SONG LEARNING

Inception of memories that guide vocal learning in the songbird

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Animals learn many complex behaviors by emulating the behavior of more experienced individuals. This essential, yet still poorly understood, form of learning relies on the ability to encode lasting memories of observed behaviors. We identified a vocal-motor pathway in the zebra finch where memories that guide learning of song-element durations can be implanted. Activation of synapses in this pathway seeds memories that guide learning of song-element duration and can override learning from social interactions with other individuals. Genetic lesions of this circuit after memory formation, however, do not disrupt subsequent song imitation, which suggests that these memories are stored at downstream synapses. Thus, activity at these sensorimotor synapses can bypass learning from auditory and social experience and embed memories that guide learning of song timing.

We learn to emulate many social and communicative behaviors with seemingly minimal effort. A wide range of behaviors, including those related to speech and language, are initially learned by observing the behavior of teachers or other more experienced social models. Long-term memories of observed behaviors can guide procedural learning by providing internal benchmarks for evaluating the quality of future performances (*1*–*4*). Unlike episodic memories, memories used to guide imitation—referred to here as behavioral-goal memories—are not thought to require the hippocampus, and instead are thought to be directly encoded in cortical circuits (*2*, *5*–*15*). However, the synaptic pathways encoding these memories have yet to be identified. We sought to define elemental circuits capable of encoding behavioral-goal memories in the young male zebra finch, a songbird that learns its adult song by observing, memorizing, and then slowly learning to copy the singing behavior of its song "tutor" during a developmental sensitive period (Fig. 1, A and B).

Adult zebra finch song is well defined by its temporal and spectral features, both of which are learned from song tutors and can be adaptively modified (*16*–*21*). Yet the manner in which auditory signals are engaged to form memories of specific temporal or spectral features of tutor songs is still poorly understood (*4*). A premotor pallial region necessary for song production, HVC, has been implicated in learning from auditory experience with a tutor (*7*, *8*, *11*, *12*, *22*) (Fig. 1C). The pallial sensorimotor nucleus interfacialis of the nidopallium (NIf) is the single largest source of auditory input to HVC and has also been implicated in song learning (*7*, *23*–*26*). Disruption of activity in NIf or HVC during tutoring experiences disrupts encoding of tutor song behavioral-goal memories (*7*).

Neurons in NIf mark the beginning and ending of song elements with sharp increases and decreases in their activity during singing (*25*), suggesting a potentially simple neural mechanism for marking the duration of vocal elements in song. Moreover, HVC has been broadly implicated in the motor control of song temporal structure (*18*). However, it is not known how behavioral-goal memories for timing or duration of a tutor's song elements are encoded in the brain of a young pupil or if NIf and HVC are specifically involved. We used optogenetics to manipulate activity at NIf-HVC synapses in juvenile birds to test whether this manipulation could implant memories capable of bypassing auditory and social experience from a vocal model and guide learning of song temporal structure.

Opto-tutoring in young birds shapes the temporal structure of their adult song

We established methods to selectively manipulate NIf's inputs to HVC in juvenile male zebra finches using an axon-targeted channelrhodopsin-2

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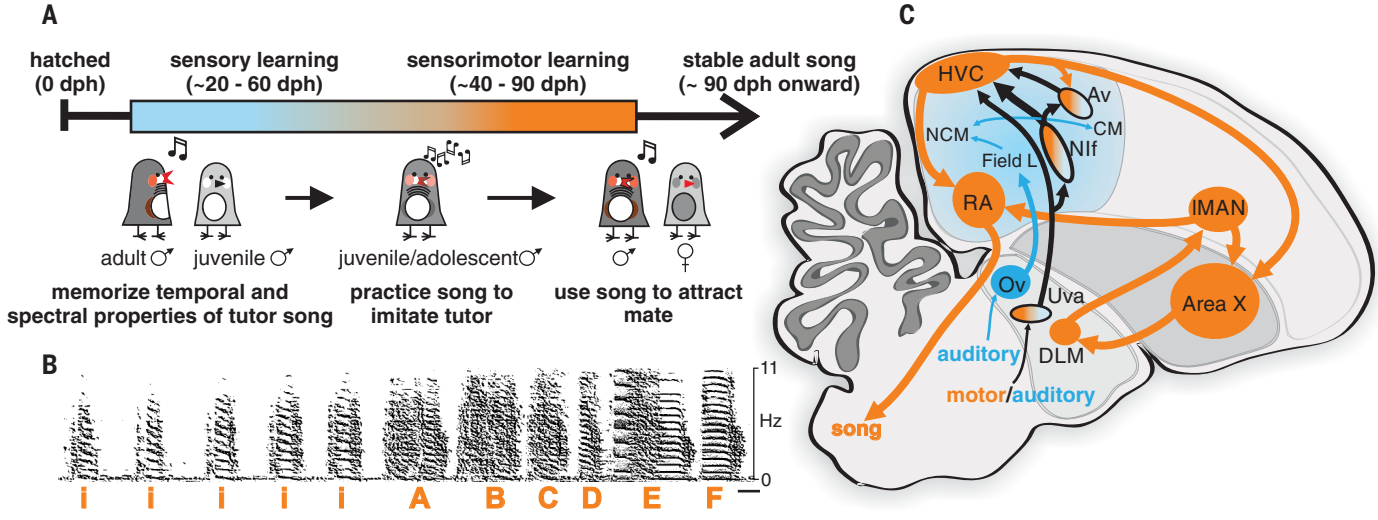

Fig. 1. Overview of song learning and neural circuits for song. (**A**) Timeline for song learning in juvenile male zebra finches. (**B**) Spectrogram of an adult zebra finch song; introductory notes (i) and individual elements in the song (A to F) are noted. Scale bar, 50 ms. (**C**) Parasagittal schematic of auditory (blue) and song motor circuits (orange). Area X, striato-pallidal basal ganglia nucleus; Av, nucleus avalanche; CM, caudal mesopallium; DLM, dorsolateral thalamic nucleus; field L, primary auditory forebrain; HVC, premotor song nucleus; IMAN, lateral magnocellular nucleus of the anterior nidopallium; NCM, caudomedial nidopallium; NIf, nucleus interfacialis of the nidopallium; Ov, thalamic nucleus ovoidalis; Uva, nucleus uvaeformis; RA, robust nucleus of the arcopallium.

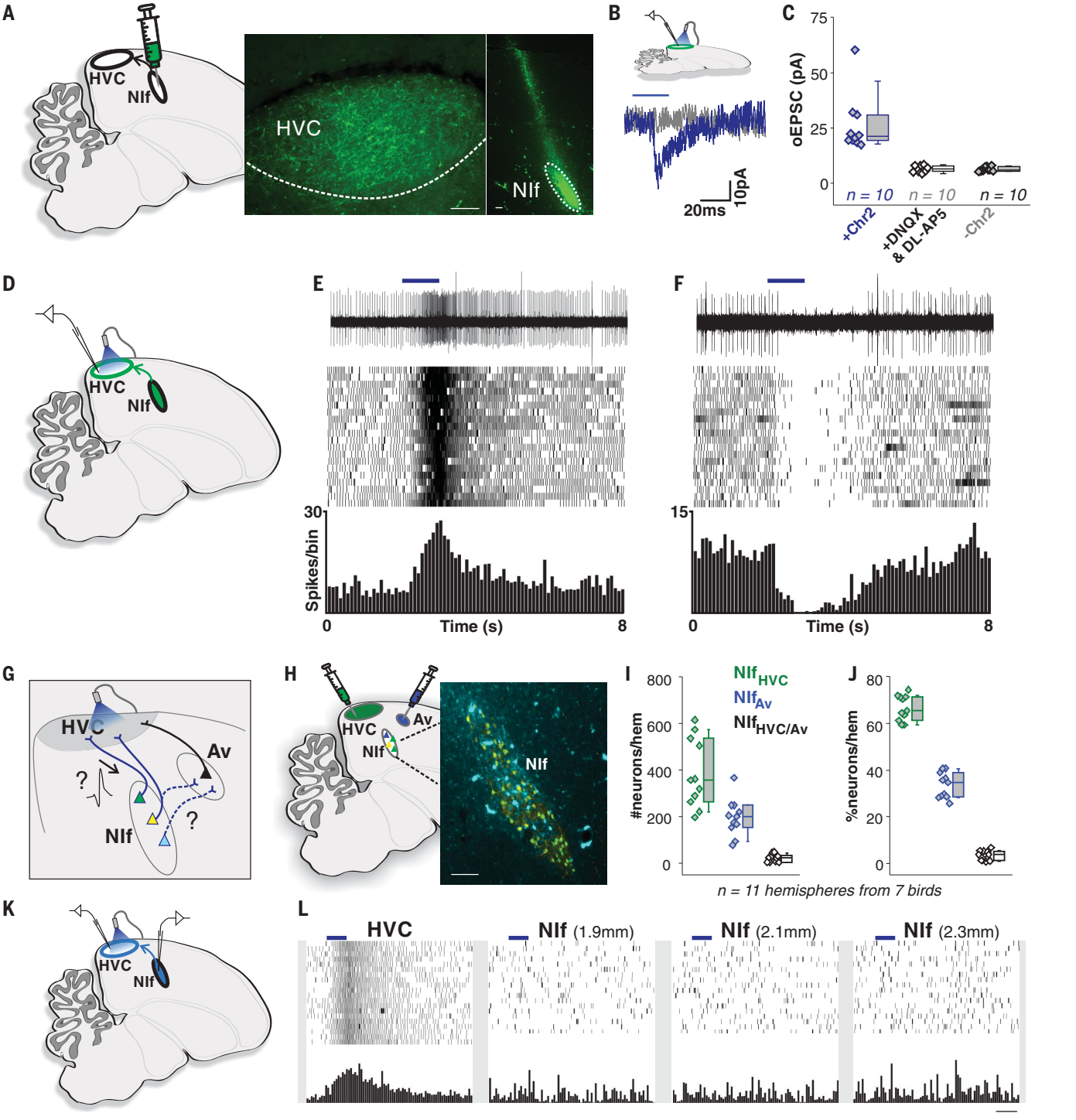

Fig. 2. Selective manipulation of the NIf-HVC pathway. (**A**) Left: Schematic of viral injections of scAAV2/9-NX-hChR2-YFP into NIf. Right: Parasagittal sections through HVC and NIf showing axon terminals labeled by tracer injections into NIf. Scale bars, 75 μm. (**B**) Light-evoked optogenetic excitatory postsynaptic currents (oEPSCs) recorded at −80 mV [20 ms, 1 Hz pulse (blue trace)] from an HVC neuron, compared to the same neuron when applying glutamate blockers (gray trace). (**C**) oEPSCs recorded from HVC neurons in response to light stimulation of NIf axon terminals (blue, gray fill) are blocked by DNQX (20 μM) and DL-AP5 (100 μM) (black, no fill) and are nonexistent in birds not injected with ChR2 in NIf (black, gray fill). (**D**) Schematic of in vivo multi-unit recordings in HVC. (**E** and **F**) Multi-unit recordings in HVC [top, representative single trials; middle, raster plots of 20 trials; bottom, histograms of 20 trials (100-ms bins)] showing excitatory (E) and inhibitory (F) light-evoked response. (**G**) Schematic illustrating questions regarding the NIf-Av-HVC circuit. (**H**) Left: Schematic of retrograde tracer injections. Right: Parasagittal section through NIf showing retrograde labeling. Scale bar, 100 μm. (**I** and **J**) Quantification of NIf neurons labeled by tracer injections [(I), actual numbers of neurons labeled; (J), percentages of neurons labeled]. (**K**) Schematic of in vivo recording in HVC and NIf. (**L**) Raster plots of multi-unit light-evoked response from HVC and from three sites in NIf in the same hemisphere while HVC is light-stimulated (100-ms bins).

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(ChR2) construct (*27*) delivered with a self-complementary adeno-associated virus (scAAV). Activation of NIf axon terminals elicited monosynaptic excitatory input to HVC neurons mediated by AMPA/NMDA receptors (Fig. 2, A to C). Optical excitation of NIf terminals in vivo produced reliable yet complex polysynaptic responses in HVC, with most neurons exhibiting strong increases in activity (257/341 recording sites) and a small percentage exhibiting strong suppression (Fig. 2, D to F, 18/341 recording sites).

Because NIf may also relay auditory information to HVC via a second sensorimotor region (*17*, *28*), Avalanche (Av) (Fig. 2G), we tested the selectivity of our optogenetic manipulations of NIf axon terminals. We first mapped efferent projections from NIf using anatomical and physiological methods (Fig. 2, H to J, and figs. S1 and S2). Approximately 70% of NIf projection neurons exclusively innervate HVC, ~30% exclusively innervate Av, and only <5% project to both HVC and Av. We next made in vivo extracellular recordings in NIf while optogenetically exciting its terminals in HVC to examine

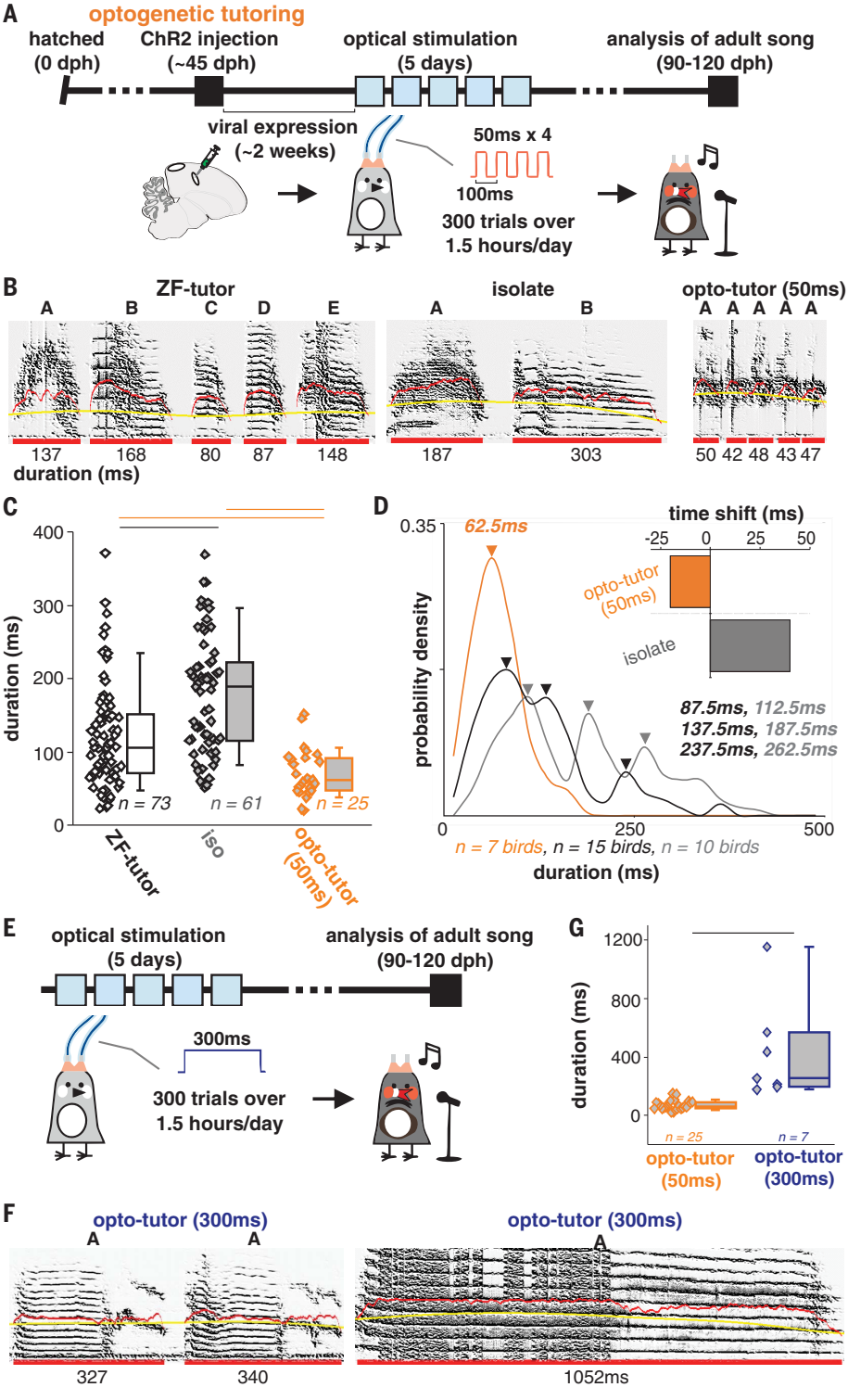

Fig. 3. Optogenetic tutoring affects learning of song temporal structure. (**A**) Timeline for the optogenetic tutoring experiment using 50-ms light pulses. (**B**) Representative spectrograms from a bird tutored by a zebra finch tutor (ZF-tutor, left), a bird reared without a song tutor (isolate, middle), and a bird opto-tutored by 50-ms light pulses (right). Song elements were quantified by thresholding (yellow curves) the amplitude (red curves) of sounds using SAP2011 (see supplementary materials). Segmented song elements and their durations are labeled by solid red lines and numbers at bottom. (**C**) Song element duration of birds tutored by a zebra finch tutor (ZF-tutor, n = 73 elements, 15 birds), isolate birds (iso, n = 61 elements, 10 birds), and birds 50-ms opto-tutored birds (opto-tutor, n = 25 elements, 7 birds). Mann-Whitney U tests: ZF-tutor birds versus isolate birds (by song element duration, $P = 2.83 \times 10^{-5}$; by bird, $P = 0.0025$); ZF-tutor birds versus 50-ms opto-tutored birds (by song element duration, $P = 3.86 \times 10^{-4}$; by bird, $P = 0.0031$); isolate birds versus 50-ms opto-tutored birds (by song element duration, $P = 4.67 \times 10^{-9}$; by bird, $P = 2.06 \times 10^{-4}$). (**D**) Song element duration distribution of birds shown in (C). Arrowheads and numbers show the peak positions for each curve (black, ZF-tutor; gray, isolate) and the corresponding duration. Inset: Time shift to achieve maximum cross-correlation with normally reared birds is shorter for 50 ms–stimulated birds (orange bar) and longer for isolate birds (gray bar). (**E**) Timeline for the optogenetic tutoring experiment using 300-ms light pulses. (**F**) Spectrograms of representative songs of two birds opto-tutored with 300-ms light pulses. (**G**) Song element durations of birds opto-tutored with 300-ms light pulses (n = 7 song elements from 4 birds) are significantly longer than those of birds tutored with 50-ms light pulses (median duration = 323 ms). Mann-Whitney U tests: 300-ms opto-tutored birds versus 50-ms opto-tutored birds (by song element duration, $P = 2.54 \times 10^{-6}$; by bird, $P = 0.0061$).

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whether terminal stimulation antidromically excites NIf neurons (Fig. 2, K and L, $n = 3$ hemispheres from two birds). Although optogenetic activation of axon terminals reliably evoked postsynaptic responses in HVC, they failed to drive antidromic responses in NIf (nine recording sites from three hemispheres). To examine whether optogenetic excitation of HVC might also directly excite NIf terminals innervating Av, which is located ~1.5 mm from the end of optic fibers over HVC, we measured the depth from the surface of the brain at which we could elicit excitatory responses. Compiling data from all of our in vivo recordings (257 recordings exhibiting excitatory responses), we found that we could only optogenetically excite cells within the first 500 μm from the surface of the brain, hence our optogenetic manipulations were unlikely to directly excite NIf axon terminals innervating Av. Together, these findings indicate that we can selectively manipulate activity at NIf-HVC synapses in juvenile birds.

To begin testing whether manipulation of the activity of NIf axon terminals in HVC can implant behavioral-goal memories, we raised young males without any social or auditory experience of adult song tutors, then optically tutored them using light pulses designed to mimic short song elements near the end of their song sensory learning phase. Light stimulation was delivered at time intervals derived from natural song tutoring patterns while experimental birds were alone in acoustic chambers (Fig. 3A and fig. S3). Adult zebra finch song typically contains ~3 to 6 unique song elements lasting ~100 ms each, separated by brief periods of silence (73 unique song elements measured from 15 birds, average 4.9 elements per bird, median duration = 106 ms). When birds are raised without any social or auditory exposure to a song tutor, their adult song, referred to as isolate song, contains ~4 to 7 song elements that are significantly longer than normally reared birds with free access to a tutor (*19*, *29*) (Fig. 3, B and C, 61 unique song elements measured from 10 isolate birds, average 6.1 elements per bird, median duration = 171 ms). We should note that isolate birds in this context are best described as untutored birds and differ from "true" isolate birds that are hand-raised by people and never exposed to other conspecifics. Birds opto-tutored as juveniles with repeated 50-ms light pulses produced adult vocalizations with significantly shorter song elements than normally reared or isolate birds (Fig. 3, B and C, and fig. S4). We found that the majority of 50-ms opto-tutored birds produced simple songs with only one to three unique elements that were repeated or trilled at a high rate (audio file S1). Song element durations in opto-tutored birds clustered near 50 ms (median = 62.3 ms), whereas both normally reared and isolate birds produced song elements spanning a significantly broader distribution of durations (Fig. 3D).

To further test whether opto-tutoring implants memories that guide learning of song element duration, we next tutored juvenile birds with long-duration light pulses instead of short-duration pulses (Fig. 3E). Birds opto-tutored with 300-ms excitation of NIf-HVC synapses produced adult vocalizations with one to three song elements. The duration of these song elements was significantly longer than for 50-ms opto-tutored birds (Fig. 3, F and G, fig. S5, and audio file S2).

To examine whether 50-ms or 300-ms opto-tutored birds learned vocal parameters other than song element duration from manipulation of NIf-HVC axon terminals, we measured acoustic features typically imitated during song learning, including pitch, mean frequency, goodness of pitch, and entropy (*20*, *30*, *31*). We found that

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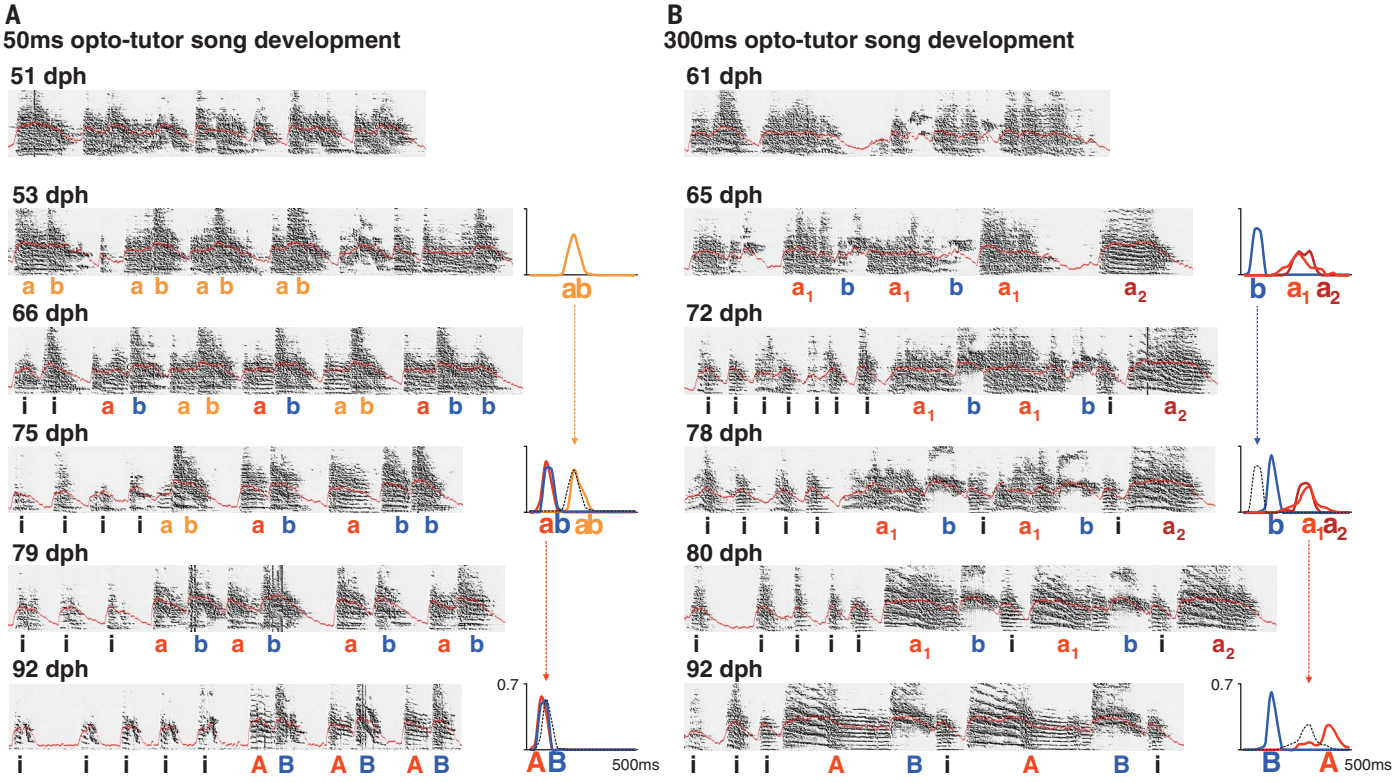

Fig. 4. Optogenetic tutoring implants a memory that guides song learning. (**A** and **B**) Spectrograms of representative vocalizations produced by a 50-ms opto-tutored bird [(A), opto-tutored on 49 to 51 dph and 53 to 54 dph] and a 300-ms opto-tutored bird [(B), opto-tutored on 56 to 57 dph and 61 to 63 dph] on different days during song development. Capital letters denote song element types present in the adult songs; lowercase letters denote precursors of each song element. Red curves on spectrograms show the original sound amplitude without segmentation. Plots at the right of the spectrograms show the probability density of song element durations; dotted black lines allow for comparison of song element durations from the previous stage in development.

opto-tutored songs did not systematically differ from songs of normally reared or isolate zebra finches (fig. S6), suggesting an acoustic phenotype that falls between these two groups.

We next explored whether opto-tutored birds used their vocalizations appropriately during social interactions. Zebra finches use their song to court female birds in a behavior commonly referred to as directed singing (*32*), and they spend extended periods of time practicing their song when alone. Likewise, opto-tutored birds practiced their song when alone and produced a range of other call types typically produced by zebra finches (*33*). When presented with female birds, opto-tutored birds readily performed directed singing behavior using the short or long vocal elements shaped by opto-tutoring (movies S1 and S2). Together, these findings indicate that opto-tutoring in juvenile birds selectively shapes the temporal structure of their adult courtship song.

Opto-tutoring implants a memory that guides song learning

Opto-tutoring could shape adult song by implanting a behavioral-goal memory that guides developmental learning of song element duration. Alternatively, opto-tutoring might directly imprint or entrain patterns of activity on the HVC network, thereby constraining the production of vocalizations to those with a specific temporal structure. To help discern these possibilities, we examined the developmental trajectory of song elements of our opto-tutored birds (Fig. 4 and fig. S7). We found that opto-tutored birds exhibited complex learning trajectories similar to those observed in normally tutored birds (*30*, *34*). Opto-tutored birds showed initial changes in vocal elements within 2 to 3 days of opto-tutoring, similar to birds that are first song-tutored near the end of their sensitive period for sensory learning (*12*, *35*). Similar to normal song learning, many changes in song elements also slowly accrued over the month of sensorimotor learning that followed the opto-tutoring experience. Birds began to modulate the amplitude of the initially noisy, long, unstructured subsongs in response to the duration of light pulses they received. Birds opto-tutored with 50-ms light pulses increased the amplitude modulation of their long vocal elements, eventually learning to produce trilled, short-duration song elements and in some

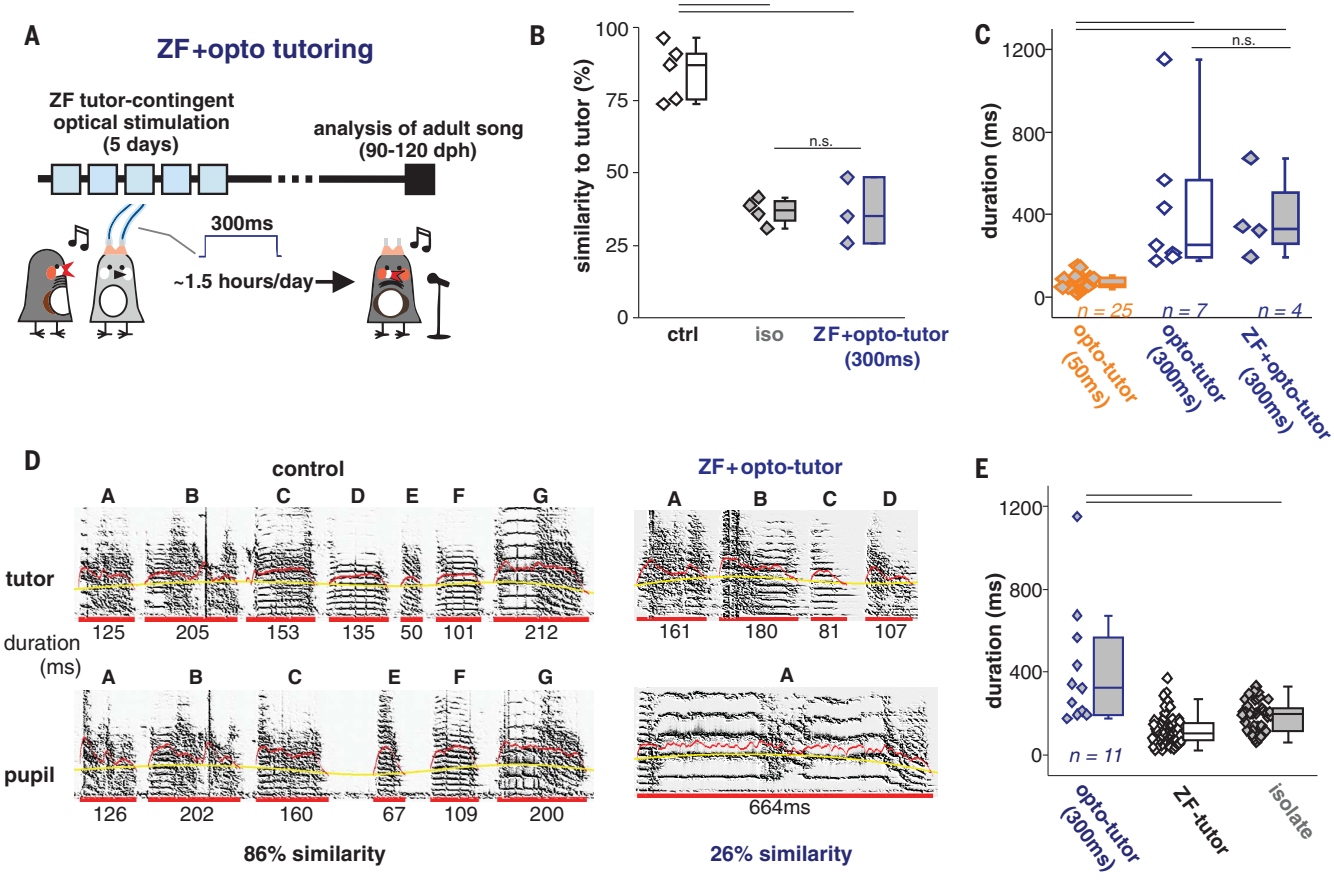

Fig. 5. Optogenetic tutoring overrides natural tutoring. (**A**) Timeline for the experiments in which birds received both zebra finch and optogenetic tutoring (ZF + opto tutoring). (**B**) Birds tutored by zebra finch tutors (black, no fill) show high percent similarity to the tutor song. ZF + opto-tutored birds (blue, gray fill) do not imitate the song of their tutor and are on par with isolate birds (black, gray fill). Mann-Whitney U tests: control birds versus ZF + opto-tutored birds, $P = 0.03$; isolate birds versus ZF + opto-tutored birds, $P = 0.8$. n.s., not significant. (**C**) Song element duration shows no difference for ZF+opto-tutored birds (blue, no fill) and birds only receiving opto-tutoring (blue, gray fill). Mann-Whitney U tests: 300-ms opto-tutored birds ($n = 7$ song elements from 4 birds) and ZF + opto-tutored birds ($n = 4$ song elements from three birds), $P = 0.9$. (**D**) Spectrograms of tutor-pupil songs of control birds (good copy, 86% similarity) and a ZF + opto tutoring pair using 300-ms pulses (poor copy, 26% similarity). (**E**) Song element durations for birds opto-tutored with 300-ms pulses (blue, gray fill) and comparisons with song element durations from normally reared (black, no fill) and isolate birds (black, gray fill). Mann-Whitney U tests: 300-ms opto-tutored birds versus isolate birds (by song element duration, $P = 0.0010$; by bird, $P = 2.0568 \times 10^{-4}$); 300-ms opto-tutored birds versus ZF-tutored birds (by song element duration, $P = 3.2549 \times 10^{-6}$; by bird, $P = 2.4684 \times 10^{-4}$).

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instances learning to produce gaps between these vocal elements (Fig. 4A and fig. S7). Birds opto-tutored with 300-ms light pulses, on the other hand, slowly learned to decrease the amplitude modulation across vocal elements, leading to the gradual emergence of longer and more harmonic vocal elements (Fig. 4B and fig. S7). Opto-tutored birds also crystallized their songs starting at 85 to 90 dph (days post-hatching). Their songs before 80 dph exhibited variable vocal durations and acoustic features, whereas songs after 90 dph were increasingly stereotyped, like those resulting from song crystallization in normally reared birds.

These results suggest that opto-tutoring implants memories that guide learning, rather than directly entraining a specific motor program in young animals. However, it is also possible that opto-tutoring simply biases or selects among precursor or innate vocalizations to specify the production of vocal elements with certain durations in adulthood. For example, optical stimulation of NIf axon terminals in HVC could bias the 50-ms birds to only sing the short introductory notes that typically precede the bird's normal song motif and bias the 300-ms birds to only sing long isolate-like vocal elements or calls. Such a scenario could point to circuit mechanisms for how innate vocal repertoires are selected or reinforced by activity during development. However, we found that both 50-ms and 300-ms opto-tutored birds produced songs with distinct introductory notes and song motifs (Fig. 4 and fig. S7); moreover, the vocal elements of 300-ms opto-tutored birds were significantly longer than those of isolate birds (Fig. 5E). Together, these findings suggest that optogenetic excitation of NIf-HVC synapses implants behavioral-goal memories that guide learning of a bird's courtship song.

The NIf-HVC pathway is necessary to form a song memory

A longstanding view is that behavioral-goal memories for song are encoded in auditory regions presynaptic to the NIf-HVC pathway, such as in the caudomedial nidopallium (NCM) and primary and secondary auditory forebrain (field L) (*6*, *36*–*38*). Memories encoded presynaptic to HVC might be capable of guiding song learning independent of behavioral-goal memories encoded via NIf-HVC synapses. In addition, auditory information entering HVC via other routes, such as Av projections into HVC, may also be capable of encoding these memories. To evaluate these ideas, we paired opto-tutoring with normal song tutoring as juvenile birds socially interacted with live tutors. Optical activation of NIf-HVC synapses was contingent on the tutors' singing behavior (Fig. 5A), providing the juvenile with two simultaneous potential sources of information from which to learn: ascending auditory information from a live zebra finch tutor and light-evoked activity at NIf-HVC synapses.

These birds failed to imitate the songs of their tutors, exhibiting levels of similarity to their song tutor that were indistinguishable from the songs of isolate birds (Fig. 5B). Instead, they learned from the 300-ms optical stimulation, displaying song element durations similar to those of birds that were opto-tutored but never tutored by adult zebra finches (Fig. 5, C and D). Grouping all birds opto-tutored with 300-ms light pulses (n = 7 birds producing 11 song elements), we found that their song element durations were significantly longer than those of isolate or normally reared birds (Fig. 5E). Therefore, even when provided with a normal song model, birds learn from opto-tutoring. This finding suggests the possibilities that (i) activity at NIf-HVC synapses overrides learning from auditory experiences with a social model, or (ii) the memories that might be encoded presynaptically or independent of NIf-HVC synapses are insufficient to guide song imitation.

To clarify whether the pathway from NIf to HVC is necessary for a young bird to acquire a behavioral-goal memory during social experiences with a tutor, we genetically lesioned neurons in NIf projecting to HVC using an intersectional viral approach for cre-dependent expression of caspase3 (*17*, *39*). NIf neurons

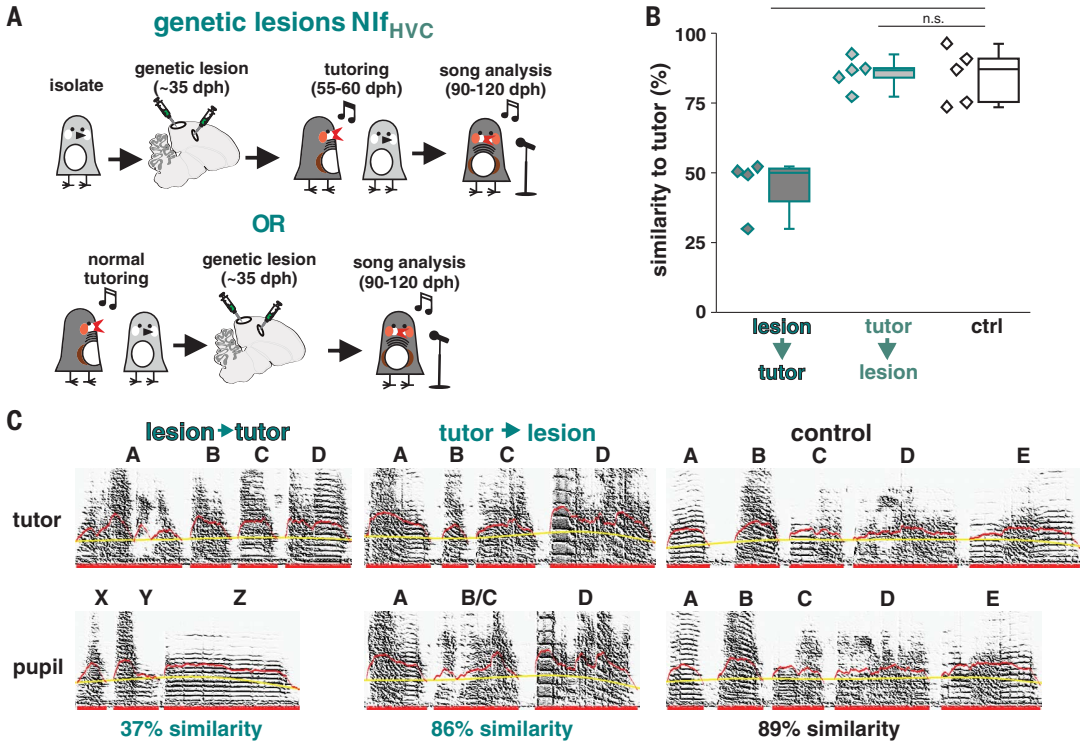

Fig. 6. NIf-HVC synapses are necessary for acquisition of a tutor song memory but not for vocal imitation. (**A**) Schematic of the NIf-HVC lesion experiments. Genetic lesions of NIf neurons projecting to HVC using viral expression of a cre-dependent caspase3 were performed either after birds had memorized the song of their tutor, or prior to song tutoring. Song learning outcomes were examined when pupils reached adulthood. (**B**) Birds with NIf-HVC neurons lesioned before tutoring (green with dark gray fill) failed to copy the tutor songs. Controls tutored before having their NIf-HVC neurons lesioned (green with light gray fill) copied the tutor songs as well as nonlesioned birds (black with no fill). Mann-Whitney U tests: lesioned then tutored birds versus control birds, P = 0.02; lesioned then tutored birds versus isolate birds, P = 0.3429. (**C**) Spectrograms of representative tutor-pupil song comparisons shown in (B).

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projecting to HVC were lesioned in 35- to 40-day-old isolate birds and then they were housed with a tutor for 5 days starting at 55 days of age. A separate group of birds were raised with a tutor prior to lesioning NIf neurons projecting to HVC at 35 to 40 days of age (Fig. 6A). We found that birds with NIf-HVC lesioned before tutoring failed to imitate the song of their tutors (Fig. 6, B and C), exhibiting similarity scores (with respect to their tutor) that were indistinguishable from those of isolate birds. It has previously been shown that nonselective lesions or inactivation of NIf prior to song tutoring can disrupt subsequent vocal imitation (*7*). Our current findings show that lesions of only the NIf-HVC pathway are sufficient to disrupt song learning. However, it is not clear whether these lesions disrupt acquisition of tutor song memories, or whether they disrupt subsequent sensorimotor learning. We found that lesions to this pathway after birds had an opportunity to learn from their tutor did not affect the ability of juvenile birds to accurately imitate the song of their tutor (Fig. 6, B and C, $P = 1.0$, Mann-Whitney U test), which suggests that the NIf-HVC circuit plays a specific role during the acquisition of behavioral-goal memories. Together with our opto-tutoring results, this indicates that the NIf-HVC circuit is necessary for forming a memory used to guide learning of song-element duration, but that this circuit is dispensable once this behavioral-goal memory is acquired. This suggests that at least certain aspects of tutor song memories used to evaluate vocal performances are ultimately stored downstream of synapses between NIf and HVC.

Discussion

Episodic memories are initially dependent on the hippocampus but are more broadly distributed at latter time points (*13*, *40*, *41*). Experiments in recent years have used activity-dependent tagging and optogenetic manipulations to dissect the cellular constituents of episodic memories, identifying "engram cells" and how they can be manipulated to affect recall and behavior (*42*–*44*). Our understanding of procedural memories, and of the behavioral-goal memories that guide motor imitation, has lagged far behind. Procedural memories are thought to be formed and directly represented in the circuits involved in their performance (*12*, *14*, *15*, *45*). Using optogenetic tutoring of juvenile songbirds, we found that aspects of behavioral-goal memories can be implanted through manipulation of sensorimotor synapses that convey information from a social model to motor circuits, but that this synaptic pathway is not necessary for evaluating vocal performances during sensorimotor learning. This suggests that, like episodic memories, lasting memories of observed behaviors used to guide imitative learning are initially dependent on a specific brain circuit for their encoding but may be more broadly distributed at latter time points when they are used to influence behavior.

Premotor cortical circuits involved in speech production are activated by listening to speech, even in pre-verbal infants (*46*), which suggests that tight coupling between sensory and premotor cortical circuits exists at the earliest stages of learning. Sensorimotor pathways may therefore provide a general substrate for encoding behavioral-goal memories during social interactions and learning. The substrate for long-term storage of behavioral-goal memories is still unknown. Previous studies have suggested that tutor song memories encoded in higher-order auditory regions function as an "auditory template" used to guide vocal imitation (*6*, *36*). Our findings indicate that behavioral-goal memories formed during social interactions require sensorimotor transformation of pertinent auditory experiences prior to consolidation. HVC provides feedback to the auditory system via its projection to Av, and this pathway may facilitate long-term distributed encoding of behavioral-goal memories during development (*17*).

This research focused on how juvenile birds form memories that guide learning of song temporal structure. How young birds memorize other aspects of tutor song, such as the spectral and syntax structure, is not known. HVC receives input from at least three pallial and thalamic regions aside from NIf (*28*). One possibility is that unified representations of tutor songs only emerge through sensorimotor transformations and different inputs to HVC carry unique streams of information, allowing birds to form memories of temporal as well as spectral and syntactical features of their tutor. Understanding the role of other sensorimotor pathways in forming behavioral-goal memories is likely to yield insights into how more complex or integrated memories of vocal models are learned during social interactions.

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Science

Inception of memories that guide vocal learning in the songbird

Wenchan Zhao, Francisco Garcia-Oscos, Daniel Dinh and Todd F. Roberts

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An imitation circuit

Animals, including humans, rely heavily on imitation and social learning, yet we know little about how this process operates in the brain. Zhao *et al.* used optogenetic manipulation of a synaptic pathway connecting auditory and vocal motor circuits to implant song memories sufficient to guide song learning into young zebra finches (see the Perspective by Clayton). Activation of this circuit overrode learning from live tutors. These experiments define circuits essential for social learning of songs from tutors and show that such memories can be localized.

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