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LETTERS

Rapid spine stabilization and synaptic enhancement at the onset of behavioural learning

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Behavioural learning depends on the brain's capacity to respond to instructive experience and is often enhanced during a juvenile sensitive period. How instructive experience acts on the juvenile brain to trigger behavioural learning remains unknown. *In vitro* studies show that forms of synaptic strengthening thought to underlie learning are accompanied by an increase in the stability, number and size of dendritic spines, which are the major sites of excitatory synaptic transmission in the vertebrate brain[1–7]. *In vivo* imaging studies in sensory cortical regions reveal that these structural features can be affected by disrupting sensory experience and that spine turnover increases during sensitive periods for sensory map formation[8–12]. These observations support two hypotheses: first, the increased capacity for behavioural learning during a sensitive period is associated with enhanced spine dynamics on sensorimotor neurons important for the learned behaviour; second, instructive experience rapidly stabilizes and strengthens these dynamic spines. Here we report a test of these hypotheses using two-photon *in vivo* imaging to measure spine dynamics in zebra finches, which learn to sing by imitating a tutor song during a juvenile sensitive period[13,14]. Spine dynamics were measured in the forebrain nucleus HVC, the proximal site where auditory information merges with an explicit song motor representation[15–19], immediately before and after juvenile finches first experienced tutor song[20]. Higher levels of spine turnover before tutoring correlated with a greater capacity for subsequent song imitation. In juveniles with high levels of spine turnover, hearing a tutor song led to the rapid (~24-h) stabilization, accumulation and enlargement of dendritic spines in HVC. Moreover, *in vivo* intracellular recordings made immediately before and after the first day of tutoring revealed robust enhancement of synaptic activity in HVC. These findings suggest that behavioural learning results when instructive experience is able to rapidly stabilize and strengthen synapses on sensorimotor neurons important for the control of the learned behaviour.

Investigating structural correlates of song learning requires repeated imaging of dendritic structure as a juvenile bird learns to sing. We used lentivirus/green fluorescent protein (GFP) constructs to label neurons fluorescently[21,22], retrograde tracers to localize the boundaries of HVC and two-photon microscopy to image dendritic spines on individual HVC neurons through a surgically implanted cranial window in male zebra finches (Fig. 1a). We focused on spinous HVC neurons, which are projection neurons important for singing and song learning[23,24]. To minimize interference with the bird's behaviour, images were collected during its subjective night time. Initial experiments, in which neurons in either juveniles (aged 60–90 d, $N = 2$ birds) or adults (120–130 d, $N = 3$) raised with normal access to a tutor were repeatedly imaged, revealed that the dendritic arbors of HVC projection neurons remained stable over the course of several nights (Fig. 1b). Significantly, repeated imaging (2-h interval) within a single night also detected a subset of dendritic spines that underwent turnover ($N = 9$ (six aged 60–90 d, three aged 130 d); Fig. 1c, d). Imaging on consecutive nights revealed that 91.75 ± 2.19% of the spines maintained over 2 h also were maintained over 24 h (Supplementary Fig. 1). The observed 24-h survival fraction was significantly

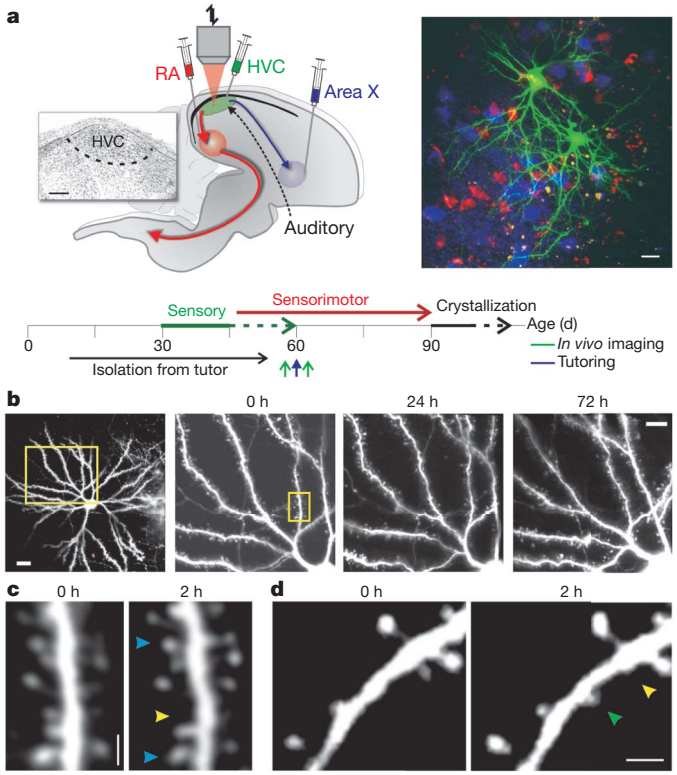

Figure 1 | Examining how tutor song affects spine turnover in juvenile zebra finches. a, Left and bottom: schematic of the zebra finch song system, experimental protocol and timeline of the experiments. Inset, Nissl-stained image of HVC in parasagittal section, showing its location on the floor of the lateral telencephalic ventricle, ~100 μm below the pial surface. RA, robust nucleus of the arcopallium; area X, striatal component of the song system; scale bar, 200 μm. Right: *in vivo* two-photon image of GFP-labelled spinous HVC neurons amid retrogradely labelled RA-projecting (HVC_{RA}, red) and area-X-projecting (HVC_X, blue) neurons. Scale bar, 20 μm. **b**, Repeated *in vivo* imaging of dendritic branches from an HVC neuron of a 130-d zebra finch over several days. The rightmost three images (scale bar, 10 μm) show the boxed region in the leftmost image (scale bar, 20 μm) at the times indicated. **c**, Views of the dendritic segment shown boxed in the 0-h image in **b**, imaged 2 h apart. **d**, View of another dendritic segment of an HVC neuron, showing the gain and loss of dendritic spines across a 2-h imaging interval. In **c** and **d**, arrowheads point to stable (blue), lost (yellow) and gained (green) spines. Scale bars, 2 μm.

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higher than estimates based on the 2-h turnover measurements ($P = 3.8 \times 10^{-5}$; Supplementary Fig. 1a), indicating that HVC neurons possess two populations of dendritic spines[25]: a larger (>90%) stable population and a smaller (<10%) transient population, the dynamics of which can be captured in a 2-h time window. Therefore, viral labelling of HVC neurons with GFP combined with two-photon imaging offers a means of examining rapid learning-related structural changes to sensorimotor neurons.

Songbirds learn to sing during a juvenile sensitive period by memorizing a tutor song (sensory learning) and using auditory feedback to match their own song to this memorized model (sensorimotor learning). In juvenile male zebra finches, sensory learning occurs during the period ~30–60 d after hatching and sensorimotor learning occurs during the period ~45–90 d after hatching[13,14]. To examine whether experience with a tutor affects spine dynamics in HVC, spine turnover levels were quantified in juveniles raised without access to a tutor. Initial experiments focused on untutored 60-d juveniles, an age when zebra finches raised with a tutor become refractory to copying new song models[13]. Imaging HVC neurons in untutored 60-d birds revealed a wide range of spine turnover levels, with some birds (six of 14) exhibiting levels substantially higher (>2 s.d.) than in age-matched juveniles raised with a tutor, and others (eight of 14) showing levels similar (±1 s.d.) to the control animals (Fig. 2a). One possibility is that this variation reflects individual differences in when the sensitive period for sensory learning ends. To test this idea, we quantified spine turnover in 45-d and 90-d untutored birds as well as age-matched birds raised with a tutor. Spine turnover in all untutored 45-d birds was significantly higher than in age-matched control birds ($P < 0.01$) and overlapped with the upper end of the turnover distribution in untutored 60-d birds. By contrast, spine turnover in all untutored 90-d birds was in the range of age-matched control birds ($P = 0.3$) and overlapped with the lower end of the turnover distribution in untutored 60-d birds. These observations support the idea that tutor experience acts during early stages of normal development to decrease spine turnover in HVC, while also showing that spine turnover can eventually decrease even in the absence of tutor experience. Because untutored birds use auditory feedback to learn and stabilize their 'isolate' songs, this transition to lower levels of turnover may reflect a commitment to a vocal behaviour learned in reference to an innate model.

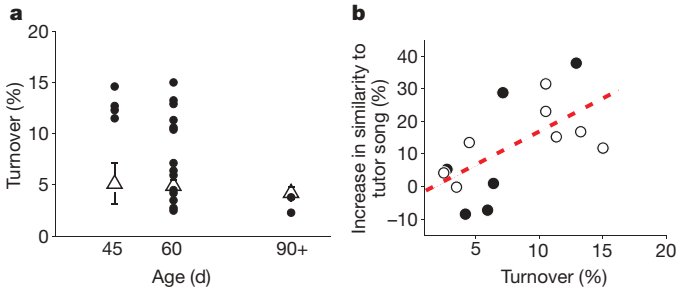

Figure 2 | Levels of HVC dendritic spine turnover correlate with song imitation. **a**, Mean 2-h HVC spine turnover levels are greater in 45-d ($P = 0.009$) and 60-d ($P = 0.04$), but not 90-d, untutored birds (circles) than in age-matched control birds (triangles) (45-d untutored, 180 spines from six cells in four birds; 45-d control, 621 spines from 11 cells in three birds; 60-d untutored, 1,419 spines from 17 cells in 14 birds; 60-d control, 1,579 spines from 23 cells in 16 birds; 90-d untutored, 188 spines from four cells in two birds; 90-d control, 1,544 spines from 23 cells in 11 birds). Error bars, s.e.m. **b**, Levels of turnover in HVC before tutoring correlate with subsequent song imitation. The scatter plot shows the relationship between levels of 2-h HVC dendritic spine turnover in 60-d birds measured the night before the first exposure to a song model and the total increase in similarity to the tutor song over song development ($P = 0.02$, $r = 0.63$; 1,419 spines from 17 cells in 14 birds). Each circle represents a single bird. The eight open circles correspond to birds in which post-tutoring dendritic spine turnover measurements were made. The red dashed line is plotted by linear regression.

In this light, the transition to low levels of spine turnover in untutored birds could reflect a diminished ability to learn from a tutor. If this view is correct, then the variable levels of turnover in untutored 60-d birds would be expected to correlate with the ability to copy a tutor song. To test this idea, we exposed previously untutored 60-d juveniles ($N = 14$) to either a live tutor or operant tutoring methods for three consecutive days and tracked their song development into adulthood. Consistent with prior observations[26], delayed tutoring resulted in a wide range of song-learning outcomes, with some birds copying elements from the tutor song (an increase of up to 37.8% in similarity to the tutor song by adulthood) and other birds copying little or not at all (Fig. 2b and Supplementary Fig. 2). Spine turnover levels measured in HVC before tutoring correlated positively with the subsequent increase in similarity of the pupil's song to the tutor ($P = 0.02$; correlation coefficient, $r = 0.63$). Therefore, the capacity for learning a new behaviour is associated with enhanced spine dynamics in sensorimotor circuits important for that behaviour.

The observations that the capacity for song learning is associated with enhanced spine dynamics in HVC and that spine turnover in all untutored 45-d birds was significantly higher than in age-matched controls suggest that experience of tutor song resulting in learning may stabilize dendritic spines in HVC. To test this idea, we repeatedly imaged the same dendritic regions in HVC at least one night before and one night after the first day of exposure to tutor song ($N = 8$, a subset of the 14 untutored 60-d birds described previously; Fig. 2b). Five of these birds, termed high-turnover birds (HTBs), displayed pre-tutoring levels of spine turnover >2 s.d. higher than in age-matched controls, and the other three, termed low-turnover birds (LTBs), displayed pre-tutoring levels of spine turnover commensurate to controls.

In all five HTBs, spine turnover in HVC decreased significantly by the night following the first day of either live ($N = 3$) or operant ($N = 2$) tutoring ($P < 0.01$; Fig. 3a). The finding that decreased spine turnover could occur with operant tutoring indicates that these structural effects did not depend on social interactions with the tutor, and instead are related to hearing a tutor song. Furthermore, in one live-tutoring experiment, the tutor failed to sing until the third day after it was housed with the juvenile. Notably, spine turnover only decreased after the tutor began to sing (Fig. 3b), further underscoring the importance of auditory rather than social experience in triggering structural changes in HVC. Moreover, in this live-tutored juvenile, it was possible to repeatedly image the same dendritic regions for 30 d following tutor exposure, a period encompassing the remainder of sensorimotor learning. In this case, the decrease in turnover over the first 48 h after tutoring accounted for the bulk (67%) of the decrease that occurred during sensorimotor learning (Fig. 3b). In addition to displaying rapid spine stabilization, four of five HTBs had increased spine density the night following the first day of tutoring ($P = 0.03$; Fig. 3c, d and Supplementary Fig. 3). In contrast, LTBs showed no changes in either spine turnover or spine density following live or operant tutoring (Fig. 3a, d). Finally, increased syllable entropy variance, which is an early indicator of song imitation, was detected in HTBs but not LTBs by the end of the first day of tutoring (post hoc Tukey test, significance threshold (α) of 0.05 in three of four HTBs; Fig. 3e), and, as adults, HTBs had copied more from their tutors than had LTBs (HTBs, 19.6 ± 3.5% increase in similarity to the tutor over song development; LTBs, 6.2 ± 4.0% increase). These findings support the idea that instructive experience leads to the stabilization and accumulation of dendritic spines on sensorimotor neurons important for the learned behaviour.

In other systems, increased spine stability and the accumulation of new spines have been linked to synaptic strengthening[2,4,6,7]. Another structural hallmark of synaptic strengthening is a dynamic increase in the volume of pre-existing (that is, stable) spines[1,8,27]. We tracked individual HVC dendritic spines maintained across pre- and post-tutoring imaging sessions and measured changes in their fluorescence intensity, a feature monotonically related to spine volume[28] (81 spines, eight

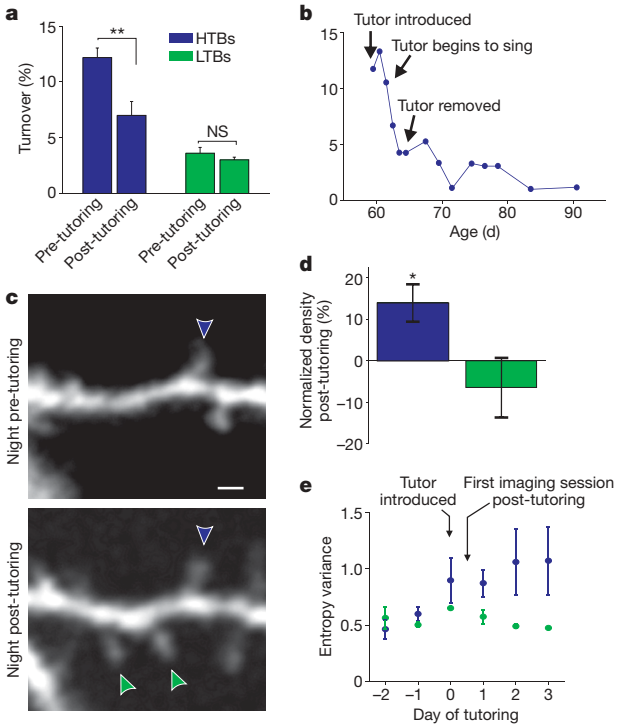

Figure 3 | Tutoring can trigger rapid stabilization and accumulation of dendritic spines on HVC neurons. **a**, Tutoring triggers a rapid decrease in the level of HVC dendritic spine turnover in HTBs ($P < 0.01$) but not LTBs ($P = 0.3$). The graph shows levels of HVC dendritic spine turnover measured the night before and the night after the first day of either live ($N = 5$) or operant ($N = 3$) tutoring in HTBs and LTBs (HTBs: $^{**}P < 0.01$, 468 spines from five birds; LTBs: $P = 0.3$, 449 spines from three birds). Furthermore, spine turnover levels measured in HVC before tutoring correlated positively with the post-tutoring decrease in spine turnover ($P = 0.02$, $r = 0.79$, $N = 8$; not shown). NS, not significant. **b**, The bulk of the decrease in turnover levels (67%) observed during sensorimotor learning occur during the first 48 h after a tutor is heard for the first time. The graph shows the level of HVC dendritic spine turnover in a HTB measured over a 30-d period following initial exposure to a tutor. The tutor did not sing during the first 2 d with the high-turnover juvenile; thereafter, turnover levels markedly and persistently decreased. **c**, Example of stable spines (green arrowheads) that formed during the first day of tutoring in a bird with high pre-tutoring levels of spine turnover. Scale bar, 2 μm. **d**, Tutoring triggers a rapid increase in HVC dendritic spine density in HTBs ($P = 0.03$) but not LTBs ($P = 0.5$). The graph shows the percentage change in dendritic spine density by the first night following tutoring (HTBs: $^{*}P = 0.03$; LTBs: $P = 0.5$; 1,198 spines from four HTBs and three LTBs). Furthermore, spine turnover levels measured in HVC before tutoring correlated positively with the post-tutoring increase in spine density ($P = 0.04$, $r = 0.71$, $N = 8$; not shown). **e**, Mean afternoon entropy variance scores increase by the afternoon of the first day of tutoring in HTBs (blue), but not LTBs (post hoc Tukey test, $\alpha = 0.05$ in three of four HTBs; data shows mean afternoon entropy variance value for four HTBs and three LTBs). Error bars, s.e.m.

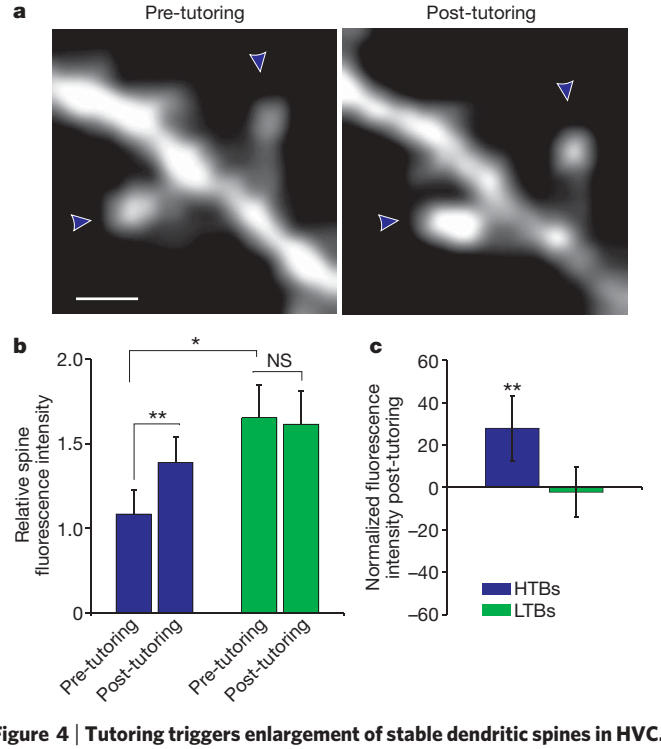

Figure 4 | Tutoring triggers enlargement of stable dendritic spines in HVC. **a**, Example of two stable spines (blue arrowheads) that exhibited increased fluorescence intensity following tutoring, indicating an increase in dendritic spine volume[28]. Scale bar, 2 μm. **b**, The size of stable spines increased in HTBs but not LTBs by the first night following tutoring, as revealed by measurements of relative integrated fluorescence intensity (81 spines, eight birds; HTBs: $^{**}P = 0.001$, $n = 47$ spines, Wilcoxon signed-rank test for paired samples; LTBs: $P = 0.4$, $n = 34$ spines). Moreover, HTBs had smaller stable dendritic spines before tutor exposure ($^{*}P = 0.02$). **c**, The first night following tutor exposure, the mean size of stable spines increased in HTBs but did not change in LTBs (HTBs: $^{**}P = 0.001$; LTBs: $P = 0.4$).

birds). The night following the first tutoring session, the size of stable dendritic spines increased by 28% in HTBs but remained unchanged in LTBs (HTBs, $P = 0.001$; LTBs, $P = 0.4$; Fig. 4 and Supplementary Fig. 3). Additionally, before tutoring, stable dendritic spines in HTBs were 52% smaller than in LTBs ($P = 0.02$; Fig. 4b), a difference that had disappeared by the night following the first tutoring session ($P = 0.3$). The smaller stable spines and higher levels of turnover in HTBs observed before tutoring may reflect functionally weaker excitatory synaptic connections to HVC neurons[1,27]. Thus, instructive experience can act on more dynamic and presumably weaker dendritic spines to increase their size, number and stability, all of which are hallmarks of functional enhancement of synaptic transmission.

Indeed, tutoring rapidly enhanced synaptic activity in HVC, consistent with the idea that structural changes to spines elicited by tutoring are associated with functional changes to synapses. By adapting the windowing methods used for *in vivo* imaging, we were able to obtain sharp intracellular recordings from electrophysiologically identified projection neurons[23] in the same small region of HVC one night before and one night after a juvenile bird's initial exposure to a tutor (Fig. 5). To maximize the likelihood that HVC dendritic spines of all birds were in a high-turnover state, experiments were conducted in ~45-d juveniles previously raised without a tutor. Comparing spontaneous synaptic activity recorded before and after the first day of tutoring revealed a marked increase in the amplitude of depolarizing synaptic activity (24 cells in three birds, $P < 0.00001$; Fig. 5 and Supplementary Fig. 4a) and the emergence of prolonged (~1-s) bursts of synaptic activity. These functional changes were not paralleled by changes in resting membrane potential ($P = 0.6$) or action-potential firing rate ($P = 0.3$). Therefore, the synaptic enhancement was unlikely to arise from increased driving force on synaptic currents or increased firing of HVC projection neurons, which are a major source of synaptic input into other HVC projection neurons. Rather, the functional and structural changes observed in HVC following tutoring are suggestive of rapid synaptic strengthening, although another mechanism that cannot be excluded is increased excitability of neurons afferent to HVC. The rapid enhancement of synaptic activity was even detected in a juvenile that failed to sing during its first tutoring session ($P < 0.00001$; Supplementary Fig. 4a), suggesting that it could occur in the absence of vocal practice and its associated auditory feedback. Lastly, the cumulative distributions of spontaneous synaptic events recorded in HVC the night following the first tutoring session were similar to those from age-matched juveniles raised with access to a tutor ($P = 0.1$; Supplementary Fig. 4b), consistent with the idea that tutoring results in a rapid physiological strengthening of initially weaker synaptic inputs into HVC projection neurons.

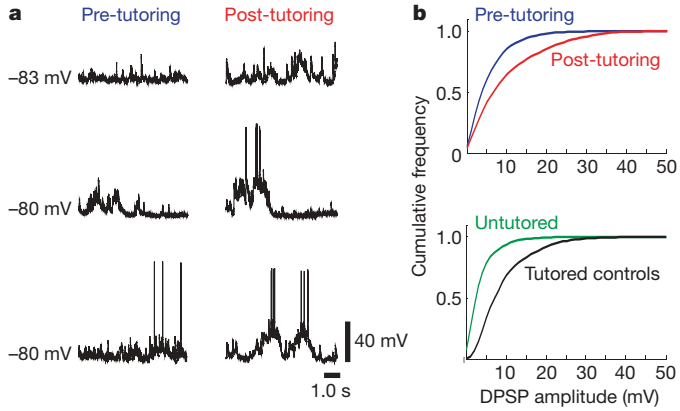

Figure 5 | Tutoring triggers enhancement of spontaneous synaptic activity in HVC. a, *In vivo* recordings of intracellular membrane potential made in six different HVC neurons in a juvenile bird one night before and one night after initial tutor exposure, showing that tutoring drives a rapid increase in the amplitude of spontaneous depolarizing synaptic activity. Numbers at left refer to resting membrane potential. **b**, Top: cumulative frequency distribution of spontaneous synaptic activity showing the amplitude of depolarizing postsynaptic potentials (DPSPs) recorded intracellularly in HVC immediately before and after tutoring ($P < 0.00001$). Data collected from 24 cells in three birds. Bottom: cumulative frequency distribution of spontaneous synaptic activity showing the amplitude of depolarizing synaptic events recorded intracellularly in HVC on two consecutive days from a 45-d untutored bird (five cells on each night) and a normally reared 45-d bird (two cells on each night).

Our findings show that behavioural learning is favoured when instructive experience rapidly stabilizes structurally dynamic dendritic spines and enhances synaptic activity of sensorimotor neurons, thereby forging a link between experience, structural and functional properties of synapses, and behavioural learning. Prior studies in juvenile mammals established that the quality of sensory experience can affect the structural dynamics of dendritic spines in the corresponding regions of sensory cortex[9,11]. Our findings show that the consequences of a single instructive signal—a tutor song—are rapidly manifested as structural changes to dendritic spines as well as functional changes to synapses in HVC, a sensorimotor region important for the control of learned vocalizations[18]. A variety of evidence implicates HVC as the source of precise timing signals for song patterning[19], the source of corollary discharge signals harnessed to allow song imitation[15], and as a primary site where auditory signals merge with these song motor representations[15,16,19]. The location of HVC at the sensorimotor interface suggests that the large-scale structural changes to dendritic spines we observed following tutoring can have direct consequences for how the HVC network translates auditory and motor-related activity into song. Consistent with this view, tutoring rapidly enhanced spontaneous synaptic activity in HVC and also led to rapid changes in vocal behaviour. Moreover, spontaneous bursting activity in song premotor neurons immediately downstream of HVC has been found to increase drastically during the initial stages of tutor-song imitation[29], a functional change that is likely to be the consequence of the structural and functional changes occurring in HVC. Overall, our findings suggest experience can act in the juvenile brain to rapidly stabilize and strengthen a structurally dynamic sensorimotor network, providing a foundation for learning new behaviours.

METHODS SUMMARY

We raised juvenile male zebra finches either in isolation from an adult song model (isolates) or with normal access to adult male song (controls). We used repeated *in vivo* two-photon optical imaging in the sensorimotor song nucleus HVC to assess structural plasticity of dendritic spines before and after the onset of song learning. Lentivirus coding for enhanced GFP injected 14–20 d before cranial windowing was combined with retrograde labelling of HVC projection neurons to genetically label and identify HVC neurons for *in vivo* two-photon laser scanning microscopy (Zeiss LSM 510 laser scanning microscope, Tsunami Ti:sapphire laser (Spectra-Physics) at 910 nm, ×40 infrared Zeiss Achroplan objective). Changes in turnover, density and fluorescence intensity of dendritic spines on HVC projections neurons were calculated (Methods) using data from 2-h imaging sessions (total of 7,048 spines from 103 cells in 61 birds). We used sharp intracellular recordings from electrophysiologically-identified HVC projection neurons made the night before and the night after initial exposure to a song model to analyse associated changes to synaptic activity (Methods). To quantify song learning, we measured how much the pupil's song gained in similarity to the tutor over song development using percentage similarity score in SOUND ANALYSIS PRO 1.04a (adult percentage similarity minus pre-tutoring percentage similarity; http://ofer.sci.ccny.cuny.edu/sound_analysis_pro). To measure the onset of changes in song with tutoring, we calculated the entropy variance of identified proto-syllable clusters from afternoon recording sessions, starting two days before tutoring and during the three days of tutoring (Methods). Entropy variance is a measure of song complexity and an early indicator of tutor-song imitation. Standard non-parametric and parametric statistical methods were used to detect significant differences ($\alpha = 0.05$).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions T.F.R. and R.M. designed the study and wrote the manuscript. T.F.R. and K.A.T. collected and analysed the imaging and behavioural data. T.F.R. and M.E.K. designed the lentiviral construct and M.E.K. made the lentivirus. T.F.R and R.M. collected the electrophysiological data.

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METHODS

We raised finches in our breeding colony or in isolation from an adult tutor and imaged them beginning at 45, 60, 90 or 130 d after hatching, in accordance with a protocol approved by the Duke Institutional Animal Care and Use Committee. All birds were placed on reverse day–night cycles (14 h of light, 10 h of darkness) ≥15 d before imaging. Control birds were raised in our breeding colony and given full access to adult tutors until ≥43 d after hatching. Experimental juvenile zebra finches were isolated from adult male tutors by 10 d after hatching and then housed in nesting groups and cared for by two adult female zebra finches or female Bengalese finches. Beginning at ~45 d (40–50 d after hatching), juvenile males from the nesting groups were separated and housed individually in visual isolation from each other until adulthood (115–130 d). Some of these juvenile birds were then tutored beginning on day 60 (59–62 d after hatching). For tutoring, juvenile birds were allowed to trigger playback of the song of an adult zebra finch or were introduced to an adult male zebra finch starting the morning following the initial night of imaging. Birds were given access to key-peck-triggered tutoring or a live singing tutor for three consecutive days. Vocalizations of juvenile birds subjected to delayed tutoring were recorded continuously using automated methods (SOUND ANALYSIS PRO 1.04a; http://ofer.sci.ccny.cuny.edu/sound_analysis_pro). Songs were recorded daily beginning 2–7 d before and ending ≥3 d after tutoring, and then were recorded either continuously or semi-continuously (every 3–10 d) into adulthood.

Viral and tracer injections. We anaesthetized male zebra finches using isoflurane inhalation (2%) and placed them in a stereotaxic apparatus. Injection target sites were located using stereotaxic coordinates and multi-unit neural recordings. A glass pipette attached to a pressure injection unit (Drummond Nanoject II) was used to deliver the lentivirus, expressing enhanced GFP under the control of the Rous sarcoma virus long terminal repeat (FRGW)[21], to HVC. Similar methods were used to deliver the neuronal retrograde tracer Fast Blue to area X and Alexa Fluor 594 conjugated dextran amines to RA (lentivirus: 32.2 nl per injection and ~30 injections, for a total of ~1 μl; tracers: 32.2 nl per injection and 2–5 injections). Lentiviral injections were made 15–20 d before imaging and retrograde tracer injections were made 5–7 d before imaging to optimize labelling of HVC neurons.

***In vivo* two-photon imaging.** We longitudinally imaged dendritic spines on GFP-expressing HVC neurons in male zebra finches aged 41–130 d (7,048 spines, 61 birds; Supplementary Table 1). Birds were injected with mannitol (10 μl g^{-1}, intramuscular), anaesthetized by isoflurane inhalation (2%) and positioned in a stereotaxic apparatus. The scalp overlying HVC was removed and the scalp margins were sealed to the surface of the skull using Vetbond (*n*-butyl cyanoacrylate). Bilateral craniotomies (~1–1.5 mm^2) were made in the skull overlying HVC. The dura mater was excised, leaving intact the pia mater, the 60–150-μm-thick layer of neural tissue and the lateral telencephalic ventricle overlying HVC. A custom-cut coverslip (no.-1 thickness) was placed directly on the pial surface or on a thin layer of agarose covering the brain, then sealed to the skull with dental acrylic. A head post was also affixed to the skull with dental acrylic. Birds were placed onto a custom stage under a Zeiss LSM 510 two-photon laser scanning microscope. Only GFP-labelled neurons located within a field of retrogradely labelled HVC_{RA} and/or HVC_X neurons were classified as HVC neurons and imaged. Dendritic segments of HVC neurons were imaged at high resolution during the bird's subjective night time (1024 × 1024 pixels, 76 × 76 μm^2, 3.2 μs per pixel, averaging two samples per pixel with 1-μm *z* steps, focused through a ×40, NA 0.8 Zeiss IR-Achroplan immersion objective). Birds were returned to a darkened holding cage and allowed to sleep until being re-imaged 2 h later. Two-hour imaging sessions were repeated during the same period each night for 1–5 nights.

Image analysis. Three-dimensional image stacks were auto-aligned and smoothed using a Gaussian filter (IMAGEJ; http://rsbweb.nih.gov/ij/) and the same dendritic segment, imaged twice with a 2-h or 24-h interval, was selected. Images exhibiting changes in fluorescence or rotational artefacts were excluded from further analysis. All sets of selected three-dimensional image stacks were coded and scored by researchers blind to the experimental condition. To assess spine growth and retraction, we compared individual dendritic spines across 2-h time intervals and calculated spine stability ($100N_{stable}/N_{total}$), spine elimination ($100N_{lost}/N_{total}$), spine addition ($100N_{gained}/N_{total}$) and spine turnover ($100(N_{gained} + N_{lost})/2N_{total}$), where N_{stable} is the number of spines that were stable over the 2-h interval, N_{lost} is the number of spines lost over the 2-h interval, N_{gained} is the number of spines gained over the 2-h interval and N_{total} is the total number of spines from the first imaging time point. Changes in spine density (N_{total} divided by dendritic length in micrometres) and spine fluorescence intensity were measured from the same dendritic segments used to assess spine turnover. Spine fluorescence intensity (integrated GFP intensity) was measured by summing all pixels for a dendritic spine, subtraction-corrected for the mean background fluorescence measured from a non-labelled region of equal area located adjacent to the dendritic spine. This value was normalized by dividing by the mean dendritic fluorescence measured on a segment of dendrite at the base of the spine, subtraction-corrected for the background fluorescence. Standard non-parametric and parametric statistical methods were used to detect significant differences ($\alpha = 0.05$).

Repeated intracellular recording. Sharp intracellular recordings were made on two consecutive nights from the same small location in HVC using repeated electrode penetrations through the same opening in the dura. During recording sessions, birds were lightly anaesthetized with diazepam (50 μl, 2.5 mg ml^{-1}). Electrode impedances were 100–150 MΩ when filled with 2 M KAc. Between nightly recording sessions, the craniotomy overlying HVC was filled with silicone oil (Advance Weight Systems) and covered with a glass coverslip and a silicone adhesive (Kwik-Sil, World Precision Instruments). Intracellular electrophysiological data was captured as previously described[23]. Briefly, recordings were amplified using an AxoClamp 2B amplifier (Axon Instruments), low-pass filtered at 3 kHz and digitized at 10 kHz. Analysis of depolarizing postsynaptic potential amplitudes from spontaneous electrical activity was conducted on median-filtered traces using custom event detection software (written by K. Hamaguchi using MATLAB, version 2009).

Behavioural analysis. We quantified the amount that juvenile birds copied from their tutor using the percentage similarity score in SOUND ANALYSIS PRO. Acoustic similarity is calculated by measuring 'pitch', amplitude modulation, frequency modulation, Weiner entropy and goodness of pitch. This aggregate score reflects the percentage of elements in the pupil's song that are similar to those in the tutor's song. We first calculated how much the pupil copied from the tutor by analysing similarity scores from recordings of the pupils' adult song (115–130 d). Next we measured how similar the pupil song was to the tutor immediately before tutor exposure (58–59 d), by retrospectively identifying syllable clusters in the juvenile song that were proto-syllables for the adult song motif ($n \approx 45$ comparisons per bird per time point). From these measurements, we calculated how much the pupil gained in similarity to the tutor over song development (adult percentage similarity minus pre-tutoring percentage similarity). This approach was used instead of absolute similarity because some juvenile songs before tutor exposure (58–60 d) were sufficiently structured to return spuriously high similarities to a tutor song. To measure the onset of changes in song with tutoring, we calculated the entropy variance, which is a measure of song complexity and an early indicator of tutor-song imitation[20,26,29], of identified proto-syllable clusters from afternoon recording sessions, starting 2 d before tutoring and during the 3 d of tutoring.