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Functional identification of sensory mechanisms required for developmental song learning

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Abstract

A young male zebra finch (*Taeniopygia guttata*) learns to sing by copying the vocalizations of an older tutor in a process that parallels human speech acquisition. Brain pathways that control song production are well defined, but little is known about the sites and mechanisms of tutor song memorization. Here we test the hypothesis that molecular signaling in a sensory brain area outside of the song system is required for developmental song learning. Using controlled tutoring and a pharmacological inhibitor, we transiently suppressed the extracellular signal-regulated kinase signaling pathway in a portion of the auditory forebrain specifically during tutor song exposure. On maturation, treated birds produced poor copies of tutor song, whereas controls copied the tutor song effectively. Thus the foundation of normal song learning, the formation of a sensory memory of tutor song, requires a conserved molecular pathway in a brain area that is distinct from the circuit for song motor control.

Young male zebra finches learn to sing from adult tutors during a critical period in juvenile life. Developmental song learning occurs in two phases¹. First, the young bird creates an auditory memory, or 'template', of his tutor's song. Then the young bird begins to vocalize, and, through a process of sensorimotor error correction, modifies his own song to resemble the tutor template. As an adult, each male zebra finch sings a unique set of unchanging syllables (the bird's own song) that reflects his earlier song exposure.

The neural circuitry that controls song production is relatively well understood^{2–4}, but it has been much more difficult to find the neuroanatomical and molecular basis for the sensory component of song learning: tutor song memorization. Recent studies suggest that the forebrain auditory lobule⁵, the functional homolog of mammalian primary and secondary auditory cortices, may be important in forming learned song representations. In adult songbirds, immediate-early gene induction and distinct patterns of cellular activity occur in the auditory lobule during song recognition learning^{6–9}. Similar measures can be correlated with the fraction of tutor song that is copied^{10–13}, implying that a tutor song trace persists in the auditory lobule. The induction of one immediate-early gene, *zenk* (also known as *Egr1*, *Zif268*, *Ngfi-a* and *Krox24*), is regulated by the extracellular signal-regulated kinase (ERK) in

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the zebra finch auditory lobule⁵ and in other models^{14–16}. This connection to ERK, part of a molecular pathway that is integral to memory^{14,17}, led us to hypothesize that the ERK cascade in the auditory lobule was necessary for tutor song memorization. We tested this hypothesis using controlled tutor experience and a specific inhibitor of ERK activation¹⁶, and found that molecular processing in the auditory lobule during tutor exposure is required for accurate tutor song copying.

Results

Controlled tutoring

Young male zebra finches were socially housed^{18,19}, but after removal from the aviary at approximately posthatch day 21 (P21, before the onset of the sensory learning period)²⁰, their only exposure to song was between P40–50 (Fig. 1). We used one tutor, Y13, for all of the birds. Experimental males were divided into five groups. The ‘live with’ group resided with Y13 to measure the levels of tutor song copying that resulted from normal daily song presentations after earlier song deprivation^{21,22}. The ‘tutor/CD’ group was exposed to Y13 from an adjacent cage for 1.5 h each day, during which time recorded Y13 song bouts were played through a speaker. This tutoring protocol was designed to control the timing and amount of tutor song experience, while maintaining social interactions with Y13. The ‘isolate’ group was never exposed to tutor song.

Birds in the main experimental group ‘U0126’ were exposed to the tutor as described above for the tutor/CD group, but had been previously implanted with bilateral cannulae into the auditory lobule for drug infusions. We injected the drug U0126 bilaterally into the auditory lobule 30 min before each tutoring session. U0126 is a potent, specific and transient inhibitor of mitogen-activated protein kinase/ERK kinase (MEK)¹⁶. It prevents ERK activation, but does not prevent synaptic transmission in animals as diverse as *Aplysia* and rodents^{23,24}. The final group, ‘U0124’, was identical to the U0126 group, except that these birds received bilateral infusions of U0124, an inactive compound that is structurally similar to U0126. This group measured the effects of surgery, handling and injections. We recorded the birds' songs 2 months after tutoring, when the birds had reached adulthood (P110–120).

Song analysis

We used a previously described method²⁵ to assess the fidelity of tutor song copying by the birds in each treatment group (Fig. 2a,b and Tables 1 and 2). This method uses computational algorithms (Sound Analysis Pro software) to compare two digital sound records, evaluating both their global properties and individual sonic features. Initially, we compared each bird's song to the song of the tutor, Y13, and calculated the means for each group. The live with group, who received the most natural tutoring experience, showed high levels of syllable copying (78.2%) and a high song similarity score (50.4), which was on par with accepted levels for normal tutor song copying^{12,22,26}. Similarly accurate song copies were produced by two other treatment groups: the tutor/CD (80.2%, 47.1) and U0124 groups (74.3%, 44.8).

In contrast, the songs of the U0126 (39.0%, 21.7) and isolate (31.7%, 17.4) groups were much less similar to Y13's song. Comparison of all groups by ANOVA revealed a significant effect of treatment for both measures (percentage of syllables copied, $P < 0.001$; song similarity score, $P < 0.001$). The distribution similarity, another global measure of song similarity that ignores temporal information, was also significantly different between experimental groups ($P < 0.001$; Table 2). By Tukey *post hoc* analysis, the U0126 and isolate groups were both significantly different ($P < 0.008$) from the other tutored groups (Fig. 2b).

These results indicated that ERK inhibition in the auditory lobule during tutoring had the same magnitude of effect on song copying as the absence of tutoring. To assess whether it had the same qualitative effect, we directly compared the U0126 and isolate songs with each other. By visual inspection of the spectral derivatives, the songs of the two groups appeared to be different from each other (see examples in Fig. 2a; all isolate and U0126 spectral derivatives are shown in Supplementary Figs. 1 and 2 online). We also carried out a quantitative analysis using Sound Analysis Pro. With the parameters set for comparison to the Y13 tutor song, U0126 songs were as different from isolate songs (32.6%, 20.7) as they were from Y13 song (39.0%, 21.7, as reported above). An alternative analysis performed with the parameters adjusted to reflect that one song is not a template for the other (Supplementary Methods online) resulted in higher scores for similarity between U0126 and isolate song (62.9%, 30.1), although they were still lower than the scores for closely related songs (Table 1).

To investigate whether U0126 treatment during tutoring affected some song properties more than others, we analyzed individual measures of sound frequency, amplitude, pitch and structure of song elements²⁵, using the Y13 song as the reference. The only individual sound trait that showed a significant difference between U0126 and other groups was frequency modulation ($P = 0.002$; Table 2), which is a measure of how much the frequency changes across the song bout.

To measure whether the quantitative differences in song similarity scores in adult songs were evident early in developmental song learning, we compared the Y13 song to the songs of birds shortly after completion of tutor exposure (mean age per group: live with, P53.2; tutor/CD, P52.8; U0126, P53.1; U0124, P51.3; isolate, P51.4). The groups did not differ significantly ($P = 0.314$) in their similarity to the Y13 song at this early age, suggesting that U0126 did not have a direct adverse effect on vocal production.

Histological analysis of cannula placement and damage

We analyzed nissl-stained sections from each U0126 and U0124 bird to determine cannula placement (Fig. 3). The average medial-lateral position of the cannula tip was 341.5 μm from the midline in the left hemisphere and 338.5 μm from the midline in the right hemisphere. All of the cannula tracks appeared to terminate in the caudomedial nidopallium (NCM) or caudomedial mesopallium (CMM) (Fig. 3b,c). There was no significant difference in the extent of auditory lobule tissue damage between U0126 and U0124 groups when analyzed as area of damage ($P = 0.383$) or as a ratio of damaged area to the auditory lobule area ($P = 0.801$). We checked cannula track positions relative to HVC, a major song nucleus in the nidopallium dorsal and lateral to the auditory lobule. All of the cannula tracks were medial to HVC, and in no case did the cannula tracks intersect with HVC. We cannot be certain that cannulae did not disrupt fibers passing to or from HVC, but as the cannula placements for U0124 and U0126 birds were distributed roughly equivalently, this probably does not account for the difference in song structure between these two groups.

The cannula of one bird, bl68, did not penetrate the auditory lobule, but was inserted into the hyperstriatum dorsal to the auditory lobule, disrupting the structural anatomy of the telencephalon. We did not record the bird's song during recording sessions between P110–120. This may simply be because he did not sing those days during the isolated song recording sessions. It is also possible that his cannula placement interfered with a brain structure or pathway that prevents song production, although this bird did vocalize with calls both during development and as an adult. Because this is only one bird, however, it is not possible to distinguish between a deficit in sensory or motor processes or between a physical (cannula) or molecular (U0126) disruption.

Previous studies have suggested distinct functions for NCM, CMM and other subdivisions of the auditory lobule (for example, see ref. ²⁷). We analyzed the relationship between cannula tip position in the auditory lobule and the song similarity score (Fig. 4). Scores were consistently lower for U0126-treated birds compared with controls at all cannula positions along both rostral and ventral axes. We detected no significant linear relationship between cannula position and song similarity score for either treatment group, in either hemisphere (rostral dimension: U0126 left, $R^2 = 0.051$, $P = 0.628$; U0126 right, $R^2 = 0.479$, $P = 0.085$; U0124 left, $R^2 = 0.009$, $P = 0.826$; U0124 right, $R^2 = 0.001$, $P = 0.943$; ventral dimension: U0126 left, $R^2 = 0.020$, $P = 0.764$; U0126 right, $R^2 = 0.147$, $P = 0.395$; U0124 left, $R^2 = 0.381$, $P = 0.103$; U0124 right, $R^2 = 0.319$, $P = 0.144$).

***zenk in situ* hybridization analysis**

To assess the anatomical spread of U0126 inhibition, we carried out *zenk in situ* hybridization on sections from a separate set of birds injected with U0126 and then exposed to song playbacks. Our results suggested that the radius of U0126 effect extended for 0.5–1 mm around the injection site (compare Fig. 3d and e). Notably, the U0126 injections did not suppress the *zenk* response to song in more distant parts of the auditory lobule (Fig. 3d), suggesting that the birds could hear and process song despite the local disruption of ERK function.

The approximate volume of the auditory lobule is 3.5 mm³; therefore, drug injections probably affected about 15–25% of the auditory lobule. The volumes of NCM and CMM are each approximately 45% of the total auditory lobule volume (field L is approximately 10% of the total volume). Thus, depending on the location of the injection (Fig. 3b,c), different auditory lobule subregions were affected by U0126. It is possible that brain structures adjacent to the auditory lobule were affected by injections; however, medial-lateral cannula tip positions for all U0126 and U0124 birds were very similar, and neither the rostral nor the ventral cannula tip positions showed a simple relationship to song similarity scores (Fig. 4). Therefore, any injection spread outside of the auditory lobule probably did not substantially contribute to the accuracy of tutor song copying.

To test whether the cannula and injections had lasting effects on the ability of the auditory lobule to process song, we played Y13 song to each experimental bird in the *zenk* induction procedure⁸. The presence of high levels of *zenk* mRNA in the auditory lobule after induction would indicate that the cellular and molecular machinery of song processing was not permanently damaged in any group. *zenk in situ* hybridization staining intensity was equivalent across experimental groups in adulthood and greater than in birds experiencing only silence (Supplementary Table 1 online).

Controls for specificity of U0126 effect on song memorization

Three additional experiments were conducted to rule out alternative explanations for the effect of U0126 on accuracy of tutor song copying. One possibility was that U0126 treatment had a detrimental effect on auditory lobule development and function that was unrelated to tutor song memorization. To control for this possibility, we raised six birds as described for the main experiment. These birds (U0126/live with) were given daily U0126 infusions from P40–50 exactly as described for the U0126 group, but they lived with the tutor during the treatment period so that they experienced tutor song at times when the drug was not active. The U0126/live with songs were compared with the Y13 song; this group accurately copied the tutor song (76.0%, 49.1) at levels that were not statistically different from the live with, tutor/CD or U0124 groups ($P \geq 0.531$ for all comparisons). Therefore, U0126 did not significantly reduce the accuracy of tutor song copying if administered at times other than during tutor exposure.

A second possibility was that U0126 treatment disrupted the processing of auditory information, not tutor song memorization. To test for immediate effects of U0126 on auditory processing, we carried out two experiments. In one, we trained birds in an operant procedure and determined whether they could perform a learned song discrimination task under the acute influence of the drug. This was a moderately difficult task; two unfamiliar songs were used to increase task stringency²⁸ and birds required an average of 950 trials (one trial = bird-initiated peck of the middle key) to carry out discrimination at a consistent level^{28,29}. We detected no obvious detrimental effect of U0126 on the performance of this discrimination (Fig. 5). In the other experiment, we measured auditory-evoked potentials (AEPs) in response to song playback at the injection site before and after infusion of U0126. In both birds, we measured robust AEPs of similar overall magnitude and pattern as those that we observed before drug infusion 30 min after drug infusion (Supplementary Fig. 3 online). Thus, U0126 did not appear to grossly interfere with normal auditory processing on either behavioral or neurophysiological levels.

Discussion

Since the initial identification of song control nuclei^{2,30}, songbirds have been widely embraced as models for studying the neural basis of vocal learning; studies of songbird vocal learning can have wider implications for understanding human speech learning³¹. With their large size and discrete boundaries, the song control nuclei make compelling targets for physiological investigation. Functional roles for some song nuclei have now been established in adult song production^{4,32} and developmental song learning^{26,33–35}. However, attempts to locate the tutor song template, the basis for the emergent song structure, in the song control nuclei have been much less rewarding³⁶.

On the basis of evidence from auditory perception studies in adult songbirds^{5–13}, we hypothesized that molecular processing in the auditory forebrain would be necessary for tutor song memorization in juveniles. Specifically, our experiment focused on the ERK signaling cascade in auditory lobule. The ERK pathway is essential for learning and memory formation in a number of model systems^{14,15,17}, and changes in ERK and *zenk* activities have been correlated with adult song-recognition learning in adult zebra finches^{5,7,8,37}. The auditory lobule is a brain area that has no known direct role in motor behavior. It is not considered part of the traditional song system, but its two main components, NCM and CMM³⁸, are highly specialized to process complex sounds, especially conspecific birdsongs (for example, see refs. 6–8,12,13,39). We used a well-established pharmacological inhibitor of ERK activation to achieve a transient, reversible disruption of memory processes in the auditory lobule. Our experiment targeted the sensory processes of developmental song learning by manipulating the auditory lobule only during tutor exposure and before the young birds had developed their own songs. Using adult song structure as a read-out of the fidelity of tutor song memorization, our results establish that ERK signaling in the auditory lobule is required for tutor song memorization. Although the ERK signaling cascade is surely not the only one involved in developmental song learning, our study demonstrates that it is essential for accurate tutor song copying, and, to our knowledge, this is the first functional demonstration that a brain area outside of the song control system is required for developmental song learning.

We carried out a series of control experiments to exclude alternatives to our conclusion that ERK interference in the auditory lobule selectively disrupted tutor song memorization. First, we considered the possibility that U0126 infusion into the auditory lobule acutely disrupted the ability of the birds to hear and recognize song. Arguing against this, all of the birds in the U0126 group vocalized in response to the tutor's vocalizations during tutor sessions. Three control experiments further excluded a major disruptive effect of U0126 on hearing. First, regions of the auditory lobule surrounding the U0126 injection site showed a *zenk* response to

song stimulation, a response that occurs only when the bird hears a complex auditory stimulus⁸. Second, song playbacks continued to evoke complex auditory potentials at the injection site after infusion of U0126. Third, birds infused with U0126 were still able to perform an operant song discrimination task that they had previously learned. These control experiments were not designed as fine-grained dissections of perceptual discrimination, and, in any case, it is not yet understood how song experience is represented in the auditory lobule^{6,9}. However, across three levels of analysis (molecular, neurophysiological and behavioral), these control experiments all indicated that birds are able to hear and recognize songs after U0126 infusion into the auditory lobule.

Next, we considered the possibility that U0126 infusion in the auditory lobule could have somehow altered the birds' rate of singing during the tutor sessions or later in vocal development and that this difference could affect the accuracy of tutor song copying. Although we did not carry out an exhaustive analysis of singing rates or song structure across development, birds in all groups responded with calls to the other birds during tutor sessions and they all sang and called during adulthood. Also, when measured early in vocal song development, the singing patterns of all groups were equally similar to the tutor song. Notably, the birds in the U0126/live with group produced accurate tutor song copies. Collectively, these behaviors suggest that overt motor deficits did not occur in U0126 birds compared to the other groups and that U0126 injections did not functionally alter the rate of vocal practice.

We also considered the possibility that U0126 treatment could have caused a more general disruption of auditory lobule development and function that is not necessarily linked to tutor song memorization. To test this, we injected young control birds with U0126, but then allowed them to experience the tutor at times when the drug was not active. As adults, these birds produced accurate copies of the tutor song. It is formally possible that U0126 disrupted auditory lobule development in a way that was overcome by extended live with tutor exposure, but not by the tutor/CD experience. However, there is no evidence that the live with condition provides stronger tutoring than the tutor/CD condition (Fig. 2), and a previous study demonstrated that more tutor exposure does not increase the extent of tutor song copying²².

Finally, we considered the possibility that tutoring would be less effective in birds that had just been handled and infused (for example, see refs. ^{40,41}), especially given evidence that restraint and isolation⁴² and stimulus context³⁷ can have major effects on the normal molecular response to hearing song in adult birds. To control for this, we included a group that was infused on the same schedule as the U0126 birds but was administered U0124, an inactive compound that is structurally similar to U0126. The U0124-treated birds produced songs that were as similar to the tutor song as those produced by unmanipulated birds exposed to the tutor. Therefore, cannula insertion, stress of handling and drug injection cannot account for the deficits in U0126 bird's tutor song copying.

Both U0126 and isolate groups produced songs that were equally poor copies of tutor song as assessed by quantitative song analysis, but direct comparison of U0126 and isolate songs suggested that they were also different from each other. It may be that U0126 birds acquired some limited information from tutor experience that was not fully captured by the analysis algorithm. For example, five of the seven U0126 birds had an element that by eye is similar to syllable E in the tutor song (Fig. 2a and Supplementary Fig. 2). Perhaps the U0126 birds formed a partial tutor song memory because U0126 infusions did not spread throughout the entire auditory lobule. In addition, the half-life of the U0126 injections was 2 h (Supplementary Methods), permitting some ERK signaling recovery as the tutor sessions progressed. Furthermore, it may be that, unlike the isolate birds, the U0126 birds gained nonauditory information from their social interactions during tutor sessions that could have influenced the development of U0126 songs by, for example, demonstrating body position and rhythm during

singing. To further investigate these mechanisms, it would be interesting to test whether reversible deafening during tutoring (for example, lidocaine infusion into Field L) has the same effect on song development as social isolation.

The auditory lobule is an intricate neuroanatomical structure³⁸, and distinct functions have been ascribed to subregions on the basis of molecular and physiological responses to tutor and other songs^{10–13,27}. Our analysis did not reveal a substantial effect of cannula placement on tutor song copying, but it is possible that future experiments injecting smaller amounts of U0126 into specific auditory lobule subregions would uncover an effect of spatial location on tutor song memorization. The current results, however, are not entirely surprising, given the extensive interconnections between auditory lobule subregions³⁸, and are consistent with an earlier study that showed that localized inhibition of caspase-3 activity in the auditory lobule blocks song-specific *zenk* response habituation across the extent of auditory lobule⁴³.

Our results demonstrate that memory-related functions in the auditory lobule are required for tutor song memorization, but the auditory lobule is clearly not the only brain area involved in developmental song learning. Previous studies of song development focused on the nuclei of the traditional song control system^{26,33–35}, a neural circuit that seems to have evolved concomitantly with the ability of birds to produce learned vocalizations⁴⁴. These studies showed that two major song nuclei, area X and the lateral magnocellular nucleus of the anterior medial nidopallium (LMAN), must be intact for a normal song to develop, but are not immediately required for song production in adulthood^{33,34}. Furthermore, disruption of NMDA receptors²⁶ or *FoxP2* gene expression³⁵ in these areas causes deficits in tutor song copying. Thus, complete developmental song learning must rely on the coordinated functions of several brain areas.

How might the auditory lobule and the song control circuit work together during developmental song learning? On the basis of our current results, we propose that the tutor song is initially memorized via activities in the auditory lobule during sensory learning. The representation in the auditory lobule is sensory^{5–13,27}, but it can influence nuclei controlling song production via established efferent projections from the auditory lobule to the song system³⁸. Integration of tutor song memories and the bird's own vocal performance is probably mediated by the basal ganglia feedback pathway, leading through area X and LMAN³. These nuclei are necessary for developmental song learning^{33,34} and their neurophysiological activities can influence motor output^{3,45–48}; they are less clearly influenced by auditory feedback^{47,49}. It may be that a representation of the tutor song passes from the auditory lobule to the area X and LMAN pathway, where it is required for the subsequent integration of sensory and motor information during song rehearsal. Without this tutor song representation, the sensorimotor error correction mechanisms necessary for accurate tutor song copying are deficient. Therefore, disruptions of either the auditory lobule or area X and LMAN^{26,33–35} during tutoring prevent the development of an accurate copy of the tutor's song.

Our study shows that the ERK function in the auditory lobule is required for the complex process of developmental song learning. To fully understand developmental song learning, it will be necessary to investigate brain areas outside the evolutionarily unique song control nuclei⁴⁴ and to determine how their functions are coordinated with those of the song system. Although this may seem to further complicate what was once considered a 'simple' model of neural circuit design, it may be essential for uncovering how important species-specific behaviors emerge from multiple interacting systems in the brain.

Methods

All procedures involving animals were approved by the University of Illinois, Urbana-Champaign Institutional Animal Care and Use Committee.

Rearing

Males hatched in the Beckman Institute Animal Facility breeding colony were removed from their parental cages 1 d after fledging between P20–23 to prevent them learning the aviary songs. Birds were moved into a room where no songs were audible and housed with an adult foster female and one or two age-matched foster siblings to replicate a relatively normal social-rearing situation. By P35, young males were placed with a single female and housed in an acoustic chamber until song crystallization (P110–120) to prevent uncontrolled exposure to other male songs (more details in the Supplementary Methods).

Tutor/CD sessions

Tutor/CD sessions were designed to provide sufficient song exposure for template formation in the 2-h half-life of the active drug used (below), balance live tutor exposure with similar song bout quality and number, and standardize the tutor song to ensure that experimental birds' song variation stemmed from unique templates and singing ability, not tutor song structure. Therefore, a single tutor, Y13, was used for all tutor sessions. A CD was constructed with a total of 65 bouts (243 motifs) of Y13 song that were randomly distributed over 1.5 h, and experimental birds experienced a combination of live and pre-recorded Y13 song throughout the tutor/CD sessions (see more details in the Supplementary Methods).

Behavioral groups

Five experimental groups were included ($n = 8$ per group). All birds were P40–50 when exposed to the tutor (Supplementary Methods). The main experimental group (U0126) had a bilateral cannula implanted into the auditory lobule (see below) and active drug infusions before all tutor/CD sessions. The other four groups controlled for all aspects of the tutor experience to determine the levels of tutor song copying that could occur with the sessions and the experience of being stressed before the tutor sessions, which could have altered the accuracy of tutor song memorization (for examples, see refs. 40,41). The tutor/CD group received tutor exposure only in the form of tutor/CD sessions and did not have a cannula or drug administration. This group controlled for the efficacy of tutor/CD sessions. The U0124 group had a bilateral cannula implanted into the auditory lobule and were given infusions of an inactive molecule, U0124, before all tutor/CD sessions. This group controlled for the stress of catching, handling and injection that was also experienced by the U0126 group, factors which could modify the extent of learning during the tutor sessions. The live with group resided with the tutor from P40–50 to control for song learning at the age of tutor exposure after prior developmental song isolation. Lastly, the isolate group did not receive tutor exposure to control for the extent of similarity between two zebra finch songs that might arise from chance.

Surgery and drug infusions

At P38, birds in the U0124 and U0126 groups had bilateral cannulae with an intercannula distance of 1 mm surgically implanted into the auditory lobule, as described previously in adult zebra finches⁵ (coordinates, 70 μ m anterior to Y_0 (the anterior-most boundary of the cerebellum at midline), 50 μ m lateral to midline, 35° head angle), with isoflurane anesthesia. Birds awoke 5–15 min postsurgery and resumed normal activity.

The structure of U0126 makes its action highly specific¹⁶ for blocking both active and inactive MEK without inhibiting other protein kinases. MEK is the only known activator of ERK, which

controls *zenk* transcription. Because there is no indication of off-target and toxicity effects of U0126 in other animals and zebra finches (below and Supplementary Methods), we injected U0124, an inactive molecule that is structurally similar to U0126, under conditions that were identical to those of the U0126 group to control for the impact of the injection and handling stress that was experienced just before the tutor learning experience (for examples, see refs. 40,41).

Birds in the U0126 and U0124 groups were infused with 0.5 μ l of U0126 or U0124 (20 μ g μ l⁻¹, Calbiochem), respectively, into both sides of the cannula 30 min before the start of tutor/CD sessions. Both U0124 and U0126 were dissolved in DMSO. After every injection, a sharp noise was made outside of the visual field of the juvenile males to ensure that the birds reacted to the sound and had therefore not been deafened.

Song recording and analysis

To record adult songs, we placed individual experimental males into an acoustic chamber with a microphone and recorded undirected songs into Sound Analysis Pro (http://ofer.sci.cuny.cuny.edu/html/sound_analysis.html). Crystallized song bouts from P110–120 were analyzed in Sound Analysis Pro for similarity to Y13's song, using both individual sound elements and more global song measures (Supplementary Methods). The average of six scores for each bird ($n = 8$, $n = 7$ for U0126) was used for two-tailed one-way ANOVA analysis (data met $F_{\max}$ criteria for homogeneity of variance, $\alpha = 0.05$) with Tukey *post hoc* analysis (SPSS). In addition to adult songs, songs from each bird at ~P50 were compared with Y13 song with Sound Analysis Pro to evaluate potential early differences in song similarity scores between the groups.

To determine whether U0126 songs were different from isolate songs, we compared song samples from all birds in the U0126 group pairwise with songs from all birds in the isolate group using Sound Analysis Pro (Supplementary Methods).

Histological analysis

For U0126 and U0124 birds, we stained sections spanning approximately 1 mm lateral to midline in both hemispheres with cresyl violet acetate to identify cannula placement. The rostral-caudal and dorsal-ventral (that is, depth) of the cannula tip was noted (Supplementary Methods). Linear curve fit analysis (SPSS) was carried out on the rostral and ventral cannula positions and song scores assuming independent, constant and normally distributed error to test whether there was a simple relationship between injection site and song similarity score. This analysis would reveal whether there was an effect of, for example, NCM versus CMM injections, or a marked reduction in tutor song copying with cannula placements near field L.

To quantitate the extent of damage resulting from cannula insertion in U0124 and U0126 birds, we identified all of the sections containing a visible cannula track in cresyl violet-stained sections. The area of the cannula track, representing the residual tissue damage of the cannula, and the total auditory lobule area were measured on each section (Image J 1.38x, US National Institutes of Health). We statistically analyzed the areas of damage in U0126 and U0124 birds by one-way ANOVA (SPSS) (Supplementary Methods).

zenk induction and *in situ* hybridization

Acute U0126 injections were carried out in another set of zebra finches to ascertain an effective U0126 dose and its half-life in zebra finch auditory lobule (Supplementary Methods). Results showed that the half-life of U0126 in zebra finch auditory lobule was 2 h, dictating the 30-min postinjection recovery time and 1.5 h tutor/CD sessions that we used in the main experiment.

When all experimental birds were over P150, their brains were processed for *zenk in situ* hybridization to ensure that long-term *zenk* induction and auditory lobule neuroanatomy was unaffected. *zenk in situ* hybridization was carried out on sections throughout the entire auditory lobule in the right hemisphere for all birds, as previously described¹⁸ (Supplementary Methods).

***In situ* hybridization analysis**

In situ hybridization sections were digitally captured with an Epson Perfection V750 scanner at 1,000 dpi (Epson). Sections of roughly equivalent medial-lateral positions of each bird were grouped and the hybridization intensity of the auditory lobule was measured with ImagePro Plus (MediaCybernetics; Supplementary Methods). One-way ANOVA (SPSS; data met $F_{\max}$ criteria for homogeneity of variance; $n = 8$, $n = 7$ for U0126, $n = 6$ for silent; $\alpha = 0.05$) was carried out first to test that all experimental groups had greater *zenk*-staining intensity than the silent control group, and then to analyze for differences in hybridization intensity across experimental groups.

Controls for specific effect of U0126 on song memorization

To test for the possibility that the U0126 administration and blockade of ERK activation had a detrimental effect on auditory lobule development and function unrelated to tutor song memorization, we reared an additional six birds as described for the birds in the main experiment. These birds were implanted with cannula at P38 and injected with U0126 from P40–50 as described above for the U0126 group. In contrast to the main U0126 group, however, the birds in this group (U0126/live with) resided with the tutor from P40–50 so that they experienced both the effects of U0126 and tutor exposure sufficient for accurate tutor song copying. The adult songs of U0126/live with birds were quantitatively compared with Y13's song, and their song similarity scores and percent syllables copied were compared with those of the live with, tutor/CD and U0124 groups in a one-way ANOVA (SPSS) to determine whether U0126 had detrimental effects on tutor song copying that were independent from tutor song memorization.

To test for the possibility that ERK blockade via U0126 administration interfered with normal auditory processing and not song memorization, we trained adult males ($n = 3$) in an operant song discrimination task and tested them in the presence of U0126. The goal of the two-alternative choice song-discrimination task was to determine whether U0126 had acute effects on auditory processing that could explain the large impact of U0126 treatment on the accuracy of tutor song copying; the task performed was of moderate difficulty (Supplementary Methods). Once the birds performed at the same level of song-discrimination accuracy two mornings in a row (9–10 d; training done all day on 13:11-h light:dark cycle), they were implanted with bilateral cannulae into the auditory lobule as described above. Birds were allowed to recover for 2–3 d, during which food was available *ad libitum*, and then they began retraining until they reached presurgery performance levels (2–3 d). Once they were at presurgery performance levels, birds were bilaterally injected with either 0.5 μ l of U0126 or U0124 (20 μ g μ l⁻¹) and tested with the song-discrimination task. The next day, birds were injected with the molecule that was not administered on the previous day (Supplementary Methods). The percentage of correct key pecks for the first 1.5 h after re-entry into the chamber after injections was compared with the percentage of correct key pecks for the same period of the morning of the first day of song-discrimination training (to show that poor or random performance is detectable), the day before surgery (to show high levels of performance before any brain manipulations) and the day before injections began (to show noninjection performance levels after cannula implantation).

As additional confirmation that auditory lobule cells were able to maintain a complex response to hearing song after drug infusion, AEPs from adult birds ($n = 2$) were recorded both before and 30 min after U0126 infusion into the auditory lobule (Supplementary Methods). Two unfamiliar zebra finch songs were used in the recording session: one to identify a recording site and the other to record the AEPs before and after U0126 infusion. The second song was played once every 5 s for 20 repetitions to establish the preinfusion auditory response. Then U0126 was infused as described above. The postinfusion AEP recording was initiated 30 min after the injection, the delay between drug infusion and tutor sessions experienced by the birds in the main experiment. Recordings were amplified (Dagan 2400 Extracellular Preamplifier), bandpass filtered (Krohn-Hite Model 3700) and extracted with BrainWare (Tucker Davis Technologies). AEPs were processed in Matlab (Supplementary Methods).

Statistical analysis

All statistical analysis was carried out with SPSS (SPSS) after checking that data conformed to the assumptions for either one-way ANOVA or linear-fit analysis. All tests had $\alpha = 0.05$ and one-way ANOVA was two tailed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental timeline. Birds were deprived of nontutor song starting P20–23. All tutor song exposure occurred from P40–50 and injection groups were surgically implanted with bilateral cannula at P38. Groups controlled for the age of tutor exposure (live with), limited Y13/CD tutor sessions (tutor/CD), cannula surgery, handling stress and injection (U0124), and innate zebra finch song structure (isolate). The main experimental group (U0126) was injected with a MEK inhibitor before tutor sessions. Adult songs were recorded between P110–120 for tutor song similarity analysis.

Figure 2. Quantitative song analysis results. **(a)** Representative spectral derivatives of each group. A capital letter marks each unique tutor (Y13) song syllable; the same letter marks similar syllables in experimental songs. Letter primes identify syllables similar to, but not the same as, the corresponding syllable in the tutor song. **(b)** Bar graph shows the mean song similarity scores and percent syllables copied for each experimental group $\pm$ s.e.m. ($n = 8$, $n = 7$ for U0126). Open circles indicate song similarity scores and percent syllables copied for individual birds. Asterisk indicates that live with, tutor/CD and U0124 groups had scores significantly different than the U0126 and isolate groups ($P < 0.008$).

Figure 3.

Auditory lobule cannula placements and *zenk in situ* hybridization. **(a)** Representative nissl-stained sagittal section showing the location of cannula placement. Arrows are to the left of the cannula track into NCM. **(b,c)** Schematic of auditory lobule anatomy showing NCM, CMM, control area hippocampus (HP) and cerebellum (C). Open circles represent U0126 birds and filled circles represent U0124 birds. Dashed lines outline the position of field L at the level of the auditory lobule shown. The positions of cannula tips in left hemisphere **(b)** and the positions of cannula tips in right hemisphere **(c)** are shown. **(d)** *zenk in situ* hybridization after U0126 injection into the auditory lobule showing only partial disruption of *zenk* transcription. Asterisk denotes injection position. **(e)** *zenk in situ* hybridization in noninjected auditory lobule to show normal distribution of *zenk* induction. Scale bar represents 1 mm.

Figure 4.

Plots of song similarity scores and position of cannula tips. **(a)** Song similarity score and the rostral cannula tip position (measured from the caudal-most boundary of the auditory lobule) for all U0126 and U0124 birds. **(b)** Song similarity score and the ventral cannula tip position (measured from the dorsal-most boundary of the auditory lobule). In both panels, U0126 birds are represented with circles and U0124 birds with squares. The left hemisphere is represented with open symbols and the right hemisphere with filled symbols.

Figure 5.

Test of U0126 effects on song discrimination. Graph shows the percentage of correct pecks after song playback in a song discrimination task. PI, performance the day before the first injection; PS, performance before cannula-implantation surgery; T1, performance on the first day of training (low, chance levels of song discrimination accuracy); U0124, performance after U0124 infusion into the auditory lobule; U0126, performance after U0126 infusion into the auditory lobule. Bars are the mean performance levels and symbols indicate individual birds.

Table 1

Results of multiple song similarity measures

Song measure	Experimental group			Isolate
	Live with	Tutor/CD	U0124	U0126
Similarity score	50.43 ± 4.40	47.09 ± 4.53	44.81 ± 2.42	21.73 ± 4.63
Percent similarity	78.19 ± 5.81	80.21 ± 4.25	74.33 ± 1.86	39.00 ± 9.71
Mean accuracy	75.56 ± 1.54	73.54 ± 1.48	74.14 ± 0.79	55.09 ± 10.05
Sequential match	69.76 ± 7.78	58.34 ± 6.38	63.66 ± 4.40	53.28 ± 10.22
Distribution similarity	71.50 ± 5.88	68.00 ± 8.97	61.88 ± 8.01	12.85 ± 2.86
K-S statistic	214.88 ± 18.31	269.00 ± 39.68	231.63 ± 16.54	316.86 ± 33.88
Pitch	219.00 ± 40.25	262.00 ± 37.37	276.88 ± 44.68	269.00 ± 58.63
Frequency modulation	109.75 ± 16.66	149.25 ± 28.27	119.88 ± 19.63	219.29 ± 16.79
Entropy	217.50 ± 41.21	210.25 ± 54.45	234.88 ± 33.47	312.00 ± 58.41
Amplitude	409.13 ± 30.02	586.63 ± 81.57	442.50 ± 71.06	483.00 ± 114.23
Mean frequency	182.00 ± 39.07	226.38 ± 52.52	180.25 ± 26.71	350.43 ± 30.58
Goodness of pitch	149.88 ± 17.33	177.75 ± 23.57	135.38 ± 26.30	266.43 ± 39.84
Motif length	591.88 ± 26.62	646.00 ± 52.06	642.38 ± 65.88	421.57 ± 101.00

Means and s.e.m. for comparisons of tutor versus experimental bird song characteristics on multiple song measures ($n = 8$, $n = 7$ for U0126).

Table 2
Statistical analysis of multiple song similarity measures

Song measure	Experimental group			
	Post hoc comparison to U0126			
	Main effect	Live with	Tutor/CD	U0124 Isolate
Similarity score	<0.001*	<0.001*	0.002*	0.005*
Percent similarity	<0.001*	0.003*	0.002*	0.008*
Mean accuracy	0.004	0.115	0.185	0.162
Sequential match	0.589	0.606	0.992	0.892
Distribution similarity	<0.001*	<0.001*	<0.001*	<0.001*
K-S statistic	0.026	0.114	0.766	0.248
Pitch	<0.001	0.106	0.352	0.100
Frequency modulation	0.002*	0.023*	0.266	0.047*
Entropy	0.267	0.752	0.699	0.863
Amplitude	0.253	0.955	0.862	0.995
Mean frequency	0.819	0.932	1.000	1.000
Goodness of pitch	0.026	0.147	0.386	0.080
Motif length	0.087	0.449	0.191	0.204
				0.953
				0.944
				0.951
				1.000
				0.999
				0.999
				0.514
				0.999
				0.985
				0.770
				0.997
				1.000
				0.994

One-way ANOVA results of song measures from tutor versus experimental bird song comparisons across experimental groups ($n = 8$, $n = 7$ for U0126). Left column lists main effect P values for each song measure; asterisks denote effects that contain significant differences between U0126 and other groups. Right columns list the *post hoc* Tukey P values for each group compared with the U0126 group; asterisks denote comparisons that are significantly different from U0126.