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Mirrored patterns of lateralized neuronal activation reflect old and new memories in the avian auditory cortex

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Abstract

In monolingual humans, language-related brain activation shows a distinct lateralized pattern, in which the left hemisphere is often dominant. Studies are not as conclusive regarding the localization of the underlying neural substrate for language in sequential language learners. Lateralization of the neural substrate for first and second language depends on a number of factors including proficiency and early experience with each language. Similar to humans learning speech, songbirds learn their vocalizations from a conspecific tutor early in development. Here, we show mirrored patterns of lateralization in the avian analog of the mammalian auditory cortex (the caudomedial nidopallium [NCM]) in sequentially tutored zebra finches, in response to their first tutor song, learned early in development, and to their second tutor song, learned later in development. The greater the retention of song from their first tutor, the more right-dominant the birds were when exposed to that song; the more birds learned from their second tutor, the more left-dominant they were when exposed to that song. Thus, the avian auditory cortex may preserve lateralized neuronal traces of old and new tutor song memories, which are dependent on proficiency of song learning. There is striking resemblance in humans: early-formed language representations are maintained in the brain even if exposure to that language is discontinued. The switching of hemispheric dominance related to the acquisition of early auditory memories and subsequent encoding of more recent memories may be an evolutionary adaptation in vocal learners necessary for the behavioral flexibility to acquire novel vocalizations, such as a second language.

Keywords

songbird; birdsong; speech; memory; sensorimotor; *Taeniopygia guttata*

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AUTHOR CONTRIBUTIONS

S.M.H.G. conceived, S.M.H.G. and E.O. designed, S.M.H.G., E.O., and R.K.M. performed the study, analyzed the data, and wrote the paper.

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INTRODUCTION

Many human behaviors, including speech, are learned during a juvenile ‘sensitive’ or ‘critical’ period and retained throughout life (Knudsen, 2004). Early-formed language representations are maintained in the brain even if exposure to that language is discontinued, supporting the hypothesis that once a memory trace is established for language, it is maintained indefinitely (Bjork and Bjork, 2006, Pierce et al., 2014).

There is considerable variation in lateralization of the neural substrate that supports language, especially when comparing monolinguals with bilinguals. In children and adults who are acquiring language or learning a second language, brain regions activated during speech perception are generally large and right-dominant or bilateral (Dehaene et al., 1997). In monolingual humans, language-related brain activation often shows a distinct lateralized pattern; activity shifts to the left side of the brain or becomes less dispersed with increased language proficiency, and left-dominance is positively correlated with age and more advanced language abilities (Dehaene-Lambertz et al., 2002, Conboy and Mills, 2006, Friederici, 2011). A left-dominant neural substrate for language seems to be advantageous to the individual, as right-dominance has been associated with several speech and language disorders (Sommer et al., 2001, Oertel et al., 2010, de Guibert et al., 2011, Eyler et al., 2012, Johnson et al., 2013, Berl et al., 2014). Studies are not as conclusive regarding the localization of the underlying neural substrate for first and second language in bilinguals (Minagawa-Kawai et al., 2011). Lateralization of the neural substrate for the two languages depends on a number of factors including proficiency and early experience with each language (Perani et al., 1996, Perani et al., 1998, Raboyeau et al., 2010).

Speech acquisition in human infants and birdsong learning share many behavioral, neural and genetic characteristics (Doupe and Kuhl, 1999, Bolhuis and Gahr, 2006). Songbirds and humans acquire their vocalizations during sensitive (or optimal) periods early in development. Some birds (“open-ended learners”) are able to learn vocalizations beyond the sensitive period, whereas the zebra finch (*Taeniopygia guttata*, an “age-limited learner”) learns and crystallizes its song during a definite time period (Fig. 1D) (Bolhuis and Gahr, 2006). Early in development, juvenile male zebra finches form a representation of the song of an adult tutor (usually their father) and subsequently acquire their own song, which resembles the tutor’s song, through a process of trial-and-error learning akin to human babbling. Left-hemispheric dominance for memory of the bird’s own song has been observed in a Wernicke-like region (caudomedial nidopallium, NCM) in the zebra finch brain (Fig. 1A), similar to the brain lateralization associated with human language (Moorman et al., 2012).

Like human infants who may be exposed to more than one language during development, wild zebra finches are exposed to several conspecific song models at the same time in their natural environment (Immelmann, 1969). Despite subsequent conflicting experiences, early exposure can exert lasting effects on neural structure and function. In swamp sparrows, a songbird species that imitates many different songs learned from multiple tutors during development, the pre-motor nucleus HVC (acronym used as proper name) maintains a neural representation of the acquired tutor songs, including those that are not part of the adult

repertoire (Prather et al., 2010). This raises the possibility that neural representations of several relevant auditory models can be stored in the adult zebra finch brain.

In the laboratory, we can manipulate the early auditory environment by exposing juvenile zebra finches to two adult song tutors to investigate whether brain regions contain a neural representation of tutor song (Yazaki-Sugiyama and Mooney, 2004). Selective auditory responses for tutor song in the lateral magnocellular nucleus of the anterior nidopallium (LMAN; part of a cortico-basal ganglia loop) are lost as the juvenile copies a new song, suggesting that the adult LMAN does not permanently store information regarding previously learned song models (Yazaki-Sugiyama and Mooney, 2004). Whether learning from a second tutor results in overwriting of the original memory engram in the secondary auditory areas or generates parallel traces of two tutor song memories remains to be elucidated. Here, we use a dual tutor paradigm to investigate memory-related neuronal activation (measured as the expression of the immediate early gene *ZENK*, an acronym for *zif268*, *egr-1*, NGFI-A and *krox-24*) in the secondary auditory areas of sequentially tutored zebra finches. We measured the number of Zenk positive neurons and determined lateralization ratios in response to exposure to the first tutor song, learned early in development (before 33 days post-hatching, or dph), and to the second tutor song, learned later in development (61 dph – 90 dph) (Fig. 1B), in the NCM and in the caudomedial mesopallium (CMM). In the NCM of sequentially tutored songbirds, memory of song learned later in development was encoded predominantly in left-hemispheric circuits, whereas song learned early in development displayed a right-lateralized pattern. These results are consistent with previous findings, and elaborate on these by demonstrating that the NCM can be flexible in response to novel auditory experiences (Bolhuis et al., 2000, Phan et al., 2006, Gobes and Bolhuis, 2007, London and Clayton, 2008, Gobes et al., 2010, Moorman et al., 2012).

EXPERIMENTAL PROCEDURES

Animals and rearing protocol

Eighteen male juvenile zebra finches (*Taeniopygia guttata*) were reared in the animal facility at Wellesley College with controlled auditory and social exposure to adult song tutors. Birds were maintained on a 16:8 light: dark cycle, lights on at 10:00 am. All birds were reared in breeding cages with their father, mother, and siblings, and each clutch was housed in acoustically isolated single-clutch holding cages until 29–33 dph (32.21 ± 1.44 mean $\pm$ SD, $N = 18$). After the first month, the father was removed from the clutch while the juveniles and their mother remained in isolation. At 33 dph (33 ± 0.32 , mean $\pm$ SD), juvenile males were transferred into individual, acoustically isolated holding cages (Fig. 1B). Birds remained in these cages until 61 dph, when they were paired with a second tutor (an adult male zebra finch that was not the biological father), with whom they were housed for the next 29 days. At approximately 89 dph, two days prior to stimulus exposure, birds were separated from their second tutor and transferred into acoustically isolated chambers set-up for playback of sound stimuli. Experimental procedures were in accordance with US law and approved by the Institutional Animal Care and Use Committee of Wellesley College (IACUC #1106 and #1405).

Behavioral analysis

Sound data was collected continuously from each experimental bird, beginning at time of separation, when birds were placed in the soundproof chambers, through time of sacrifice, and included data from birds individually and together with the first or second tutor. Vocalizations were monitored and digitally recorded with directional microphones (Shure SM93, Shure Incorporated, Niles, IL, USA) using custom written software. Songs used for similarity analysis were taken from days that the birds were housed individually between tutoring experiences (at dph 59) and right before the behavioral experiments (at dph 91). Four comparisons were made for each bird: similarity to first tutor post-exposure at dph 59, similarity to second tutor pre-exposure at dph 59, similarity to first tutor at dph 91, and similarity to second tutor at dph 91. Second tutor pairings were determined based on analysis of similarity scores (see below), and tutors were selected to optimize learning based on low similarity scores between second tutor and first tutor. For four animals, song data of sufficient quality for further analysis could not be collected on days of interest due to technical issues with the recording equipment.

We used “Sound Analysis Pro (2011)” (Tchernichovski and Mitra, 2004) to measure the overall similarity of the bird’s own song (BOS) to either of its tutors’ songs. The “percentage similarity” that is calculated by Sounds Analysis Pro, is an objective quantification of the fidelity of song imitation based on multiple acoustic parameters (pitch, Wiener entropy, frequency modulation (FM), and spectral continuity) (Tchernichovski et al., 2000). To compare the song of a juvenile zebra finch to its tutors, we identified the most frequently repeated single motif from a day of sound data and randomly selected 10 examples of this motif. Using paired comparisons between tutor and tutee, we compared 10 single motifs from the tutee to 10 motifs from each tutor (excluding introductory notes) to calculate the average percentage similarity. To determine the baseline level of similarity to non-related birds at 59 dph, we calculated the similarity to the second tutor before the birds were paired with this tutor. The baseline level of similarity to non-related birds at 91 dph was determined by comparing 10 motifs from each tutee at 91 dph to 10 motifs each taken from five adults unrelated to the birds used in this experiment (50 comparisons total per tutee).

Procedures for playback experiments

One day prior to stimulus exposure, birds were put in a cage measuring $40 \times 35 \times 35$ cm and placed within a soundproof chamber with water and food available *ad libitum*. Mean age at the day of the experiment was 92.84 dph (± 1.42 SEM; range 91 – 96 dph).

On the day of the experiment, birds were presented with a 30 min song stimulus (see (Chirathivat et al., 2015) for experimental procedures, stimulus preparation and broadcast methods). The stimulus consisted of a recording of the song of their first tutor (father, TUT1), or a recording of the song of the second tutor (TUT2). Birds were divided over the two groups (TUT1 exposure or TUT2 exposure) semi-randomly as to maximize diversity in each group. That is, we made sure each group represented a range of learning outcomes with respect to retention of the song learned first or the degree of switching over to the second song.

Tissue collection

One hour after stimulus onset, the experimental subjects were anesthetized with 0.03 mL Natriumpentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) and subsequently perfused with phosphate buffer (PB, pH 7.4) containing 0.2% heparin, followed by fixation with 2% paraformaldehyde. Whole brains were dissected out, separated by hemisphere and post-fixed at 4°C in 2% paraformaldehyde in PB overnight. Parasagittal sections (50 µm) were made on a vibratome and stored in PB overnight at 4°C or in cryoprotectant at -18°C.

Immunocytochemistry & Image Analysis

We performed immunohistochemistry for Zenk (Santa Cruz Biotechnology, Cat. No. sc-189) and quantified the number of Zenk-positive cells in three sections at the medial position (<600 µm from the midline) and three section at the lateral position (between 600 and 1000 µm from the midline) using identical methods to those described previously (Chirathivat et al., 2015). Birds of different experimental groups (first tutor exposure, second tutor exposure) were run in parallel with one another on the same well plate with control sections for which the primary or secondary antibody were omitted.

Statistical analysis

To test whether birds had learned from their first or second tutor, we used paired t-tests. We conducted repeated-measures analysis of variance (ANOVA) to examine the effects of playback stimulus on the Zenk response in the different brain regions and experimental groups. Pearson's correlation coefficient with Bonferonni correction for multiple testing was used to test for correlations between lateralization ratios and song similarity with tutor 1 and tutor 2 at ~91 dph, because activity-induced Zenk expression is directly related to playback at ~93 dph and we were interested in the effects of re-exposure to each tutor's song in adulthood, after the learning process had been completed. Data were analyzed using SPSS 22.0.0 (IBM Corporation).

RESULTS

Song learning

We followed a rearing protocol similar to that developed by Yazaki-Sugiyama and Mooney (2004), raising male zebra finches with a first tutor and subsequently with a second tutor, which resulted in successful extension of the sensory acquisition phase beyond 60 days post hatching (dph). At 59dph, right before the birds were paired with their second tutor, most birds had already started to copy elements from their first tutor in their songs (Fig. 2), which showed significantly higher similarity with their first tutor than with their second tutor (paired t-test, $t(13) = 5.37$, $p < 0.001$; Fig 2). As similarity to the second tutor was determined before the birds were paired with this tutor, this measurement can be seen as a 'baseline' level of similarity between a 59 dph bird and an unfamiliar adult (Fig 2). After 30 days with their second tutor, at 91 dph, these same birds had now learned from their second tutor, showing significant increases in similarity scores with their second tutor between 59 dph and 91 dph (paired t-test, $t(12) = 2.66$, $p = 0.021$) and a trend towards decreased similarity with their first tutor over this same time period (paired t-test, $t(12) = 1.98$, $p = 0.071$). At 91 dph,

the tutees' songs significantly resembled tutor songs as compared to the baseline levels with unrelated birds ($t(16)=5.63$, $p<0.001$ for similarity scores with the first tutor, and $t(16)=5.23$, $p<0.001$ for similarity scores with the second tutor). There was no (inverse-) relationship between first- and second- tutor song similarity at 91 dph (Pearson's correlation = -0.17 , $p = \text{n.s.}$). Thus, the lack of an increase in similarity to the first tutor between 59 dph and 91 dph shows that the birds really learned from their second tutor, and that increases in similarity scores are not the result of 'normal' song maturation independent of imitative learning.

Different patterns of immediate early gene expression are related to behavioral flexibility

Next, we conducted a nested repeated measures ANOVA with factors Stimulus (T1 or T2), Region (NCM or CMM), Hemisphere (Left or Right), and Level (medial or lateral). This analysis showed a significant interaction between Stimulus and Region ($F_{(1,15)} = 5.12$, $p = 0.039$). The factor Level (medial or lateral) had a significant effect on the number of Zenk-positive cells ($F_{(1,15)} = 5.21$, $p = 0.038$) and this factor interacted with Stimulus ($F_{(1,15)} = 6.82$, $p = 0.02$), Hemisphere ($F_{(1,15)} = 6.44$, $p = 0.023$), and Region ($F_{(1,15)} = 4.89$, $p = 0.043$). There was no overall effect of Stimulus ($F_{(1,15)} = 0.022$, $p = \text{n.s.}$, see Fig. 3). To further investigate the interaction between Stimulus and Region, we proceeded with analysis of lateralization of Zenk expression in each region separately. The lateral and medial levels of NCM and CMM were considered separately, based on the nested repeated measures ANOVA and previous reports (Terpstra et al., 2004, Gobes et al., 2009, Gobes et al., 2010, Moorman et al., 2012).

The lateralization ratio ($[L-R]/[L+R]$) is a measurement used to normalize the relative activation levels of the left and right hemisphere in order to compare birds to each other independent of absolute numbers of Zenk positive neurons. It has been shown that the lateralization ratio correlates with the fidelity of song imitation when juvenile male zebra finches are exposed to the song of the tutor (Moorman et al., 2012). When previously isolated zebra finches are exposed to the song of a conspecific for the first time in their life, the lateralization ratio in NCM is zero, indicating equal levels of Zenk expression in each hemisphere (Chirathivat et al., 2015). We used Pearson's correlation coefficient to investigate whether the lateralization ratio was related to behavioral flexibility in learning from two tutors in the medial and lateral parts of NCM and CMM. In the medial part of the NCM, we found a mirror-image pattern of immediate early gene expression between birds exposed to their first or second tutor: the more the birds had retained from their first tutor, the more right-lateralized they were when exposed to that tutor (Fig. 4A, Pearson's $r = -0.91$, $p = 0.001$; significant at Bonferonni corrected $\alpha = 0.0031$ adjusted for performing 16 tests); the more birds had learned from their second tutor, the more left-lateralized they were when exposed to that tutor (Fig. 4D, Pearson's $r = 0.89$, $p = 0.003$; significant at Bonferonni corrected $\alpha = 0.0031$ adjusted for performing 16 tests). There were no significant correlations (with uncorrected $\alpha = 0.05$) in the other brain regions (medial and lateral CMM and lateral NCM), or when investigating the reverse relationships (i.e., fidelity of song imitation with the second tutor in birds exposed to a stimulus of their first tutor, Fig. 4B & C).

DISCUSSION

After learning a new song in the second half of vocal development, zebra finches exhibited mirrored lateralization patterns in the NCM to first tutor and second tutor song, represented by the number of Zenk expressing neurons. The more the birds retained from their first tutor, the greater the molecular neuronal response in the right NCM when exposed to that song (Fig. 4A); the more the birds' song resembled the song of their second tutor, the greater the response in the left NCM in response to playback of that song (Fig. 4D). In contrast, there was no relationship between molecular neuronal activation in response to playback of the first tutor song and song similarity with the second tutor and vice versa (Fig. 4 B and Fig. 4 C). This suggests that the reported pattern of molecular neuronal activation is specifically related to reactivation of tutor song memory, and perhaps reconsolidation of that memory (Bozon et al., 2003). The present results suggest that the zebra finch brain may hold a timeline of song memories, in which one hemisphere is more flexible and may serve as the neural substrate for the representation of more recent songs, while the other hemisphere may allow for the representation of older song memories. Whether these lateralized patterns of Zenk expression reflect a purely sensory representation of the tutor's song or perhaps guide the birds' own vocal development through interaction with other regions in the songbird brain remains to be determined (Gobes and Bolhuis, 2007, London and Clayton, 2008, Roberts et al., 2012).

Previous studies investigating the neural substrate for tutor song memory in juvenile zebra finches have identified the NCM as a region that shows stronger molecular neuronal activation in response to tutor song than to novel song in the middle of the sensori-motor learning phase (Gobes et al., 2010, Moorman et al., 2012). More specifically, neuronal activation in the NCM, as well as the degree of left lateralization, are both related to the fidelity of song imitation in juvenile as well as in adult male zebra finches; this lateralization is not innate, but rather develops with experience (Bolhuis et al., 2000, Bolhuis et al., 2001, Terpstra et al., 2004, Moorman et al., 2012, Chirathivat et al., 2015, Moorman et al., 2015). In these studies, subjects learned to imitate a single conspecific song. The correlation between left-lateralized NCM responses and song similarity to the second tutor replicates the relationship between song learning and activation that is seen in birds tutored with a single song (Bolhuis et al., 2000, Bolhuis et al., 2001, Terpstra et al., 2004, Moorman et al., 2012, Moorman et al., 2015). Thus, in normally reared juveniles, as well as in zebra finches exposed to multiple tutors, left-dominant molecular neuronal activation is related to successful song imitation from the most recent social interaction.

The capacity to learn from a second tutor may depend on the ability to adapt to novel acoustic features. In a group of adult male zebra finches that were trained to distinguish between two conspecific song stimuli in a GO/NoGO paradigm, birds that were faster in learning the paradigm exhibited greater neural responses and faster adaptation rates to novel conspecific stimuli compared to slow learners (Bell et al., 2015). Furthermore, these fast learners demonstrated left-lateralized responses in NCM (Bell et al., 2015). In our study, "fast learners" may be the birds that showed greater song similarity to the second tutor, as they were faster to acquire aspects of this tutor. Zebra finches whose song was most similar to the second tutor showed greater left-lateralization in NCM in response to second tutor

song, which suggests that the left NCM may be more flexible in acquiring a representation of memory for a newly learned song. Perhaps this flexibility is supported by the addition of new neurons, as the degree of left-hemispheric dominance in adult neurogenesis correlates with the similarity between the bird's song and the tutor song to which it was exposed during development (Tsoi et al., 2014). Left hemispheric dominance in good learners has also been reported during sleep: birds with high similarity to tutor song demonstrate greater neuronal activation in the left NCM, while poor learners show higher expression in the right NCM (Moorman et al., 2015). Left-lateralized activation in the NCM of juvenile songbirds during wake and sleep may facilitate memory consolidation and song learning.

Sequentially-tutored zebra finches in our experiment demonstrate a right-lateralized response to first tutor song in NCM. Because the song from the first tutor is learned much earlier in development than the song from the second tutor, it is possible that 'older' memories are consolidated in the right hemisphere, while the left hemisphere provides the neural substrate for acquisition of new memories. Right-lateralized responses to conspecific auditory stimuli in the NCM can be reversed with exposure to a novel auditory environment (Yang and Vicario, 2015), which suggests a reorganization of lateralization patterns in the NCM in response to song stimuli consistent with our results of exposure to a novel song tutor. In birds raised in isolation from adult song tutors, neuronal responses in the left NCM are greater to song than to a behaviorally irrelevant stimulus (rhythmic white noise) when they are first exposed to song, while responses in the right hemisphere are not modulated (Chirathivat et al., 2015). Without any previous experience, the left NCM may be recruited by isolate birds upon first exposure to song due to the novelty of the stimulus and the sharp change in the auditory environment, and this process may be replicated with the introduction of a second song tutor.

There are limitations inherent to our experimental design. Zenk expression can only be quantified post-sacrifice, thus the findings presented here are based on two separate groups of animals representing a range of learning outcomes from the first and second tutor, rather than comparisons made within the same animal. Future studies using in-vivo measures of neuronal activity within the same animal, such as functional Magnetic Resonance Imaging, electrophysiology, and calcium imaging experiments, may be used to investigate how the 'switch' from one tutor song memory to the next is encoded in neural circuits of the left and right hemispheres. In parallel with our findings, Yang & Vicario (2015) demonstrate that activity in the NCM switches from right- to left-lateralized as a result of novel auditory exposure within the same animal, albeit in a different set of auditory contexts, using electrophysiological methods. Furthermore, although there are different time intervals between learning from the first and second tutor and song re-exposure to evoke recognition-dependent Zenk expression, song similarity (as a proxy for strength of song memory) was determined for both groups on the same day right before playback experiments were performed. These measures thus reflect memory for first and second tutor song at the same time point in adulthood. The finding that song similarity in adulthood to the first and second tutor song correlates with right- and left-lateralization, respectively, demonstrates that this phenomenon is not due to a recency-effect, but rather represents tutor-song-memory-dependent neuronal activation.

These data are consistent with studies in human sequential language learners showing that traces of a first language remain in the brain, even after the individual is completely dependent on a different language later in development. Studies performed on humans who had early but limited exposure to their first language demonstrate that circuits recruited for more general language processing, such as processing sentences, do not maintain memory traces for the language learned early in life. However, unique aspects of the first language that are encoded early in development, such as accent and discrimination of phonemes, are skills that remain intact in adulthood (Au et al., 2002, Pallier et al., 2003, Oh et al., 2010). The encoding of lexical tone, characteristic to tonal languages, is preserved at the neural level; international adoptees show similar brain activation to lexical tone compared to native controls (Pierce et al., 2014). The duration of exposure to the early-learned language affects the maintenance of the memory representations: the more experience with the first language the stronger the neural representation of that language (Hyltenstam et al., 2009, Pierce et al., 2014). We found a similar correlation between elements of the first tutor song preserved in the bird's current song and the extent of a neural representation for that first tutor song; this supports the hypothesis that the maintenance of a memory for song learned early in development depends on the strength of memory acquisition for that song. Thus, the greater the skill level acquired early in life, the more likely that aspects of auditory-vocal memory will be retained throughout life in humans as well as birds.

When two languages are learned in sequence, this can result in losing some while preserving other aspects of a neural representation of the first language. Regions recruited early in development to encode unique aspects of language may serve as the neural substrate for a representation of this language, whereas regions recruited later in development, when schemas and top-down language mechanisms are developed to faster process speech with current behavioral relevance, may be dedicated to processing the most recent language. In songbirds, sequential learning may take advantage of lateralized auditory processing and vocal production circuits, allowing for simultaneous preservation of memory for elements of early-learned song and acquisition of memory for novel song. Thus, left-hemispheric dominance in neuronal responses and plasticity of the brain during vocal development may be an adaptive mechanism to maintain flexibility and efficiency for vocal learning in responding to novel auditory environments.

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ABBREVIATIONS

CM	caudal mesopallium
CMM	caudomedial mesopallium

dph	days post hatching
HVC	acronym used as proper name
LMAN	lateral magnocellular nucleus of the anterior nidopallium
MLd	mesencephalicus lateralis pars dorsalis
NCM	caudomedial nidopallium
Ov	ovoidalis

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Highlights

- Sequentially learned songs elicit unique lateralized responses in zebra finches.
- Right hemispheric activation correlates with memories from early in development.
- Left hemispheric activation correlates with memories acquired more recently.

Figure 1. Parallels between neural systems for auditory learning, and between behavioral development in both humans and zebra finches (normally raised or sequentially tutored)

(A): Simplified diagram (not to scale; left) of the avian auditory system. The ascending auditory pathway (dark grey arrows) includes a midbrain nucleus (MLd), a thalamic nucleus (Ov) and several cortical regions. The primary auditory cortex (Field L2) connects to the secondary auditory regions Field L3 and L1, which in turn are reciprocally connected to regions analogous to the mammalian auditory association cortex (NCM and medial and lateral CM). Adapted, with permission from: (Chirathivat et al., 2015).

(B): Developmental time line of vocal learning with sequential tutors. Juvenile male zebra finches are exposed to the first tutor (Tutor Song 1) until 33 dph and then individually isolated for 28 days. At 61 dph, they are exposed to a second tutor (Tutor Song 2) until 90 dph. Between 90 and 93 dph, zebra finches are individually isolated. At 93 dph, zebra finches were exposed to a 30-minute playback of either Tutor 1 or Tutor 2.

(C) Block diagram highlighting parallels between mammalian and songbird circuitry. Adapted, with permission from: (Chirathivat et al., 2015).

(D): Developmental time line of human speech perception and production and songbird auditory learning and song development. Top: first, zebra finches acquire an auditory memory (or “template”) of their tutor’s song (between 25 and 65 days post hatching). Between days 35 and 90, zebra finches practice their vocalizations through a process of trial-and-error learning (sensorimotor learning phase). By day 90, zebra finches produce a “crystallized” song, which remains relatively stable throughout their adult life. Bottom: During the first 3 months, human infants produce non-speech sounds, which progresses to babbling by 7 months. By 12 months, infants are beginning to produce their first words, which will be retained and used throughout their lifetime. Adapted, with permission from (Doupe and Kuhl, 1999).

Figure 2. Zebra finches can sequentially learn from a first and a second tutor

Song similarity was calculated by comparing 10 motifs of both Tutor 1 (left) and Tutor 2 (right) to 10 motifs of the tutee at 59 dph, before exposure to the second tutor, and at 91 dph, after exposure to the second tutor, with Song Analysis Pro. Dashed line indicates birds exposed to Tutor 1 song at playback; solid line indicates birds exposed to Tutor 2 song at playback. Each bird is demarcated with a number; numbers correspond to the same birds in both graphs. The horizontal, dotted lines represent the average similarity (baseline level) at 59 dph and at 91 dph with non-related birds. There was significant learning from the first tutor as compared to the second tutor at 59 dph ($t(13)=5.37$, $p<0.001$) as well as learning from the second between 59dph and 91 dph (paired t-test, $t(12)=2.66$, $p=0.021$). Learning scores at 91 dph were significantly different from baseline levels ($t(16)=5.63$, $p<0.001$ for similarity scores with the first tutor, and $t(16)=5.23$, $p<0.001$ for similarity scores with the second tutor).

Figure 3. Neuronal activation (number of Zenk-positive neurons) in birds exposed to songs from their first or second tutor

Mean ($\pm$ SEM) number of Zenk-positive nuclei per mm² in the left and right medial and lateral NCM (left) and CMM (right) in response to the song of the first tutor (grey) or the song of the second tutor (black) playback.

Figure 4. Song similarity correlates significantly with lateralization ratios in NCM in response to playback of the first and second tutor song

Scatter plots of lateralization ratios ($([L-R]/[L+R])$) of Zenk-positive nuclei in the medial NCM in response to Tutor 1 (A, B; grey) or Tutor 2 (C, D; black) playback and song similarity (percentage of song elements copied from tutor song) to Tutor 1 (A, C) and Tutor 2 (B, D) song at 91 dph. Lateralization ratios greater than 0 indicate left-lateralized activation; lateralization ratios less than 0 indicate right-lateralized activation.

The correlations in A and D are significant (A: $r = -0.91$, $p = 0.001$; D: $r = 0.89$, $p = 0.003$); no significant correlations were found for B and C.