Bilateral neurotoxic lesions in NCM before tutoring onset do not prevent successful tutor song learning

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Abstract
Sensorimotor learning crucially depends on the ability to acquire a sensory memory for shaping motor commands. Such learning is conveniently studied in young songbirds when they memorize the song of an adult singer and gradually transform their own vocalizations toward the memorized target song. Here we study the involvement of the Caudal Medial Nidopallium (NCM), a higher auditory cortical area, in acquisition of a song memory. NCM has previously been shown to be involved in tutor song memorization. To study the necessity of NCM in this process, we perform large irreversible NCM lesions using ibotenic acid injections in about 40 days old juvenile zebra finches, before their first exposure to tutor song. Surprisingly, NCM-lesioned juveniles successfully copied the tutor song at least as well as untreated control animals, showing that a fully intact NCM is not required for tutor song memory formation and normal song development.

Introduction
Similarly to speech learning in humans, developmental birdsong learning involves perception and memorization of a sensory stimulus within a critical sensory period [2] [3]. In zebra finches, this critical period lasts until about 60 days-post-hatch (dph) [4] [5]. During this period, a young bird must be exposed to a singing adult bird to develop a normal song. The neuronal substrate for storing this tutor song memory is still largely unknown.

Many brain areas are involved in tutor song memorization, among which are the premotor nucleus HVC and the caudal medial nidopallium (NCM). HVC is important for the temporal control of birdsong. Optogenetic, electrical, or pharmacological disruption of neural activity in HVC specifically during exposure to tutor song prevents successful tutor song copying [6]. With regards to NCM, there exists both correlative and causal evidence for its involvement in song memorization. The correlative evidence comes from studies of NCM gene expression [7] and electrophysiology [8]. The causal evidence comes from pharmacological manipulations, which reveal that transiently suppressing the extracellular signal-regulated kinase signaling pathway in NCM, specifically during tutoring, severely impairs song learning [9].

To probe for possible behavioral differences between transient and irreversible brain manipulations [10], we test whether NCM neural circuits are required in their full integrity for successful memorization of tutor song. We perform large irreversible and bilateral lesions in NCM of very young birds before we start tutoring them. Thereafter, we examine the juveniles’ developing songs and quantify their similarity with tutor song. We compare our findings to normally developing songs in a control group of birds that do not receive any lesions.

Objective
We test whether a fully intact NCM is needed for formation of a song memory during an early phase of song development. In 43 days old juvenile zebra finches we study the effects of very large ibotenic acid injections into NCM. The lesions are made before tutoring onset and before closure of the sensory song learning phase (around 60–80 dph).
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Figure Legend

Figure 1. Birds with pre-tutoring bilateral lesions in NCM learn good copies of tutor song.

(A) Sample sagittal Nissl-stained brain section confirming the lesion placement (black arrowheads) in NCM (red dashed line). The dashed circle shows the volume of the injected acid as a rough estimation of the initial lesion volume, see also [1]. The bird was perfused at age 143 dph, 101 days after lesioning NCM. Dorsal is up, rostral is right. Note that we show histology from the oldest bird in the lesion group because any lesion present at 143 dph must have been present at 80 dph when the other birds were perfused.

(B) Average similarity scores of juvenile song motifs with themselves (self), with tutor motifs (tutor), and with motifs from unfamiliar birds (unfamiliar). The bars show the mean ±1 standard deviation of all birds and the circles indicate the scores of individual birds. The scores for the NCM-lesioned bird shown in panel a are highlighted in red and the four birds shown in panel d are marked with according colors. The 3 similarity scores are each indistinguishable between NCM-lesioned birds (dark grey bars) and untreated control birds (light grey bars), revealing that NCM-lesioned birds are not impaired in their ability to memorize and subsequently learn a song. The 3 asterisks indicate that the average similarity score between lesioned juveniles and their tutor is significantly larger than the average similarity score between the lesioned juveniles and unfamiliar adult birds, showing that NCM-lesioned juveniles specifically imitated their tutor.

(C) The song motifs of NCM-lesioned birds and of control birds at 75 dph exhibit roughly identical percent similarity, sequential match, and accuracy in relation to tutor song.

(D) Sample song-motif spectrograms from 3 birds with NCM lesions and from one control bird (right). The motifs shown on top (‘early’) were recorded at 50 dph, after 7 consecutive days of tutoring. The motifs in the middle (‘late’) were recorded at 75 dph, after 25 days of isolation from the tutor. Despite the NCM lesion and the isolation, the ‘late’ motifs in NCM-lesioned birds strongly resemble the tutors’ motifs (bottom, ‘tutor’).

(E) The song changes in the 3 NCM-lesioned birds and the control bird in d occurring between 50 dph and 75 dph (arrows) point more towards tutor song than towards unfamiliar song, demonstrating that within a week after NCM lesions, birds can memorize...
a tutor’s song and subsequently learn to imitate it. 

**Birds**

We used a total of 33 male zebra finches (Taeniopygia guttata) from our breeding facility. 12 juveniles were lesioned and 5 were used as controls. Another 16 adult males were used either as tutors or as unfamiliar males (one of the birds was excluded from the analysis because of low motif self-similarity score). Birds were normally raised with both their parents until 15 dph, which is before the onset of the sensory phase in which a memory of the target song is acquired [18]. At 15 dph, the young birds were transferred together with their mothers to a different room without singing males; thus, after 15 dph they were raised without song exposure (note that young zebra finches need to be fed by at least one of the parents until about 30 dph; only afterwards can they be housed individually). Control and NCM-lesioned birds were housed individually in a sound isolation chamber on a 14/10 h day/night schedule. Starting from 1–5 days after surgery, lesioned birds (N = 12) were tutored by placing an adult singing male zebra finch in the same recording chamber 24 h/day. The corresponding controls (N = 5) were identically tutored with tutoring onset at a comparable age (43–46 dph). Birds were tutored on average for 20.6 ± 8.4 days (range 7 to 28 days, N = 17). We did not find a significant correlation between motif similarity scores and the number of days the birds had been exposed to a singing tutor (Pearson’s correlation coefficient 0.22, p = 0.40, N = 17 including 5 controls). All but 3 birds were raised in a recording chamber alone with the tutor. 3 birds from one clutch (2 with lesions and 1 control) were instead raised together with a tutor in a large cage. Tutor song similarity in these 3 birds fell within the range of that of the other birds; we therefore included these birds in the analysis. 

**Surgery**

Bilateral excitotoxic lesions targeting NCM were made as previously described [19] [1]. Birds were anesthetized using isoflurane and head fixed in a stereotaxic apparatus. In each hemisphere we injected 500 nl ibotenic acid solution (7 mg/ml in ddH₂O). Injections were made between 1500 and 2500 μm ventrally from the brain surface at 760 ± 91 μm anterior to the bifurcation of the mid-sagittal sinus and 452 ± 91 μm lateral from the midline (N= 12 birds). Because we found uncompromised learning in lesioned birds, we decided not to perform sham injections in control birds. All birds resumed normal behavior a few hours after surgery. Canopoli et al. [19] previously showed that this lesion protocol effectively lesions a volume in NCM comparable in size to the volume of the injected acid (Panel A).

**Histology**

To assess lesion extent in histological brain sections we perfused NCM-lesioned birds between 75 and 83 dph (average 78.6 ± 2.5 dph, N = 10 birds). Birds were given an overdose of sodium pentobarbital, followed by a perfusion via the left ventricle first with ringer solution, then with a 4% paraformaldehyde solution. 2 birds were perfused at 143 dph. In these 2 birds and in one control bird, we recorded songs at 120 dph and found a mere 3.9% increase of average similarity score relative to their average score measured at 75 dph (N = 3 birds). Thus, we expect that the songs recorded at 75 dph are good approximations of the songs that juveniles would have produced as adults. 

**Song motif selection and analysis**

We selected and analyzed song motifs as described in [1] [20]. Song motifs of juveniles were taken at an age of 76.2 ± 3.0 dph (range 72 to 82). The motif similarity scores between NCM-lesioned birds and their tutors did not correlate with the age of the juveniles on which their songs were recorded (Pearson’s correlation coefficient 0.24, p = 0.44, N = 12 birds). 

**Results & Discussion**

We made bilateral ibotenic acid lesions in NCM of song-isolate birds when they were 42.5 days old (range 39 to 45, N = 12 birds) and thereafter we exposed the juveniles daily to a singing adult tutor (see Methods). At the end of the experiments (mean 76.3 dph, range 72 to 82 dph, N = 12 birds), juveniles with NCM lesions showed a high song stereotypy (mean 66.9 ± 11.9, range 36.1 to 77.3, N = 12), comparable to the stereotypy in age-matched untreated control juveniles (60.9 ± 7.6, range 53.9 to 70.9, N = 5, p = 0.32, t-test, Panel B).
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NCM-lesioned birds successfully imitated tutor song motifs with an average similarity score of 50.7 ± 10.9 (range 29.1 to 65.3, N = 12 birds), which was not significantly different from similarity in control birds and their tutors (average score 45.1 ± 13.0, range 26.8 to 58.3, N = 5 birds, p = 0.37, t-test). None of the individual measures were significantly different between lesioned birds and control birds: percent similarity (p = 0.61, t-test), sequential match (p = 0.08, t-test), and accuracy (p = 0.81, t-test). NCM-lesioned birds specifically imitated the songs of their tutors and no other songs: The average motif-similarity score in NCM-lesioned birds with their tutors (50.7 ± 10.9) was much higher than the average score of 36.4 ± 6.0 with 14 unfamiliar adult birds (range 29.7 to 49.0, N = 12, p = 0.0006, t-test, Panel B).

We did not find evidence that song learning happened mainly on the days on which the tutor was present with the juvenile. We tested whether pupils’ songs improved in the absence of direct interactions with the tutors. In 4 birds (3 with lesions and 1 control) that were tutored for only 7 days, we calculated tutor-pupil motif similarity scores both on the first day after tutoring offset (53 dph) and at the end of the experiment (75–77 dph). We found that during this period of isolation, the song motifs in all 4 pupils became more similar to their tutors’ motifs than to motifs in 14 unfamiliar adult birds, panel D and E. Thus, birds could improve songs without the presence of their tutors, implying that they used a memory of tutor song that they acquired within a week after the bilateral lesions in NCM.

Conclusions
To our surprise, NCM lesions did not impair tutor song learning, showing that tutor song memory formation does not require a fully intact NCM. These findings contrast with findings presented in [9] where impaired song learning was reported following a more subtle (transient) manipulation in NCM. Given that London et al. injected identical volumes of pharmacological agent as we did, it is unlikely that our manipulation affected smaller portions of NCM than were affected in their study (note that we are not aware of any NCM-manipulation study in which injected volumes were larger than in our study). A possible explanation for the discrepancy with [9] could be an indirect effect of the transient manipulation, triggering a homeostatic imbalance of downstream circuits, as reported in [10] for a different brain area. That is, the impact of transient inactivation of NCM on song learning could be a result of disrupting downstream areas instead of a more direct involvement of NCM in tutor song memorization. For example, tutoring could normally trigger a genetic program causing NCM neurons to change their firing mode, e.g. from spiking to bursting. Downstream neurons would only form a long-term song memory if they receive bursting input from NCM (explaining result from [9]), but when NCM does not support any spiking at all (our study), then reliance on bursting is dropped. According to this view, the effect of transient inactivation may not be directly caused by the inactivation of the targeted area itself, but by the effect on a downstream area (not to provide spike-burst input).

Additional Information

Methods

Birds
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**Supplementary Material**

Please see https://sciencematters.io/articles/201612000007.

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**Ethics Statement**

All the experimental procedures were in accordance with the Veterinary Office of the Canton of Zurich.

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**Citations**


