal activation might thus be a reflection of auditory memory, sensorimotor learning, or both. Female zebra finches do not sing, but do also form a memory representation of the song of their father. Thus, research in females allows studying the neuronal mechanisms that are used for auditory memory formation exclusively. Here, we investigated memory formation in juvenile female zebra finches and compared it to a similar previous study in males (chapter 4 of this thesis). As we did previously in juvenile males, we housed female songbirds with an unrelated adult male (tutor) for two or ten days. Then, tutor song recognition was measured in phonotaxis preference tests, a method in which adult females have been shown to prefer tutor song over unfamiliar song. Four days later, neuronal activation in response to tutor song was measured in two brain regions that are involved in song perception, recognition and memory: the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM), two higher-order auditory brain regions. The NCM is crucially involved in tutor song memory in males, while the CMM was previously demonstrated to be important for father's song memory in female songbirds. In contrast to significant song learning in juvenile males (chapter 4 of this thesis), there was no evidence for tutor song recognition in the juvenile females in the current experiment; neither in the two- nor ten-day tutored birds. However, there was substantial neuronal activation in both the NCM and CMM. More specifically, in the females that had been housed with the tutor for two days, there was great neuronal activation in response to tutor song in the NCM, while birds that had been housed with the tutor for ten days showed significant tutor-song induced neuronal activation in the CMM. This suggests that the NCM is more important for initial song memory acquisition, while the CMM is more important during later stages of tutor song memory.

Introduction

Human infants and juvenile songbirds learn vocalisations from adult conspecifics, usually their parents. Both humans and songbirds have a separate memorization and sensorimotor phases in which vocalisations are learned. In male zebra finches, experiments showed that there is a sensitive phase in which memorization (ca. day 25-60) and sensorimotor learning (ca. day 30-90) most efficiently occurred (Eales 1985; Böhner 1990; Roper & Zann 2006; Braaten 2010). Also the neural mechanisms of tutor song memory and sensorimotor learning in male songbirds are relatively well understood. However, song memory in males probably also guides song imitation, and neuronal activation might thus be a reflection of auditory memory, sensorimotor learning, or both. Female zebra finches do not sing, but like males, they also form a memory of the song of their father (Bolhuis & Eda-Fujiwara 2003). When females' stimulus preference was measured using playbacks of tutor song and an unfamiliar conspecific song on two sides of a cage, it was demonstrated that adult females preferred to spend more time at the speaker where the father's song was played (Terpstra et al. 2006). Furthermore, in operant tests, females peck more at the key that elicits playback of the father's song than other songs (Riebel et al. 2002). This shows that females recognize the song of their father, implicating that they have formed a memory of the father's song. Similar to males, females probably also have a sensitive phase for song memory that starts when birds are around 25 days of age (Riebel 2003).

Since female zebra finches do not sing, research in females allows studying the neuronal mechanisms that are used 'purely' for auditory memory formation (Bolhuis & Eda-Fujiwara 2003). There are two higher-order auditory brain regions that are crucially involved in tutor song memory: the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM). The NCM was found to be important for tutor song memory in male songbirds (Mello et al. 1995; Chew et al. 1996; Bolhuis et al. 2000b; Terpstra et al. 2004; Gobes & Bolhuis 2007; London & Clayton 2008; Canopoli et al. 2014). In adult females, it was shown that the CMM was highly activated upon father's song exposure (Terpstra et al. 2006). The CMM was also activated in response to tutor song in males, but the activation was uncorrelated to song learning, in contrast to activation in the NCM that was correlated to the level of tutor song imitation (Terpstra et al. 2004). Although it is clear that the NCM and CMM are important for tutor song memory, it is not fully understood what their exact individual roles are, especially not of the CMM, how the NCM and CMM relate to each other, or how their roles develop during memory formation.

Here, we investigated neuronal activation in the NCM and CMM during early tutor song memory formation in juvenile female zebra finches. To this extend, we housed juvenile females with an unrelated adult male (tutor) for two or ten days, in a similar way as in a previous experiment in males (chapter 4 of this thesis; figure 5.1). Then, tutor song recognition was measured in phonotaxis preference tests, a method in which adult females have been shown to prefer tutor song over unfamiliar song. Four days later, birds were sacrificed to study

neuronal activation. As we were interested in a possible role for sleep in memory formation (Diekelmann & Born 2010), birds were sacrificed either directly after re-exposing them to the tutor song or keeping them in silence, or in the subsequent night when the animals were asleep (figure 5.1). We analysed neuronal activation in three brain regions bilaterally (figure 5.2): the CMM, NCM, and the hippocampus, a region that might play a role in song perception and tutor song memory in adult females (Bailey et al. 2002; Bailey & Wade 2005; Bailey et al. 2009; Bailey & Saldanha 2010). We compared these results in females with previous findings in similarly treated juvenile male zebra finches (chapter 4 of this thesis).

Materials and methods

Animals. The 59 juvenile female zebra finches were bred at the Central Animal Facility of Utrecht University. Experimental procedures were in accordance with European law and approved by the Animal Experiments Committee of Utrecht University. More details on housing, tutoring, stimuli, brain tissue collection and Zenk expression analyses can be found in chapter 4 of this thesis (and see figure 5.1). Mean age at the day of the experiment was 55.13 days post hatching (range 54-56 days).

Preference tests. Females were tested in a phonotaxis preference test at day 51 (see figure 5.1; mean age at the day of preference testing=51.39, range=50-53 days), in which we alternately presented tutor song and novel song (unfamiliar conspecific song) to the birds at opposite sides of a cage. Previously it was shown that birds spent more time at the side where tutor song was played (e.g., Miller 1979a; Terpstra et al. 2006). Therefore, using this paradigm, we could test whether the juveniles recognized the tutor song. We placed the birds in the test cage on the day before the preference test. The test cage was divided into two approach zones (left and right), and a central zone in which food and water were available ad libitum. For logistical reasons, we used two different cages for preference testing; one had three compartments that were each 40 cm wide (cage size: 120 x 40 x 40 cm), the other had a larger middle compartment of 60 cm, while the approach zones were 30 cm wide (cage size: 120 x 40 x 45 cm). There was no difference in approach zone preference between the birds that were tested in different cages ($F_{1,41}=0.013$, $p=0.910$). In both cages, the birds could fly freely between all zones. A speaker was placed at either side of the cage. During 10 minutes, one of the songs (tutor or novel; chosen randomly for each bird) was broadcast on the left, and alternately the other song on the right (whether left or right played first was also determined randomly). Ten different tutor song bouts were selected for making the stimuli for the phonotaxis tests. These songs were high-pass filtered (0-400 Hz) and the root-mean-square amplitude of all songs was equalized using Praat software (Boersma & Weenink 2005). Each song playback consisted of 5 song bouts and lasted 30 seconds. The presentations of songs alternated, with 30 seconds of silence in between (for example, playbacks started on the left side with tutor song for 30 seconds, then there was 30 seconds of silence, and then the novel song was played on the right side of the cage). After a silent interval of 10 minutes, another session of song playbacks followed, in

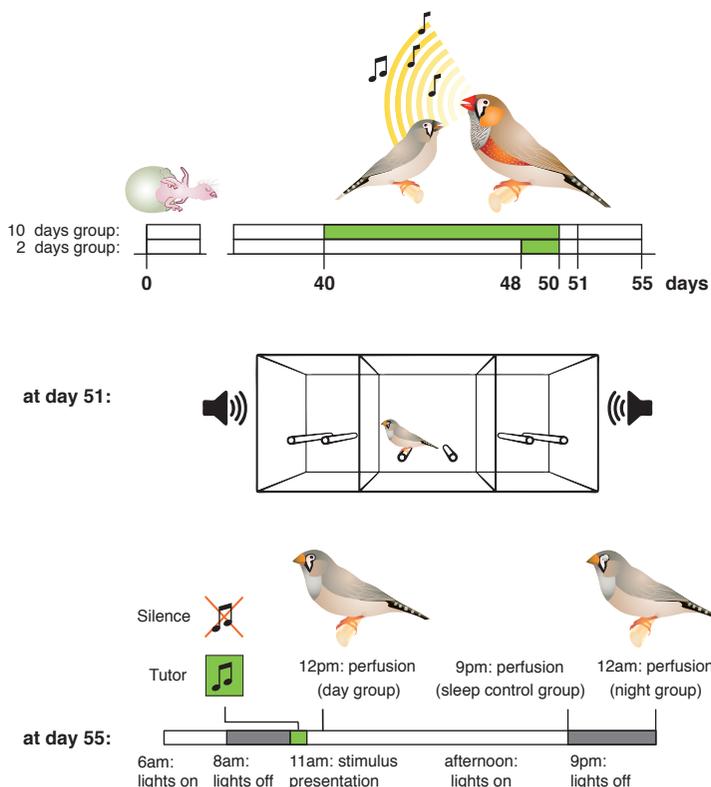


Figure 5.1. Schematic of experimental design. Top: Birds were raised in isolation until 40 or 48 days post hatching. Then, an adult male was moved to their cage for a period of either two or ten days, so that the juveniles could memorize their songs. Middle: At day 50, birds were moved to a preference test cage to adjust to the new environment. At day 51 a preference test was taken, in which tutor song and an unfamiliar conspecific song were played alternatively (see text). Bottom: At day 55 half of the experimental subjects did not receive auditory stimulation ('silence'), while the other half was re-exposed to their tutors' song. Half of the animals were sacrificed after stimulus presentations, the other half during the subsequent night, when the birds were asleep. Therefore, there were 8 experimental groups: 10SD, 10TD, 10SN, 10TN, 2SD, 2TD, 2SN and 2TN - housed with the tutor for a period of 10 or 2 days, stimulus is silence (S) or tutor (T), sacrifice during the day (D) or night (N).

which the sides from which the two songs were played were reversed (in the same example as before, the playbacks would start with novel song on the left side of the cage). The birds' behaviour was scored using the computer programme JWatcher (Blumstein et al. 2000). We scored the time spent in each zone, and the number of long distance calls that the birds made, because juvenile female white-crown sparrows were previously shown to call more in response to tutor stimuli than unfamiliar songs (Nelson et al. 1997). Behaviour was scored blind as to the order and sides of the cage at which tutor and novel songs were played. For each bird, an approach zone preference score was calculated, by dividing time spent in the part where the tutor song was played by the total time spent in the two approach zones; call ratios

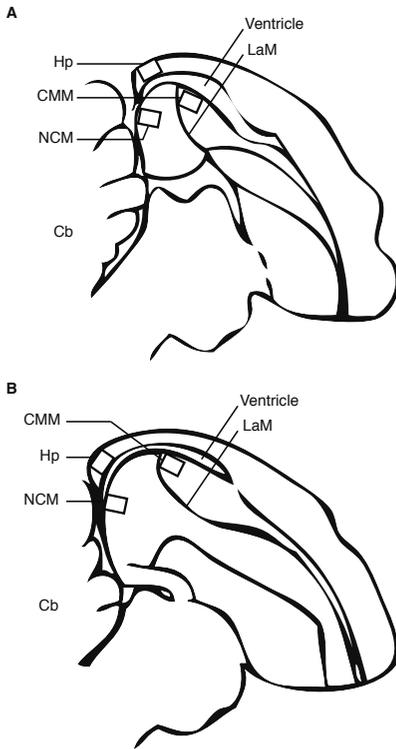


Figure 5.2. Schematic sagittal brain slices showing neural anatomy. Immediate Early Gene (IEG) expression was measured bilaterally in the brain regions NCM, CMM and hippocampus in juvenile female zebra finches. Photomicrographs were made (A) medially and (B) laterally, locations are indicated by the squares. Abbreviations: Cb, cerebellum; CMM, caudomedial mesopallium; Hp, hippocampus; LaM, lamina mesopallialis; NCM, caudomedial nidopallium. Adapted, with permission, from Terpstra et al. (2006).

were calculated by dividing the number of calls emitted during tutor song presentation or in the 30 seconds immediately after tutor song playbacks by the total number of calls emitted by the bird. Furthermore, we calculated the latency (time) between stimulus onset and the first move and first vocalisation of each bird. Birds who failed to reach the following three activity criteria were considered too passive to give any insight on their stimulus preference, and were therefore excluded from analysis: (1) spend more than 10% of the total test time in one of the approach zones, and (2) make at least two choices for the approach zones per ten minutes stimulus playbacks. Based on these criteria, we excluded 7 birds, and we missed preference analyses of 14 birds due to experimental procedural problems or because we did not conduct a preference test for logistical reasons.

Stimuli preparation. We selected 18 adult males that readily sang when they were moved to the recording chambers as tutors for the experimental birds. The songs of these tutors were recorded prior to the experiment. Recordings were made digitally with Sennheiser MKH 50P48 directional microphones with the recorder of Sound Analysis Pro (Tchernichovski et al. 2000). We calculated how many motifs birds sang in each song bout across a large number of recordings that we made. The songs that were selected for the tutor song re-exposure stimuli consisted of a similar number of motifs. Therefore, the stimuli were a reflection of the natural song pattern of the birds. Ten different song bouts were selected

for making the tutor song stimuli. These songs were high-pass filtered (0-400 Hz) and the root-mean-square amplitude of all songs was equalized using Praat software (Boersma & Weenink 2005). Each song was played twice, in random order, so that a total of 20 song bouts was presented to the juveniles. During a 30-minute stimulus exposure session, we used 5 cycles of song playbacks with 7 minutes of silence in between each rendition. Each playback lasted 24 seconds and contained 4 song bouts.

Statistical analyses.

Preference test data. We conducted one-way ANOVA and 4 subsequent one-sample t-tests to find out whether the approach zone preference ratio or call ratio were different between the training groups, and for each group and ratio whether they were different from chance level (0.5).

Neural data. Raw data were square-root transformed, because the data were not normally distributed (z-values for Skewness and Kurtosis >1.96). Repeated measures ANOVA revealed significant differences between brain regions. Therefore, we conducted 5 separate repeated measures ANOVAs to compare the effect of stimulus exposure on the Zenk response in the left and right lateral NCM, medial NCM, lateral CMM, medial CMM and hippocampus. Data were analyzed using SPSS 20.0.0.

Results

No behavioural song preference. There were large inter-individual differences in preference ratios, ranging from a tutor preference, to no preference or a preference for the unfamiliar song (figure 5.3a). Call ratios were uncorrelated with approach zone ratios (figure 5.3a), showing it was unpredictable whether a bird would express a stimulus preference through approaching the speaker, vocalizing, or both. On average, neither the juvenile females that had two days of tutoring experience (approach zone ratio: $t(17) = -0.546$, $p = 0.592$; calls ratio: $t(17) = -0.319$, $p = 0.754$), nor the birds that had been housed with the tutor for ten days (approach zone ratio: $t(23) = -0.878$, $p = 0.389$; calls ratio: $t(22) = 0.711$, $p = 0.485$) showed a preference for tutor or unfamiliar song (figure 5.3b). One-way ANOVA did not reveal differences in approach zone preference ratio ($F_{1,41} = 0.054$, $p = 0.818$) or call ratio ($F_{1,40} = 0.444$, $p = 0.509$) between the two tutoring-experienced groups.

Previously it was shown that birds temporally suspended their activities when they heard song playbacks. The duration of response latency was longer for novel than familiar stimuli (Stripling et al. 2003). However, in our current experiment, there were no differences in response latencies between 2- and 10-day trained females (latency to move: $F_{1,37} = 1.769$, $p = 0.192$; latency to vocalise: $F_{1,37} = 0.107$, $p = 0.745$).

Overall, we were unable to demonstrate any song preferences. This indicates that either the juvenile females did not recognise tutor song as familiar song; or they did recognise the tutor song, but did not show it in our preference tests.

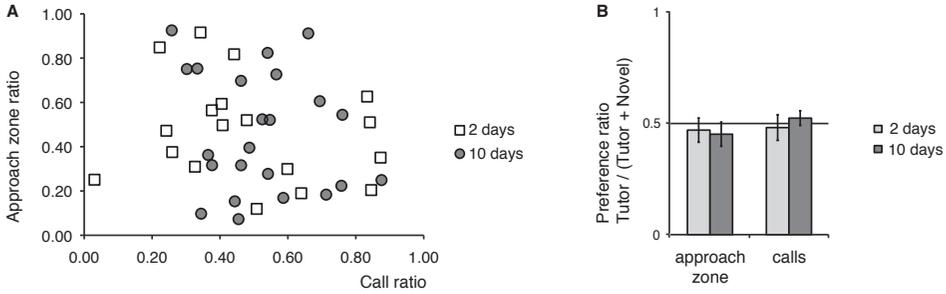


Figure 5.3. Preference tests. A: The distribution of the approach zone and call ratios of each two- or ten-day trained female are shown. The ratios are uncorrelated. B: Mean approach zone ratios and call ratios for both training groups were around chance level (0.5). Error bars represent the SEM. In both graphs A and B, a ratio closer to 1 indicates a preference for the tutor song, a ratio around 0.5 indicates there is no preference, and a ratio closer to 0 indicates a preference for the novel song.

Neuronal activation differs between brain regions. There was a significant effect of Brain Region (lateral CMM, medial CMM, lateral NCM, medial NCM, and hippocampus; figure 5.4; $F_{4,10}=12.415$, $p=0.001$). Therefore, we analysed brain regions separately. As previous research indicated lateralisation of tutor song memory-related neuronal activation in the NCM (chapters 2, 3 and 4 of this thesis), we also analysed possible differences between neuronal activation in the left and right hemispheres. However, we found no lateralisation effects in these juvenile females (figure 5.4).

Tutor song re-exposure induced great neuronal activation during the day in the CMM of birds with ten days of tutoring experience. Since research in awake adult female zebra finches suggested that the CMM is important for tutor song memory in females (Terpstra et al. 2006), we first analysed neuronal activation in the CMM. Neuronal activation in the medial part of the CMM was significantly different from the lateral part ($F_{1,43}=19.805$, $p=0.000$), with higher activation levels in the lateral CMM (figure 5.4a, b). However, there were no significant effects or interactions in the lateral CMM, while there was a significant interaction between Stimulus and Day/Night in the medial CMM ($F_{1,43}=8.282$, $p=0.006$). There was a significant effect of Stimulus in the medial CMM during the day ($F_{1,21}=5.023$, $p=0.036$), with more neuronal activation in birds that had been re-exposed to the tutor song than in birds kept in silence on the experimental day, especially in birds who had been housed with the tutor for 10 days (10TD and 2TD, figure 5.4b). Indeed, post-hoc tests showed a difference between 10TD and 10SD, in both the medial and lateral CMM (although not significant after correction for multiple testing: medial, $p=0.019$, lateral, $p=0.030$; $\alpha=0.05/28=0.0018$). Furthermore, there was more neuronal activation in the medial CMM during the day than night in birds that had been housed with the tutor for 2 days, and had been re-exposed to tutor song on the day of the experiment (2TD versus 2TN; although not significant after correction for multiple testing: $p=0.022$, $\alpha=0.05/28=0.0018$).

Neuronal activation in the CMM during sleep. ANOVA revealed a non-significant effect of Stimulus during sleep in the medial CMM ($F_{1,20}=3.094$, $p=0.094$), with an opposite

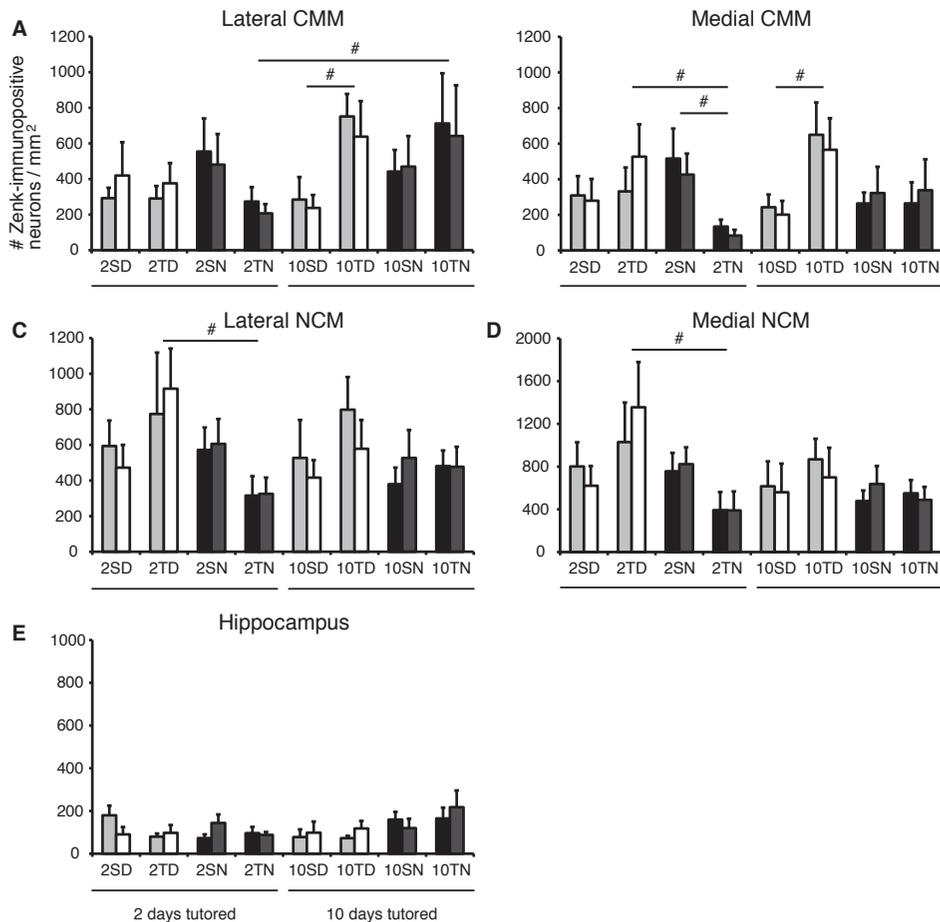


Figure 5.4. Mean Zenk expression levels. The mean number of Zenk-immunopositive neurons per square millimeter is shown for the different brain regions. Results are shown for all eight experimental groups, and both the left and right hemispheres. **Abbreviations:** 2SD, 2TD, 2SN, 2TN, 10SD, 10TD, 10SN and 10TN: housed with the tutor for a period of 2 or 10 days, stimulus is silence (S) or tutor (T), sacrifice during the day (D) or night (N). The light bar-combinations indicate groups that were sacrificed during the day (grey, left hemisphere; white, right hemisphere) and the dark bar-combination indicate groups that were sacrificed during the night (black, left hemisphere; dark grey, right hemisphere). Error bars represent the SEM. A hash sign (#) indicates a difference between experimental groups, with $p < 0.05$ but uncorrected for multiple testing. Note the different y-axis scales. **A:** Neuronal activation in the lateral CMM were highest in birds that had been housed with the tutor for ten days and were re-exposed to the tutor song on the day of the experiment. **B:** Neuronal activation in the medial CMM is lower than in the lateral CMM, but as in the lateral part, there was a significant higher neuronal response to tutor song than silence during the day in females that had been tutored for 10 days. **C:** Neuronal activation in the lateral NCM of juveniles, showing a significant difference between 2TD and 2TN. **D:** Neuronal activation in the medial part of the NCM was higher than in the lateral part (note the different y-axis scales). Similar to the lateral NCM, 2TD had the highest level of neuronal activation, and there was a significant difference between 2TD and 2TN. **E:** Low levels of neuronal activation in the hippocampus.

direction compared to the day (darkly versus lightly coloured bars in figure 5.4b). In birds that had been housed with the tutor for 2 days, neuronal activation in the CMM during the night was greater in birds that were kept in silence on the experimental day than in birds that had been re-exposed to the tutor song (2SN versus 2TN). Indeed, post-hoc tests revealed a difference between these two experimental groups in the medial CMM (although not significant after correction for multiple testing: $p=0.009$, $\alpha=0.05/28=0.0018$). In the lateral CMM, there was a high level of neuronal activation during sleep in birds that had been housed with a tutor for ten days, and were re-exposed to the tutor song on the experimental day (10TN, figure 5.4a). Indeed, there was more neuronal activation in the lateral CMM during sleep in the 10- than 2-day tutored group (post-hoc test 10TN versus 2TN, although not significant after correction for multiple testing: $p=0.036$, $\alpha=0.05/28=0.0018$).

The NCM was highly activated in two-days tutored, awake birds that were re-exposed to the tutor song. There was a significant difference between neuronal activation in the medial and lateral part of the NCM ($F_{1,42}=6.547$, $p=0.014$). There were no significant effects or interactions in the lateral NCM, but ANOVA revealed a significant interaction between Stimulus and Day/Night in the medial NCM ($F_{1,42}=5.785$, $p=0.021$). Neuronal activation in both the medial and lateral part of the NCM during the day differed from activation during sleep in birds who had been housed with the tutor for two days, and were re-exposed to tutor song on the day of the experiment (2TD vs 2TN, although not significant after correction for multiple testing; lateral, $p=0.028$, medial, $p=0.009$; $\alpha=0.05/28=0.0018$).

Low levels of neuronal activation in the hippocampus. Activation levels in the hippocampus of the juvenile females were generally low (mean was approximately 117 Zenk-immunopositive cells per mm^2). There was no significant effect of Stimulus ($F_{1,23}=0.054$, $p=0.818$), but there was a significant interaction between Day/Night and Tutoring experience (2 or 10 days; $F_{1,23}=5.031$, $p=0.035$). However, post-hoc tests did not reveal any significant differences between experimental groups (all $p>0.05$).

Discussion

Females do memorize their fathers' song, but do not sing themselves, and therefore provide a powerful model to study birdsong memory formation (Bolhuis & Eda-Fujiwara 2003). Here we investigated two early stages of memory formation in juvenile female zebra finches (2 or 10 days of tutoring experience). We explored the amount of tutor song exposure that is needed to memorize song, the neural activation characteristics that are involved in tutor song memory formation, and memory consolidation during sleep; and compared these with a similar study in juvenile males (chapter 4 of this thesis).

No evidence for tutor song recognition. We could not demonstrate tutor song recognition in juvenile females that were housed with a tutor for 2 or 10 days during the sensitive period. Neither the females who had been housed with a tutor for two days, nor ten days,

showed a preference for the tutor's song. Others previously showed that early life auditory input is necessary for zebra finch males and females to develop stimulus-specific neural responses or auditory discrimination abilities (Sturdy et al. 2001; Lauay et al. 2004; Maul et al. 2010). Females form a memory representation of the tutor song during a sensitive phase between ~25-70 days post hatching (Riebel 2003). It was shown that generalized features of this song are used for mate selection later in life (Clayton 1990; Lauay et al. 2004; Riebel 2009). In males, song exposure of only 9 days during the sensitive phase is sufficient to recognize songs (Braaten 2010), and just 5 or 10 days of tutoring will result in successful tutor song imitation (Tchernichovski et al. 2001; Roper & Zann 2006). Juvenile females that were raised with an adult male until 35 days post hatching developed a preference for the tutor song as adults, but females that were raised with an adult male until 25 days post hatching did not (Miller 1979a; Clayton 1988; Neubauer 1999; Riebel 2003; Holveck & Riebel 2014). This suggests that, similar to males, 10 days of tutoring during the sensitive period is sufficient for females to be able to recognise tutor song.

Therefore, the question is: did the juvenile females not recognize their tutor, or was our way of testing inappropriate to test recognition in juvenile females? We used a phonotaxis preference test to test for tutor song recognition (song familiarity). The same test previously successfully demonstrated significant tutor preferences in adult females (e.g., Miller 1979a; Miller 1979b; Clayton 1988; Eda-Fujiwara et al. 2003; Lauay et al. 2004; Holveck & Riebel 2007; Bailey et al. 2009). In addition to phonotaxis where physical approach towards the speakers is used to calculate the stimulus preference, we tested other possible indicators of song preference: response latencies and call ratios (c.f. Nelson et al. 1997; Stripling et al. 2003). However, these scores also failed to indicate tutor song recognition. It may be that juvenile and adult females respond differently to song playbacks, for example because of sexual maturation, or juveniles might be more curious towards the unfamiliar song than adult birds. To our knowledge, no preference tests were done in juveniles before. One previous study showed song preferences of juvenile females in an operant test: female zebra finches that were even younger birds than the birds in the present experiment (28-53 dph) had a preference for conspecific song over heterospecific song (Braaten & Reynolds 1999). Also, juvenile female white-crowned sparrows called more in response to tutor song (Nelson et al. 1997). These studies used a different way of testing than we did, or a different songbird species, respectively, which may explain the different results compared to our experiment. Thus it is not clear whether a preference test can demonstrate song recognition in juvenile female zebra finches. A future study could address this by raising juvenile females as in our experiment, but postpone the preference test until the birds are adults. Alternatively, a different tutor song recognition test such as an operant task (Riebel et al. 2002; Terpstra et al. 2006) might have yielded different results.

Brain activation in early stages of female song memory formation. Even though we cannot be sure whether the females had learned to recognize the song of their tutor, we did find significant differences in neuronal activation between the juveniles that had been housed with their tutor for two or ten days. In juvenile females that had been housed with

their tutor for two days, there was greater neuronal activation in the NCM during the day after being re-exposed to the tutor compared to silence (2TD versus 2SD). In the CMM, there was more neuronal activation in birds with ten days of tutoring experience (10TD versus 10SD). These results suggest that in a very early stage of memory formation, the NCM is involved, while in a later stage of memory formation, the CMM becomes involved. The CMM is greatly and selectively activated in response to tutor song in adult females (Terpstra et al. 2006). Thus, adult-like neuronal activation in females seems to develop somewhere between two and ten days of tutoring experience.

Similarly, in adult starlings it was shown that the CMM was more important for song memory in a learned auditory discrimination task, but the NCM was involved in the acquisition of new song discriminations (Gentner et al. 2004). Our current results seem to fit this division of labour between the NCM and CMM. Furthermore, auditory information that enters the avian brain in the primary auditory regions (field L) is first projected to the NCM, and from there to the CMM (Vates et al. 1996). In this light, it is plausible that in early stages of learning, the NCM is involved, while later, the CMM might be more important.

The hippocampus might not be involved tutor song memory formation. The hippocampus is a region that was suggested to play a role in song perception and tutor song memory in adult females (Bailey et al. 2002; Bailey & Wade 2005; Bailey et al. 2009; Bailey & Saldanha 2010). However, here we found low levels of activation in the hippocampus of juvenile females. There were no significant effects differences between experimental groups, suggesting that the hippocampus is not involved in early stages of tutor song memory formation.

Comparison with previous results in juvenile males. Previously, juvenile females showed similar levels of neuronal activation to males (Braaten 2010). We also found neural similarities between juvenile males (chapter 4 of this thesis) and females. Males and females both showed the highest level of neuronal activation in the NCM when they had been housed with their tutor for two days, were re-exposed to the tutor song on the day of the experiment, and sacrificed during the day (2TD). There was a large difference between the high levels of activation in this group compared with neuronal activation during sleep (2TD versus 2TN in both males and females). Juvenile males showed a left-dominant neuronal activation pattern in the NCM, whereas the females in the current experiment showed bilateral activation. It may be that, for lateralisation to develop in females, longer tutoring experience is necessary. Indeed, a left-dominant neuronal activation pattern was previously demonstrated in adult female zebra finches when auditory and visual stimuli of a courting male were presented to the birds (Avey et al. 2005). Alternatively, females may never develop lateralised neuronal activation for tutor song memory (which might be a very different stimulus to the bird than a courting male). Lateralisation of tutor song memory-related neuronal activation was demonstrated in the NCM of juvenile males, but not of adult males (chapter 2 of this thesis). Therefore, lateralisation in the NCM might be a reflection of using the tutor song memory representation as a template for the juveniles' own song, which does not happen in adult males who already imitated the tutor song or females that do not sing.

A role for sleep in tutor song memory consolidation? There was great neuronal activation in the CMM of females that had been housed with a tutor for ten days, were re-exposed to the tutor song on the experimental day, and sacrificed during the night (10TN). This might indicate the occurrence of tutor song memory consolidation during sleep. Since the females did not show tutor song recognition in preference tests, ten days of tutoring experience might have been the moment of initial tutor song memory formation in females. In contrast, juvenile males showed significant tutor song imitation after both two and ten days of tutoring and low levels of neuronal activation during sleep (chapter 4 of this thesis). Thus, tutor song memory consolidation during sleep might have already occurred after the first or second day of tutoring in males. Indeed, a recent study in juvenile male zebra finches showed behavioural evidence of song learning after only one day of tutoring (Deshpande et al. 2014). To further study the role of sleep in memory formation, neuronal activation in males should be measured at an even earlier time point, on the first night after tutoring, while in females it may be worthwhile to add a later time point to the experiment. Alternatively, it may be that memory consolidation occurs mainly during the day, possibly during day-time naps (Margoliash & Schmidt 2010; Whitaker et al. 2012). Sleep deprivation studies could help elucidating the role of sleep in song memory consolidation.

Conclusions. Juvenile female zebra finches did not show evidence of tutor song recognition in a preference test after two or ten days of tutoring experience. However, we found high levels of neuronal activation in two higher-order auditory brain regions. Neuronal activation in females was bilateral, in contrast to juvenile males of similar age and experimental conditions in a previous study, where neuronal activation in the NCM was left dominant. The lack of lateralisation in females suggests that male lateralisation could be involved in using the memory template for tutor song imitation. There was significant neuronal activation in response to tutor song during the day: females that had been housed with the tutor for two days showed high levels of neuronal activation in the NCM, while birds that had been housed with the tutor for ten days showed strong tutor-song induced neuronal activation in the CMM. This suggests that the NCM could be more important for initial song memory acquisition, while the CMM is more important during later stages of tutor song memory.

6

Memory-related brain lateralisation in birds and humans

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Abstract

Visual imprinting in chicks and song learning in songbirds are prominent model systems for the study of the neural mechanisms of memory. In both systems, neural lateralisation has been found to be involved in memory formation. Although many processes in the human brain are lateralised – spatial memory and musical processing mostly involves right hemisphere dominance, whilst language is mostly left hemisphere dominant – it is unclear what the function of lateralisation is. It might enhance brain capacity, make processing more efficient or prevent occurrence of conflicting signals. In both avian paradigms we find memory-related lateralisation. We will discuss avian lateralisation findings and propose that birds provide a strong model for studying neural mechanisms of memory-related lateralisation.

Introducing lateralisation

Lateralisation is the asymmetric representation of functions in the body. This may be expressed overtly, as in the case of left- and right-handedness in humans and lateralised limb preferences in other animals (Rogers 2009). A more extreme example in an invertebrate is claw asymmetry in male fiddler crabs (*Uca pugnax*). These crabs have one major claw that is large and predominantly used in conflict, and a smaller minor claw. This asymmetry is also reflected in the motor control of the claws; nerves to the major claw are enlarged and outnumber those of the minor claw (Young & Govind 1983).

Cerebral lateralisation is a difference in the functional contribution of the two brain hemispheres. Anecdotal accounts characterising human phenotypes as “left-brain dominant” (logical, rational, analytical, objective, and sensitive to component parts of concepts) or “right-brain dominant” (random, intuitive, subjective, and sensitive to the whole concept) are widespread. However, while there is considerable evidence for lateralisation in numerous cognitive functions, there is little evidence for differential representation of such functions according to personality type (Nielsen et al. 2013). There is commonly a right-sided dominance for spatial cognition in primates (Oleksiak et al. 2011). Music perception occurs predominantly in the right hemisphere, although the different components of music (rhythm, pitch, tonality, etc.) have different neural representations and lateralisation patterns (Parsons 2001). Another well-known example of functional brain asymmetry is speech and language lateralisation in humans. In the majority of people, language structure and meaning are processed in the left hemisphere predominantly, while syllable-stress cues for example are processed in the right-hemisphere predominantly (Friederici 2011). Also many other animals show lateralisation for production and perception of vocalisations (Ocklenburg et al. 2013).

Functional lateralisation is an ancient phenomenon, occurring both in subcortical structures and throughout vertebrate phylogeny (Harris et al. 1996; Bisazza et al. 1998; Vallortigara et al. 1999; Frasnelli et al. 2012). Examples of asymmetry in behaviour and in brain function have been reported in numerous lower vertebrate species. It should therefore be expected to benefit the individual or population. So what are the advantages conferred by neural lateralisation? It has been suggested that lateralisation might enhance the brain's capacity for neural processing, by reducing conflict and interference between simultaneous neural processes, and thereby enhancing task performance (Vallortigara & Rogers 2005). As an illustration of the principle, in teleost fish the escape response is mediated by two large, reticulospinal neurons, the Mauthner cells, which project to motor neurons innervating the contralateral body musculature. The response involves activation of the muscles of one side while inhibiting the other. The crucial function of the response is to distance the fish from the source of threat by fast contraction of the muscles along one side and simultaneous inhibition of the contralateral side. The direction of escape is secondary. Clearly the process would be restricted by bilateral activation, and this is prevented by a spinal inhibitory pathway (Korn & Faber 2005) In the Goldbelly topminnow (*Girardinus falcatus*) the escape

response is lateralised, the majority of adults escape in a rightwards trajectory on initial exposure to a potentially threatening stimulus (Cantalupo et al. 1995). In the Shiner perch (*Cymatogaster aggregate*) escape reactivity is fastest for individuals in which the response is most strongly lateralised (Dadda et al. 2010).

Visual information processing is commonly lateralised in species in which the visual fields of the two eyes have little or no overlap, as documented in both vertebrate species (Vallortigara 2000) and invertebrates (Jozet-Alves et al. 2012). Lateralisation of visual brain regions was first investigated in the domestic chick (*Gallus gallus domesticus*) by closing one or other eye. With the left eye occluded, the left hemisphere of chicks and pigeons, receiving visual input largely from the right eye, has been found better at processing visual details, such as small food particles, while the right hemisphere (right eye occluded) is more efficient at scanning for predators (figure 6.1; Güntürkün et al. 2000; Rogers et al. 2004; Concha et al. 2012; Rogers 2012). Another example is eye and foot lateralisation in a cognitive task in parrots. The strongly lateralised individuals (either left-dominant or right-dominant) performed better than symmetrical individuals (Magat & Brown 2009). However, a study in humans found that lateralisation did not facilitate parallel processing of a typically right-hemispheric face recognition task and simultaneously a typically left-hemispheric language recognition task. Instead, subjects with symmetric neural activation outperformed asymmetric subjects (Hirnstein et al. 2008).

Lateralisation also occurs in memory processes across a broad phylogenetic range. In honeybees, initial olfactory memory recall is lateralised towards the right antenna, while later, from six hours after training, the memorised odour is recalled more efficiently when the left antenna is used (Rogers & Vallortigara 2008). In human subjects, motor control of arm movements is lateralised; while the left hemisphere is more important in learning new movements, the right has a more important role in real-time updating and control of established movements (Mutha et al. 2012). Birds in particular show pronounced lateralisation in a wide range of mnemonic functions. It has been suggested that while the left hemisphere is associated with memorized tasks and environments, the right is involved in novelty detection (MacNeilage et al. 2009; Rogers 2012).

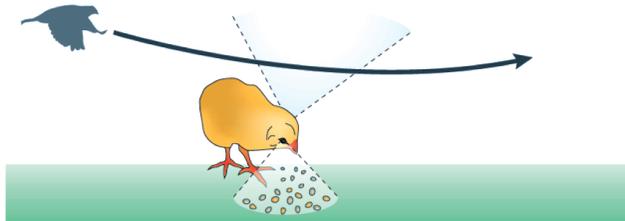


Figure 6.1. Visual lateralisation in domestic chicks. The right eye (left hemisphere) is better at tasks such as discriminating grain and the left eye (right hemisphere) is better at tasks such as detecting moving predators. Figure is reproduced, with permission, from Concha et al. (2012).

In this review we will focus on song learning and visual imprinting (figure 6.2a, f). There are strong similarities between visual imprinting in the chick and song learning in zebra finches (chapter 2 of this thesis), which are shared also with language acquisition in human infants (Peña et al. 2003; Dehaene-Lambertz et al. 2006). Each is guided by predispositions, for appropriate sounds in vocal learning (Doupe & Kuhl 1999), and for appropriate visual cues in imprinting (Bolhuis & Honey 1998; Vallortigara et al. 2005). In each case sleep is important in the consolidation process (Dave & Margoliash 2000; Derégnaucourt et al. 2005; Gómez et al. 2006; Jackson et al. 2008; Hupbach et al. 2009; Shank & Margoliash 2009; Brawn et al. 2010; Gobes et al. 2010; Henderson et al. 2012; Brawn et al. 2013; chapter 3 of this thesis). Foremost here, in each case there is pronounced lateralisation of function (Horn 1985; Bolhuis & Honey 1998; Tervaniemi & Hugdahl 2003; Horn 2004; Friederici 2011; Moorman et al. 2012; Price 2012; Ocklenburg et al. 2013; chapter 3 of this thesis).

Song learning

Human infants need to hear their parents speak in order to acquire language. Songbirds, hummingbirds and parrots also have to hear other birds singing in order to learn to sing themselves. The ability to imitate vocalisations from conspecifics is not a very widespread trait in the animal kingdom (Doupe & Kuhl 1999; Hauser et al. 2002; Bolhuis & Gahr 2006). Therefore, songbirds, for example zebra finches (*Taeniopygia guttata*), are considered as the best existing model for human speech acquisition (Bolhuis et al. 2010). In zebra finches, only the males sing. Juvenile male zebra finches form a memory representation of the song of an adult tutor (usually their father) and use this as a template for development of their own song (figure 6.2f; Woolley 2012). Even though zebra finch song crystallizes when the birds are three months old, which means that their song is stable and stereotyped and the bird cannot learn to sing new song elements, the birds maintain a long-term memory representation of the tutor song far beyond that time (Funabiki & Konishi 2003).

The neural mechanisms of song production and song learning and memory have been studied extensively (Brainard & Doupe 2002; Mooney 2009; Bolhuis et al. 2010). Two networks of nuclei in the songbird brain are involved in song production (figure 6.2d), both involving song nucleus HVC (a letter-based name). HVC receives input from three auditory brain regions: NIF (the interfacial nucleus of the nidopallium), Uva (nucleus uvaeformis) and CLM (caudolateral mesopallium). The song motor pathway (1), connecting HVC through RA (the robust nucleus of the arcopallium) to vocal-motor and respiratory centres in the midbrain and hindbrain, is important for generation of song motor signals (reviewed in Zeigler & Marler 2008; Bolhuis & Everaert 2013). The anterior forebrain pathway (2), connecting HVC to Area X in the striatum, to DLM (medial subdivision of the dorsolateral nucleus of the anterior thalamus) and via LMAN (the lateral magnocellular nucleus of the anterior nidopallium) to RA of the song motor pathway, is important during sensorimotor learning and is involved in generating vocal plasticity (reviewed in Zeigler & Marler 2008; Bolhuis & Everaert 2013). There is a third

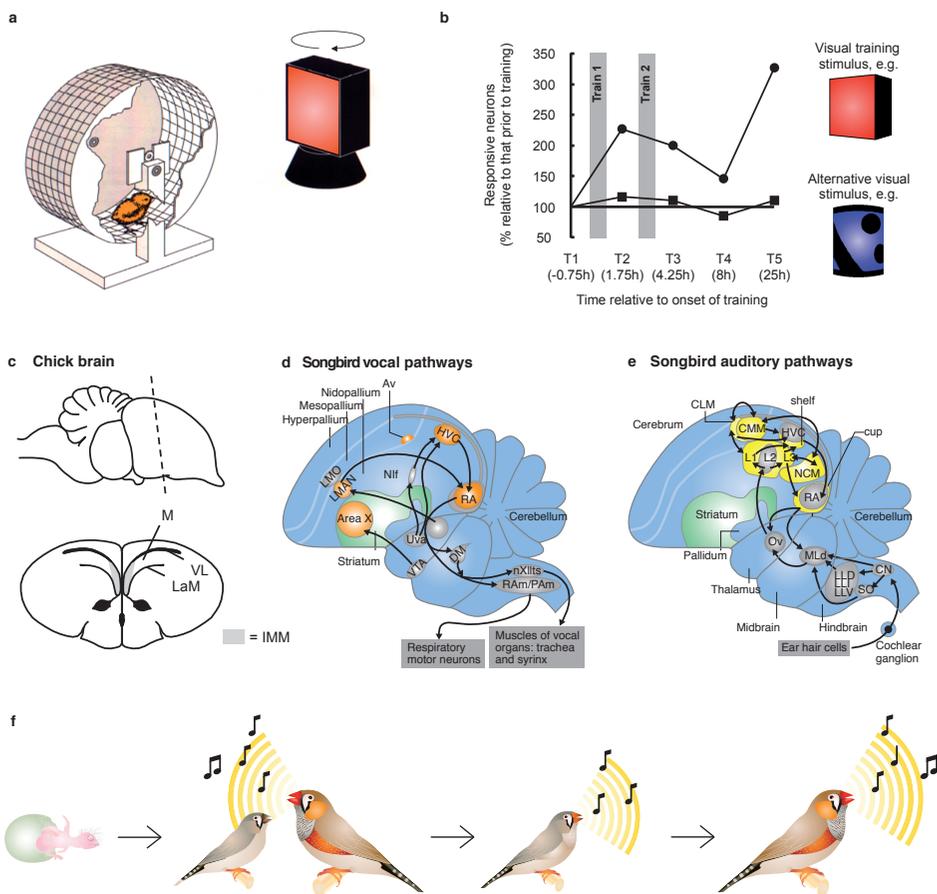
network of other brain regions that is involved in auditory perception and song learning and memory (figure 6.2e). The auditory pathway (3) includes field L, the primary termination site of the ascending auditory pathway in the nidopallium, the NCM (caudomedial nidopallium), CLM and CMM (caudomedial mesopallium), HVC shelf (a brain region adjacent to song nucleus HVC), RA cup (a brain region surrounding song nucleus RA) and MLd (the dorsal part of the lateral nucleus of the mesencephalon) (Mello & Ribeiro 1998; Woolley 2012). In the auditory network, the NCM was found to be a crucial region for tutor song memory in male zebra finches (Bolhuis et al. 2000b; Gobes & Bolhuis 2007; London & Clayton 2008), while the CMM might be involved in tutor song memory in female zebra finches (Terpstra et al. 2006) or in other types of auditory memory in starlings (Gentner & Margoliash 2003). The localization of a song memory representation allows for further understanding of the underpinnings of memory formation (Bolhuis & Gahr 2006).

A role for lateralisation in singing

Language-related brain activation in humans shows a very distinct lateralised pattern, with left-dominant processing of most language-related production and perception. Song learning in songbirds is a model for human speech acquisition, but do songbirds show similar hemispheric lateralisation? Most evidence on lateralisation in songbirds covers song production. Birds can use the two halves of their vocal organ (the syrinx) independently, and

Figure 6.2. Visual Imprinting and Song Learning. In visual imprinting and song learning, young birds learn by exposure to (A) a conspicuous visual stimulus (a rotating red box, RB), and (F) the paternal song respectively. **B:** The responsiveness of IMM neurons to the imprinting stimulus (e.g. RB) increases through two periods of training. This increase is initially unstable, but reaches a maximum after a period of consolidation. Responsiveness to an alternative stimulus (e.g. a rotating blue cylinder) remains unchanged. **C:** Recognition memory for visual imprinting is stored in the intermediate medial mesopallium (IMM), shown as the shaded area in both hemispheres on the cross-section through the forebrain at the level indicated by the dotted line. Abbreviations: M, mesopallium; VL, lateral ventricle; LaM, mesopallial lamina. **D, E:** Schematic side views of songbird vocal (**D**) and auditory (**E**) pathways. Brain regions depicted in (**D**) are activated when the bird is singing, while regions in (**E**) are involved in song perception and memory. Figures (**D, E**) are reproduced, with permission, from Moorman et al. (2011). Abbreviations: Area X, Area X of the striatum; Av, avalanche; CLM, caudolateral mesopallium; CMM, caudomedial mesopallium; CN, cochlear nucleus; DLM, medial subdivision of the dorsolateral nucleus of the anterior thalamus; DM, dorsomedial subdivision of nucleus intercollicularis of the mesencephalon; HVC, a letter-based name; L1, L2 and L3 are subdivisions of Field L; LLD, lateral lemniscus, dorsal nucleus; LLI, lateral lemniscus, intermediate nucleus; LLV, lateral lemniscus, ventral nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; LMO, lateral oval nucleus of the mesopallium; MLd, dorsal part of the lateral nucleus of the mesencephalon; NCM, caudomedial nidopallium; Nlf, interfacial nucleus of the nidopallium; nXILts, tracheosyringeal portion of the nucleus hypoglossus (nucleus XII); Ov, nucleus ovoidalis; PAm, nucleus para-ambiguus medullaris; RA, robust nucleus of the arcopallium; RAm, nucleus retroambiguus medullaris; SO, superior olive; Uva, nucleus uvaeformis; VTA, ventral tegmental area. (**F**) Juvenile songbirds imitate songs that they are exposed to early in life (usually father's song). After hatching, the juvenile zebra finch is exposed to adult song during the memorisation phase. When the bird is approximately one month old, it starts singing, and song performance improves through practise. When the bird is approximately three months old, it sings a stable, adult song that resembles the model song. ►

both halves receive projections from the ipsilateral song motor pathway (Suthers & Zollinger 2004). Zebra finch juveniles imitate the air sac pressure pattern for singing from the father, so also the physical aspects of song motor production have a learned component (Suthers & Zollinger 2004). In some songbird species, such as the inbred Belgian ‘Wasserschlager’ or ‘Waterslager’ canary strain (*Serinus canaria*) and the chaffinch (*Fringilla coelebs*), the left syrinx is dominant during song production (Nottebohm & Nottebohm 1976). Another canary strain, the outbred and common or domestic canary uses both sides of the syrinx for song production (Suthers et al. 2004), as does the European starling (*Sturnus vulgaris*; Uchida et al. 2010). In the zebra finch, a lesion experiment showed that the right side of the syrinx is more important during singing (Williams et al. 1992). However, bronchial airflow recordings in zebra finches, made to detect airflow through the two sides of the syrinx, indicated that most syllables are produced bilaterally under more natural circumstances (Goller & Cooper 2004). Thus, even though disabling one half of the syrinx can deteriorate song more than disabling the other half, it does not mean that the other half does not



normally contribute to song production. The tracheosyringal portion of the hypoglossal nucleus (nXIIIts, see figure 6.2d) that controls muscles of the syrinx is left dominant in *Wasserschlager* canaries (Nottebohm & Nottebohm 1976) but right dominant in zebra finches (Floody & Arnold 1997). Thus, there are species-differences in lateralisation of the syrinx and its innervations (table 6.1).

Differential vocal complexity and the type of notes that the birds sing could explain the lateralisation differences between species, at least in part. The left and right syrinx might be specialized for different frequency ranges, and because different songbird species have different song characteristics, it might result in different lateralisation patterns (Suthers & Zollinger 2004). This might explain the lateralisation difference between *Wasserschlager* and common canaries – the first is selectively bred for its complex songs. A lesion to RA in the left hemisphere of a *Wasserschlager* canary had a stronger deteriorating effect on song than a lesion to the right RA (Nottebohm et al. 1976). Interestingly, the fundamental frequency range decreased from 1-4 kHz to 1.7-2.1 kHz in the bird in which the left RA was lesioned, which could be further support for the hypothesis that the two sides of the syrinx are specialized for different frequency ranges. In zebra finches, lesions of left and right RA are equally deteriorating for their song (Ashmore et al. 2008), which is in accordance with the bilateral bronchial airflow findings in this species (Goller & Cooper 2004).

Table 6.1. Lateralisation of song perception and production in songbirds. Lateralisation results are separated for brain regions (NCM, HVC or other). In HVC, we separated production, auditory, and anatomical studies. Symbols indicate that information is from the same reference (but different brain areas). In the summary column, 'L=R' means that no hemispheric differences were found; 'L' means that the left hemisphere was dominant; 'R' that the right hemisphere was dominant. Abbreviations: A, adult

Brain region	Summary	Reference	Songbird	Age	Gender	Stimulus
	R	Voss et al. (2007)	Zebra finch	A	M	BOS/tut/con/pure tones
	R	Phan & Vicario (2010)	Zebra finch	A	M+F	A mixture of different songs and calls
	L/R	Moorman et al. submitted Δ	Zebra finch	J	M	Tut/nov/sil
	L=R	Chew et al. (1996)	Zebra finch	A	M+F	Conspecific songs and calls, heterospecific songs, and human speech
NCM Perception	L=R	Moorman et al. (2012) \star	Zebra finch	A	M	Tut/nov/sil
	L	Avey et al. (2005)	Zebra finch	A	M+F	Courtship songs +/- videos
	L	Remage-Healey et al. (2010)	Zebra finch	A	M	BOS/con
	L	Moorman et al. (2012) \star	Zebra finch	J	M	Tut/nov/sil
	L	Van Der Kant et al. (2013) \clubsuit	Zebra finch	A	M	BOS/tut/con

In the *Wasserschlager* canary, the left HVC is dominant for production, in accordance with the left-sided dominance of its syrinx (Nottebohm et al. 1976). The left HVC is also dominant for singing in common canaries (Halle et al. 2003), although their syrinx on both sides contributes equally during singing. A lesion experiment showed that right HVC was dominant for song production in zebra finches (Williams et al. 1992), in accordance with the earlier described study that found that the right syrinx was dominant (Floody & Arnold 1997). However, using electrical stimulation to briefly disrupt HVC signalling, Wang and colleagues (2008) showed that left and right HVC contribute to song production equally, with rapid alternation between the two hemispheres ('hemispheric switching'). Furthermore, using a cooling device to slow down HVC signalling, Long and Fee (2008) showed that left and right HVC contribute to different parts of the song (syllables or long subsyllabic elements). Also, it was shown that there is communication between the left and right HVC: activation of HVC in one hemisphere affects the contralateral HVC (Vu et al. 1998; Schmidt et al. 2004). These latter experiments are in agreement with conclusions concerning bronchial airflow (Goller & Cooper 2004) and RA lesion studies (Ashmore et al. 2008), suggesting bilateral song production mechanisms in zebra finches.

As a hypothesis, both the right-sided dominance *and* bilateral contributions of the song system in zebra finches might be true. Both left and right HVC might contribute equally to singing,

birds; Beng song, Bengalese finch song; BOS, bird's own song; CMM, caudomedial mesopallium; Con, conspecific song; CSD, copulation solicitation display; F, females; fMRI, functional Magnetic Resonance Imaging; Het, heterospecific song; ICC, immunocytochemistry; J, juvenile birds; L, left hemisphere; M, males; Nov, novel song; NCMd, dorsal NCM; NCMv, ventral NCM; R, right hemisphere; Sil, silence; Tut, tutor song; ZF song, zebra finch song.

Method	Behavioural state	Lateralisation result
fMRI	Anaesthetised	R auditory areas: response different for stimuli, L no discrimination
Electrophysiology	Awake	Some groups R>L (based on early experience)
Zenk ICC	Sleeping	Good learners are L-dominant, poor learners R-dominant
Electrophysiology	Awake	Habituation rates were similar in the left and right NCM
Zenk ICC	Awake	Similar neuronal activation in the left and right NCM
Zenk ICC	Awake	CMM, NCMv, NCMd: All Left dominant
Retrodialysis and preference tests	Awake	Inhibiting estrogens in L-NCM (not R) eliminates BOS preference
Zenk ICC	Awake	L>R for tutor song only
fMRI	Anaesthetised	L-NCM activation (not R) during tutor song is correlated to song learning strength

Table 6.1. Continued

Brain region	Summary	Reference	Songbird	Age	Gender	Stimulus
HVC Singing	R	Williams et al. (1992) §	Zebra finch	A	M	Singing
	L=R	Vu, Schmidt & Mazurek (1998)	Zebra finch	A	M	Singing
	L=R	Long & Fee (2008)	Zebra finch	A	M	Singing
	L=R	Sakata & Brainard (2008) ∞	Bengalese finch	A	M	Distorted auditory feedback during singing
	L=R	Wang et al. (2008) ¥	Zebra finch	A	M	Singing (males could see females)
	L	Nottebohm et al. (1976) †	Wasserschlagler canary	A	M	Singing
	L / L=R	Greenspon & Stein (1983)	American Singer canary	A	F	Singing after testosterone treatments
L	Halle et al. (2003)	Common European canary	A	M	Singing	
HVC Perception	R	Poirier et al. (2009) €	Zebra finch	A	M	BOS/con/het
	R	Moorman et al. submitted Δ	Zebra finch	J	M	Tut/nov/sil
	L=R	Halle et al. (2002)	Common European canary	A	F	Canary song
	L=R	George et al. (2005) ø	Starling	A	M	White noise/pure tones/BOS/con
	L=R	Sakata & Brainard (2008) ∞	Bengalese finch	A	M	Distorted auditory feedback during quiescence
	L	Okanoya et al. (2001)	Bengalese finch	A	M	Discrimination task Beng/ZF song
	L	George et al. (2005) ø	Starling	A	M	White noise/pure tones/BOS/con
	L	Moorman et al. (2012) ★	Zebra finch	J	M	Tut/nov/sil
L	Moorman et al. (2012) ★	Zebra finch	A	M	Tut/nov/sil	
HVC Anatomy	L=R	Wilbrecht et al. (2006)	Zebra finch	J	M	-
	L=R	Burek et al. (1994)	Zebra finch	J+A	M+F	-
	L=R	Wang et al. (2002)	Zebra finch	J+A	M	-
	L=R	Gil et al. (2006)	Zebra finch	A	M	-
	L=R	Roberts et al. (2008)	Zebra finch	A	M	-

Method	Behavioural state	Lateralisation result
Lesions	Awake	R-HVC lesions (not L) affected temporal patterning of song
Electrical stimulation in HVC	Awake	Premotor activity in either R/L HVC was rapidly altered by stimulation of contralateral HVC
Cooling HVC	Awake	L/R HVC contribute to different parts of the song
Electrophysiology	Awake	Transient feedback perturbation decreased L&R HVC activity
Electrical stimulation in HVC & LMAN	Awake	Rapid switching of dominant hemisphere
L/R nerve & brain lesions	Awake	L-HVC and L-hypoglossus lesions deteriorated song more than R lesions
L/R HVC lesions	Awake	L-HVC lesions deteriorated song more than R lesions; L=R during song acquisition (when testosterone was injected after the lesions were made)
L/R HVC lesions	Awake	L-HVC lesions deteriorated song more than R lesions
fMRI	Anaesthetised	R-HVC specific for BOS (not L)
Zenk ICC	Sleeping	R-HVC always active (more than L)
Lesions & CSD	Awake	Unilateral lesions: normal CSD, bilateral HVC lesions: no CSD any more
Electrophysiology	Anaesthetised	No lateralisation
Electrophysiology	Awake	Transient feedback perturbation decreased L&R HVC activity
L/R HVC lesions	Awake	L-HVC lesions had a more disruptive effect on song discrimination learning than R lesions
Electrophysiology	Awake	R-HVC is more responsive, L-HVC more selective
Zenk ICC	Awake	L-HVC always active (more than R)
Zenk ICC	Awake	L-HVC always active (more than R)
Neurogenesis	Awake	Similar level of neurogenesis L&R HVC
Neurogenesis	Awake	Similar level of neurogenesis L&R HVC
Neurogenesis	Awake	Similar level of neurogenesis L&R HVC
Size of nuclei	Awake	L&R HVC same size
Retrograde labeling	Awake	L&R HVC connected to RA

Table 6.1. Continued

Brain region	Summary	Reference	Songbird	Age	Gender	Stimulus
Area X	R	Poirier et al. (2009) €	Zebra finch	A	M	BOS / con / het
field L	R	George et al. (2004)	Starling	A	M	Bird's own - / familiar - / unfamiliar whistles
field L	L	Hauber et al. (2007)	Zebra finch	A	M	Con / tones / white noise
LMAN	R	Wang et al. (2008) ¥	Zebra finch	A	M	Singing (males could see females)
MLd	R	Poirier et al. (2009) €	Zebra finch	A	M	BOS/con/het
MLd	R	Van Der Kant et al. (2013) ♣	Zebra finch	A	M	BOS/tut/con
nXIIts	R	Floody & Arnold (1997)	Zebra finch	A	M	Singing
nXIIts	L	Nottebohm et al. (1976) †	Wasserschlagler canary	A	M	Singing
Ov	L	Cynx et al. (1992)	Zebra finch	A	M	BOS / cage mate / unfamiliar conspecific
RA	L=R	Paton et al. (1981)	Bengalese finch	A	M+F	-
RA	L=R	Wild, Williams & Suthers (2000)	3 species	A	M	Anatomy
RA	L=R	Ashmore et al. (2008)	Zebra finch	A	M	Singing
RA	L	Nottebohm et al. (1976) †	Wasserschlagler canary	A	M	Singing
syrix	R	Williams et al. (1992) §	Zebra finch	A	M	Singing
syrix	L=R	Goller & Cooper (2004)	Zebra finch	A	M	Singing
syrix	L=R	Suthers et al. (2004)	Common European canary	A	M	Singing
syrix	L=R	Uchida et al. (2010)	Starling	A	M	Singing

but with different specific roles for the two hemispheres, where the role of the right hemisphere might be more noticeable in the song, in accordance with the lesion findings (Williams et al. 1992). One reason to come up with such a hypothesis is a recent finding in juvenile zebra finches. Lateralisation of HVC was investigated using immunocytochemistry to label Zenk (the protein product of the immediate early gene ZENK, which is an acronym of *zif-268*, *egr-1*, *ngf-1a* and *krox-24*), a marker for neuronal activation. The left and right HVC were activated equally during singing (chapter 3 of this thesis), which confirms the idea that both hemispheres contribute to singing. However, the left HVC was spontaneously activated during the day (chapter 2 of this thesis), while during sleep, the right HVC was predominantly activated (chapter 3 of this thesis). This shows that there is state-dependent lateralisation in HVC in juveniles, where neuronal activation patterns of the right HVC during singing are consolidated or replayed during sleep. One could therefore think of a representation of the song motor pattern in the right hemisphere, and song motivational control in the left, or some other form of hemispheric specialisation.

Method	Behavioural state	Lateralisation result
fMRI	Anaesthetised	R-Area X specific for BOS (not L)
Electrophysiology	Awake	R-field L is more responsive to familiar whistles and bird's own whistles; L-field L is more responsive to unfamiliar whistles
Electrophysiology	Anaesthetised	Left hemisphere is more selective for conspecific song
Electrical stimulation in HVC & LMAN	Awake	Right LMAN stimulations: truncation, distortion, song stoppings or early song restarts
fMRI	Anaesthetised	R-MLd specific for BOS (not L)
fMRI	Anaesthetised	R-MLd response different for stimuli, L no discrimination
L/R nerve lesions	Awake	R-nXIIts lesions deteriorated song more than L lesions
L/R nerve & brain lesions	Awake	L-nXIIts lesions deteriorated song more than R lesions
Ov lesions	Awake	L-Ov song discrimination, R-Ov harmonic profile
Electrophysiology	Anaesthetised	RA has bilateral projections to L&R nXIIts
Retrograde & anterograde labeling	Awake	Contralateral projections from RA to nXIIts were substantial in canaries, not in zebra finches
Unilateral lesions	Awake	Adults: L&R RA eliminated normal singing, juveniles: no effect
L/R nerve & brain lesions	Awake	L-RA (not R) lesions result in reduction of song frequency range
Lesions	Awake	Denervation of the R-syrinx affected song (more than L)
Airflow measurements	Awake	Both sides of the syrinx contribute to sound production
Airflow measurements & bronchus occlusion	Awake	Both sides have specific contributions to song
Muscle histology & EMG	Awake	No clear L or R dominance

Lateralisation of songbird's auditory perception

Apart from its role in song production, HVC is also involved in song perception; especially in perception and recognition of the bird's own song (BOS; Margoliash & Konishi 1985). HVC is activated bilaterally during song perception in the common canary (Halle et al. 2002). It was investigated how HVC lesions affected a social response of female canaries to male song, the courtship solicitation display. Complete unilateral HVC lesions on either side affected courtship solicitation display, while incomplete bilateral lesions did not influence this behavioural measure of song perception (Halle et al. 2002). In contrast, song perception-related activation in HVC was lateralised in other songbird species (table 6.1). For example, starlings produce a diverse range of vocalisations, among others, 'warbles' and 'whistles'. Complex warble sequences are used for individual recognition on a short distance, while whistles are used for recognition of birds that are further away. It was found that there is a hemispheric specialization for the two types of vocalisations: the right HVC responds more

to warbles, while the left HVC is more responsive to whistles (George et al. 2005b). Also, neural responses in field L were lateralised: the right field L of starlings was more responsive to familiar whistles, while the left field L responded more to unfamiliar whistles (George et al. 2004). In zebra finches, neural mechanisms for song discrimination abilities were also lateralised. Ov (nucleus ovoidalis, the thalamic auditory relay nucleus) receives auditory input from MLd and projects to field L (Vates et al. 1996). After lesions were made in the left Ov in zebra finches, song discrimination abilities were affected (BOS from cage-mate), while lesions in the right Ov affected discriminating differences in harmonic profile (Cynx et al. 1992).

During song perception, neural responsiveness in secondary auditory regions has been found to be lateralised (table 6.1). For example, when estrogen levels were suppressed in the left NCM, the BOS preference – a behavioural preference measured in a phonotaxis test for bird's own song over song of other zebra finches – disappeared, showing that the BOS preference was estrogen-dependent. Suppressing estrogen levels in the right NCM did not influence BOS preference, showing that the left NCM is crucial for BOS preference (Ramage-Healey et al. 2010). Furthermore, exposure to a digital movie of a courting zebra finch male evoked higher responses in the left than right NCM and CMM in male and female zebra finches. The lateralisation effect was not very strong within each experimental group, but the effect was very consistent among brain regions, experimental groups and individual birds (Avey et al. 2005). Another experiment showed right-sided dominance in the NCM during song perception. The absolute response magnitude (ARM), a measure for neuronal activation recorded with electrophysiology, was greater in the right NCM than left in response to a mixture of several auditory stimuli in both male and female zebra finches (Phan & Vicario 2010). This pattern of lateralisation was only present in birds that had auditory input early in their life, showing that lateralisation was experience-dependent. In the same experiment, also adaptation rates were calculated. These rates reflect stimulus familiarity: unfamiliar songs give faster adaptation rates than familiar songs (Phan et al. 2006). The adaptation rates were generally faster in the right than left NCM (Phan & Vicario 2010). However, it is hard to interpret the adaptation rate results from this experiment. Because familiar and unfamiliar song presentations were mixed, the adaptation rate that was measured was a total response to both familiar and unfamiliar songs. One interpretation is that the faster adaptation rate in the right NCM could imply that the right NCM is more important for song perception, but another possibility is that the left NCM adapts mostly to familiar songs (slow adaptation rate), while the right side adapts mostly to unfamiliar songs (fast adaptation rate).

Lateralisation of songbird's auditory memory

If, as we have discussed, the left NCM indeed adapts more to familiar songs, while the right adapts more to unfamiliar songs, this would mean that there is lateralisation of auditory memory in the NCM, where the left NCM has a dominant role in providing a neural substrate for memorized song. Empirical support for this hypothesis comes from

using immunocytochemistry to visualise Zenk expression in the NCM in juvenile zebra finch males that had been exposed to tutor song, unfamiliar conspecific song or silence (chapter 2 of this thesis). The left NCM was dominant over the right during tutor song perception specifically. In juveniles that had been exposed to unfamiliar song or silence, NCM activation was not lateralised. Also, in adult males who sing crystallized song, the left and right NCM were activated equally during tutor song perception, further indicating that lateralisation was memory-specific. Moreover, the lateralisation ratio (neuronal activation in the left NCM minus neuronal activation in the right NCM divided by the total) in response to tutor song in the juvenile birds was correlated to song learning strength (chapter 2 of this thesis). In addition, in juveniles of the same age, the NCM was activated during sleep. In juveniles that had copied the song of their tutor well ('good learners'), the NCM was more active in the left hemisphere than in the right. In juveniles that had not imitated many elements of the tutor song ('poor learners'), the NCM was activated more in the right hemisphere (chapter 3 of this thesis).

In contrast, others found right-sided dominance in the NCM. Voss and colleagues (Voss et al. 2007) used playbacks of tutor song, bird's own song, songs of unfamiliar conspecific and pure tones and measured neural activation in adult zebra finch males with fMRI. They analysed neural responses per coronal brain views, so for several regions together. A medial slice (as analysed from the fMRI data, not an actual brain slice) in the right hemisphere, comprising the NCM and field L, was selective for BOS and tutor song over unfamiliar conspecific songs and pure tones. The same slice on the left showed higher responses to tones and tutor song than the right hemisphere. However, memory-related effects might be expected in the NCM specifically, and analysing a whole slice might conceal NCM-specific effects (Voss et al. 2007). In another fMRI experiment in adult male zebra finches, the left and right NCM showed equal BOLD responses during song perception (BOS, tutor song or conspecific song), and there was no difference between three types of song stimuli. However, the neural response to tutor song in the posterior lateral left NCM, and not in the right NCM, was correlated to song learning strength. In addition, lateralisation was found in MLd, a region in the midbrain that transmits auditory information to higher-order auditory brain regions. In the right MLd, BOLD responses were more selective for stimulus type than in the left. The neural selectivity for BOS and tutor song over conspecific song in the right MLd was correlated to song learning strength (Van Der Kant et al. 2013; table 6.1).

There are several methodological differences between the studies that found left-dominance and those that found right-dominance in the NCM. One important factor is the type of stimuli that were used. During song perception, usually one hemisphere is selective for some song types over others (e.g. bird's own song vs. conspecific song or conspecific vs. heterospecific song), and the other hemisphere responds more to birdsong in general, although the direction of lateralisation is not consistent. This directional ambiguity could be due to the behavioural state of the animal (awake, sleeping or under anaesthesia). Often, birds are under anaesthesia or mild sedation in electrophysiological or fMRI studies. Also,

because lateralisation of the NCM seems to be memory-specific (chapter 2 of this thesis), experiments in which novel songs are used might not find lateralised activation patterns.

HVC might also be involved in songbird's auditory memory, and because of its role in BOS perception (Margoliash & Konishi 1985), HVC would especially be expected to be of interest for BOS memory. Using fMRI in adult zebra finch males, it was found that the right HVC was more selective than the left for perception of BOS contrasted with perception of the song of the bird's cage-mate (Poirier et al. 2009). However, in Bengalese finches, birds that had a lesion in the left HVC required more time to learn to perceptually discriminate two novel songs in an operant discrimination task than birds with a lesion in the right HVC, showing that in this case the left HVC was dominant (Okanoya et al. 2001). The different results are perhaps caused by the anaesthesia in the former study. HVC selectivity for BOS was enhanced by estrogen-dependent signalling from the NCM (Ramage-Healey & Joshi 2012). Even though in that experiment lateralisation was not investigated, it was shown previously that a behavioural preference for BOS was dependent on the presence of estrogens in the left NCM, but not the right NCM (Ramage-Healey et al. 2010). Perhaps it is the left NCM specifically that transmits information about BOS to HVC. There are no direct pathways known between the NCM and HVC, but there are several possible ways in which the NCM could project to the ipsilateral and the contralateral HVC.

In conclusion, lateralisation for song memory was demonstrated in the NCM, CMM and HVC in songbirds. Lateralisation of auditory-responses is memory-specific, and might be present only during song acquisition. Also, lateralisation during sleep was demonstrated in juvenile songbirds. Sleep is important for memory consolidation (Diekelmann & Born 2010), and therefore especially important during the learning phase. Most studies measuring neural activation during anaesthesia or sleep show a right-dominant pattern, while studies in awake animals mostly show left-sided dominance, both in the NCM and HVC. This behavioural-state dependent lateralisation pattern might further indicate different roles of the two hemispheres, showing consolidation under different conditions.

Visual Imprinting

Imprinting is a learning process common in the young of precocial species. It was first studied empirically by Konrad Lorenz (Lorenz 1937) who described imprinting in ducklings and goslings as a learning process occurring soon after hatching, whereby the young birds learn to recognise the first conspicuous object to which they receive prolonged exposure. They form a filial attachment to that stimulus, typically the mother bird, and subsequently follow it in preference to other stimuli. The memory formed is robust, and continues into adult life, for example influencing sexual preferences (Bateson 1966). Within limits, the strength of learning increases with the duration of exposure to the imprinting object (Bateson 1974; Bolhuis et al. 2000a).

Domestic chicks may be hatched in darkness prior to imprinting, so that they have no previous visual information stored in the brain (Horn 1985). Hence, an advantage to this form of learning and memory in studies of the underlying mechanisms is that the experimenter can carefully control visual experience of the subject animal. In the laboratory, the dark-reared chicks can be trained, again in darkness, by placing them individually in running wheels and exposing them to a conspicuous object (see figure 6.2a). Once trained, the chicks express their preference for the training stimulus by approaching that stimulus more than an alternative stimulus. When tested with serial stimulus presentations, the amount of approach behaviour directed towards the training stimulus relative to that towards an alternative provides a measure of the strength of imprinting. Thus, a further valuable property of imprinting in studies of memory and its underlying mechanisms is that the chick can demonstrate behaviourally how much it has learned during training.

Visual imprinting is subject to certain predispositions. Chicks imprint more strongly on a naturalistic stimulus, such as an adult hen, than a similarly conspicuous artificial stimulus. Thus, rather like the innate preference in human infants for face-like stimuli (Johnson &

6

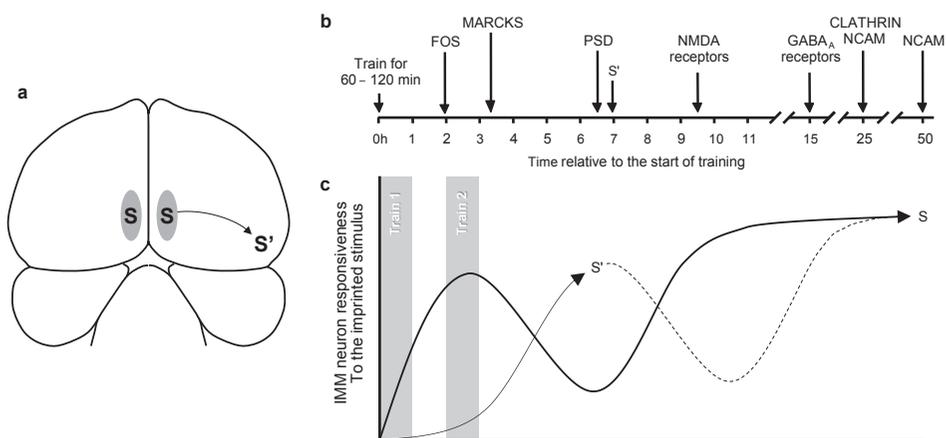


Figure 6.3. The time course of imprinting-related changes in IMM. A: The recognition memory for visual imprinting is stored in left and right IMM (S). Right IMM is necessary for establishing an additional store (S') outside IMM. B: Shown are the times, relative to the onset of training at 0h, of a representative cross-section of reported molecular and structural changes in IMM, and the approximate time when S' is known to have formed. C: Also shown is a diagrammatic representation of storage dynamics for imprinting memory (see also Fig 2b). The responsiveness of IMM neurons increases through two 1h training sessions, but is unstable after training, and 6h after the onset of training there is no greater responsiveness to the imprinting stimulus than before training started. After a period of consolidation, storage (S) in IMM stabilises at its optimal level. The time at which S' is formed coincides with the time when storage in IMM is unstable, so at this time S' is capable of maintaining the chick's preference for the imprinting stimulus. Assuming the formation of S' is subject to the same constraints influencing the formation of S, then its continued development, extrapolated in the broken line, will fluctuate similarly (Horn 2004). It is interesting to note that intracerebral injection of glutamate to the right IMM, but not left, in the period when S' is being formed disrupts retention of imprinting memory (Johnston & Rogers 1998).

Morton 1991), chicks' preferences are guided by a composition of features representing the conspecific. More specifically, this predisposition is focussed on the head and neck region of the adult, and in this region the presence of two eyes is particularly salient (Bolhuis & Honey 1998). The innate preference may even overcome the preference for an artificial stimulus acquired through imprinting training. To avoid this potential confound, artificial stimuli are often used in laboratory studies of imprinting (Horn 1998; see figure 6.2a).

There is strong evidence that implicates the intermediate medial mesopallium (IMM) in the forebrain of the chick as a store for the recognition memory that underpins imprinting (Horn 2004). The region is polysensory, receiving inputs from all sensory systems, and also projects to descending motor areas (Horn 1985). These changes include increases in (i) the number of neurons responding to the imprinting stimulus (Brown & Horn 1994; Nicol et al. 1995; Horn et al. 2001; Jackson et al. 2008; see figure 6.2b); (ii) the length of the postsynaptic density (PSD) on axospinous synapses (Bradley et al. 1981; Horn et al. 1985); (iii) the number of NMDA receptors (McCabe & Horn 1991); (iv) NMDA receptor binding (Johnston et al. 1995); (v) Fos expression (McCabe & Horn 1994), a majority of the Fos-expressing neurons also expressing GABA and taurine (Ambalavanar et al. 1999); (vi) clathrin heavy chain protein (Solomonina et al. 1997); (vii) all three major NCAM isoforms (Solomonina et al. 1998); (viii) amyloid precursor protein (Solomonina et al. 2003) and (ix) calcium/calmodulin protein kinase II (Solomonina et al. 2005). Further learning-related changes are listed in Table 6.2.

The progression of learning-related change in neuronal responsiveness to the imprinting stimulus is a dynamic process – the responses of individual neurons wax and wane before becoming stable through a process of consolidation (Horn et al. 2001). Through each of two training sessions of one hour, a cohort of IMM neurons becomes responsive to the imprinting stimulus. However, neurons that respond to the imprinting stimulus shortly after completion of training are unlikely to be those that responded after the first training session. Rather, it seems that the responsiveness acquired by IMM neurons to the imprinting stimulus in each training session is subsequently lost, only to be resurrected after consolidation (Horn et al. 2001; Jackson et al. 2008). A period of undisturbed rest shortly after the end of training, when the chicks spend much of their time sleeping, is important in this consolidation process (Jackson et al. 2008; Nicol & McCabe 2013).

In many studies of visual imprinting, a recording of a hen's maternal call was played whenever the visual stimulus was presented during training. The presence of the maternal call during training selectively enhances the chick's subsequent preference for the visual training stimulus (Vankampen & Bolhuis 1993). If the maternal call is not played during the preference test, the test provides a measure of visual learning (Bateson 1966; Horn 1985; Bolhuis 1991). As mentioned previously, the IMM is a polysensory region, and there are IMM neurons that respond to auditory stimuli, including the maternal call. However, while chicks can acquire a preference for an auditory stimulus (Vankampen & Bolhuis 1991), responsiveness of IMM neurons to the maternal call presented in training declines dramatically through training, and through the period when elevated responsiveness to

the visual imprinting stimulus becomes consolidated (Nicol et al. submitted; Nicol & Horn 2011). Thus the learning-related changes in responsiveness of IMM neurons to the imprinting stimulus are selective for the visual component of that stimulus. In fact the decline in auditory responsiveness to the maternal call is also found in responsiveness to a visual stimulus that was not seen in training, and even to the combination of the visual training stimulus and the maternal call, but with one important caveat; the proportion of IMM neurons responsive to any of these stimuli *increases* if those neurons also respond to the imprinted stimulus (Nicol et al. submitted; Nicol & Horn 2011). Consistent with these findings, reduced responsiveness of IMM neurons to the presentation of video recordings of familiar chicks combined with recorded chick calls (Town 2011) further suggests habituation to non-imprinted stimuli, although in this study, the responses to the separate visual and auditory component stimuli were not investigated.

There is compelling evidence for the existence of an additional store for imprinting. This was demonstrated in a series of lesion studies (Horn 1986). In chicks that were imprinted by exposure to an artificial stimulus, and tested to confirm that they had acquired a preference for that stimulus, the impact of subsequent bilateral lesions to IMM was found to be time-dependent. If the lesions were made shortly after training (<3h), the chicks were rendered amnesic for the imprinting stimulus, showing no preference for the stimulus presented in training. However, if a six-hour interval was allowed before making the lesions, the chicks retained their preference for the training stimulus, demonstrating that, over a period of time (3 – 6h post-training), the memory for the imprinting stimulus had been transmitted from IMM to an additional store elsewhere in the brain. Whilst this additional store, known as S' ("s-dash"; Bolhuis & Honey 1998), has yet to be localised, it has been the key to understanding the functional lateralisation of IMM in imprinting.

Lateralisation in Visual Imprinting

The imprinting-related biochemical and morphological changes in IMM are more prevalent in the left IMM than in the right (Horn 2004). Such asymmetries include the increases in (i) PSD length (Bradley et al. 1981; Horn et al. 1985), (ii) number of NMDA receptors, (iii) NMDA receptor binding (Johnston et al. 1995), and (iv) the three major isoforms of neural cell adhesion molecule. These changes are significant in the left, but not the right IMM. None have been reported with the opposite lateralisation. Further examples are presented in Table 6.2.

However, unilateral damage to IMM in a newly hatched chick *does not* prevent imprinting, irrespective of which side remains intact. Thus, both left and right IMM are capable of supporting the learning process (Cipolla-Neto et al. 1982). The effect of subsequently lesioning the remaining IMM is both side- and time-dependent. Chicks trained with only the left IMM intact are rendered amnesic by subsequently lesioning that IMM regardless of the time elapsed since imprinting. Chicks trained with only the right IMM intact are rendered amnesic by the second lesion only if that lesion is made sooner than six hours after

Table 6.2. Lateralisation in visual imprinting in the domestic chick. The table presents a representative sample of studies addressing the functions of the three stores for imprinting memory: left IMM (L), right IMM (R), and S'. The Store column indicates whether findings were localised to one store only (L in every case), two stores and equivalent (L = R), two stores and different (L ≠ R, L ≠ S'), or whether both sides were treated, but not considered separately (L + R). Studies of L and R which implicated S' are denoted (S').

Store	Reference	Method	Result
L = R	McCabe et al (1981)	Pre-training bilateral electrolytic lesions	Bilateral lesions impair acquisition of imprinting
L = R	McCabe et al (1982)	Post-training bilateral electrolytic lesions	Bilateral lesions impair retention of imprinting
L = R (S')	Cipolla-Neto et al (1982)	Sequential electrolytic lesions	L + R IMM both capable of supporting imprinting. Additional store (S') outside IMM.
L ≠ R (S')	Horn et al (1983)	Sequential electrolytic lesions	L + R IMM capable of independently supporting imprinting. R necessary for formation of S'
L ≠ S'	Honey et al (1995)	Unilateral electrolytic lesions	Different representations of imprinted stimuli in L & S'
L ≠ R	Johnston & Rogers (1998)	Unilateral intraventricular glutamate administration	Disruption of imprinting memory by post-training administration to R, not L
L	Horn et al (1985)	PSD length	Increase in L, not in R
L	McCabe & Horn (1991)	NMDA receptor number	Increase in L, not in R
L	Johnston et al (1995)	NMDA receptor binding	Increase in L, not in R
L	Meredith et al (2004)	Ca ²⁺ -dependent, K ⁺ -stimulated neurotransmitter release	Increased GABA and taurine in L, not in R
L	Sheu et al (1993)	MARCKS phosphorylation	Increased phosphorylation of MARCKS (a PKC substrate protein) in L, not in R
L	Solomonias et al (1997)	Clathrin heavy chain protein	Increased clathrin (associated with synaptic vesicle release/uptake) in L, not in R
L	Solomonias et al (1998)	Neural cell adhesion molecules	Increase in the three major isoforms of neural cell adhesion molecule in L, not in R
L	Solomonias et al (2003)	Amyloid precursor protein	Elevated amyloid precursor protein in L, not in R
L	Solomonias et al (2013)	AMPA receptor phosphorylation	Increase in phosphorylation of the GluA1 receptor subunit in L, not in R
L = R	Ambalavanar et al (1999)	Fos-immunoreactivity	Fos expression, co-localised with GABA-containing neurons, in L and R
L	Brown & Horn (1994)	Electrophysiology	Elevated responsiveness to an imprinted stimulus in L
L = R	Nicol et al (1995)	Electrophysiology	Elevated responsiveness to an imprinted stimulus in R, and comparison to L
L = R	Jackson et al (2008)	Electrophysiology	EEG elevation in undisturbed rest following imprinting
L ≠ R	Nicol et al (in preparation)	Electrophysiology	More selective responsiveness in L, more generalised responsiveness in R

the end of training (Horn et al. 1983). Thus, S', the additional store for imprinting memory mentioned above, is formed only when the right IMM is intact. Intracerebral injection of glutamate to the right IMM, but not left, in the period when S' is formed, disrupts retention of imprinting memory (Johnston & Rogers 1998).

This property of IMM has allowed investigation of the functions of S' dissociated from those of IMM. After imprinting, chicks can be prepared either by making a lesion in the right IMM immediately after training, or by making bilateral lesions after a delay sufficient to allow formation of S'. In the former case, S' cannot be formed and memory for the imprinting stimulus is held only in the left IMM; in the latter, IMM is expunged in both hemispheres, and the memory remains only in S'. Chicks were imprinted jointly on two stimuli, in two training sessions, each of one hour, in which the stimuli were either alternated regularly throughout each session (mixed), or the stimuli were presented separately, one in each session (separate). Chicks trained using the separate procedure learn to recognise both stimuli and are able to discriminate between them, whereas those trained using the mixed procedure form a combined representation of the two stimuli, and are less able to discriminate between them (Bateson & Chantrey 1972). In a subsequent operant task where one of these stimuli was rewarded (warm air, in an otherwise cooled environment), chicks that had been imprinted using the mixed procedure were less able to discriminate between the two stimuli only when the memory for those stimuli was held in S' but not left IMM (Honey et al. 1995). Chicks with memory for the imprinted stimuli held in left IMM were equally capable of discriminating between the two when trained using either procedure. The implication of this is that left IMM forms separate representations of stimuli presented during imprinting, whereas the features of those stimuli are combined in S'.

A number of studies examining the responses of IMM neurons to visual stimuli have reported little difference between hemispheres in responsiveness to an imprinted stimulus (e.g., Nicol et al. 1998; Jackson et al. 2008). Indeed, Gabriel Horn was often mystified by the apparent similarity between left and right IMM in our electrophysiological studies, given the asymmetry apparent from other studies. However, analyses that were ongoing at the end of his life, regarding the specificity of responses of individual IMM neurons across a range of stimuli, including the visual imprinting stimulus, the auditory imprinting stimulus, alternative visual stimulus, and the combined visual and auditory imprinting stimulus, have revealed subtle, but important differences between hemispheres (Nicol et al. submitted). Individual IMM neurons may be responsive to one or more of these stimuli. Right IMM neurons are significantly more likely to respond across a range of stimuli than left IMM neurons. Those exclusively responsive to the imprinted visual stimulus occur more in the left than the right. Given the different properties of left IMM and S', and the role of right IMM in establishing S', the greater propensity of right IMM neurons for polymodal responsiveness would be an important feature in establishing S' as a store with capacity for storing combined representations of multiple stimuli.

Further, given that neurons in the IMM of either hemisphere become less responsive to stimuli if they are also unresponsive to the visual imprinting stimulus, increased

responsiveness to the imprinting stimulus in the left IMM against relatively lower background responsiveness than that in the right may in part accommodate the relative paucity in quantified biochemical and morphological changes influencing levels of sensory activity in the right IMM compared to the left.

Overtly, the left and right IMM appear similarly capable of supporting visual imprinting. However, careful analysis of the underlying dynamic processes both elucidates and explains lateralisation of function in these regions. There is a need for distinct representations of the recognition memory for imprinting, which, if co-localised might be functionally conflicting. So there is additionally a need for separate representations of the imprinted stimulus in memory.

Common Themes in Song Learning and Imprinting

Both in songbirds and domestic chicks, there is a predisposition to learn conspecific songs over heterospecific vocalisations, or naturalistic features over artificial objects, respectively. It is known which brain regions are involved in the memory representation, and especially in visual imprinting in chicks, there is detailed knowledge on the temporal dynamics of the brain regions and molecular mechanisms involved. Interestingly, in both songbirds and chicks, lateralisation is an important phenomenon in memory formation. The results discussed can pave the road towards understanding brain mechanisms of sensory memorisation, such as important for language acquisition in humans.

Avian models of lateralisation

Lateralisation of brain function is by no means an exclusively human phenomenon, as once widely believed (cf. Walker 1980). Its prevalence throughout vertebrate phylogeny, and beyond, suggests there is a profound adaptive advantage conferred by lateralisation in the brain. In the case of the Mauthner cell mediated escape response in fish, as described in "1. Introducing lateralisation" (Cantalupo et al. 1995), this advantage is clear – the response is optimal when one hemisphere is dominant. However, in more complex cognitive processes the adaptive nature of lateralisation is more subtle. In visual imprinting, memory for the visual features of the imprinting stimulus is stored in the IMM of both hemispheres. However, this information is handled differently in each side. While the left IMM retains a fixed representation of the imprinting stimulus, the right is important for establishing an additional store, S', in which the imprinting stimulus may be integrated with other learned stimuli. The neurophysiological properties of IMM neurons in the two hemispheres appear consistent with this partitioning of function: more selective responsiveness to the imprinting stimulus in the left IMM, and more generalised, polymodal responsiveness in the right. In this respect, the asymmetry of function between left and right IMM in imprinting is consistent with behavioural analyses across a range of avian species, and behavioural paradigms, whereby the left hemisphere seems important for recognition under routine conditions, and the right is

sensitive to change or novelty (Rogers 2012). Under this convention, the function of the right hemisphere would require continual appraisal across a broad range of sensory input. The responsiveness of right IMM neurons, relative to those in the left, seems consistent with this. Also in songbirds, there is some evidence for high responsiveness in the right hemisphere, and stimulus specificity in the left hemisphere. This was demonstrated in the HVC of awake starlings (George et al. 2005a). However, others found an opposite result in anaesthetised songbirds, where the right hemisphere was more selective, and the left more responsive (Voss et al. 2007; Poirier et al. 2009). Therefore, lateralisation of responsiveness versus selectiveness might be dependent on the behavioural state (sleep/anaesthesia or awake).

While the *known* circuitry for visual imprinting is less complex than that for song learning, it should be noted that the anatomical location of S' is as yet unknown. The regions involved in song learning are well established, and here, as in visual imprinting, the partitioning of functions is lateralised. In the zebra finch, song learning occurs in the juvenile, and neuronal activation related to tutor song memory formation is left hemisphere dominant (chapter 2 of this thesis). This is consistent with left hemisphere dominant activation in regions related to language acquisition in the human brain, Broca's area and Wernicke's area (e.g., Dehaene-Lambertz et al. 2010; Table 6.3). Evidence for lateralisation of perception of conspecific sounds is contradictory (Heffner & Heffner 1984; Poremba et al. 2004; Gil- Da-Costa et al. 2006; Tagliatalata et al. 2009), and there is no evidence for lateralisation of memorized sounds in non-human primates, which do not show vocal learning.

In the avian brain there is no direct commissural connection between the hemispheres of the forebrain. This property has been used to great effect in numerous studies of visual perception in birds. When required to discriminate food grains from similarly sized pebbles (the pebble floor task), birds in a range of species perform well when allowed to use their right eye (left hemisphere), but peck at random when allowed only to use the left eye (Rogers 2012). However, when required to detect a small change in an imprinted visual stimulus, chicks performed well when using their left eye, but poorly using their right eye (Vallortigara & Andrew 1991). Again, these studies point to pronounced differences in function between hemispheres: the left favouring consistency of information, the right sensitive to modification.

In the mammalian brain there is extensive commissural connectivity, permitting regulation between hemispheres. Moreover, this interhemispheric connectivity may itself be asymmetric. In mice, postsynaptic spines of CA1 pyramidal cells targeted by projections from the right CA3 are enlarged relative to those targeted by projections from the left CA3, and contain higher GluN2B, a receptor sub-unit contained by NMDA receptors supporting induction of long-term potentiation (Shinohara et al. 2008). Recent studies, using optogenetic techniques to selectively stimulate CA3 projections in one or other hemisphere, have demonstrated a left to right bias in cross-hemisphere CA3 to CA1 projections. More long-term potentiation may be induced by stimulating projections from the left CA3 than by stimulating those from the right hemisphere (Kohl et al. 2011). This asymmetry is also manifested behaviourally. With the corpus callosum and hippocampal commissure

Table 6.3. Human speech- and language lateralisation. Examples of lateralisation results in Broca's and Wernicke's areas are separated for age- and clinical groups. In the summary column, 'L=R' means that no hemispheric differences were found; 'L' means that the left hemisphere was dominant. This is not an all-inclusive literature review, but we aimed to provide a representative overview of the current literature status on lateralisation of human speech and language. Adults or more proficient subjects generally had left-dominant patterns of brain activation, while younger or less proficient subjects show larger networks of brain

Group	Brain region	Summary	Age	Task
Preterm newborns	Broca's area	L	30 weeks gestational age	Listening to human speech (syllables)
	Wernicke's area	L	30 weeks gestational age	Listening to human speech (syllables)
Newborns	Broca's area	L	2-5 days old	Listening to human speech vs. hummed, flattened speech or non-speech
	Wernicke's area	L / L=R	2-5 days old	Listening to human speech vs. hummed, flattened, reversed speech or non-speech
		L	0-1 day old	Listening to mother's vs. stranger's speech
Babies	Broca's area	L	3-6 months old	Listening to human speech vs. rest, two different languages or non-speech
		L	12 months old	Listening to human speech vs. non-speech
	Wernicke's area	L / L=R	2-4 months old	Listening to human speech or mother's speech vs. rest, non-speech sounds or stranger's speech
		L	6-9 months old	Listening to audio + visual human speech vs. visual only or human speech vs. non-speech
		L	12 months old	Listening to human speech vs. non-speech
Children	Broca's area	L	4-6 years old	Listening to human speech, correct vs. incorrect sentences
		L / L=R	7-9 years old	Listening to human speech, correct vs. incorrect sentences or covert word generation task
		L / L=R	10-18 years old	Listening to human speech, correct vs. incorrect sentences, covert word generation task or word reading
	Wernicke's area	L	4-6 years old	Listening to human speech, correct vs. incorrect sentences
		L	7-9 years old	Listening to human speech, correct vs. incorrect sentences
		L	10-12 years old	Listening to human speech, correct vs. incorrect sentences, word reading

activation, which can be large areas in the left hemisphere, or right dominant or bilateral activation. In newborn infants, listening to human speech already evokes left-dominant brain activation. Strong lateralisation of language-related brain activation in Wernicke's area can be found at a younger age than in Broca's area. In disorders in which language abilities are affected, brain activation is less left dominant than in healthy subjects. Abbreviations: EEG, Electroencephalography; fMRI, Functional Magnetic Resonance Imaging; MEG, Magnetoencephalography; fNIRS, Functional Near Infrared Spectroscopy; PET, Positron Emission Tomography.

Method	Lateralisation result	References
fNIRS	Activation is left-dominant	Mahmoudzadeh et al., 2013
fNIRS	Anterior auditory cortex right-dominant, posterior (Wernicke's area) left-dominant	Mahmoudzadeh et al., 2013
fMRI, MEG	Large area of activation especially in the left hemisphere; Broca's area not yet involved in speech perception	Perani et al., 2011; Imada et al., 2006
fMRI or optical topography	Auditory cortex in both hemispheres is activated, or left auditory cortex was more activated than right	Perani et al., 2011; Pena et al., 2003; Imada et al., 2006
EEG	Left auditory cortex was more activated than right	Beauchemin et al., 2011
fMRI, EEG MEG	Activation is left-dominant or mildly left-dominant	Dehaene-Lambertz et al., 2006; Friederici et al., 2007; Imada et al., 2006
MEG	Activation is left-dominant	Imada et al., 2006
fMRI, fNIRS	Auditory cortex in both hemispheres is activated for human speech vs. rest; Larger areas in the right hemisphere; More posterior area (Wernicke's area) is left-dominant; Left-dominant for mother's speech	Dehaene-Lambertz et al., 2006; Dehaene-Lambertz et al., 2010; Minagawa-Kawai et al., 2011
fNIRS, MEG	Activation is left-dominant	Bortfeld et al., 2009; Imada et al., 2006
MEG	Activation is left-dominant	Imada et al., 2006
fMRI	Activation is mildly left-dominant, some children show right-dominance or bilateral activation	Berl et al., 2014
fMRI	Activation is left-dominant, some children show bilateral activation	Berl et al., 2014; Holland et al., 2001
fMRI, EEG	Activation is left-dominant or mildly left-dominant or bilateral	Berl et al., 2014; Gaillard et al., 2000; Holland et al., 2001; Spironelli and Angrilli, 2010
fMRI	Activation is mildly left-dominant	Berl et al., 2014
fMRI	Activation is left-dominant	Berl et al., 2014
fMRI, EEG	Activation is left-dominant	Berl et al., 2014; Spironelli and Angrilli, 2010

Table 6.3. *Continued*

Group	Brain region	Summary	Age	Task
Adults	Broca's area	L	19-48 years old	Covert word generation task or word reading
	Wernicke's area	L	22-59 years old	Covert word generation task or word reading
Adults learning a new language	Broca's area	L	23 years old	Daily learning sessions to learn 80 new words; early (not proficient) and later phase (proficient)
	Wernicke's area	L=R	21-32 years old	Not very proficient in new language
		L	19-50 years old	Proficient learning level
Autistics	Wernicke's area	R	2-4 years old	Forward vs. reversed speech
Dyslectics	Wernicke's area	L=R	8-12 years old	Dichotic pitch stimuli
Schizophrenics	Wernicke's area	L=R	27 years old	Verb-generation and reversed-reading task

transected, and with visual input restricted to one eye, mice are reported to be better able to perform spatial tasks using the left eye (right hemisphere) than with the right eye, while non-spatial hippocampus-dependent tasks are unaffected (Shinohara et al. 2012).

Functional lateralisation has been demonstrated in both avian and mammalian brains. Interestingly, interhemispheric connectivity is very different between the two brain systems, with either multiple small connections between hemispheres or with small connections and in addition a major corpus callosum, respectively. Therefore, there are at least two ways in which interhemispheric communication can be organised. One possible mechanism of lateralisation is that one hemisphere actively inhibits the other; another possibility is that specialisation of the dominant hemisphere leads to higher levels of activation, while the other hemisphere is less efficiently activated. If inhibition were the most important mechanism for lateralisation, there should be strong connections between the hemispheres. However, avian lateralisation suggests that an interhemispheric connection that is as efficient as the corpus callosum is not necessary for lateralisation to occur. Experiments in split-brain birds and mammals could further clarify the role of interhemispheric connections in lateralisation.

There is considerable variation in lateralisation between human individuals. Most people have strongly left-lateralised language functions, and strongly right-lateralised spatial orientation brain activation, but others have weaker lateralisation patterns, or bilateral patterns (Risse et al. 1997; Knecht et al. 2001). These alternative patterns of lateralisation do not seem to have

Method	Lateralisation result	References
fMRI, EEG, Doppler ultrasonography	Activation is strongly left-dominant; No differences in linguistic abilities of left-dominant, bilateral or right-dominant subjects	Gaillard et al., 2000; Spironelli and Angrilli, 2010; Knecht et al., 2001
EEG, Doppler ultrasonography	Strongly left-dominant; No differences in linguistic abilities of left-dominant, bilateral or right-dominant subjects	Spironelli and Angrilli, 2010; Knecht et al., 2001
fMRI	Activation is left-dominant; Non-linguistic brain regions involved in early learning phase when not proficient yet	Raboyeau et al., 2010
PET scans	Reduced activation relative to native language	Perani et al., 1996
PET scans	Second language same activation characteristics as native language	Perani et al., 1998
fMRI	Reduced activation relative to typically developing children; Activation is right-dominant	Redcay and Courchesne, 2008; Eyler et al., 2012
EEG, MEG	Reduced left-dominance relative to typically developing readers	Johnson et al., 2013
fMRI	More activation in language-related brain regions relative to healthy controls; More right-sided activation, and thus reduced left-dominance; The less left-dominant, the more auditory hallucinations	Sommer et al., 2001

a direct reflection on linguistic abilities (Knecht et al. 2001) or cognitive performance (Lust et al. 2011). On the contrary, weakly lateralised or bilateral people might have an advantage over strongly lateralised individuals: it was shown that strongly lateralised individuals are more susceptible to a loss of function when the dominant hemisphere is inhibited than individuals that have more bilateral brain activation for the function (Knecht et al. 2002). However, most people are left dominant for language processing, and it follows that this may reflect a fitness advantage, at least at the population level. Indeed, individuals that showed left dominant brain activation for language and right dominant brain activation for spatial orientation performed better than people with atypical lateralisation patterns when they had to do a linguistic and spatial task simultaneously (Lust et al. 2011). When children become more proficient in their language, left-dominance of brain activation increases (e.g., Imada et al. 2006; Berl et al. 2014; Table 6.3), further suggesting that it is beneficial to be left lateralised. Indeed, abnormal lateralisation in the human brain may have pathological implications. Auditory perception is significantly less lateralised to the left hemisphere in dyslexic children than in healthy controls (Johnson et al. 2013). In autism, language skills can vary from very poor to outstandingly excellent. Temporal lobe activation was right dominant for language perception in autistic children with impaired language comprehension skills (Eyler et al. 2012). In schizophrenic patients who suffered from hearing voices, it was found that the right hemisphere had increased neural activation levels relative to healthy controls. Auditory hallucinations were most severe in patients with the most activation in the right

hemisphere (Sommer et al. 2001; Oertel et al. 2010). Therefore, it seems that left-dominance of language functions is advantageous to the individual. Similarly, stronger left-dominance of song memory was correlated to better song imitation in juvenile zebra finches (chapter 3 of this thesis). Research in zebra finches and domestic chicks will promote our understanding of mechanisms of memory-related lateralisation. Furthermore, future studies should investigate the neural causes of individual lateralisation- and performance differences.

We have speculated here on the adaptive functions of lateralisation. Through many levels of complexity and phylogeny, lateralisation is a conserved and recurring feature. Lateralisation of human language and song learning in birds suggests evolutionary convergence. However, a genetic basis for left or right dominance in any cognitive or behavioural function remains elusive. Right-handedness occurs in approximately 90% of people, and there is compelling evidence for handedness being influenced by genetic factors (Ocklenburg et al. 2013). However, cultural and epigenetic factors are sufficiently powerful to obscure familial tendencies (Laland 2008). In other animal models of lateralisation, these factors may be similarly difficult to disentangle. For example, the preweaning offspring of altricial species will be susceptible to environmental factors that may shape hemispheric bias. The chicks used in our studies of visual imprinting are hatched and reared in darkness and in isolation, and so are relatively immune to such influences. As such, it may be a safe assumption that the direction of lateralisation in imprinting is genetically determined, and this may be a valuable resource in a quest to find a genetic basis to hemispheric asymmetry.

Lateralisation might be crucial during learning and memory formation. Spatial separation of a consolidated memory from the site of ongoing analysis of sensory information may prevent interference. Thus, in chicks, the consolidated memory for an imprinted stimulus might be held in left IMM while *S*₁ in conjunction with right IMM, continues to update representation of the memorised stimulus with new information. In songbirds, a similar outcome may be accomplished by lateralisation in the NCM. In parallel, lateralisation is important during human language acquisition. In human infants, language lateralisation increases with advancing proficiency. In addition, impaired neural lateralisation of linguistic processing is correlated with language deficits in humans, but the functional mechanism of language lateralisation is unknown (Bishop 2013). Because of our considerable understanding of lateralisation in song learning and visual imprinting, these avian models are ideal candidate systems for providing a thorough understanding of the role of lateralisation and its involvement in memory formation.

7

General Discussion

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Discussing the neural mechanisms of song memory formation in juvenile zebra finches

There are many parallels between the acquisition of spoken language in human infants and birdsong learning, at the behavioural, neural, genetic and cognitive levels (Doupe & Kuhl 1999; Bolhuis et al. 2010). Birdsong provides an excellent model for vocal learning and auditory memory formation, because song learning and imitation is a behaviour that comes very natural to the juvenile songbird, happens in a period early in life and can be experimentally manipulated. In this thesis, I have explored neuronal mechanisms of early memory formation in juvenile zebra finches. I have studied neuronal activation in the NCM, a higher-order auditory brain region that is involved in song perception, recognition and memory (see the introduction of this thesis, chapter 1). Here, I will discuss the evidence for a neural substrate for tutor song memory first, and then continue with the role of sleep for tutor song memory consolidation. The importance of lateralisation for song learning and memory was already discussed in chapter 6 of this thesis.

7

Roles for the song system?

The role of the NCM as the neural substrate for tutor song memory has been critically addressed recently, because experiments showed that HVC activation was important for song imitation. When HVC activation was disrupted in juvenile zebra finches during tutoring, the birds were unable to learn the tutor song (Roberts et al. 2012). This was not caused by missing time to practise singing, because when HVC activation was disrupted outside the tutoring sessions, song imitation was unaffected. Disruption of NIf activation also impaired tutor song imitation. NIf is the major source of auditory information to HVC, therefore, the experiments suggested that auditory information concerning the tutor song is needed in HVC, while the tutor is singing, for proper song imitation (Roberts et al. 2012). It was therefore suggested that HVC is the locus of tutor song memory storage (Roberts & Mooney 2013). Indeed, these results suggest that activation of HVC during tutoring is important for song imitation, i.e., to produce a song that resembles tutor song. However, it is unclear whether or not these juveniles formed a memory representation of tutor song. To further test whether HVC is the neural substrate of long-term tutor song memory, recognition tests would have to show that the juveniles in which HVC signalling was disrupted also do not recognize tutor song in behavioural preference tests.

In juvenile zebra finches early in the sensorimotor phase, many HVC neurons were tutor song-selective when electrophysiology recordings were made in the awake bird (Nick & Konishi 2005a). When the birds were older (late sensorimotor phase or adult), the response to tutor song had decreased substantially, but there was a strong response to the bird's own song (BOS; Nick & Konishi 2005a). Interestingly, HVC responses to BOS in juvenile zebra finches were strongest to the BOS as recorded on the same day, while an earlier version of

BOS that was recorded when the bird was younger did not evoke strong responses (Nick & Konishi 2005b). This shows that in the zebra finch HVC, neuronal responsiveness to tutor song in juveniles is transient, and is replaced by responsiveness to the BOS that is also continuously updated to the most current version.

Swamp sparrows sing a large repertoire of songs, and juveniles do not imitate all of the tutor songs as adults. Neuronal responsiveness to tutor songs was tested in HVC of adult swamp sparrows, both to songs that the birds had imitated from their tutor, but also to tutor songs that were not part of the birds' own repertoires. HVC neurons were responsive to many of the tutor songs, even those that were never imitated (Prather et al. 2010). This suggests that HVC in swamp sparrows has a representation of auditory tutor song memory. Also, HVC might play different roles in swamp sparrows and zebra finches. Perhaps the difference between the two species is caused by the fact that the swamp sparrow is an open-ended learner that can sing many different songs, while the zebra finch is an age-limited learner with only one single song in its repertoire. As described above, when zebra finches have learned their song, HVC neurons are not selective for the tutor song any longer, but have become responsive to the BOS. In swamp sparrows that can learn new songs throughout their lives, the tutor song representations in HVC might be maintained.

Thus, HVC is important for song production and tutor song imitation, and perhaps therefore there is a temporal representation or neuronal selectivity for tutor song in HVC. However, future studies have to shed light on whether HVC is part of the neural substrate of tutor song memory. In swamp sparrows there was long-term tutor responsiveness (Prather et al. 2010). It would be interesting to investigate whether in swamp sparrows, HVC is involved in behavioural tutor song recognition. It may be that HVC activation is driven by inputs from higher-order auditory regions. Indeed, it was shown that auditory input from the CMM (Bauer et al. 2008), and estrogen-related input from the NCM (Remage-Healey & Joshi 2012) might drive song-selective responses in HVC. It is not known whether HVC neurons are also tutor-song selective when input from these higher-order auditory regions would be blocked.

Tutor song-selective neurons have also been found in Area X (Solis & Doupe 1997; Solis & Doupe 1999) and LMAN (Achiro & Bottjer 2013) in juvenile male zebra finches. Interestingly, Area X and LMAN of juveniles contain both cells that are tuned to tutor song and cells that are tuned to BOS. However, tutor-song selectivity was greatly reduced in adults (Solis & Doupe 1997; Solis & Doupe 1999; Achiro & Bottjer 2013), while adult songbirds can still recognize tutor song. The fact that tutor song selectivity disappears after song learning suggests that neither Area X nor LMAN are the major loci of tutor song memory. Rather, because of the importance in auditory feedback, these regions might be involved in comparing BOS to tutor song in order to improve song imitation in juveniles. Further evidence that LMAN contains a transient tutor song representation comes from an experiment in which juveniles were tutored with two different adults consecutively. LMAN neurons responded to the current tutor song, but not to the earlier tutor song (Yazaki-Sugiyama & Mooney 2004). Why would LMAN have a transient representation of tutor song? In juveniles, LMAN is important for

sensorimotor learning and vocal exploration (Bottjer et al. 1984; Scharff & Nottebohm 1991; Brainard & Doupe 2000a). Indeed, transiently blocking neuronal activation in LMAN during tutoring impaired song learning (Basham et al. 1996). The current evidence suggests that LMAN is important for integration of auditory feedback, BOS and a tutor song representation, to improve song imitation in juveniles.

Alternatively, the neuronal substrate for tutor song memory could comprise a distributed network, where auditory regions are important for tutor song recognition, while song production nuclei also have temporal representations of tutor song to guide vocal learning. Instead of having one single neural substrate, a distributed network might involve the NCM, CMM, HVC shelf, RA cup and CLM, as all of these regions have been shown to be involved in auditory perception (Mello & Ribeiro 1998; Mello et al. 2004). However, the specific roles of HVC shelf, RA cup and CLM have not been investigated to date.

Song memory in females and the role of the CMM

7

Previous work showed that in adult female zebra finches, the NCM and CMM were both activated in response to tutor song (which is usually their father's song), and the level of activation was higher than when unfamiliar song was played (Terpstra et al. 2006). However, the level of activation was much higher in the CMM than in the NCM, and the difference between neuronal responsiveness to tutor and novel song was significant in the CMM only (Terpstra et al. 2006). Neuronal activation in the CMM and NCM was also significantly greater to tutor song than novel song in female Bengalese finches (Kato et al. 2010). It has been suggested that tutor song memory in females functions as a reference frame or guidance for mate selection, perhaps as a way to recognize singers' reproductive quality or to recognize the local song when there is geographically-based variation between conspecific songs (Riebel 2003; Hernandez et al. 2008). Therefore, it would be expected that the CMM would play a role in mate selection. A lesion experiment suggested that this is indeed the case: lesions to the CMM caused female zebra finches to perform courtship displays to heterospecific song, which they normally only do in response to conspecific song (MacDougall-Shackleton et al. 1998). In female canaries, Zenk expression in the CMM was higher when the birds heard songs containing so-called 'sexy' syllables (canary syllables of which it is known that they are attractive to females), than when they heard 'nonsexy' syllables (Leitner et al. 2005). Furthermore, there was more neuronal activation in the CMM of female zebra finches that heard directed song than undirected song (Woolley & Doupe 2008). Directed song is the song that a male sings to a female; it is the same song that the zebra finch always sings (zebra finches have only one song), but it is sung faster and with less spectral variability than undirected song. Females preferred directed song over undirected song in a preference test (Woolley & Doupe 2008). However, when comparing conspecific and heterospecific songs, there was neuronal activation in the CMM in response to both song types, while the NCM was selective for conspecific song (Bailey et al. 2002;

Theunissen et al. 2004). Song complexity correlated significantly and positively with NCM activation (and only to a lesser extent with CMM activation) in female budgerigars (Eda-Fujiwara et al. 2003). In female starlings that were presented with attractive longer motifs or less attractive short ones, there was differential activation in the NCM, but not the CMM (Gentner et al. 2001). Thus, there is some support for the CMM being important for mate selection, but other data point to the NCM.

The CMM is also involved in song perception and discrimination in male songbirds (Avey et al. 2005; Bailey & Wade 2005). Similar to females, in male zebra finches, the NCM and CMM were both activated in response to tutor song (Terpstra et al. 2004). In male Bengalese finches (Kato et al. 2012) and male zebra finches (Terpstra et al. 2004; Bauer et al. 2008), the CMM was strongly responsive to BOS. Furthermore, neurons in the CMM were selectively activated by auditory perception of learned conspecific vocalisations in European starlings (Gentner & Margoliash 2003; Gentner et al. 2004). In male European starlings that were trained in an auditory discrimination task, the CMM, but not the NCM, was activated during task performance. Interestingly, when the birds learned to discriminate two novel songs (same task, new stimuli), both the CMM and NCM were strongly activated (Gentner et al. 2004). Similar to the present results in juvenile females (chapter 5 of this thesis), it suggests that the CMM may be more important for long-term memory storage, while the NCM is involved in early memory formation, in the acquisition and encoding phase, and perhaps also for providing input to the song system for vocal imitation in males.

Song memory formation in juvenile songbirds

In juvenile females that had been housed with a tutor for only two days (chapter 5 of this thesis), the CMM was not as strongly activated upon tutor song re-exposure as in adult female zebra finches (Terpstra et al. 2006). However, the juvenile females did show significant levels of neuronal activation in the NCM (chapter 5 of this thesis), suggesting that the NCM may be important during early stages of tutor song memory formation, while the CMM might be important in later stages. Neuronal activation characteristics in the NCM in juvenile females were very similar to those of juvenile males (chapter 4 of this thesis). There are only a few studies in which auditory discrimination or memory in juvenile males and females was compared, but these studies also suggest that neuronal activation is similar in both genders (Bailey & Wade 2005; Tomaszycycki et al. 2006). In adult zebra finch males and females, both the CMM and NCM were activated when tutor song was presented. However, in females, the CMM showed more neuronal activation than the NCM, and the difference between tutor song and unfamiliar conspecific song was significant in the CMM only (Terpstra et al. 2006), while in males, a correlation between memory strength and neuronal activation was found in the NCM only (Bolhuis et al. 2000b; Bolhuis et al. 2001; Terpstra et al. 2004; Phan et al. 2006). Thus, while in both males and females the same neural structures may be involved in tutor song memory formation early in life, at an older

age there is a differential neuronal activation pattern between males and females. This may be because the two sexes use tutor song memory for different purposes - males as a template on which they base their own song, females as reference for mate selection.

Miller-Sims and Bottjer (2014) recorded multi-unit activity from awake, restrained adult and juvenile male zebra finches, using single electrodes in one position in the NCM. They compared neuronal habituation (as in Phan et al., 2006) across the different ages. High rates of neuronal habituation (a steep decline from first to later response strength) are thought to reflect the novelty of the stimulus. In contrast to what was previously found in adults, the juveniles showed high habituation rates for tutor song, even though the song was already very familiar to them (Miller-Sims and Bottjer, 2014). Neurons in the NCM of juveniles were not selectively activated by tutor song, in contrast to what was found in adults. However, juveniles showed higher rates of neural habituation to tutor song than to other familiar conspecific songs, which suggests that the NCM has a special response for tutor song during song learning, in which the habituation rate does not decrease, even though the song is very familiar. For the special case of tutor song, memory formation might be more detailed and a stronger, long-term memory trace has to be formed. Therefore, the adult-typical reduction in habituation rate might only occur after the song has been fully and thoroughly memorized.

Recently, Yazaki-Sugiyama and colleagues (personal communication) investigated the responsiveness of neurons in the NCM to tutor song and to a number of alternative stimuli. Flecke and Yazaki-Sugiyama (manuscript in preparation) performed multi-electrode electrophysiological recordings in the dorsomedial part of the NCM of anaesthetised zebra finch males. The juvenile males were isolated from their father before 12 days after hatching. Starting at 55 days after hatching, the males were tutored by a male zebra finch for five days, after which multi-unit recording started. The birds were exposed to tutor song, or a number of other song stimuli including BOS and unfamiliar zebra finch songs. In tutored birds, 7.6% of all units preferentially responded to tutor song, which was significantly greater than the biased responsiveness to any of the other song stimuli. In age-matched isolated controls that had not been exposed to tutor song, only 2.3% of all units preferentially responded to the same song, which was not significantly different from preferential responsiveness to any of the other stimuli. In a subsequent electrophysiological study in awake juvenile zebra finches, Yanagihara and Yazaki-Sugiyama (2014) recorded single-unit activity in the NCM of freely moving males before and after tutor song exposure. A number of auditory stimuli were presented during neural recording, including tutor song, BOS, unfamiliar conspecific song, heterospecific song, male calls or female calls. Before tutoring, NCM neurons did not exhibit preferential responsiveness to any of the song stimuli that were presented. However, after 4-5 days or more of exposure to a live tutor, a group of 19 neurons (out of 246) showed highly selective responses to the tutor song. Another group of 12 neurons responded selectively to BOS, and 5 other neurons selectively responded to both tutor song and BOS. Taken together, these findings suggest that relatively brief exposure to tutor song shapes the response properties of a subset of neurons in the NCM of juvenile zebra finch

males. Importantly, learned songs appear to be sparsely represented in the NCM, with about 15% of cells responsive to tutor song, BOS, or both. This may explain why Miller-Sims and Bottjer (2014) did not find preferential responsiveness to tutor song in the NCM of juvenile males in their study. The results from Yazaki-Sugiyama's laboratory are consistent with a role for the NCM in the representation of tutor song memory, as was previously hypothesised (Bolhuis and Gahr, 2006; Bolhuis et al., 2010).

Subregions within the NCM

The NCM “is a big place”, according to Maney and Pinaud (2011). In the literature, the NCM is often divided into sub-parts, mainly on the basis of functional considerations. In our laboratory, and throughout the project described in this thesis, we measure Zenk activation at the extreme caudal pole of the NCM, and we make a distinction between the “medial” and “lateral” NCM, on the basis of differential activation (Terpstra et al. 2004). The caudal part of the NCM has neuronal projections, electrophysiological responses and neurochemical characteristics that are different from those in the larger rostral part of the NCM (Maney & Pinaud 2011). Furthermore, the caudal part of the NCM has greater neuronal activation in response to song than the rostral part (Maney & Pinaud 2011; personal observations). The medial NCM is generally more responsive to tutor song (Terpstra et al. 2004; Gobes et al. 2010; chapters 2, 4 & 5 of this thesis; see table 7.1). The habituation rate to tutor song measured with electrophysiology was found to correlate with strength of tutor song imitation in the medial NCM (Phan et al., 2006). However, correlations between strength of tutor song imitation and neuronal activation (measured as immediate early gene expression) were found in the lateral NCM in four separate experiments (Bolhuis et al. 2000b; Bolhuis et al. 2001; Terpstra et al. 2004; chapter 3 of this thesis). In two studies in which the lateral part of the NCM was lesioned or blocked, males showed impaired tutor song recognition (Gobes & Bolhuis 2007), or they could not successfully imitate the tutor song (London & Clayton 2008; see table 7.1). However, neither study found that lesions had a 100% complete detrimental effect on song memory. Although being severely impaired, there was some tutor song recognition or song imitation left. Perhaps this was the case because the medial NCM was left intact. In a training paradigm where birds had altered their song to avoid negative reinforcement, large lesions extending the medial and lateral NCM were very effective in preventing recovery to the original song once song interference was withheld (Canopoli et al. 2014; see table 7.1).

In the present study, juveniles who had had more than a month of tutor song experience (chapter 2 of this thesis), exhibited tutor song-specific responses in the medial and lateral NCM, with greater activation levels in the medial NCM. Neuronal activation in the left or right NCM and song similarity percentage were not correlated. Then we calculated the relative neuronal activation levels between hemispheres in a lateralisation ratio (activation in the left NCM minus right, divided by the total). The lateralisation ratio correlated with song similarity, in both the lateral and medial NCM (only the results in the medial NCM were presented in

Table 7.1. Examples of literature on the lateral and medial sub-parts of the NCM and their roles in tutor song memory

Sub-part of the NCM	Result	Stimulus / treatment	References
Medial NCM	Neuronal activation levels Medial > Lateral	Auditory perception: Tutor song playbacks	Terpstra et al. 2004; Gobes et al. 2010; chapters 2, 4 & 5 of this thesis
	Impaired recovery to the original BOS (see text)	Lesions to the medial NCM in adult males, extending well into lateral NCM	Canopoli et al. 2014
	Correlation between habituation rate and song similarity	Repeated tutor and unfamiliar song playbacks	Phan et al. 2006
	High habituation rates for tutor song	Repeated tutor and unfamiliar song playbacks	Miller-Sims & Bottjer 2014
	Selective responses to tutor song in a small subset of neurons	Tutoring and re-exposure to tutor song	Flecke & Yazaki-Sugiyama 2014; Yanagihara & Yazaki- Sugiyama 2014
Lateral NCM	Correlation with song similarity with the tutor song	Neuronal activation in the lateral NCM	Bolhuis et al. 2000b; Bolhuis et al. 2001; Terpstra et al. 2004; chapter 3 of this thesis
	Tutor song recognition was impaired	Lesions to the lateral NCM in adult males	Gobes & Bolhuis 2007
	Tutor song imitation was impaired	ERK signalling blocked in the lateral NCM in juvenile males	London & Clayton 2008
	Impaired recovery to the original song (see text)	Lesions to the medial NCM in adult males, extending well into lateral NCM	Canopoli et al. 2014

chapter 2). During sleep, there was a correlation between spontaneous neuronal activation and song similarity (chapter 3 of this thesis), but this correlation was found in the lateral NCM only (only the results in the lateral NCM were presented in chapter 3). In the juveniles described in chapter 4 of this thesis, who had only two or ten days of tutoring experience, the difference between response to tutor song and silence was greater in the lateral than medial part of the NCM, especially during sleep. Furthermore, activation in the lateral NCM was left hemisphere dominant, but this was not true for the medial NCM. In adult females, there was no difference between the lateral and medial NCM (Terpstra et al. 2006), but in juvenile females the medial NCM was significantly more activated than the lateral part (chapter 5 of this thesis).

In conclusion, it seems plausible that both the medial and lateral part of the NCM are involved in tutor song memory. Possibly, the lateral part might be most important for vocal tutor song imitation, for example by providing tutor song information to the song system.

Neuronal habituation

When zebra finch males are repeatedly exposed to an auditory stimulus, neuronal responsiveness in the medial NCM rapidly decreases relative to the initial neural response. This decreased responsiveness is not due to sensory adaptation, and has been called neuronal habituation (Chew et al. 1995; Mello et al. 1995; Chew et al. 1996; Stripling et al. 1997; Phan et al. 2006; Thompson & Gentner 2010). If a different song was played subsequently, there was increased neuronal responsiveness, at the initial level, showing that habituation was stimulus-specific (Mello et al. 1995; Chew et al. 1996). Habituation occurred immediately; the neuronal response to the second stimulus exposure was already much lower than to the first stimulus playback. When playbacks were continued, the neuronal response continued to decrease until it was about half of the initial response after about 50 auditory stimulus presentations. From that point on, if playbacks were continued, the response did not decrease any further (Chew et al. 1995; Stripling et al. 1997). Unlike traditional habituation as defined by psychologists, the effect described here was a long-term effect: if the auditory stimulus to which the bird was habituated was presented again after a pause in which the bird remained in silence or was exposed to another song, the neuronal response started at the habituated level again. This was true if the stimulus was presented up to 48 hours later (Chew et al. 1996), and could even last until up to two months (Woolley & Doupe 2008). Habituation rate is related to song familiarity: novel songs show a faster habituation response than familiar songs (Phan et al. 2006). Similarly, in a study in starlings, it was shown that NCM neurons had reduced responsiveness to songs that the birds had been trained on in an operant recognition task, regardless of the specific details of those songs (Thompson & Gentner 2010). There was also behavioural habituation. Territorial responses decreased when a song was repeated (Dong & Clayton 2009; Geberzahn et al. 2013). In zebra finches, playbacks of a novel song caused the birds to stop all ongoing behaviours and sit silently. When playbacks were repeated, birds resumed their activities, a phenomenon called response latency (Stripling et al. 2003).

Habituation could be a reflection of memory formation occurring at some other level, but it could also be one of the mechanisms that directly underlies memory formation. Indeed, one can imagine that habituation could be beneficial for song memory formation. Once a bird detects a new song, it will memorize the song, for short or longer time spans, so memory encoding will occur. However, neuronal responses to continued song exposure might interfere with encoding. Habituation might prevent such interference, because it reduces neuronal activation in response to ongoing songs. Accordingly, it was shown that experimental manipulations blocking neuronal habituation also prevented song learning. When ERK activation was inhibited during repeated song playbacks (“training”), the Zenk response to the same song was high on the next day, as if the bird heard a novel song, showing that ERK activation was necessary for neuronal habituation (Dong & Clayton 2008). The same lack of Zenk habituation was caused by inhibiting caspase-3 during song training (Huesmann & Clayton 2006). Although caspase-3 is known for its role in programmed cell death (apoptosis), it is also crucial for long-term spatial memory in rats (Dash et al.

2000; Markina et al. 2009). Thus, after ERK or caspase-3 inhibition during song training, neuronal responsiveness occurred as if the bird had never heard the stimulus before. Does a lack of habituation response mean that there is also no behavioural or cognitive memory of the song? This was tested by measuring behavioural response latencies in birds in which ERK was inhibited during training. However, after cannulae were implanted, the behaviour of both ERK inhibited and control animals was affected, with none of the birds showing long response latencies to novel stimuli. Neuronal responses were conforming to the predictions, with high responsiveness to novel stimuli, and habituation to the trained stimulus in control animals, and high responsiveness to both novel and familiar stimuli in birds in which ERK signalling was inhibited (no habituation). Since the behaviour was probably influenced by confounding factors related to the technique (Dong & Clayton 2008; Dong & Clayton 2009), further experiments should clarify whether neuronal and behavioural habituation are related. Furthermore, future research should investigate the role of neuronal habituation in memory formation.

The role of sleep in birdsong memory formation

During sleep, the brain is not quiet; rather, there is considerable neural activity during sleep that travels through the brain (Beckers et al. 2014). As discussed in the introduction of this thesis (chapter 1), sleep is important for memory consolidation after learning (e.g., Gobes & Bolhuis 2008; Jackson et al. 2008; Van Der Werf et al. 2009; Beyaert et al. 2012). The importance of sleep has been demonstrated in a number of different learning paradigms, including language acquisition in humans (Gómez et al. 2006; Hupbach et al. 2009), and also birdsong learning (e.g., (Dave & Margoliash 2000; Hahnloser et al. 2006; Crandall et al. 2007; Brawn et al. 2010).

A phenomenon observed during sleep is neuronal replay: brain structures that were active during memory encoding are also activated during sleep, with similar temporal activation patterns (Pavlidis & Winson 1989; Wilson & McNaughton 1994; Peigneux et al. 2001). In songbirds, spontaneous neuronal replay during sleep that resembled motor patterns during day-time singing was demonstrated in the song system nucleus RA (see figure 1.6; Dave & Margoliash 2000; Shank & Margoliash 2009), Area X (Yanagihara & Hessler 2012), HVC (Hahnloser et al. 2006), and NIf (Hahnloser & Fee 2007). Neuronal activation during sleep was also demonstrated in the NCM, where the level of activation correlated positively with the strength of song learning (Gobes et al. 2010; chapter 3 of this thesis).

The changes in the brain, neurons and synapses that occur during learning and consolidation are not yet fully understood. It is supposed that learning and memory formation involves gene expression and protein synthesis within neurons, changes in synaptic connectivity and transmission between neurons, and during consolidation the neural substrate of memory might be relocated to another brain region (Horn 2004; Born & Wilhelm 2012). What happens during sleep? What is neuronal replay? Although other explanations are also possible, such as energy restoration after neuronal activation during the day (homeostasis),

spontaneous neuronal activation (such as neuronal replay) during sleep might be a reflection of the process of memory consolidation (e.g., Hennevin et al. 2007). Furthermore, it has been suggested that reactivation of the memory trace allows for its relocation, a process that has been called systems consolidation (Cipolla-Neto et al. 1982; Honey et al. 1995; Buzsaki 1998; Vassalli & Dijk 2009; Born & Wilhelm 2012). An additional mechanism called synapse consolidation might involve synaptic strengthening by reactivating neuronal networks, and/or a reduction of synapses through synaptic pruning (Vassalli & Dijk 2009). Although synaptic strengthening and pruning seem to be two contradictory hypotheses at first, they might actually work in concert, for example as global synaptic downscaling in combination with local synaptic strengthening (Vassalli & Dijk 2009).

Neuronal reactivation during sleep is thought to strengthen existing synaptic connections in the brain. Synaptic strengthening involves enlargement of the active zone (postsynaptic density) at the synapse and an increase in the number of release sites for synaptic vesicles, receptors and signalling proteins. Synaptic strengthening might thereby make signalling more efficient (Benfenati 2007; Newpher & Ehlers 2009). Other mechanisms that might be involved in synaptic strengthening are spine clustering (Fu et al. 2012) and formation of new synaptic connections (Moczulska et al. 2013). Indeed, in cats that received visual input in one eye only, there was sleep-dependent synaptic strengthening in the corresponding hemisphere (Aton et al. 2009). However, in visual imprinting in domestic chicks, it was shown that although the number of neurons that responded to the imprinted stimulus increased after training, there was no increase in functionally coupled neuron pairs as measured with electrophysiology (Horn 2004). Horn proposed that a large set of uncoupled neurons is likely to have a larger storage capacity than a highly interconnected set of neurons would have. As not all neurons are intrinsically activated by the imprinted stimulus, uncoupled neurons can transmit their signal to those neurons instead of to each other, and thereby increase neuronal responsiveness to the imprinted stimulus (Horn 2004).

In contrast to synaptic strengthening, synaptic pruning is a process of elimination of synapses, also called synaptic downscaling. In different studies in rodents and fruitflies, it was shown that after sleep, there are fewer synapses, synapses are smaller and less electrically active, and they have fewer glutamate receptors (Tononi & Cirelli 2006; Bushey et al. 2011). Although it seems contradictory that removal of synaptic connections is beneficial for memory consolidation, the idea is that reducing the number of synapses to baseline levels is energetically efficient and provides space for new learning to occur (Tononi & Cirelli 2006; Vassalli & Dijk 2009; Maret et al. 2011). It is proposed that pruning might occur proportionally to synaptic strength. Strong connections might only be downscaled to some extent, while weaker connections might return to a baseline level, thus resulting in a high signal-to-noise ratio for the stronger connections (Tononi & Cirelli 2006; Tononi & Cirelli 2014). Alternatively, synaptic pruning might not be selective for synaptic strength, but neuronal replay might protect some selection of networks against synaptic pruning (Wang et al. 2011).

In song learning, new synaptic connections might be formed or existing synaptic connections might be strengthened during song acquisition. If some of those synapses are removed during sleep during a process of synaptic pruning, at first the result might be behavioural deterioration, thus poor song quality. However, since the capacity to form new synaptic connections is also increased by pruning, there is ample opportunity to form new connections and improve song imitation again. If not all synaptic connections disappear during sleep this system should lead to gradual improvement of the neuronal network and song imitation. Indeed, in a behavioural experiment in juvenile songbirds it was found that there was a cycle in song learning in which song imitation improved during the day, while song performance had deteriorated the next morning, after a night of sleep. However, during the day song performance increased again, and each day it reached a higher level of tutor song imitation than it had on the day before (Derégnaucourt et al. 2005). Birds with the strongest morning song deterioration effect eventually sang the best imitations of the tutor song (Derégnaucourt et al. 2005). These behavioural findings seem to be consistent with a neural mechanism of synaptic pruning during sleep in songbirds.

However, in contrast, it was shown that tutoring induced synaptic strengthening in HVC (Roberts et al. 2010). Before tutoring started, there were substantial inter-individual differences in spine dynamics in HVC. Spines are small protrusions on the dendrites of neurons that receive input from axonal synapses of other neurons. These spines are very plastic and can readily appear or disappear from the dendrites. The rate at which spines are gained or lost is called the spine turnover rate. Some juvenile zebra finches had high levels of spine-turnover in HVC, others lower levels. The birds with high turnover levels demonstrated better tutor song imitation after tutoring than low-turnover birds. In juveniles with high spine turnover rates, exposure to a tutor song stabilized and enlarged spines in the subsequent night, so that their number increased and the dendritic spines had increased in size. At the same time, spontaneous neuronal activation increased (Roberts et al. 2010). As suggested before in other memory paradigms (Vassalli & Dijk 2009), both synaptic pruning and synaptic strengthening could occur during song learning, and further research is needed in order to understand more about the mechanisms underlying song memory consolidation during sleep.

Memory consolidation occurs during song learning in juvenile songbirds, and therefore we expected high levels of neuronal activation during sleep in juveniles in early stages after tutoring. In this thesis, I demonstrated neuronal activation during sleep in juvenile zebra finches (chapter 3). However, in juvenile zebra finches that were exposed to tutoring for just two or ten days, in contrast to being raised with their father until 47 days post hatching as in chapter 3, there was little neuronal activation during sleep, and it was unrelated to the level of song imitation (chapters 4 and 5 of this thesis). It may be that there was no consolidation during sleep in birds that had very little exposure to tutoring. Alternatively, even though the levels of neuronal activation were lower than during the day, they might have been sufficient for replay of the neuronal networks used for song acquisition or synaptic strengthening.

We expected to find a difference between neuronal activation in the two- and ten-day training groups (chapter 4) because the two-days trained birds were earlier in the learning process (see chapter 4 for a more detailed discussion). However, there was no difference in neuronal activation during sleep between the two tutoring groups, which could indicate that sleep might not be involved in early song memory consolidation. Or perhaps we were too late, and learning occurred within two days already (indeed, these birds had already significantly copied elements of the tutor song), so perhaps one should compare trained to more naïve or even untrained birds, or measure neural activation during the first sleeping opportunity after learning. Alternatively, ten days might not be sufficient to “overtrain” the birds, so a much longer-trained group might have been useful. In comparison, Shank and Margoliash (2009) found increased bursting activity in RA immediately after song training started, which lasted until 11 nights after training at least. Even though RA and the NCM supposedly have very different roles in song imitation, it is an example in which neuronal activation did not differ between two- and ten-days tutored juveniles either.

Furthermore, we found little neuronal activation in HVC of juveniles with only two or ten days of tutoring experience (chapter 4 of this thesis), in contrast to HVC activation in birds of the same age with longer tutoring experience (chapters 2 and 3 of this thesis). This is consistent with previous work (Crandall et al. 2007), that showed that sleep activity in HVC increased with age and song experience, and shows an interesting parallel to Broca’s area in human infants, which is increasingly activated during speech perception when infants get more speech exposure (Imada et al. 2006; table 6.3). In order to understand more about neuronal consolidation during tutor song memory formation in juveniles, a more elaborate approach should explore neuronal activation during sleep throughout the tutoring process.

We found high levels of neuronal activation in the NCM during the day though (chapter 4). Not only was the NCM activated in response to tutor song, but also spontaneously when the birds remained in silence. This could reflect memory consolidation taking place during the day. This could either happen when the birds are awake, or while they are taking day-time naps. It is described that juvenile songbirds frequently take day-time naps (Margoliash & Schmidt 2010). Behaviourally, it was shown that naps can even induce an additional cycle of song degradation and recovery, similar to what was found after a night of sleep (Derégnaucourt et al. 2005). Furthermore, juveniles were observed to nap after their first tutoring session in the lab (T. Lints and O. Tchernichovski, unpublished results, as cited in Margoliash & Schmidt 2010). Moreover, the birds that took a nap after the first tutor song playback were anecdotally observed to eventually imitate the song with better accuracy than birds who did not nap after the first playback (Whitaker et al. 2012). Taken together, these results suggest that naps promote memory consolidation. Similarly, in humans, it was shown that afternoon naps promote episodic learning: subjects who stayed awake performed worse in a learning task in the late afternoon compared to noon, while subjects who napped had similar learning abilities at the two testing times, or even slightly increased after napping (Mander et al. 2011). Also in a texture discrimination task in humans, memory performance increased after napping,

even to a similar extent as after a full night of sleep (Mednick et al. 2003). In addition, SWS promoted strengthening of declarative memories in infants (Wilhelm et al. 2012).

Memory consolidation could also occur during the day, when the subject is awake (Staresina et al. 2013). To prevent interference of activities, this could happen during “off-line” periods, or alternatively, awake replay could be triggered by sensory input from the environment. Both of these consolidation events (off-line and sensory-triggered) were demonstrated in the rat hippocampus after a spatial learning task (Karlsson & Frank 2009; Carr et al. 2011). Electrophysiological recordings in freely behaving juveniles could help elucidating occurrence and mechanisms of day-time memory consolidation by measuring brain oscillations to analyse whether birds are awake or asleep and the corresponding neuronal activation.

Conclusions and future perspectives

In humans, language-related brain activation is generally strongly lateralised, not only in adults, but also in infants who are in the acquisition phase. There are some indications that an altered lateralisation pattern is related to linguistic deficits, such as in dyslectics, autistics or schizophrenics, but the exact role of lateralisation is poorly understood. Investigating the role of lateralisation would benefit tremendously from the ability to manipulate input, experience and the use of brain regions during acquisition, and investigation of neuronal mechanisms. This is not possible in humans, but a good animal model would provide these opportunities. The present research has shown that songbirds have human-like lateralisation during tutor song learning (chapter 2). Avian research has already indicated an important role for lateralisation in sensory learning (chapter 6 of this thesis). As songbirds are a well-established model to study mechanisms of auditory-vocal learning, juvenile male zebra finches provide an animal model to further study the role of lateralisation in learning and memory. It was previously shown that sleep is beneficial for memory in different learning tasks, including birdsong learning. I have studied neuronal activation during sleep in juvenile songbirds, and shown that the activation is differentially lateralised between birds that sing a very good imitation of the tutor song, and birds that sing a poor imitation (chapter 3). Furthermore, I studied juveniles who had only brief periods of tutoring experience. However, contradictory to our expectations, these birds did not show high levels of neuronal activation during sleep (chapters 4 and 5). Therefore, memory consolidation might also occur during the day when birds are awake, or perhaps during napping. Sleep deprivation studies could be conducted to study the importance of sleep and napping for memory formation and song learning. I have shown that just two days of tutoring experience is sufficient for juvenile male zebra finches to significantly imitate tutor song (chapter 4). Studying memory consolidation in early stages of song tutoring seems a very promising research direction, which can improve our understanding of the mechanisms of memory consolidation.

8

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English summary

There are many parallels between the acquisition of spoken language in human infants and song learning in songbirds, at the behavioural, neural, genetic and cognitive level. Both human infants and juvenile songbirds are able to imitate sounds from adults of the same species (often their parents), called vocal learning. Vocal learning is a relatively rare ability in the animal kingdom that even appears to be absent in our closest relatives, non-human primates. In contrast, almost 5,000 avian species are vocal learners.

This thesis encompasses vocal learning in the zebra finch (*Taeniopygia guttata*), a songbird that has a very stereotyped song. Birdsong learning provides an excellent model for vocal learning and auditory memory formation, because it is a behaviour that comes very natural to the juvenile songbird, and can easily be experimentally manipulated. Juvenile songbirds imitate the song of any adult that sings in close proximity, which, under natural circumstances, is usually their father. In the lab we can raise juveniles with another unrelated male, called a ‘tutor’, which will lead to song imitation. Zebra finches memorise tutor song in a sensitive phase from about three weeks to two months after hatching, and start imitating when they are about one month old, gradually shaping it into adult-like song until they are about three months old.

Neuronal activation differs between hemispheres

I studied early stages of song memorisation in juvenile zebra finches that were raised with their father for the first one-and-a-half month (**chapters 2 & 3**) and in juveniles that received only two or ten days of exposure to a tutor song (**chapters 4 & 5**). I used immunocytochemistry to quantify an immediate early gene called *Zenk*, that is used a marker for neuronal activation (Moorman, Mello & Bolhuis, 2010). I specifically looked at neuronal activation in response to tutor song playbacks and during sleep in two brain regions: the NCM and HVC. The NCM (nidopallium caudomediale) is a higher-order auditory brain region, involved in song perception, recognition and memory. HVC (used as a proper name) is a premotor nucleus and an important node in the song system network; it is important for song production and sensorimotor learning. The song system including HVC is considered functionally similar to Broca’s area in humans, and the NCM is comparable to Wernicke’s area. Both are regions in the human ‘language brain network’. During linguistic processing in human brains, regions in the left side of the brain are often higher activated than the regions in the right hemisphere. In **chapters 2, 3 and 4**, I demonstrated similar lateralisation for tutor song in juvenile male zebra finches. In **chapter 4**, birds that had only two or ten days of tutoring experience showed slight but consistent left-dominant activation in the NCM. In **chapter 2**, juveniles that had more experience with the tutor song but were still in the song-learning phase, showed left sided dominance of NCM activation that was positively correlated with the quality of tutor song imitation. During sleep in similarly raised birds (**chapter 3**), there was a difference between birds that imitated the tutor song well and birds that did not: good learners showed left-lateralised NCM activation, while



poor learners showed right-lateralised neuronal activation in the NCM. In **chapter 6**, the evidence for lateralisation as an important feature of learning and memory is discussed in more detail. Lateralisation in HVC depended on the behavioural state of the juvenile male: during the day, HVC was activated higher in the left hemisphere (**chapter 2**); while during sleep the right-sided HVC was higher activated (**chapter 3**), which might reflect differential roles of the left- and right-sided HVC in singing (**chapter 6**).

Song memory in juvenile males and females

Next, we studied the amount of tutoring that is sufficient to imitate or recognize tutor song, and the accompanying neuronal activation characteristics. We demonstrated significant song imitation in juvenile males after only two days of tutor exposure (**chapter 4**). The NCM was highly activated in the two-day experienced birds, more than in birds that received ten days of tutoring and had learned the tutor song slightly better already, suggesting the importance of the NCM in especially the initial stages of song memory formation. Indeed, it is becoming clear that song imitation occurs very rapidly, and only little experience with tutor song is necessary to imitate the song (Deshpande et al., 2014). I compared results in juvenile male zebra finches to similarly raised females (**chapter 5**). Female zebra finches do not sing, but do form a memory representation of the tutor song. We could not demonstrate significant song memory in phonotactic song preference tests after two or ten days of tutoring, indicating that the learning process might be slower in females than males. Even though song recognition could not be demonstrated behaviourally, there was substantial neuronal activation in the NCM in juvenile females. As in the male juveniles, activation in the NCM was higher after two days of tutor song exposure than after ten days, suggesting a role for the NCM in initial song memory encoding.

Neuronal activation during sleep

Sleep is beneficial for many memory processes, including song learning in songbirds (Deregnacourt et al., 2005; Brawn et al., 2010). In **chapter 3**, I show that the NCM is activated during sleep in juvenile zebra finches that were raised with their fathers. Likewise, there was great brain activation during sleep in juvenile females that had been housed with a tutor for ten days (**chapter 5**). In contrast, and in contrary to what we expected, juvenile males with two or ten days of tutoring experience did not show much neuronal activation during sleep (**chapter 4**). One important difference between males (**chapter 4**) and females (**chapter 5**) was that the females did not demonstrate tutor song recognition, while the males already significantly imitated tutor song after both two and ten days of tutoring. Therefore, the females might still have been in the learning process, involving neuronal activation during sleep, while male tutor song memory consolidation might have already occurred during night-time sleep after the first or second day of tutoring. Alternatively, juveniles might take daytime naps in early stages of learning, and might therefore have consolidated tutor song memory during the day (as suggested by Margoliash & Schmidt, 2010), while night-time consolidation occurs later in learning (as we demonstrated in

chapter 3). To further study the role of sleep in memory formation, neuronal activation in males should be measured at an even earlier time point, during the day of tutoring and the first night of sleep afterwards, while later time points (more than ten days) should be chosen for an experiment in females. Sleep deprivation studies could help further elucidate the role of sleep in song memory consolidation.

Conclusions

In conclusion, sleep might be most important immediately after the first song learning opportunity. In addition, juveniles might consolidate tutor song memory during the day. Studying neuronal activation in juvenile females might be very useful to disentangle auditory memory formation from song-motor learning, because they do memorise song of their father, but do not sing. Juvenile females show very similar neuronal activation to males during initial song memory formation. Furthermore, we showed left-sided dominance of neuronal activation in the NCM of juvenile males. Thus, in addition to behavioural, genetic and neural parallels that were found between speech in humans and song in zebra finches (Bolhuis et al., 2010), our findings suggest that there is an additional parallel of lateralised brain activation. Songbird research will allow to further study the functions of sleep and lateralisation for memory formation, and a possible link between sleep and lateralisation characteristics and individual differences in learning performances.



Nederlandse samenvatting

Zangvogels leren hun lied van hun ouders, net zoals kinderen leren spreken. Zonder ooit een soortgenoot gehoord te hebben, kunnen zangvogels niet leren zingen. Dit is heel anders dan bij de meeste dieren: een hond hoeft niet van een andere hond te leren blaffen, of een cavia van een andere cavia te leren piepen. Zelfs de nauw aan ons verwante (mens-)apen hoeven hun geluiden niet van een soortgenoot te leren. Daartegenover zijn er bijna 5.000 soorten vogels (die allemaal onder de zangvogels, kolibries en papegaaien vallen) die hun lied wel van soortgenoten imiteren. Het leerproces van zangvogels en mensen vertoont op meerdere niveaus gelijkenissen: zangvogels en kinderen hebben een voorbeeld nodig en imiteren geluiden van een soortgenoot, de hersenprocessen die bij dit leerproces betrokken zijn lijken op elkaar, en er zijn genetische parallellen. Net als mensen, hebben zangvogels een gevoelige periode waarin ze het beste kunnen leren. Ze onthouden eerst de geluiden die ze hun ouders (of 'tutor') horen maken, en gaan daarna oefenen met het nadoen van die geluiden. Bij mensen zijn de hersengebieden van Broca en Wernicke heel belangrijk voor taal en spraak. Deze gebieden zijn ingebed in een groter netwerk dat actief is tijdens spreken en luisteren, en de linker hersenhelft is vaak actiever dan de rechter tijdens taalverwerking. Het gebied van Broca ligt in het oppervlak van het brein iets boven de slaap, en is belangrijk voor spreken. Het gebied van Wernicke ligt ook in de buitenste hersenschors, maar dan iets naar achteren, ongeveer boven het oor, en is vooral betrokken bij het verstaan en begrijpen van spraak. Zebravinken hebben ook een heel netwerk van hersengebieden dat betrokken is bij zingen. Een deel van de gebieden is vooral belangrijk voor het zingen, en een ander deel voor de perceptie en herkenning van zang, zoals een tutorlied. Ik heb deze hersenmechanismen van zangherkenning onderzocht tijdens mijn promotieonderzoek. Meer specifiek, ik heb geheugen van het tutorlied onderzocht in jonge zebravinken, zowel in mannetjes, als in vrouwtjes die zelf niet zingen, maar wel het tutorlied onthouden. De jonge zebravinken werden tijdens de leerfase aan een tutorlied blootgesteld. Daarna heb ik door de zangimitatie te beoordelen bepaald hoeveel de jongen van hun tutor geleerd hadden, en de hersenenactiviteit onderzocht.

Zanggeheugen in jonge vogels

Onder natuurlijke omstandigheden onthouden mannetjes zebravinken het lied van hun vader in een memorisatie fase die duurt vanaf dat ze ongeveer drie weken tot twee maanden oud zijn, en gaan ze het lied proberen na te zingen in een leerfase als ze tussen één en drie maanden oud zijn. Ik heb onderzocht of twee maanden oude vogels die slechts enkele dagen bij een tutor hebben doorgebracht het lied al hadden onthouden. De jonge mannetjes konden het tutorlied al nazingen na twee dagen tutor-ervaring (**hoofdstuk 4**). Zebravink vrouwtjes zingen niet, maar onthouden het lied van de vader wel. Er wordt gedacht dat dit belangrijk is voor vrouwtjes om een goede partner te selecteren. Mijn onderzoek lijkt uit te wijzen dat jonge vrouwtjes er iets langer over doen om het lied van de tutor te onthouden: we zagen nog geen bewijs voor memorisatie in het gedrag van de vrouwtjes na twee en tien



dagen tutor-ervaring (**hoofdstuk 5**). Dit zou te maken kunnen hebben met dat er minder noodzaak is voor vrouwtjes om snel te leren, omdat ze niet hoeven te oefenen met nazingen.

Lateralisatie van zanggeheugen

Hersenactiviteit kan onderzocht worden door middel van een kleuring die hecht aan een stofje dat alleen aanwezig is in actieve hersencellen. Onder een microscoop zijn de gekleurde cellen zichtbaar en de hoeveelheid gekleurde cellen in een hersengebied is een maat van hersenactiviteit. Ik heb vogels het tutorlied laten horen en vervolgens de hersenactiviteit geanalyseerd. Ik heb hierbij speciaal gekeken naar een hersengebied dat de 'NCM' heet (nidopallium caudomediale). Dit gebied lijkt in functionaliteit erg op het gebied van Wernicke bij mensen, en is belangrijk voor de herkenning van het tutorlied.

Omdat spraak-gerelateerde hersenactiviteit bij mensen links-dominant is, vroeg ik mij af of de hersenactiviteit bij vogels verschillend verdeeld was over de twee hersenhelften (lateralisatie). Inderdaad was de hersenactiviteit in de NCM van jonge mannelijke zebra-vinken links-dominant (**hoofdstukken 2 en 4**). Daarmee heb ik ontdekt dat lateralisatie (verschillend gebruik hersenhelften) een nieuwe parallel is tussen taal leren bij mensen en zang leren bij zebra-vinken. Bovendien was lateralisatie gerelateerd aan het leervermogen van de vogels: hoe beter een jong het lied kon nazingen, hoe meer links-dominant de hersenactiviteit was (**hoofdstuk 2**). Mogelijk is lateralisatie een belangrijk mechanisme voor het leren van vogelzang en menselijke spraak, en voor sensorische geheugenprocessen in het algemeen (**hoofdstuk 6**). Nu kunnen we de precieze rol van lateralisatie, die nog relatief onbekend is, verder gaan onderzoeken in vogels.

Rol van slaap op geheugenvorming

Ook heb ik hersenactiviteit tijdens de slaap onderzocht. Uit eerder onderzoek bleek dat slaap belangrijk is voor het in het geheugen opslaan van dingen die je overdag leert. Dit geldt voor leren fietsen of piano spelen, voor het onthouden van woordjes als je een nieuwe taal leert en nog veel meer. Ook bij zangvogels was het eerder al aangetoond dat slaap het nazingen van het tutorlied beïnvloedt. In **hoofdstuk 3** vond ik veel actieve cellen in de NCM tijdens slaap in jonge mannelijke zebra-vinken die de eerste zes weken van hun leven bij hun vader hadden gezeten, en het lied dus al goed geleerd hadden. Interessant was dat er grote verschillen in hersenactiviteit waren tussen vogels die het lied goed konden nazingen, en vogels wiens lied wat minder op dat van de tutor leek. De mannetjes die het lied van de tutor goed konden nazingen hadden links-dominante hersenactiviteit, terwijl de NCM van de jongen die het tutorlied minder goed nazongen rechts-dominant geactiveerd was. Bij mannetjes die kortere ervaring hadden met het tutorlied (twee of tien dagen), zag ik veel minder hersenactiviteit tijdens de slaap (**hoofdstuk 4**). Het lijkt er daarom op dat jonge vogels vroeg in het leerproces vooral overdag leren. Een interessante mogelijkheid is dat rustmomenten en dutjes overdag al voldoende gelegenheid bieden om het geleerde op te slaan. Mogelijkerwijs leren vogels in eerste instantie dus vooral overdag, en gaan de

leerervaring pas 's nachts verwerken als ze al meer geleerd hebben (zoals in hoofdstuk 3, waar we wel veel hersenactiviteit tijdens slaap vonden).

Conclusies

Concluderend heb ik aangetoond dat jonge mannetjes al na twee dagen het lied van de tutor kunnen nazingen, en dat het leerproces in eerste instantie niet per sé tijdens nachtelijke slaap plaatsvindt. De bijbehorende hersenactiviteit is, net als bij mensen, verschillend in de linker en rechter hersenhelft. Dit onderzoek in zangvogels draagt bij aan het begrip van de rol van slaap en lateralisatie in auditief geheugen. Dit is van belang voor taalverwerving bij kinderen, en mijn onderzoek biedt aanknopingspunten voor vervolgonderzoek naar hersenmechanismen. Misschien wel het meest interessant is de correlatie tussen het dominante gebruik van de linkerhersenhelft en leerprestaties: dit wijst erop dat lateralisatie belangrijk is voor leren en geheugen bij zangvogels en mensen.



Persoonlijk slotwoord

“In any event, what does it cost to prove whether or not we have the ability to create original science? In the end, how can we know whether there is a gift of exceptional aptitude for science among us if we don’t try to create the opportunity for it to be expressed, under the influence of first-rate moral and technical discipline?”

– Santiago Ramón y Cajal

Al snel nadat ik “per ongeluk” bij de studie Biologie belandde, na te zijn uitgeloot voor Diergeneeskunde, ontdekte ik mijn passie voor Biologische Wetenschappen. Alles vond ik interessant: de bouw van planten, balansen in ecosystemen, evolutie, genetica, maar bovenal de hersenen. Na twee ontzettend leuke en succesvolle stages was ik er volledig van overtuigd promotieonderzoek te willen doen. En toen ik de vacature bij Johan zag, was ik gelijk verkocht. Het onderzoek zou gedrag met hersenmechanismen combineren, en de data inzicht geven in grote onderwerpen zoals complexe cognitie en evolutie. Een allround project waar ik veel potentie in zag. En dat was terecht, zo bleek. Jullie weten hoe zeer ik het naar mijn zin heb gehad tijdens dit onderzoek. Dat komt onder andere door mijn zeer toegewijde en getalenteerde promotor en dagelijkse begeleider, Professor Bolhuis. **Johan**, bedankt voor de fantastische opleiding die je me hebt gegeven, de leuke tijd en de vruchtbare samenwerking. Je hebt mij van het begin af aan overal bij betrokken en veel verantwoordelijkheid gegeven. Ik mocht zelf bedenken wat ik wilde doen, maar je hebt me ook geadviseerd en sturing gegeven. Ik heb er veel van geleerd en erg van genoten. Daarnaast was **Thijs** een hele belangrijke component van zowel het succes van mijn onderzoek als het plezier in het lab. Je hebt altijd leuke verhalen te vertellen, houdt erg van een grap en weet ook nog eens alles van immuno’s. Je bent mijn steun en toeverlaat geweest in de afgelopen vier jaar, dat heb ik zeer gewaardeerd. Ik mis je nu ik in Boston woon! Bedankt dat jij straks mijn paranimf wilt zijn. **Gabriël** kwam wat later bij de groep, maar na een paar weken “kennismakings-geklets” was het alsof je er altijd al was geweest. Een hele fijne collega, een getalenteerde onderzoeker, en altijd garant voor een goed gesprek tijdens de lunch (en op andere momenten). Ik ben heel blij voor je dat je nu een vaste baan in Utrecht hebt gekregen. **Claudio**, thanks for the fruitful collaboration for our BioEssays review paper. Also, thanks for inviting me to your lab for the catFISH protocol optimisations. It was very educational and I enjoyed it a lot! **Ali**, thanks a lot for writing the lateralisation review paper together with me. It was a very natural collaboration and I am very proud of the result! **Sharon**, je bent me voorgegaan met een PhD bij Johan en een postdoc in Boston. Ondanks dat we nooit directe collega’s zijn geweest, hebben we wel samen gepubliceerd. Ik wens je veel succes toe in je verdere carrière. Daarnaast zijn er veel hele goede, slimme en gemotiveerde studenten bij mijn onderzoek betrokken geweest, wat ik altijd heel waardevol heb gevonden. Jullie waren een gezellige sociale aanvulling op onze groep, hebben me gigantisch geholpen met alle experimenten, en ik heb heel veel geleerd van jullie. Heel erg bedankt **Sanne B.**, **Inka**, **Paula**, **Ferdi**, **Nienke**, **Tessa**, **Amber**, **Rosanne**, **Lisa**, **Nikki**, **Febe** en **Jesse**. **Carien**, je bent nu zelf AiO bij Johan



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tien jaar samen en wat hebben we het leuk hè! Het is fijn dat we steeds in dezelfde levensfase zitten, veel begrip hebben voor elkaars gewerk en soms gestress, het samen vieren als we een belangrijk resultaat hebben, en nu allebei in Boston werken. Maar veel belangrijker nog ben je mijn allerliefste vriendje, en is alles leuker als jij erbij bent!

“Today, at last, the questions that all aficionados of science ask when taking the first uncertain steps along the path of research have lost their desperate inhibitory grasp: Who cares what I do? To whom will I confide the joy that my small discovery has given me, someone who will not smile sarcastically or enter the realm of annoying compassion? If I triumph, who will applaud? And if I am uncertain, who will correct me and provide the encouragement to go on?”

– Santiago Ramón y Cajal

Jullie zijn dat voor mij.



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Curriculum Vitae

Sanne Moorman was born in Borne, the Netherlands, on December 29, 1984. In 2003, she graduated high school and went to Utrecht to study Biology (BSc) at Utrecht University.

In her second year, she joined the board of the society for Biology students in Utrecht as vice-treasurer (2004/2005, which had ca. 1,200 members then). She joined the Council of the Department Biology in 2005 and the Council of the Science Faculty in 2006. She served as vice-president in the years 2006-2009. In 2009, she organised an introduction day for ca. 200 students of Life Science Master programmes. In 2007, she enrolled in a prestigious MSc programme in Neuroscience and Cognition at Utrecht University, with a focus on behavioural neuroscience.

During this programme, she conducted a nine-months research internship in the laboratory of Professor Van Wezel in the Functional Neurobiology group, studying perception of mouth movements in humans using fMRI. There, she also collaborated with Mathijs Raemaekers on another human fMRI project, which resulted in a paper in *Human Brain Mapping*.

In 2009 she went to Miami, Florida, USA, to study molecular mechanisms of neuronal regeneration during her six-months internship. She carried out research involving primary cell cultures and protein expression assays in the laboratory of Professor Bixby, under supervision of Andrea Johnstone. Her work there became part of a paper in *The Journal of Neuroscience*.

After graduation in the Netherlands, she came back to the laboratory of Professor Van Wezel to work as a research assistant. During three months, she helped set up two-photon imaging equipment and ran the first pilot experiments involving imaging of barrel cortex activation *in vivo*, using a calcium-indicator dye in rats.

Then she joined the laboratory of Professor Bolhuis in February 2010 as a PhD student. She studied neural mechanisms of song memory formation in juvenile zebra finches, and presented her work at conferences in Europe and the USA. During this time, she was also an editor of the *Helmholtz Herald*, a bi-annual magazine of the Helmholtz Institute (2011-2014), and co-organised the 18th Benelux Congress of Zoology, an international scientific conference in 2011. She supervised eight MSc students during their research internships, and eight BSc students during small research projects and thesis writing (2010-2014). She visited Professor Mello's laboratory in Portland, Oregon, USA in 2012 to learn to do cellular compartment analysis of temporal activity by fluorescence *in situ* hybridization (catFISH). Her PhD work resulted in this thesis.

On June 1, 2014 she moved to Boston, Massachusetts, USA, to work as a postdoctoral researcher with Professor Gardner. There, she studies neural representations of song memory in zebra finches using electrophysiology.



