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Motor circuits are required to encode a sensory model for imitative learning

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Premotor circuits help generate imitative behaviors and can be activated during observation of another animal's behavior, leading to speculation that these circuits participate in sensory learning that is important to imitation. Here we tested this idea by focally manipulating the brain activity of juvenile zebra finches, which learn to sing by memorizing and vocally copying the song of an adult tutor. Tutor song–contingent optogenetic or electrical disruption of neural activity in the pupil's song premotor nucleus HVC prevented song copying, indicating that a premotor structure important to the temporal control of birdsong also helps encode the tutor song. *In vivo* multiphoton imaging and neural manipulations delineated a pathway and a candidate synaptic mechanism through which tutor song information is encoded by premotor circuits. These findings provide evidence that premotor circuits help encode sensory information about the behavioral model before shaping and executing imitative behaviors.

The cultural transmission of behavior involves observation of a behavioral model followed by imitation of the observed behavior. How the brain encodes the formative sensory experience provided by the behavioral model is not well understood. Although sensory structures are undoubtedly activated during observation of the model, premotor structures that have a role in generating imitative behaviors can also be activated during observation of another animal's behavior[1–5]. This has led to speculation that premotor circuits may help encode sensory information about the model that is important to subsequent behavioral imitation[6–9]. Birdsong is a culturally transmitted vocal behavior with strong parallels to human speech learning, including the obligatory auditory experience of a vocal model during a juvenile sensitive period followed by a phase of vocal copying[10–13]. Juvenile male zebra finches first listen to and memorize the song of an adult male tutor during a sensory learning phase (**Fig. 1a**; ~30–60 days post-hatching (dph)) and then engage in vocal practice to emulate this memorized song model during a partially overlapping and more prolonged phase of sensorimotor learning (~45–90 dph)[11]. In addition, the brain of the male zebra finch contains well-described auditory and song motor pathways that are thought to be critical to these two phases of learning (**Fig. 1b**)[13,14]. Nonetheless, how the experience of the tutor song is initially encoded in the brain of the juvenile and how this information interacts with song motor circuits to guide song development is unclear.

One possibility is that the auditory memory of the tutor song is encoded in forebrain structures that are analogous to the secondary and tertiary auditory cortices of mammals (**Fig. 1b**). In support of this idea, vocal imitation is impaired after pharmacological manipulation of these secondary auditory regions of juvenile zebra finches during tutoring[15], and mapping studies of activity-dependent gene expression and electrophysiological responses in adult zebra finches have suggested that neurons in these regions could encode a long-lasting representation of the tutor song[16–19]. However, these findings do not address whether encoding the tutor song also requires activity in downstream structures, including motor structures that directly control singing (**Fig. 1b**). Indeed, secondary auditory regions provide direct and indirect input to the telencephalic nucleus HVC[20,21], a premotor structure that is essential for song generation[22] and that contains neurons that encode precise timing information for song patterning and respond to the auditory presentation of a tutor song[23–25]. Moreover, exposing a juvenile zebra finch to a tutor song can trigger rapid structural and functional changes to synapses in its HVC that correlate with the quality of its subsequent song imitation[26]. The finding that exposure to a tutor song can rapidly alter the HVC network suggests a possible role for HVC in encoding the tutor song experience.

One challenge to testing this idea is that juvenile zebra finches often interleave periods of singing and other forms of vocal activity with periods of listening to a tutor song. Consequently, although pharmacological manipulations either upstream or downstream of HVC can affect the quality of song copying[15,27], it is unclear whether these effects are the result of interference with vocal premotor activity, auditory activity evoked by the tutor song or auditory feedback activity evoked by the pupil's own singing. To examine whether HVC has a critical role in encoding the experience of the tutor song, we sought a method that would allow us to disrupt HVC activity only when the pupil listened to his tutor's song but not at other times, including during periods of vocal rehearsal (**Fig. 1c**). The transgenic expression of light-activated cation channels (channelrhodopsins) provides a means for the precise spatiotemporal control of neural activity without the potential confound of activating fibers of passage that can accompany electrical stimulation or the typically prolonged (minutes to hours) modulation of neural activity accompanying pharmacological manipulations[28–31].

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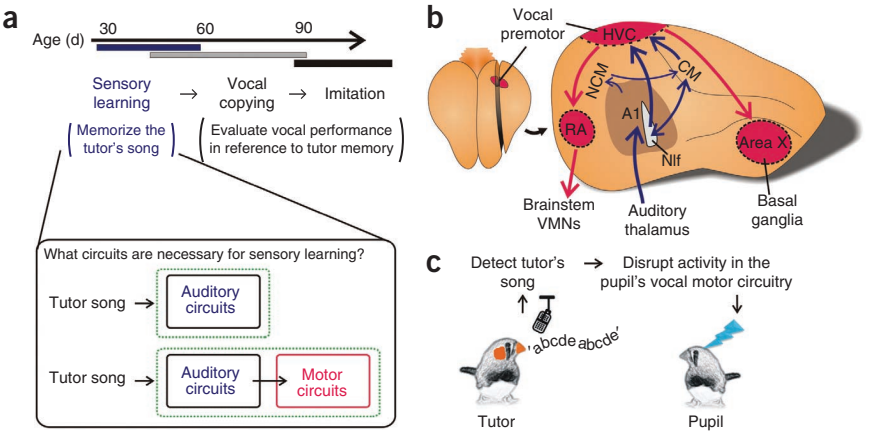

Figure 1 Testing the role of premotor circuits in sensory learning in songbirds. (**a**) Song learning (top) in juvenile male zebra finches comprises a sensory learning phase, during which the pupil memorizes the song of a tutor, and a longer sensorimotor learning phase, during which the pupil uses auditory feedback to match its song to the memorized model. The brain regions that are important to sensory learning could be restricted to auditory circuits or might also require the participation of motor circuits. (**b**) Dorsal view of the zebra finch brain (left) and a parasagittal view through the medial forebrain (right) showing song premotor circuitry (red), including HVC and auditory circuitry (blue). A1, primary auditory regions (field L); CM, caudal mesopallium; Area X, striatal component of the song system; RA, robust nucleus of the arcopallium; VMNs, vocal motor neurons. (**c**) Schematic of tutor-song–contingent disruption of neural activity in the pupil's brain.

RESULTS

Premotor circuits are essential to sensory learning

We found that viral-mediated expression of humanized channelrhodopsin-2 (hChR2 expressed using scAAV2/9-hChR2–yellow fluorescent protein (YFP) or herpes simplex virus 1 (HSV1-hChR2)) could be used in combination with light pulses to robustly alter HVC neuronal activity (**Fig. 2a–c**). Extracellular recordings made in anesthetized juvenile male zebra finches expressing hChR2 revealed that brief (50–500 ms) pulses of laser light (473 nm) applied through a fiber optic cable could alter activity across the mediolateral and rostrocaudal extent of HVC (**Fig. 1c**; $n = 13$ birds, 26 hemispheres). Notably, we only detected light-evoked responses in the dorsal aspect of HVC. Extracellular recordings in anesthetized birds confirmed that optogenetic activation of HVC did not evoke antidromic activity in auditory regions presynaptic to HVC (the telencephalic nucleus interface (NIf) or caudal mesopallium; **Fig. 1b**; 0/18 sites in $n = 3$ birds) and, similarly, did not activate neurons more ventrally in the HVC 'shelf', a distinct region that may have a role in auditory processing[32,33] (in 25/26 hemispheres, we detected light-evoked responses in only the first ~250 µm from the surface of the brain; in the remaining hemisphere, we detected responses up to a depth of ~350 µm). Illuminating the dorsal surface of HVC strongly excited neurons at some recording sites, suppressed spontaneous activity at other sites or elicited more prolonged and complex responses consisting of both suppression and excitation (**Supplementary Fig. 1**). These findings suggest that viral-mediated expression of hChR2 coupled with laser illumination can modulate the activity of both excitatory and inhibitory neurons that populate the HVC microcircuit[34,35], an idea that we confirmed using intracellular recordings from physiologically identified HVC neurons in brain slices prepared from male zebra finches previously injected with AAV2/9-hChR2-YFP in HVC (**Supplementary Fig. 2**). Therefore, a virally mediated optogenetic approach is well suited to disrupt HVC network dynamics in juvenile birds learning to sing.

To selectively disrupt HVC activity during tutoring, we used software[36] to detect recognizable features of the tutor's song and directly trigger optogenetic stimulation of neurons in the pupil's HVC (**Supplementary Video 1**). We assessed the functional expression of hChR2 in the left and right HVC of tutor-naive juvenile zebra finches with *in vivo* extracellular recordings and illumination over the recording site before implanting optical fibers (200-µm diameter) immediately dorsal to each HVC. The morning after implantation, we connected the optical fibers through an optical commutator to a 473-nm laser and introduced an adult male tutor to the holding cage with the implanted juvenile. Beginning at 43–53 dph, we exposed the juveniles to their tutors for 2 h per day for 5 consecutive days and then raised them in isolation to adulthood, with adulthood being defined as >90 dph (**Fig. 2d**). During tutoring, we used features of the tutor song to trigger laser pulses lasting 200 ms ($n = 2$ birds) or 500 ms ($n = 2$ birds) (hit rate >80%). Juveniles subjected to such tutor song–contingent optogenetic disruption of HVC activity developed adult songs that bore little resemblance to the song of their tutor (**Fig. 2e,f**; $n = 4$ birds, all exposed to the same tutor). The adult songs of the hChR2-birds and untutored birds were equally dissimilar to the tutor song (mean similarity to the tutor song: hChR2-birds, 28.7%; untutored birds, 37.6% ($n = 3$ birds); two-sample $t(5) = 1.0$, $P = 0.35$). In contrast, birds in four different control conditions all copied significantly more of the song from their tutors ($n = 6$, two-sample $t(8) = 2.3$, $P = 1.7 \times 10^{-6}$, power $(1 - \beta) = 1$). These control conditions included (i) juveniles subjected to the same temporal pattern of optogenetic stimulation in HVC immediately after the removal of the tutor (Online Methods); (ii) juveniles subjected to tutor song–contingent optical stimulation of HVC after injection with an adeno-associated virus (AAV) expressing enhanced green fluorescent protein (GFP) into HVC (**Fig. 2e,f**; $n = 2$); (iii) a juvenile expressing HSV1-hChR2 in HVC subjected to optical stimulation in the primary auditory forebrain; and (iv) a juvenile expressing HSV-hChR2 in HVC without optical activation. Indeed, as adults, these control birds and birds raised with unlimited access to the same tutor had similar copying (mean similarity to tutor song: control birds, 75.8%, $n = 6$; unlimited-access birds, 77.7%, $n = 3$). A *post hoc* analysis of the similarity scores indicated that the adult songs of birds subjected to tutor-song–contingent optogenetic stimulation of HVC and the birds from the four control groups fell into non-overlapping distributions (two-sample $t(8) = 2.3$, $P = 1.7 \times 10^{-6}$, power $(1 - \beta) = 1$). These observations indicate that the pattern of neural activity in the pupil's HVC during exposure to the tutor's song is necessary for accurate copying of that song.

HVC helps encode tutor experience with temporal precision

Adult zebra finches sing a highly stereotyped 'motif' comprising a fixed sequence of several spectrally distinct syllables whose temporal features are controlled with millisecond precision[11,24]. Various studies in singing birds have suggested that HVC precisely encodes the temporal features of song[23,24,37,38], raising the possibility that the HVC network also helps to encode tutor song experience in a temporally precise fashion.

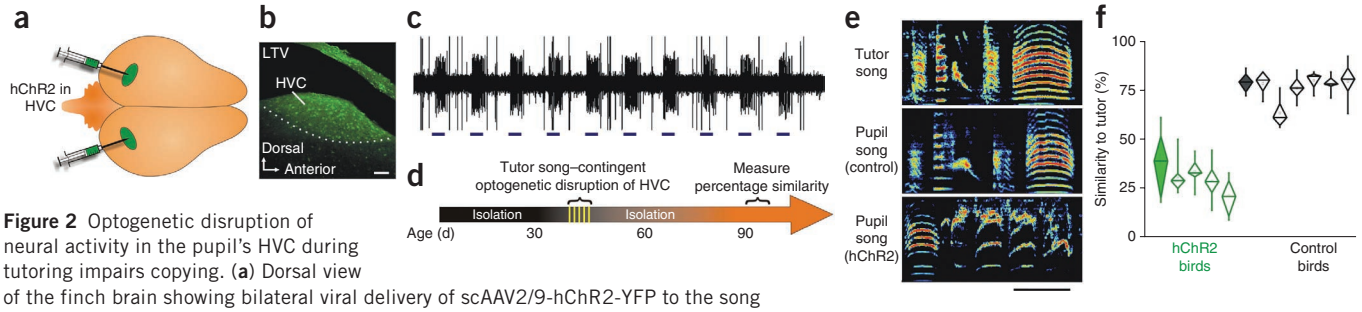

Figure 2 Optogenetic disruption of neural activity in the pupil's HVC during tutoring impairs copying. (**a**) Dorsal view of the finch brain showing bilateral viral delivery of scAAV2/9-hChR2-YFP to the song nucleus HVC. (**b**) Parasagittal section through the HVC showing neuronal expression of hChR2-YFP immunoreacted with antibodies to GFP 11 d after injection of scAAV-hChR2-YFP into the same region. Scale bar, 100 µm. LTV, lateral telencephalic ventricle. (**c**) *In vivo* extracellular recording of light-evoked action potentials (473 nm, 500 ms, 10 trials) in the HVC of a juvenile zebra finch injected with HSV-hChR2. (**d**) Sketch of the experimental timeline in which activity in the pupil's HVC is optogenetically disrupted while the tutor is singing but not at other times. (**e**) Sonograms of a tutor's song and the adult songs of two of his pupils, including a control and a pupil that received optogenetic activation of the HVC during tutoring (hChR2). Scale bar, 200 ms. Ordinate, 0–9 kHz. (**f**) Optogenetic disruption of a juvenile finch's HVC only when its tutor is singing disrupts the subsequent copying of the tutor's song ((two-sample $t_8 = 2.3$, $P = 1.7 \times 10^{-6}$ for hChR2-birds ($n = 4$) versus control birds ($n = 6$) (20–25 songs analyzed per bird); green filled diamond, average for birds raised in isolation from a tutor ($n = 3$ birds); black filled diamond, average for birds raised with free access to the same tutor used for optogenetic experiments ($n = 3$ birds); the diamonds denote the 25–75% range and diamond plot whiskers denote the 10–90% range of similarity scores for each bird, and the learning outcomes were measured in adulthood).

Testing this idea requires a method of altering HVC activity during tutoring on the time scale of individual syllables (~100 ms). Optogenetic modulation of HVC activity can lag behind light onset and persist up to several hundred milliseconds after light offset and, hence, lacks the required temporal specificity (**Supplementary Fig. 1**).

To attempt to increase the temporal specificity of our perturbation, we implanted monopolar platinum stimulating electrodes (0.1 MΩ) bilaterally in HVC of tutor-naive juveniles and used software[36] to target electrical stimulation of HVC during the utterance of a specific syllable in the tutor's song motif (**Fig. 3a**). Beginning at 43–53 dph, we subjected the juveniles to tutor song–contingent microstimulation (20 µA per HVC, biphasic pulses at 170 Hz for 200 ms) for 4 h per day for 5 consecutive days and then raised them in isolation to adulthood (>90 dph). These birds produced poor copies of the tutor song syllable that was paired with the microstimulation, even though they accurately copied syllables that came before and after the targeted syllable (**Fig. 3b**,**c** and **Supplementary Fig. 3**; $F_{4,14} = 7.508$, $P = 0.001$ by analysis of variance, $n = 4$ birds, all of which were exposed to the same tutor and received microstimulation paired with the tutor's syllable 'c'). Together with our optogenetic manipulations, these results suggest that the premotor structure that is important to the precise temporal control of birdsong also has an observational role during sensory learning by helping encode the auditory experience of the tutor song in a temporally specific manner.

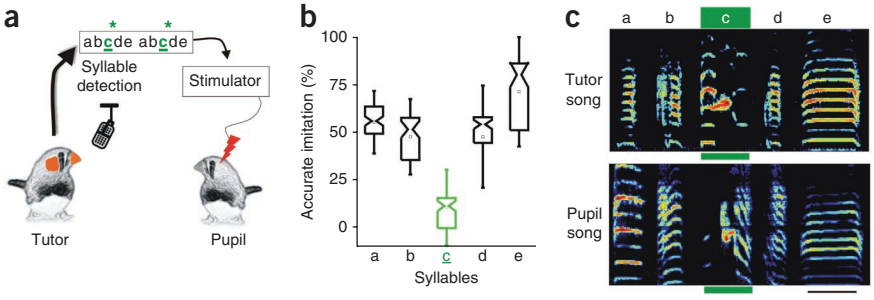

Figure 3 Microstimulation of HVC triggered by tutor song syllable disrupts copying of the targeted syllable. (**a**) Sketch of the experimental design in which the pupil's HVC is microstimulated (20 µA per HVC, biphasic pulses, 300 µs for each phase at 170 Hz for 200 ms) while the tutor is singing syllable c. (**b**) Pupils are not able to imitate the syllable that is paired with the HVC microstimulation (syllable c; $F_{4,14} = 7.508$, $P = 0.001$ by analysis of variance, 90–100 syllables analyzed per bird, $n = 4$ birds; notched box plot denotes 25–75 percentile and whiskers, 1.5 s.d.). (**c**) Sonograms of the tutor's song and the adult song of one his pupils that was microstimulated in the HVC when the tutor sang syllable c. The green bar under syllable c and the scale bar at the lower right indicate 130 ms. Ordinate, 0–9 kHz.

A candidate mechanism for encoding tutor experience

A remaining question is how the HVC network helps encode the tutor song experience. Recent *in vivo* multiphoton imaging experiments performed in juvenile zebra finches showed that tutoring can trigger the rapid enlargement of previously stable dendritic spines in HVC[26], a structural correlate of synaptic strengthening that in other systems has been shown to depend on the activation of postsynaptic NMDA receptors[39,40]. To test whether the enlargement of HVC dendritic spines after tutoring is dependent on NMDA receptors, we combined *in vivo* multiphoton imaging of HVC dendritic spines with acute pharmacological blockade of NMDA receptors on HVC neurons (**Fig. 4a**). We used a lentivirus expressing GFP to label HVC neurons and their dendritic spines, cranial windowing to provide optical access to HVC under a multiphoton microscope and retrograde tracing from the efferent targets of HVC to help visualize the borders of HVC.

To establish a baseline measurement of spine size, we imaged dendritic spines on GFP-expressing HVC neurons in tutor-naive juvenile male zebra finches during their subjective nighttime (the first imaging session occurred between 43–53 dph). The next morning, we pressure injected the NMDA receptor antagonist D-AP5 ((2*R*)-amino-5-phosphonopentanoate; 100 nl, 25 mM) into the HVC of the birds immediately before a single, brief (~1.5 h) tutoring session. We then reimaged the neurons in these recently tutored birds the following night to assess changes in the size of the HVC dendritic spines that persisted between the two nightly imaging sessions (stable spines). Spine size did not change when tutoring was preceded by an infusion of D-AP5 (**Fig. 4b**; paired, two-sample *t* test comparing relative spine brightness before and after tutoring $t\ (72) = 1.0$, $P = 0.3$, $n = 4$ birds, 73 dendritic spines). This finding indicates that the tutoring-induced enlargement of dendritic spines in the HVC depends on an NMDA receptor–dependent mechanism.

If spine enlargement in HVC helps encode tutor song experience, then blocking NMDA

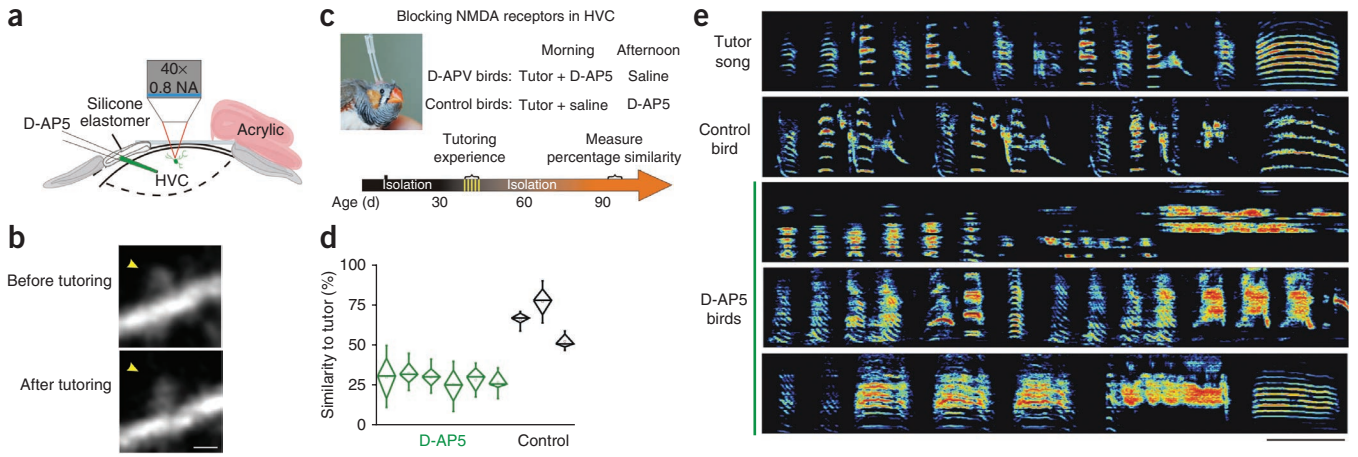

Figure 4 Blocking NMDA receptors in HVC during tutoring prevents spine enlargement and disrupts copying of the tutor song. (**a**) Schematic of the *in vivo* multiphoton imaging of dendritic spines in HVC and the pharmacological blockade of NMDA receptors achieved by injecting D-AP5 (25 mM) into the HVC immediately before tutoring. NA, numerical aperture. (**b**) Example of a stable spine (yellow arrowheads) imaged in the HVC before and after tutoring and injection of D-AP5. Scale bar, 1 µm. The spine size did not change when tutoring was preceded by infusion of D-AP5 (tutoring and D-AP5: $P = 0.30$, $n = 74$ dendritic spines from 4 birds; tutoring alone: $P = 0.001$, $n = 47$ dendritic spines from 5 birds[26]). (**c**) Schematic for reversibly blocking NMDA receptors in the HVC during tutoring. Shown are a zebra finch with reverse microdialysis probes bilaterally implanted in HVC (upper left), the treatment groups and tutoring schedule used in these experiments (upper right) and the timeline of the experiments (bottom). (**d**) Infusion of D-AP5 in the HVC during tutoring sessions (green diamond plots), but not during periods of vocal practice (black diamond plots), prevents the subsequent copying of the tutor song ($P = 0.0001$, the diamonds denote the 25–75% range and diamond plot whiskers denote the 10–90% range of similarity scores for each bird). (**e**) Sonograms of a tutor's song and the adult songs of four of his pupils in which D-AP5 was infused in the HVC during (D-AP5 birds) or immediately after (control bird) each of five morning tutoring sessions. Scale bar, 200 ms. Ordinate, 0–9 kHz.

receptors in HVC during tutoring should prevent accurate imitation of the tutor song. To test this prediction, we implanted reverse microdialysis probes bilaterally in HVC and infused D-AP5 (25 mM) during five consecutive 4-h (9 a.m. to 1 p.m.) tutoring sessions, allowing us to reversibly block NMDA receptors in HVC of juvenile zebra finches (**Fig. 4c**; $n = 6$ male juveniles, 43–53 dph, all of which were tutor-naive before the first tutoring session). After the end of the morning tutoring session, we flushed the probes with saline and isolated the bird in a sound-attenuating chamber until the next morning. After the last tutoring session (the afternoon of the fifth day of tutoring), we isolated the D-AP5–treated birds from other birds and raised them to adulthood (>90 dph), at which time we recorded their songs and compared them to their tutors' songs. Juveniles treated with D-AP5 during their tutoring sessions developed adult songs that bore little resemblance to those of their tutors based on quantitative measurements of song similarity and other comparisons of their songs' spectral and temporal features (**Fig. 4d**,**e** and **Supplementary Fig. 4**; mean similarity to the tutor songs, 29.1%, $n = 6$ birds). In contrast, juveniles that received saline in their HVC during morning tutoring sessions and D-AP5 (25 mM) in the afternoon (4 h; 1 p.m. to 5 p.m.), when they were housed in isolation, copied significantly more of their tutors' songs (**Fig. 4c–e**; mean similarity to the tutor songs, 65.3%, $n = 3$ birds, two-sample $t(7) = 7.4$, $P = 0.0001$, power $(1 - \beta) = 0.999$). Together, these findings promote an NMDA-receptor–dependent process at synapses on HVC neurons as a candidate mechanism for encoding the tutor song experience.

A pathway that conveys tutor experience to HVC

A remaining question is how tutor-song–related information is conveyed to the premotor network in HVC. Although HVC receives input from several sources[20,21,33,41], NIf has been identified as a major source of auditory input to HVC and is also the putative source of the spontaneous bursting activity that is augmented in HVC immediately after tutoring[26,42–45]. Permanent lesions of NIf in adult zebra finches do not have persistent effects on singing behavior[42] and do not interfere with auditory-feedback–dependent vocal plasticity[45]. To test whether NIf is required for sensory learning (**Fig. 5**), we made permanent bilateral lesions of NIf in tutor-naive juvenile zebra finches 1 day before their initial exposure to a tutor (**Fig. 5a**; with tutoring starting at 36–47 dph). Juveniles were housed with their tutors for 5 consecutive days and then raised in isolation to adulthood (>90 dph). Lesions to NIf severely impaired tutor song imitation, and lesion size was strongly correlated with the degree of impairment (**Fig. 5b** and **Supplementary Fig. 5**; $n = 9$ birds, $R^2 = 0.79$). To test whether the effects of NIf lesions on imitation were indeed caused by the disruption of input to HVC during tutoring rather than possible secondary effects of the NIf lesions, we reversibly inactivated NIf during tutoring in a separate set of birds. Reversibly inactivating NIf just before each of five consecutive daily tutoring sessions also severely disrupted subsequent tutor song imitation (**Fig. 5c**,**e**,**f** and **Supplementary Fig. 6**; $n = 7$ previously tutor-naive juvenile birds that received 14 nl tetrodotoxin (TTX) (50 µm) injected bilaterally into the NIf before a 1.5-h morning tutoring session, beginning at 40–45 dph, followed by an afternoon injection of 14 nl saline). In contrast, juveniles subjected to a reversed TTX and saline treatment schedule ultimately produced better copies of the tutor song (**Fig. 5c**,**e**,**f**; two-sample $t(10) = 2.9$, $P = 0.016$, $n = 5$ control birds). These findings suggest that NIf has a critical role in sensory learning by interacting with HVC during tutoring.

To better delineate the timescale of this interaction, we applied tutor song–triggered electrical microstimulation methods to NIf or an adjacent auditory region (Field L1 (ref. 46)) in juvenile zebra finches (**Fig. 5d**). We exposed previously tutor-naive juveniles ($n = 6$, 43–53 dph on the first day of tutoring) to a live tutor for 4 h per day for 5 consecutive days then raised them in isolation to adulthood (>90 dph). As adults, all of the NIf-stimulated birds produced poor copies of their tutors' songs, whereas birds stimulated in the adjacent auditory region learned the song of their tutor (**Fig. 5e**,**g** and **Supplementary Fig. 7**; mean similarity of the NIf-stimulated birds to the tutor song, 35.6%, $n = 3$; field L1–stimulated birds, 78.9%, $n = 4$; two-sample $t(5) = 2.5$,

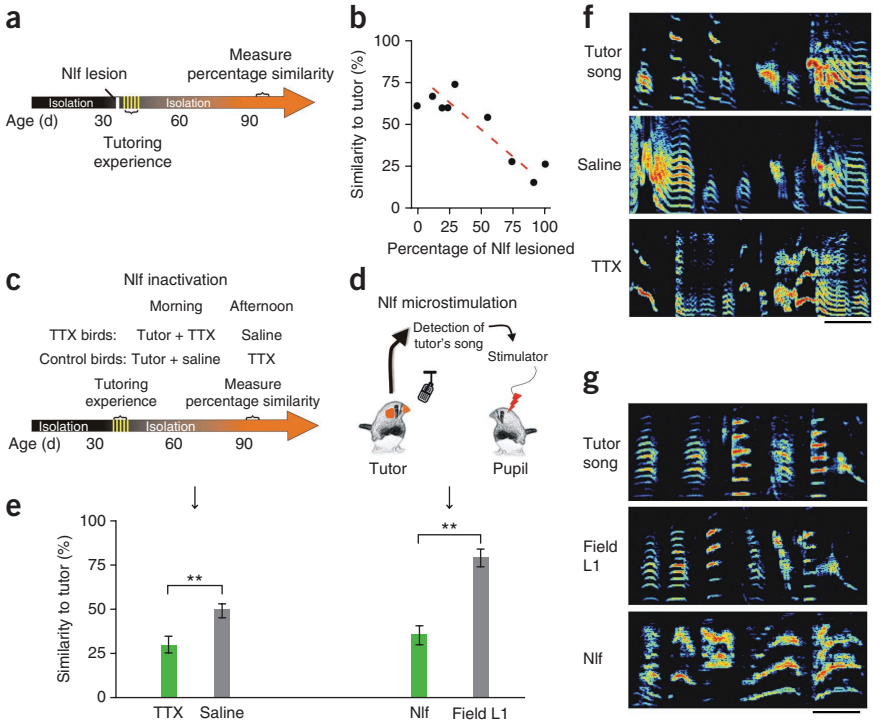

Figure 5 The tutor experience is conveyed to HVC from the nucleus NIf. (**a**) Timeline for the NIf lesion experiments. (**b**) Lesioning NIf before tutoring severely disrupts the subsequent imitation of the tutor's song ($R^2 = 0.79$, $n = 9$ birds). Birds with >50% of their NIf lesioned ($n = 4$ birds) showed severe disruption in tutor song imitation compared to birds with <30% of their NIf lesioned ($n = 5$ birds, $P = 0.001$). See **Supplementary Figure 4** for sonograms of the NIf lesioned birds. (**c**) Schematic of the NIf inactivation experiments. Shown are the treatment groups and tutoring schedule used in these experiments (top) and the timeline for the NIf inactivation experiments (bottom). (**d**) Sketch of the experimental design in which the pupil's NIf or field L1 was microstimulated (20 μA per side at 76–170 Hz for 200–400 ms) while the tutor was singing. (**e**) Reversible inactivation of NIf (left green bar; 14 nl of 50 μM TTX) during but not immediately after tutoring sessions (left gray bar) impairs subsequent copying (**$P = 0.016$; tutor and TTX, $n = 7$ birds; tutor and saline, $n = 5$ birds; error bars, s.e.m.). Tutor song–triggered microstimulation of NIf (right green bar) but not field L1 (right gray bar) disrupts the subsequent imitation of the tutor's song (**$P = 0.0067$; NIf, $n = 3$ birds; field L1, $n = 4$ birds; error bars, s.e.m.). (**f**) Sonograms of a tutor's song and the adult songs of two of his pupils in which NIf was inactivated with TTX either during (TTX) or after (saline) morning tutoring sessions. Scale bar, 100 ms. Ordinate, 0–9 kHz. (**g**) Sonograms of a tutor's song and the adult songs of two of his pupils in which tutor-triggered microstimulation was applied to either field L1 or NIf during tutoring sessions. Scale bar, 100 ms. Ordinate, 0–9 kHz.

$P = 0.001$). Together, these findings point to NIf as a critical conduit for conveying auditory information to HVC when the tutor is singing.

DISCUSSION

Here we used a combination of song-triggered optogenetic and focal electrical stimulation methods to manipulate the activity of vocal premotor neurons in juvenile zebra finches as they listened to the song of a tutor. These manipulations impaired the quality of song imitation, indicating that the pattern of neural activity in the vocal premotor circuitry during this formative auditory experience is critical to subsequent vocal motor learning. Furthermore, blocking NMDA receptors in HVC during tutoring blocked spine enlargement and also impaired vocal imitation of the tutor song, suggesting that an NMDA receptor–dependent strengthening of synapses on HVC neurons is important to encoding the tutor song experience. Along with the recent observation that tutoring rapidly stabilizes and strengthens synapses in HVC[26], our findings support the idea that synapses in HVC are sites where the experience of the tutor song is encoded in the brain and also indicate that this encoding depends on NIf, which supplies auditory input to HVC[42,43]. Although these findings do not exclude the involvement of other regions downstream of HVC in this sensory encoding process[27], they do rule out a prevailing model in which the auditory experience of the tutor song is first encoded in auditory regions upstream of the HVC and is only later used to guide changes in the vocal motor network during sensorimotor learning[15,18,47].

By using optogenetic and electrical stimulation methods to disrupt activity in HVC only during the juvenile's auditory experience of the tutor song, we delineate a role for the song motor system in sensory learning. An earlier study showed that blocking NMDA receptors in a song system nucleus downstream of HVC during tutoring also impaired the quality of song copying[27], raising the possibility that the encoding of the tutor song experience involves distributed activity in the song system, an idea that can be tested in the future with the tutor song–contingent stimulation methods developed here. Furthermore, a prior study showed that pharmacological blockade of extracellular-signal–regulated kinase activation in the secondary auditory telencephalic region caudomedial nidopallium (NCM) during tutoring disrupted song copying[15], whereas we found that tutor song–contingent electrical stimulation in field L1, an auditory region that is presynaptic to the NCM, did not disrupt song learning. Although tutor song–contingent stimulation methods will be necessary to better define the role of the NCM in song learning, these various findings may constrain the locus of tutor-song encoding to the song motor system and levels of the auditory system above field L. Moreover, a projection from HVC to secondary regions of the auditory telencephalon[20] may enable HVC to transmit information important in song learning to the auditory system, perhaps in the form of a sensorimotor registration signal. Even in these distributed models, our findings emphasize that the HVC is a critical node for encoding information about the tutor song.

We also reveal that the auditory experience of the tutor song interacts with the premotor network in a temporally precise fashion, as microstimulation targeted to a single syllable in the tutor's song disrupted copying of the targeted syllable but not adjacent syllables. Prior studies in singing birds have shown that HVC premotor neurons fire precise bursts of action potentials that are tightly linked to the temporal organization of song[23,24], raising the possibility that the same neural machinery that controls the song's temporal organization in adults also is used to encode the temporal features of the song model early in juvenile life. In this view, the auditory experience of the tutor song influences the functional organization of synaptic connections in the HVC premotor network, and this synaptic organization helps to shape the temporal structure of the pupil's song. In primates, sensory-evoked activity in premotor structures has been speculated to facilitate imitation, including speech learning[1,3,7–9,48,49]. Our results extend this

view, providing evidence that premotor circuits initially function in an observational mode to help store information about the behavioral model. Later in development, this information could help instruct these same circuits when they operate to shape and execute the motor programs underlying behavioral imitation.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

This manuscript combines the synergistic results of two independently conceived and executed research studies arriving at similar conclusions. T.F.R. and R.M. designed all experiments involving tutor song-contingent optogenetic or electrical manipulations of HVC activity, *in vivo* multiphoton imaging of HVC dendritic spines, pharmacological manipulations of NMDA receptors in HVC during tutoring and tutor song-contingent microstimulation of NIf and Field L. T.F.R. conducted all of the aforementioned experiments and analyzed resultant data with input from R.M., S.M.H.G. and B.P.Ö. designed, executed and analyzed the results of experiments involving permanent or reversible NIf lesions. M.M. adapted optogenetic methods for use in the zebra finch and characterized the effects of hChR2 activation on HVC neurons in brain slices. T.F.R. and R.M. wrote the manuscript with extensive input from B.P.Ö. and S.M.H.G. All authors read and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Tutoring and comparison of adult pupil songs to tutor songs. Juvenile male zebra finches, obtained from the Duke University or Harvard University breeding facility, were isolated from adult male song tutors at 7–12 dph and then exposed to a song tutor for 5 consecutive days starting at 40–53 dph. Before tutoring, juvenile male zebra finches were housed in nesting groups in sound-attenuation chambers and cared for by one to three adult female zebra finches. Juvenile males were removed from the nesting groups and separately housed in sound-attenuation chambers starting at 34–40 dph. After 5 days of tutoring, the juvenile zebra finches were raised to adulthood (>90 dph) in visual and acoustic isolation from other birds. Songs were recorded with microphones (Shure SM 93) preamplified and saved to a computer using Sound Analysis Pro (http://ofer.sci.ccny.cuny.edu/sound_analysis_pro) or with custom-written software (Labview, National Instruments). The adult song of each bird was then compared to the song of its tutor to measure song imitation. We quantified the amount of song that juvenile birds copied from their tutor using the percentage similarity score for whole-motif comparisons or the percentage local similarity score (percentage accuracy imitation) for syllable-level comparisons using Sound Analysis Pro ($\alpha = 0.05$). Standard parametric and nonparametric statistical methods were used to calculate significant differences ($\alpha = 0.01$), and a retrospective power analysis was used to determine the inferential power of our analyses ($1 - \beta > 0.95$; see **Supplementary Table 1** for a list of experimental manipulations and outcomes). Experimental procedures were conducted in accordance with the US National Institutes of Health guidelines and were reviewed by the Duke University Medical Center Animal Care and Use Committee (IACUC) or the Harvard University IACUC.

Viral, tracer and ibotenic acid injections. Male zebra finches were anesthetized using isoflurane inhalation (2%) and placed in a stereotaxic apparatus. Target sites in the brain were located using stereotaxic coordinates and multiunit neural recordings. A glass pipette attached to a pressure injection unit (Drummond Nanoject II, Drummond Scientific, Broomall, Pennsylvania, United States) was used to deliver the virus or a neural tracer to target brain regions. For behavioral optogenetic experiments in HVC, we used a self-complementary AAV expressing hChR2 under the control of cytomegalovirus (CMV) promoter (scAAV2/9-hChR2-YFP, UNC Vector Core, custom prepared) or HSV1 expressing hChR2 (HSV1-hChR2 BioVex). scAAV2/9-hChR2-YFP (600–700 nl) and HSV1-hChR2 (400 nl) injections were made into the HVC 5–6 d before *in vivo* electrophysiological recordings and fiber optic cable implantation. For *in vitro* optogenetic experiments in HVC, we used AAV2/9-hChR2-Venus or -mCherry (Penn Vector Core) injected 40–60 d before cutting brain slices to drive expression of hChR2. For *in vivo* multiphoton imaging of dendritic spines in HVC, we used a lentivirus expressing eGFP under the control of the Rous sarcoma virus long terminal repeat[50]. Lentiviral injections (1 µl) were made 15–20 d before imaging, and retrograde tracer injections were made into the two targets of the HVC 5–7 d before imaging (Fast Blue (Polysciences Inc.) to area X and Alexa Fluor 594–conjugated dextran amines (Invitrogen) to the robust nucleus of the arcopallium). For neurotoxic lesions of NIf, the location of NIf was verified electrophysiologically by recording antidromic responses to stimulation in the nucleus HVC (bipolar stimulation electrodes, 200-µs pulses of ~500 µA at 1 Hz). Twenty-three nanoliters of 1% ibotenic acid (Asc-041, Ascent Scientific, Princeton, New Jersey, United States) dissolved in 0.1 M NaOH were then injected bilaterally into the NIf using a Nanoject II injector.

Tutor song–triggered optogenetics. One to two days before their tutoring experience, juvenile male zebra finches (41–51 dph) previously injected in HVC with a virus expressing hChR2 were anesthetized with isoflurane (2%), and multiunit neural recordings were used to assess light-evoked optogenetic responses (473-nm light; Ikecool, IKE-473-200-OP) in HVC. In a subset of these birds, multiunit recordings from HVC auditory afferents, NIf and the caudal mesopallium were also combined with optical stimulation of HVC to examine whether optogenetic stimulation of HVC was capable of antidromically exciting NIf and caudal mesopallium. Only birds with light-evoked responses in at least three different recordings sites in each HVC were implanted with fiber optic cables (200-µm diameter core, 0.37 NA; Thor Labs (BFL37)) and used as pupils in subsequent tutor song–triggered behavioral experiments. Fiber optic cable guide cannulae (PlasticsOne, C315GS-4-SP guide 26GA cut 2 mm below the pedestal) were implanted immediately dorsal to HVC. Fiber optic cables were connected to a diode-pumped solid-state 473-nm laser (Ikecool, IKE-473-200-OP) through a 1 × 2 fiber optic commutator (Doric Lenses, FRJ_1x2i_FC-2FC). Custom software[36] was used to detect components of the tutor's song and trigger optical stimulation (200–500 ms, 5–8 mW/mm^2 per hemisphere) of HVC. Pupils were tutored for 2 h per day for 5 consecutive days and then raised in isolation to adulthood.

A separate group of birds was used as optogenetic controls and subjected to one of the four following conditions: (i) juvenile birds were tutored 2 h per day for 5 consecutive days. Immediately after each tutoring session and out of earshot of the juvenile, an audio recording of the tutor's singing behavior from that day's tutoring session was played back to voice recognition software to trigger optogenetic stimulation of the pupil's HVC. This approach ensured that the juveniles received a pattern and amount of optogenetic stimulation in HVC that was highly similar to the tutor song–contingent stimulation group except that the stimulation was not coincident with the tutor song experience. (ii) Juveniles were subjected to tutor song–contingent optical stimulation of HVC after injection into the HVC with AAV virus expressing eGFP. (iii) A juvenile was subjected to tutor-song–contingent optical stimulation in the primary auditory forebrain after injection in HVC with HSV expressing hChR2. (iv) A juvenile was tutored after injection in HVC with HSV expressing hChR2 without optical activation.

In a blind *post hoc* analysis of learning outcomes, data from all experimental and control birds were pooled and found to be bimodally distributed, constituting two nonoverlapping groups. One group ($n = 4$) showed very low similarity to the tutor, and the other group ($n = 6$) showed very high similarity to the tutor. All of the birds that received tutor song–contingent optogenetic stimulation of HVC fell into the population with very low similarity to the tutor song, whereas all of the birds that received any of our four control manipulations fell into the second population with very high similarity to the tutor song. A two-sample *t* test was used to examine statistical differences between these two groups.

Tutor song–triggered microstimulation. One to two days before tutoring, isolate juvenile male zebra finches (41–51 dph) were anesthetized with isoflurane (2%) and placed in a stereotaxic holder, and multiunit neural recordings were used to identify target structures in the brain (HVC, NIf or field L1; HVC was identified by its characteristic bursting activity, NIf was identified by antidromic stimulation from the HVC, and field L1 implants were placed anterior to the NIf). Platinum monopolar electrodes (0.1 MΩ; MPI) were bilaterally implanted in HVC, NIf or field L1 and secured in place with dental acrylic. A small grounding screw was then implanted over the cerebellum. Stimulating electrodes and the grounding screw were then wired to a custom-built adaptor and secured with additional dental acrylic. Custom software[36] was used to detect specific acoustic features associated with a given syllable in the tutor's song and trigger electrical bilateral stimulation of the HVC, NIf or field L1 (200 400 ms, 20 µA per hemisphere, 73–170 Hz biphasic pulses; A-M Systems isolated pulse stimulator model 2100). Pupils were tutored for 4 h per day for 5 consecutive days starting at 43–53 dph and then raised in isolation to adulthood.

***In vivo* multiphoton imaging.** Measurement of changes to dendritic spines on HVC neurons using *in vivo* multiphoton imaging was conducted as previously described[26] with additional modifications described below. Cranial windows were bilaterally implanted over the HVC of isolate juvenile male zebra finches (43–53 dph) that were previously injected with a lentivirus in the HVC expressing GFP and retrograde tracers in the targets of the HVC, robust nucleus of the arcopallium and area X. A 200-µm gap between the custom cut glass coverslip covering the HVC and the skull at the caudal border of the HVC was covered with Kwik-Sil (MPI) to allow targeted infusion of D-AP5 under the coverslip with a glass pipette. The juvenile zebra finches used for these experiments were maintained in a reversed day-night cycle, and images of the HVC neurons and their dendritic spines were obtained during the bird's subjective nighttime with a multiphoton microscope (Zeiss LSM 510). Immediately before the beginning of the bird's subjective daytime, the bird was briefly anesthetized with isoflurane (2%), and a glass pipette filled with D-AP5 (25 mM) attached to a pressure injection unit (Drummond, Nanoject II) was advanced at 45° to the pial surface to the center of the HVC. One-hundred nanoliters of D-AP5 was injected in the center of the HVC bilaterally. Immediately after recovery from anesthesia (~5 min) a tutor was placed with the isolate bird for 1.5 h.

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The following evening, the same neurons, stretches of dendrite and dendritic spines were reimaged under the multiphoton microscope.

Reverse microdialysis. Custom designed reverse microdialysis probes (K. Hamaguchi, Duke University Medical Center) with a 200-μm diameter dialysis membrane of which 200 μm was exposed (Spectra/Por; 13 kD molecular weight cutoff) were bilaterally implanted into HVC and secured to the skull with dental acrylic 1–2 days before tutoring. Pupils received morning tutoring sessions 4 h per day for 5 consecutive days starting at 43–53 dph. D-AP5 (25 mM) was dialyzed into HVC during morning tutoring sessions, and saline was dialyzed into HVC in the afternoon when the birds were not with their tutor. Control birds received the opposite treatment: saline was dialyzed into the HVC during morning tutoring sessions, and D-AP5 was dialyzed into the HVC in the afternoon when the birds were not with their tutor. After 5 days of tutor exposure, birds were raised to adulthood in isolation from other finches in sound-attenuating chambers. Post mortem histological analyses were used to confirm placement of the probe.

Transient inactivation. Three to four days before the tutoring experiments (age range 38–42 dph), birds were anesthetized, and small holes were made in the skull above the NIf bilaterally. A head holder was implanted on the anterior part of the skull as described previously[51]. The craniotomies were covered with Kwik-Kast (World Precision Instruments, Sarasota, Florida, United States).

In the morning and afternoon of the experimental days, birds were placed in a foam restraint, and the head holder was attached to the stereotaxic apparatus for approximately 10 min. Kwik-Kast was removed from the craniotomies, and TTX (14 nl, 50 μM; T5651, Sigma, St. Louis, Missouri, United States) or PBS was injected bilaterally into the NIf using a Nanoject II. Based on previous studies[51], we estimated the inactivation radius resulting from the TTX injections to be <200 μm. Visual inspection of the fluid level in the injection pipette confirmed successful drug injection. Dye-conjugated dextrans (D-22912 or D-22910, Molecular Probes, Eugene, Oregon, United States) were co-injected with TTX for *post hoc* verification of the injection site.

***In vitro* intracellular recordings from HVC neurons.** Forty to sixty days after injection of AAV expressing hChR2, birds were anesthetized with isoflurane (5%) and decapitated. The brain was quickly removed and moved into a solution of ice cold artificial cerebrospinal fluid. 400-μm sagittal brain slices including the HVC were cut using a vibratome (Leica, VT 1000s). Borosilicate glass electrodes (80–200 MΩ) filled with 2 M potassium acetate and 5% Neurobiotin were used to obtain sharp intracellular recordings. Membrane potential recordings were amplified with an Axoclamp 2B amplifier (Axon Instruments) in bridge mode, low-pass filtered at 1–3 kHz and digitized at 10 kHz. Data were collected using a data acquisition board (National Instruments) controlled by custom Labview software. The different HVC cell types (Area X projecting neurons (HVC_X), neurons projecting to the robust nucleus of the arcopallium (HVC_{RA}) and interneurons (HVC_{INT})) were identified by their response to families of current pulses[52] (−600 to +1,000 pA, 500 ms duration). Short collimated light pulses (3–100 ms duration) at 473 nm (3–5 mW/mm2) were delivered to the HVC by a 200-μm diameter fiber optic cable coupled to a diode-pumped solid-state laser (model BL473T3-150, Shanghai Laser and Optics). Electrophysiological data were analyzed offline using custom-written MATLAB software (K. Hamaguchi and M.M.).

Histology. Birds were anesthetized with 0.08 ml natriumpentobarbital (Nembutal, intramuscular injection) and subsequently perfused with PBS, followed by fixation with 4% paraformaldehyde in PBS (PFA). Brains were dissected out and post-fixed in 4% paraformaldehyde at 4 °C overnight. Parasagittal sections (50 μm) were cut on a Vibratome (Leica). Tissue sections were mounted and stained with cresyl violet to reconstruct the location of implanted dialysis probes, stimulating electrodes or fiber optic cables. Injection sites for the TTX inactivation experiments were verified in alternate brain slices by fluorescence microscopy (**Supplementary Fig. 5**). The remaining slices were stained with cresyl violet, and the location of the NIf was confirmed based on nucleus shape and size and its orientation between the anatomical landmarks lamina mesopallialis and lamina pallio-subpallialis[53] (**Supplementary Fig. 5a**). Photomicrographs of fluorescent injection sites were superimposed on their alternate cresyl violet sections using Adobe Photoshop to determine the location of the injection (**Supplementary Fig. 5b–e**). We measured the distance between the center of the injection and the center of the NIf with ImageJ (NIH) software (left hemisphere: 194 ± 38 μm (s.e.m.); right hemisphere: 189 ± 36 μm; **Supplementary Fig. 5e**). Outlier analyses using *z*-scores confirmed that the centers of the injections relative to the center of the NIf were not significantly different from the group mean in any of the birds for both hemispheres, and, thus, all birds were included for further statistical analyses. For lesions, location and size were determined by outlining the area of visually damaged tissue (based on loss of neurons and gliosis[53]) on photomicrographs of cresyl violet–stained sections with Spot Basic image capture software. Lesion size was expressed as percentage of intact NIf size[53]. All analyses were performed blind to experimental treatment.

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