Regulation of learned vocal behavior by an auditory motor cortical nucleus in juvenile zebra finches

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Naie K, Hahnloser RH. Regulation of learned vocal behavior by an auditory motor cortical nucleus in juvenile zebra finches. J Neurophysiol 106: 291–300, 2011. First published April 27, 2011; doi:10.1152/jn.01035.2010.—In the process of song learning, songbirds such as the zebra finch shape their initial soft and poorly formed vocalizations (subsong) first into variable plastic songs with a discernible recurring motif and then into highly stereotyped adult songs. A premotor brain area critically involved in plastic and adult song production is the cortical nucleus HVC. One of HVC’s primary afferents, the nucleus interface of the nidopallium (NIf), provides a significant source of auditory input to HVC. However, the premotor involvement of NIf has not been extensively studied yet. Here we report that brief and reversible pharmacological inactivation of NIf in juvenile birds leads to transient degradation of plastic song toward subsong, as revealed by spectral and temporal song features. No such song degradation is seen following NIf inactivation in adults. However, in both juveniles and adults NIf inactivation leads to a transient decrease in song stereotypy. Our findings reveal a contribution of NIf to song production in juveniles that agrees with its known role in adults in mediating thalamic drive to downstream vocal motor areas during sleep.

COMPLEX LEARNED MOTOR BEHAVIORS are based on coordinated interaction between motor signals and sensory feedback in the brain. These interactions can occur in single brain areas and even in single neurons, as exemplified by neurons that show traces of either sensory responses or motor signals depending on the behavioral state or neuron type (Cardin and Schmidt 2004; Dave and Margoliash 2000; Keller and Hahnloser 2009; Prather et al. 2008). Coexistence of sensory and motor functions in a brain area motivates a fine-grained analysis of its behavioral involvement. Here we investigated the motor involvement of a higher songbird brain nucleus that is distantly connected to the sensory and motor peripheries. This nucleus mediates auditory responses and sleep-related burst patterns in downstream motor areas, but its involvement in vocal production has not been fully characterized yet (Cardin et al. 2005a; Coleman and Mooney 2004; Hahnloser and Fee 2007).

Male zebra finches (Taeniopygia guttata) learn their songs during a critical 3-mo period that consists of two overlapping phases. During the sensory phase juvenile birds acquire a sensory template of tutor song, and in the subsequent sensori-motor phase they rely on auditory feedback of their singing to gradually transform their songs into copies of the memorized template (Bohner 1990; Eales 1985; Immelman 1969; Konishi 1965; Tchernichovski and Mitra 2002; Thorpe 1958). Many sensory and motor brain areas involved in song production and learning have been described (Akutagawa and Konishi 1998; Aronov et al. 2008; Bottjer et al. 1984; Nottebohm et al. 1976). Adult song is produced mainly by HVC, as shown by lesion, cooling, and single-unit recording experiments (Aronov et al. 2008; Hahnloser et al. 2002; Long and Fee 2008). However, less understood are premotor or sensory areas feeding into HVC such as the thalamic nucleus uvaeformis (Uva), the nucleus interface of the nidopallium (NIf), the medial magnocellular nucleus of the anterior nidopallium (MMAN), and the nucleus avalanche (AV) (Akutagawa and Konishi 2010; Foster et al. 1997). Here we investigated a possible premotor involvement of the cortical NIf. Anatomically, NIf is embedded in the auditory field L complex (Fig. 1). Reversible and irreversible inactivation studies have shown that NIf provides a strong auditory drive to the song generator in HVC (Bauer et al. 2008; Cardin and Schmidt 2004; Coleman and Mooney 2004). NIf’s song-related functions are somewhat controversial. In adult Bengalese finches (which sing more complex and variable songs than adult zebra finches), reversible and irreversible NIf lesions lead to changes in song syntax, apparent in reduced diversity of syllable sequences and occasional syllable stuttering at the beginning of songs (Hosino and Okanoya 2000; Okumura et al. 2007). In adult zebra finches, irreversible lesions of NIf do not lead to major degeneration of song motifs (Cardin et al. 2005b). However, NIf multiunit activity in zebra finches during singing can be prevocal because it precedes contact calls and introductory notes by several tens of milliseconds (McCasland 1987). Thus current evidence suggests that NIf in the zebra finch may have a premotor function that can be rapidly compensated when NIf is lesioned; however, this motor function has not been well characterized yet.

Here we probed NIf’s online premotor role by performing reversible NIf inactivation in singing birds. We perform our inactivation experiments in birds from different age groups because NIf’s contribution may be developmentally regulated. Our main finding is that NIf inactivation strongly affects older juveniles singing plastic songs, but less so adults singing highly stereotyped songs, and not at all very young fledglings singing subsong.

MATERIALS AND METHODS

Male zebra finches (Taeniopygia guttata), ranging from 28 to 400 days of age, were obtained from our breeding colony or from a local supplier. Adults and the bulk part of the juvenile birds used were raised with their parents and siblings. We measured song characteristics associated with normal song development in three developmental groups: a subsong group (36–50 days, n = 10), a plastic song group (51–83 days, n = 10), and an adult group (>100 days, n = 10).

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Birds received a 6-ml injection with saline (NaCl, 0.9%), and sealed with a heat-treated tungsten wire. Morphasol injection (Gräub) containing 24 mg/H9262 was pulled to a tip size of OD, 0.68-mm-ID borosilicate glass capillaries (WPI Europe) heat pulled to a tip size of ~30 μm. Before implantation they were filled with saline (NaCl, 0.9%) and sealed with a heat-treated tungsten wire (0.002 in., A-M Systems). Birds were randomly extracted from the colony and isolated for 2–3 days in a soundproof chamber to record their undirected songs. Each bird was part of only one group, with the exception of two birds that were in both the subsong and plastic song groups: To record song development over extensive time periods (Fig. 2), two juvenile birds were separated from their parents at age 30–35 days and thereafter raised in isolation (with occasional female company). Both these birds were included in both the subsong and plastic song groups. All eight juvenile birds that underwent NIf injection experiments were included in the corresponding developmental (baseline) groups (subsong or plastic song). None of the adult birds that underwent NIf injection experiments was included in the adult group (because the injection experiments in adults and the analysis of song development were performed independently).

Three juveniles received GABA injection, five juveniles received muscimol injection, and all of the adults received GABA injection. In four of the eight juveniles in which we inactivated NIf with GABA/muscimol, the effect of saline injection on song production has been tested (saline injections were made through the same injection cannula). All injections were made bilaterally into both hemispheres. Two of the three birds that underwent pipette implantation and GABA injection experiments into field L were included in the plastic song group. All procedures described here were approved by the Veterinary Amt of the Canton of Zurich, Switzerland, and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences 1996).

**Injection experiments.** Injection cannulas were made of 1.2-mm-OD, 0.68-mm-ID borosilicate glass capillaries (WPI Europe) heat pulled to a tip size of ~30 μm. Before implantation they were filled with saline (NaCl, 0.9%) and sealed with a heat-treated tungsten wire (0.002 in., A-M Systems). Birds received a 6-μl intraperitoneal Morphasol injection (Gräub) containing 24 μg of butorphanol and were anesthetized with 1.5% isoflurane (Attane, Minrad). The implantation sites (NIf and field L) were determined stereotactically and verified by multunit recordings of neural activity (0.5 MΩ, tungsten electrode, WPI Europe). The cannulas were fixed to the skull with dental acrylic (Paladur, Heraeus Kulzer) and connected after the surgery via silicon/polyurethane tubing and a two-way swivel (Instech Laboratories) to a pressure injection unit (Picospritzer II, Parker Hannifin, Fairfield, NJ). Birds were given 1–2 days to recover, during which all vocalizations were recorded. Before the experiments, the tungsten seal was removed and pressure injections (~20-ms duration at 20 PSI) were calibrated by measuring the mensural movement of the saline solution within the glass cannula. After that, the saline was removed by suction through a nonmetallic syringe needle and was replaced by either muscimol (40 mM, dissolved in 0.9% NaCl; Tocris Bioscience, Bristol, UK) or GABA (250 mM; Sigma-Aldrich, Buchs, Switzerland). Muscimol was chosen to inactivate NIf for durations of hours, whereas GABA was chosen to inactivate NIf for durations of minutes (Hahnloser et al. 2008; Olveczky et al. 2005). For muscimol experiments, we injected volumes of ~100–250 nl under visual control and released birds back into their cage. For GABA experiments, we injected similar volumes without visual control; injections were repeated at a minimum interval of 10 min as long as the bird continued to sing. The experimental sessions ended with assessment of injected volumes by measurement of meniscus displacement. At the end of the experiment, we injected 100–200 nl of red fluorescent ink (Boss refil, Stabilo International) through the chronically implanted pipettes, allowing us to verify that NIf had been hit (see Fig. 6A). Red ink was used to mark the injection site; because of large viscosity differences between red ink and saline, we did not attempt to infer the NIf inactivation volume by the ink distribution. NIf borders were assessed in unstained sections.

In two birds we histologically verified that injected drugs did not leak to HVC or robust nucleus of the arcopallium (RA) by adding Fluoro-Ruby to the GABA solution. Birds were deeply anesthetized with 0.1 ml of 50 mg/ml Nembutal sodium solution (Abbott, Baar, Switzerland) and transcardially perfused with 0.01 M phosphate-buffered saline and 4% paraformaldehyde. Brains were sectioned at 60 μm on a vibratome. Injection sites were identified by localization of the red fluorescent ink. All sections were photographed for documentation (ColorView and analySIS, SoftImaging System; see Fig. 5) and were subsequently stained with cresyl violet.

**Sound and rhythm analysis.** Vocalizations were band-pass filtered (300 Hz–13 kHz), sampled at 44 kHz, and analyzed with the Sound Analysis Pro software package (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html) (Tchernichovski et al. 2001; Tchernichovski and Mitra 2002). For each bird we adjusted the derivative power threshold manually until the automated identification of individual song syllables was consistent across all motifs. We randomly chose 240 song syllables each day and analyzed sound features including mean amplitude, mean Wiener entropy (a measure of “tonality”—from pure tone at minus infinity to white noise at zero), mean goodness of pitch, mean mean-frequency, variance Wiener entropy (a measure of tonal repertoire), variance goodness of pitch, and variance mean-frequency.

When comparing song features between different experimental conditions (for example, “treated” vs. “before injection”), we analyzed a number of syllables in each bird that was determined by the bird singing the fewest syllables or was set to 800, whichever was smaller. For the treated group we used songs produced in the first 5 min after GABA injection or produced in the first 4 h after muscimol injection. For the recovery group we used songs recorded at least 15 min after GABA injection and at least 5 h after muscimol injections. Differences in syllable features between these groups were assessed with the Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) with a significance threshold of $P = 0.01$.

Because we were not able to classify the different syllables in subsong and plastic song, we quantified the diversity of syllable durations in terms of the multimodality of syllable duration histograms (see Figs. 2C and 5A). First we smoothed these histograms with the moving average method with span 80 ms and normalized the integral to sum to 1. As a measure for syllable duration diversity we computed the derivative of the normalized histograms (to increase sensitivity to small peaks), took the absolute value, and counted the number of disjoint peaks that exceeded a fixed threshold of 0.01 (e.g., in the bottom histogram in Fig. 2C there are 5 peaks). The number of peaks depended on the width of the smoothing kernel and the threshold. However, differences between the treated and the plastic song group were very robust for smoothing kernels from 40 to 160 ms wide and for thresholds from 0.005 to 0.02. Also, similarity between the treated and subsong groups was persistent for smoothing widths from 40 to 160 ms, although for thresholds between 0.003 and 0.007 the average number of peaks in the subsong and treated groups grew apart by up to 50%. 

**Fig. 1. Schematic drawing of the song system, showing the main premotor and motor areas, the anterior forebrain pathway, as well as the main auditory areas: caudalateral mesopallium (CLM); HVC (proper name); fields L1, L2a, and L3; lateral magnocellular nucleus of the anterior nidopallium (LMAN); caudomedial nidopallium (NCM); nucleus interface of the nidopallium (NIf); supraspinatal nucleus (nXIIIs); paraamigualis (PAm); robust nucleus of the arcopallium (RA); retroamigualis (RAm); nucleus uvaeformis (Uva).**

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Fig. 2. Zebra finch song development illustrated by an example. A: spectral derivatives of subsong [31 days of age (top)] and of plastic songs [44 days (middle) and 52 days (bottom)]. Red boxes enclose renditions of the emerging stereotyped song motif. Introductory syllables are labeled i and ii and the remaining syllables A, B, and C. The sound amplitude corresponding to the bottom spectrogram in A is indicated by the blue line. The rhythm function just above (black line) was constructed by thresholding (red line) and mapping the sound amplitude to values of 0 and 1. B: spectrum of the rhythm function as a function of age. Red indicates high intensity, and blue indicates low intensity. For example, a peak around 8 Hz in the rhythm spectrum corresponds roughly to the average duration of song syllables. C: distribution of syllable duration as derived from the rhythm function at different ages [31, 35, 39, 44, 48, 52, and 56 days (d), as indicated]. Syllables A and C were often subdivided into 2 parts by the thresholding operation: A1, A2, and C1, C2. D: comparison of song features extracted from the songs sung by 10 different zebra finches in each of the groups: a subsong group, a plastic song group, and an adult group. Mean goodness of pitch (a), mean amplitude (b), variance goodness of pitch (c), and variance Wiener entropy (d) all increase significantly (P < 0.001) during development. Error bars denote means ± SD.
To quantify temporal structure of song we used a rhythm function defined as 1 if sound amplitude is above a certain threshold and 0 if sound amplitude is below threshold. The threshold was separately chosen for each bird, but was identical for all recorded songs within a given bird.

We also analyzed the rhythm spectrum, i.e., the spectral power density of the rhythm function (computed by Fourier transforming the rhythm function and squaring). In Fig. 2B and Fig. 5B, horizontal rows in the stack plot represent color-coded rhythm spectra, where each spectrum is computed from a song window of 20-s duration and the windows corresponding to adjacent lines had 15-s overlap. Vertical red-yellow lines in the stack plot each indicate a characteristic frequency of song temporal structure.

**Stereotypy score.** We analyzed the stereotypy of songs produced within the first 5 min after GABA or vehicle injection into NIf. The identity of each syllable was visually ascribed on the basis of spectrogram morphology and syllable location within the motif. The blind analysis was done by a naive observer who was instructed to classify song syllables given a few template songs in each bird. We computed linearity and consistency scores as defined by Scharff et al. (Scharff and Nottebohm 1991) from 450 syllables per experimental session. The linearity score measures how well the syllables are ordered in a song: (# different syllables / # different transition types).

The consistency score measures how often a particular sequence is sung: (# typical transitions / # transitions overall).

The stereotypy score is a combination of both of these scores and is defined as: (linearity score + consistency score)/2. Therefore, a single fixed syllable sequence yields a stereotypy score of 1, and with increasing diversity of syllable transitions the stereotypy score approaches 0.

In both juveniles and adults, NIf inactivation led to decreases in mean stereotypy scores that were composed of joint decreases in linearity and consistency scores. That is, in juveniles (n = 3), we found mean stereotypy scores before/after = 0.47/0.27, mean linearity scores before/after = 0.49/0.33, and mean consistency scores before/after = 0.41/0.20. In adults (n = 4) we found mean stereotypy scores before/after = 0.74/0.6, mean linearity scores before/after = 0.72/0.58, and mean consistency scores before/after = 0.76/0.63.

**Discarded animals.** In total, 30% of the injection experiments were unsuccessful, either because the birds did not sing for several days or because the injection pipette was clogged. We also had to discard data from one bird because the pipette was implanted at the far edge of NIf, precluding classification of the animal as either test or control.

**RESULTS**

To estimate the premotor role of NIf, we reversibly inactivated NIf in freely behaving birds and assessed the effects of inactivation on vocal output. To interpret effects in the context of developmental trends, we compared the songs of NIf-inactivated juvenile and adult birds to normal songs produced by birds in three age groups of our colony: a subsong group, a plastic song group, and an adult song group. Given that subsongs and stereotyped adult songs are produced by different motor pathways, both of which potentially receive NIf input (Aronov et al. 2008; Olveczky et al. 2005), our experiments allow us to estimate the extent to which NIf output is destined to the stereotyped song pathway or the subsong pathway.

**Song development.** We first made a statistical assessment of song development in our colony. We analyzed the songs of 30 randomly chosen birds in three developmental groups (Bohner 1990; Eales 1985; Immelman 1969): a subsong group (36–50 days, n = 10), a plastic song group (51–83 days, n = 10), and an adult group (>100 days, n = 10).

In agreement with previous findings (Tchernichovski et al. 2004), syllable durations in our colony developed from a broad and typical bimodal curve in the subsong group to a multi-peaked curve in the plastic song group (Fig. 2). The broad subsong peaks were not associated with distinct song syllables. In contrast, the plastic song peaks corresponded each to a different song syllable (or part thereof). The number n of peaks in syllable duration histograms increased with age group, from around two peaks in the subsong group [n = 2.0 ± 0.3 (SE)] to more than three peaks in the plastic and adult groups (n = 3.5 ± 0.2 plastic song group, n = 3.5 ± 0.4 adult group). This increase reflects the emergence of distinct song syllables during the sensorimotor learning phase.

In addition to syllable durations we also analyzed song rhythmicity, i.e., the temporal patterning of song elements. In agreement with previous work (Saar and Mitra 2008), we found the rhythm spectrum of song to develop from a broad and undifferentiated curve in the subsong group to a set of narrow peaks and troughs in the plastic song group, indicating a stabilization of temporal syllable patterns (Fig. 2B). We quantified the complexity of temporal rhythms in the three bird groups by the modulation of the rhythm spectrum, i.e., by the root mean square (RMS) residual of a linear fit to the rhythm spectrum in the interval 3 to 30 Hz (spanning the time scale from long notes to small groups of song syllables). The RMS residual was expressed as a percentage in reference to the average of the adult group. The developmental trend was a robust increase in rhythm complexity with age: the rhythm modulation increased from 27 ± 3% (SE) in the subsong group, to 81 ± 7% in the plastic song group, up to 100 ± 10% in the adult group (see Fig. 5D).

We also characterized spectral song features and their development. Previous work has shown that spectral aspects of song can be characterized by features such as sound amplitude, mean Wiener entropy (a measure of “tonality”—from pure tone at minus infinity to white noise at zero), mean goodness of pitch (a measure of sound periodicity), mean mean-frequency, variance Wiener entropy (a measure of tonal repertoire), variance goodness of pitch, and variance mean-frequency (Deregnaucourt et al., 2005). In agreement with this previous work we found that with increasing age there was a significant increase in mean and in variance goodness of pitch, in mean sound amplitude, and in variance Wiener entropy [P = 0.01, 1-way ANOVA; mean goodness of pitch F(2,27) = 917, mean sound amplitude F(2,27) = 1.040, variance Wiener entropy F(2,27) = 115; see also Fig. 4]. These findings reflect the known fact that adults sing more complex syllables than do juveniles.

**Song degradation following reversible inactivation of NIf in juveniles.** We chronically implanted juvenile birds between 50 and 80 days post hatch (dph) with cannulas made of glass pipettes that were targeted onto NIf and connected to a pressure injection system (see MATERIALS AND METHODS). Bilateral injection of the inhibitory neurotransmitter GABA into NIf of a 75-day-old bird in the plastic song phase resulted in transient song degradation. The degraded songs were subsong-like in the sense that they were much softer and less harmonic, and they often lacked clearly recognizable song motifs (Fig. 3A, see Supplemental Movie). In this bird, visual inspection of spec-
tral derivatives—conducted as a blind analysis (Tchernichovski et al. 2000)—revealed that 32% of immediate postinjection syllables could not be associated with preinjection syllables. Syllables recovered gradually in this bird, and in the interval 2–5 min after the injection offset roughly 83% of all syllables could again be identified, a fraction that was comparable to the 88% of recognizable syllables before the injection.

To record more songs during NIf inactivation, we injected a number of birds with the GABA-A receptor agonist muscimol, which is known to silence brain areas for several hours. The songs of muscimol-injected birds (8 birds) were degraded similarly as the songs of GABA-injected birds (5 birds). In the first 5 min after bilateral GABA injection and in the first 4 h after bilateral muscimol injection into NIf, plastic songs became degraded and were frequently composed of unrecognizable song syllables. On average only 4% of syllables were not clearly recognizable before the injection from their spectrotemporal representation (range 1–12%, median 3%; n = 8 birds), whereas on average 29% of syllables became unrecognizable after injections (Table 1; n = 8 birds).

Plastic song features in NIf-inactivated juveniles degrade in a developmentally reversed direction. Within seconds of the bilateral GABA or muscimol injections into NIf, songs underwent a transient decrease in sound features such as amplitude, (absolute) mean and variance Wiener entropy, and mean pitch. In all eight injected birds at least three of six examined features degraded (different features degraded in different birds). In all cases feature values were smaller during inactivation than before (P < 0.01), and in none of the eight birds did a feature value increase during inactivation (P > 0.01). In only 11 of 48 tested cases (6 different sound features in 8 birds) were feature values unchanged. Moreover, for 8 of these 11 cases average baseline feature values preinjection were less than 1 standard deviation above average subsong values, suggesting there was not much room for these feature values to further decrease.

By pooling the data over all birds, we found that songs produced during NIf inactivation were reduced in mean song amplitude (n = 8 GABA- or muscimol-injected birds, P = 0.001; Fig. 4A), in mean and variance goodness of pitch (n = 8 birds, P = 0.001; Fig. 4, B and F), in variance Wiener entropy (n = 8 birds, P = 0.001; Fig. 4C), and in mean mean-frequency (n = 8 birds, P = 0.001; Fig. 4D).

Table 1. Number and percentage of recognizable syllables and unrecognizable syllables before and during NIf inactivation

<table>
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Values are number and percentage of recognizable syllables (+) and unrecognizable syllables (−) before and during nucleus interface of the nidopallium (NIf) inactivation. dph, Days post hatch; Med, median values of each column.

Fig. 3. Transient degradation of song features following GABA injection into right nucleus interface of nidopallium (NIf) of a 75-day-old bird. A: spectral derivatives of songs produced roughly 4 min before GABA injection into right NIf (pre), 40 s after the injection (during), and 3 min after the injection (post). B, from top to bottom: NIf inactivation leads to transient decreases in mean sound amplitude, in variance Wiener entropy, and in mean Wiener entropy. Each circle represents the mean feature value computed over an entire song bout. Lines are cubic spline interpolations of the data circles. Dotted vertical lines indicate the time points 230 s before (i), 40 s after (ii), and 220 s after (iii) the injection, corresponding to the spectrograms in A.
Song degradation after muscimol injection in NIf was associated with flattening of the rhythm spectrum (Fig. 5). NIf inactivation led to a reduction in rhythm modulation to 32 ± 3% \((n = 3\) birds), which was comparable to the rhythm modulation in the subsong group \((P = 0.10)\). Thus NIf inactivation induced a transient loss of stereotyped syllable patterning similar to subsong.

Song degradation was specific to NIf inactivation. When we injected saline bilaterally into NIf (either before or after the NIf inactivation with GABA/muscimol), no song degradations were seen \((P > 0.01, n = 4\) birds, 500 syllables per bird and case). Even after bilateral injections of muscimol into the neighboring field L region \((P > 0.01, n = 3\) birds, 54–67 dph, 800 syllables per bird and case), no degradations of song were detectable (Fig. 6B).

Given that plastic songs under NIf inactivation were similar to subsong, we did not expect NIf inactivation during the subsong phase to produce any noticeable effects. Indeed, we found that NIf inactivation in very young zebra finches \((n = 2\) birds, 40 and 46 dph) did not result in any quantifiable visible or audible effects on subsong \((P = 0.45);\) syllable durations, song rhythmicity, and sound features).

Minor song degradation after reversible inactivation of NIf in adults. NIf inactivation in adult zebra finches \((\text{age} > 100\) days) with GABA injections did not lead to noticeable changes in song feature values \((n = 4\) birds, \(P = 0.07, 152\) syllables per bird and case). Hence, our findings agree with a irreversible NIf inactivation study in adults \((\text{Cardin et al. 2005b}),\) in which song features also remained unaffected.

Nevertheless, we observed two types of song degradations in adults. First, during NIf inactivation adult birds often produced long strings of introductory-like notes without subsequently singing a song motif, an effect reminiscent of effects seen after irreversible Uva lesions \((\text{Coleman and Vu 2005})\) and after NIf lesions in adult Bengalese finches \((\text{Hosino and Okanoya 2000; Okumura et al. 2007})\). Second, during NIf inactivation adult birds frequently interrupted their songs either in between or during song syllables. These interruptions led to a minor decrease in syllable sequence stereotypy [quantified with the stereotypy score of Scharff and Nottebohm \((\text{Scharff and Nottebohm 1991})\); Fig. 7]. In each of the four adult birds examined, NIf inactivation led to significant decreases in sequence stereotypy scores \((2\text{-proportion z-test, } P < 0.05;\) see MATERIALS AND METHODS).

By contrast, saline injections into adult NIf \((n = 3)\) did not lead to a reduction in sequence stereotypy, revealing specificity of stereotypy decrease to NIf inactivation.

Our observation of reduced sequence stereotypy in NIf-inactivated adults led us to reanalyze our data from juveniles. We found that in NIf-inactivated juveniles there was also a similar decrease of song stereotypy during periods of partial recovery lasting a few tens of seconds in which most song syllables could be identified (Fig. 7). In each of the three juvenile birds in which we gathered sufficient data for a sequence stereotypy analysis, NIf inactivation led to significant decreases in sequence stereotypy scores \((2\text{-proportion z-test, } P < 0.05;\) see MATERIALS AND METHODS). Hence, the exclusive song feature and song rhythm degradation in juveniles suggests that NIf has a premotor role mainly during a restricted developmental time window. Yet, because NIf inactivation in adults

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**Fig. 4.** Comparison of song characteristics before and during reversible NIf inactivation by GABA agonists. Each plot shows mean values of 8 birds before \(B\), during \(T\), and after \(R\) NIf inactivation. P, plastic song; S, subsong. Circles indicate the mean value of randomly chosen syllables in each bird for the features: mean amplitude \((A)\), mean goodness of pitch \((B)\), variance Wiener entropy \((C)\), mean mean-frequency \((D)\), mean Wiener entropy \((E)\), and variance goodness of pitch \((F)\). All song features significantly changed during NIf inactivation. Developmental feature values are plotted for comparison: the graded bars \((\text{black, right})\) labeled “subsong” display values measured in 36- to 50/day-old birds \((n = 10)\), and the graded bars \((\text{black, left})\) are measured in 50- to 83-day-old birds \((n = 10)\). All error bars denote means ± SD.

Average sound features measured in birds of the plastic song group during NIf inactivation could not be distinguished from values measured in the subsong group \((n = 10\) birds, \(P > 0.01)\) except for mean Wiener entropy, which overshot the mean Wiener entropy in the subsong group \((n = 8\) birds, \(P = 0.001;\) Fig. 4E). It should be noted, however, that mean Wiener entropy was not a reliable developmental indicator in our colony (Fig. 4E).

**Syllable durations and song rhythm during NIf inactivation are subsong-like.** The sound features analyzed thus far provided only information about the spectral patterns of individual song syllables but not about syllable durations and song rhythmicity. Inactivating NIf during plastic song production \((n = 3\) muscimol-injected juveniles) caused significant changes in syllable durations: The histogram of syllable durations displayed fewer peaks \((n = 2.3 ± 0.3)\) and peaks were broadened, including the appearance of song syllables of new durations (Fig. 5). The diversity of syllable types in NIf-inactivated juveniles was similar to the diversity in the subsong group (the number of peaks were indistinguishable; \(P = 0.23)\).
has an effect on song sequence stereotypy, we can ascribe a
minor premotor function of NIf also in adult zebra finches.

DISCUSSION

We found that in juvenile zebra finches NIf is involved in
generating temporal and spectral aspects of plastic songs, and
in juveniles and adults it contributes to high stereotypy of
syllable sequences. In the following, we put these findings in
the context of the existing literature and discuss the possible
functions of NIf from a broader perspective.

We found an age dependence of NIf inactivation effects. In
adults, reversible NIf inactivation has almost no effect on song,
except to destabilize syllable sequences. These findings agree
with irreversible (excitotoxic) lesions of NIf in adults that have
only minor short-term effects including syllable loss and de-
stabilization of harmonic stacks (Cardin et al. 2005a). In young
juveniles singing subsong, NIf appears to have virtually no
involvement in singing. Because NIf’s sole projection target is
HVC, our findings agree with a recent finding showing that
lesions of HVC have little influence on subsong production
(Aronov et al. 2008).

Song degradation in older juveniles and roles of lateral
magnocellular nucleus of the anterior nidopallium and audi-
tory feedback.

The strongest effects of reversible NIf inactivation were seen in older juveniles, in which plastic songs were
strongly degraded. The temporal and spectral degradations
of plastic song we observed are reminiscent of song degradations
following HVC microlesions in adults (Thompson and Johnson
2007; Thompson et al. 2007). Interestingly, whereas song
recovers gradually after HVC microlesions, recovery is almost
instantaneous when the lateral magnocellular nucleus of the
anterior nidopallium (LMAN) is lesioned in addition to HVC
(Thompson et al. 2007). Thus LMAN (the output of a basal
ganglia motor pathway) actively compensates for HVC mi-
crolesions by producing less stereotyped song. In the context of
our experiments, these findings suggest that the degraded songs
observed during NIf inactivation could be driven by LMAN
that actively overrides the more stereotyped plastic songs
normally produced by NIf and HVC. Thus a possibility to be
further studied is whether LMAN lesions in older juveniles
prior to NIf inactivation are able to prevent song degradation.
Such experiments would test whether HVC microlesions are in
a sense equivalent to NIf inactivation. If equivalence applies,
then we expect song recovery from NIf inactivation to depend on auditory feedback, as is the case for song
recovery from HVC microlesions. What speaks against this
possibility is that currently there exists no evidence that NIf
transmits auditory feedback involved in song plasticity. Song
decrystallization following vocal nerve transection does not
require NIf (Roy and Mooney 2009). NIf input to HVC could
thus reflect auditory feedback not used for song plasticity but
used for completing the ongoing song. Absence of NIf input
may thus have effects on temporal song structure similar to
absence of auditory feedback. If true, then deafened or muted
juveniles would increase the frequency of song truncations and
the diversity of syllable transitions similar to what we see

Fig. 5. Loss of song rhythm during NIf inactivation in juveniles. A: NIf inactivation alters the distribution of syllable durations produced during the plastic song
phase (80-day-old bird; black line before inactivation, red line after inactivation). For comparison, the subsong distribution is also shown in this bird (49 days
old; blue line). B: NIf inactivation at 80 days (black arrow) flattened the rhythm spectrum toward the rhythm spectrum observed during the subsong phase (49
days; same bird as in A). C: bar plot summarizing the nonuniformity of syllable duration histograms in different experimental groups. Bars indicate mean number
of peaks in transformed syllable duration histograms (see MATERIALS AND METHODS). Error bars indicate SE. D: bar plot summarizing the modulation of the rhythm
spectrum in different experimental groups. Bars indicate mean root mean square (RMS) residuals of fitted rhythm spectra (see MATERIALS AND METHODS), expressed as a percentage of the adult group. Error bars indicate SE.
during NIf inactivation. In favor of this hypothesis is the fact that song decrystallization in deafened birds is fast in young birds (Lombardino and Nottebohm 2000), in line with our finding that NIf inactivation is more deleterious in juveniles than in adults.

**NIf and control of syllable transitions.** How can we reconcile the decrease in sequence stereotypy during NIf inactivation in both juveniles and adults (Fig. 7) with studies in Bengalese finches, in which NIf lesions lead to increased sequence stereotypy (Hosino and Okanoya 2000, Okumura et al. 2007)? Part of the answer may be that zebra finches mainly sing a single stereotyped motif and do not vary syllable sequences to a similar extent. Whereas our results confirm that NIf is not required for production of crystallized song motifs, our analysis of song sequence stereotypy reveals a facilitatory influence of NIf on syllable transitions. Presumably this drive is not essential in adults and can be compensated when missing after irreversible NIf lesions.

One hypothesis about NIf is that it controls syllable transitions and their diversity (the NIf syllable sequence hypothesis). As mentioned, zebra finches produce little variability of syllable sequences and thus are not well suited to directly test this hypothesis. Yet, if our findings are to agree with this hypothesis, an interesting prediction about NIf activity and HVC development follows. Namely, the decrease in sequence stereotypy during NIf inactivation can be interpreted as impairment in generating syllable onsets and syllable transitions, suggesting that NIf activity in adults is stereotyped during production of stereotyped syllable sequences. Similarly, NIf inactivation in juveniles may have a more dramatic effect than in adults because the juvenile HVC network is not fully developed and relies more strongly on suitable trigger signals from NIf and the anterior forebrain (Foster and Bottjer 2001) than the adult network.

**Roles of NIf during sleep and input from Uva.** NIf appears to drive HVC during singing, at least in older juveniles. Such a view agrees with NIf’s involvement in sleep-related processing and in NIf’s involvement in relaying thalamic auditory inputs to HVC. Specifically, it was found that reversible inactivation of NIf in sleeping adults leads to cessation of premotor-like spike bursts in HVC neurons (Hahnloser and Fee 2007) and to suppression of HVC spike responses elicited by electrical stimulation in Uva (Hahnloser et al. 2008). Hence, NIf appears to mediate the excitatory Uva drive destined for HVC. It is questionable, however, whether in sleeping adults this drive is of relevance, because NIf is able to drive spike bursts in HVC also during NIf inactivation. In favor of this hypothesis is the fact that song decrystallization in deafened birds is fast in young birds (Lombardino and Nottebohm 2000), in line with our finding that NIf inactivation is more deleterious in juveniles than in adults.
when Uva is inactivated (Hahnloser et al. 2008). Thus NIf has a particularly important role during sleep and is currently the highest nucleus to which a driver role of sleep bursts in motor areas is ascribed. The combined driver roles of NIf during sleep and singing point to a possible involvement of NIf in shaping the developing premotor circuitry by driving activity during sleep as a function of auditory experience during the day (Roberts et al. 2010; Shank and Margoliash 2009).

In singing adults HVC seems to be driven strongly by direct Uva input, because bilateral Uva lesions have a strong influence on normal adult song and lead to degradation of the temporal structure of song and to many repeated syllables and excessively long syllable gaps (Williams and Vicario 1993). This degradation of song following Uva lesions is much stronger than the 10% decrease in stereotypy we observed during NIf inactivation. Thus, given our results, we speculate that Uva’s song involvement in adults is mediated mainly via HVC-projecting Uva neurons, whereas the frequent and excitatory bursts in NIf-projecting Uva neurons seen in sleeping zebra finches are absent or redundant during song production. Spikes in HVC-projecting Uva neurons have not been measured during singing yet, but in sleeping adults they have an inhibitory influence on bursting in HVC. It may thus well be that HVC relies on some type of inhibitory synchronization signal from Uva but is able to generate ultrasparse sequences during singing on its own, without indirect Uva input via NIf. The driver roles of NIf in singing juveniles and sleeping adults, as well as NIf’s embedding within the Uva-HVC network, will have to be considered in future network models of sensorimotor learning that involve both online and offline learning phases.

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DISCLOSURES

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