Bidirectional manipulation of mTOR signaling disrupts socially mediated vocal learning in juvenile songbirds

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Early life experiences can have long-lasting behavioral consequences because they are encoded when the brain is most malleable. The mechanistic target of rapamycin (mTOR) signaling cascade modulates experience-dependent synaptic plasticity, among other processes. mTOR has been almost exclusively examined in adult rodent learning models, but may be especially important in organizing neural circuits required for developmental acquisition of meaningful complex behaviors. It is among the most commonly implicated factors in neurodevelopmental autism spectrum disorders (ASD), characterized, in part, by distinct social and communication phenotypes. Here, we investigated mTOR in juvenile zebra finch songbirds. Much as children learn language, young male zebra finches need to interact socially with an adult tutor to learn a meaningful song. The memory of the tutor’s song structure guides the juvenile’s own song, which it uses to communicate for the rest of its life. We hypothesized that mTOR is required for juveniles to learn song. To this end, we first discovered that hearing song activates mTOR signaling in a brain area required for tutor song memorization in males old enough to copy song but not in younger males or females, who cannot sing. We then showed that both inhibition and constitutive activation of mTOR during tutor experiences significantly diminished tutor song copying. Finally, we found that constitutive mTOR activation lowered a behavioral measure of the juvenile’s social engagement during tutor experiences, mirroring the relationship in humans. These studies therefore advance understanding about the effects of experience in the context of neurodevelopmental disorders and typical neural development.

Learned behavior depends on mechanisms that reconfigure synaptic connections in response to experience. Neural plasticity is greater during development than in adulthood. Experience-dependent processes that alter synaptic function during development may therefore have particularly robust influences on lasting patterns of learned behavior, and are best studied in models in which effects of age and experience can be parsed.

The mechanistic target of rapamycin (mTOR) signaling cascade is well-positioned to direct experience-dependent synaptic plasticity. As part of two multiprotein complexes, mTOR integrates environmental signals provided by multiple upstream receptor systems. The mTOR complex 1, in particular, affects synaptic function by regulating protein synthesis via its downstream effector proteins eukaryotic initiation factor 4E-binding protein (4EBP1) and S6 kinase (S6K) (1–4) (Fig. L4). Protein synthesis is a key feature of long-term memory formation, and mTOR signaling contributes to learned behavior in a variety of paradigms in adult rodents (1, 5, 6). Some of the strongest evidence that mTOR may also be required developmentally comes from genetic mutations associated with neurodevelopmental disorders.

Mutations in the mTOR cascade are associated with multiple neurodevelopmental disorders and are among the most commonly implicated single-gene contributions to the neurodevelopmental autism spectrum disorders (ASDs); mTOR has been proposed as a shared mechanism across ASD symptoms (7–12). ASDs arise early in life and are characterized by perseverative interests and behaviors, as well as deficits in social interactions and communication (refs. 13–15; https://www.nimh.nih.gov/index.shtml). Behavioral interventions can be effective in ameliorating social and language symptoms, especially when treatment starts early in childhood (16–20). mTOR signaling may therefore encode early life experiences into organizing neural circuits required for meaningful complex behaviors. Surprisingly, little investigation has been done on the behavioral effects of mTOR function in young animals (6, 21–23).

The zebra finch songbird presents a unique opportunity to investigate mTOR signaling in a model for persistent behavioral effects of developmental experience. Juvenile males learn to sing from a “tutor” bird during one developmentally sensitive period [females cannot sing (24–26)]. Social interactions with the tutor during this period promote memorization of the tutor song, which the juvenile uses to guide the patterning of its own song. Tutor song memorization largely determines the structure of the stereotyped song the bird produces for the entirety of its adult life. We hypothesized that mTOR signaling in the auditory forebrain, a region required for tutor song memorization, was regulated during development to influence song learning (27, 28). We first used a song playback paradigm in young males and females to assay how age and experience contribute to mTOR activation upon song exposure, and to examine if mTOR signaling was sex-dependent, because there is a 4:1 bias toward boys in ASD diagnoses. We then bidirectionally manipulated mTOR cascade activation in vivo to test the contribution of experience-dependent mTOR signaling to tutor song copying. We also assessed the juvenile’s social behavior during tutor sessions. Our results are among the first to demonstrate a functional requirement for mTOR signaling during developmental learning of social behavior.

Significance

We investigated the mechanistic target of rapamycin (mTOR) signaling cascade, which is commonly implicated in neurodevelopmental autism spectrum disorders but is almost never studied in juveniles. We demonstrated that experience-dependent activation of mTOR has functional consequences for complex learned behavior using a songbird model that has strong developmental, social, behavioral, neural, and genomic parallels with human vocal communication. Our results highlight the value of investigations that integrate age and experience to understand molecular mechanisms and behavioral outcomes of neurodevelopmental disorders and typical neural development.

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complex behavior, and establish a system for meaningful investigation into mechanisms affecting behavioral outcomes of neurodevelopmental disorders and typical neural organization.

Results

mTOR Machinery Is Present in the Juvenile Zebra Finch Auditory Forebrain. We were not aware of previous reports of mTOR cascade proteins in songbirds. We therefore first established that key components of the complex 1 mTOR signaling cascade—mTOR, ribosomal protein S6K, and the 40S subunit ribosomal protein S6 (S6)—were present in the auditory forebrain of juvenile males and females reared normally (Fig. 1A). Western blots revealed bands of the expected size for each protein (Fig. 1B).

Hearing Song Playbacks Activates mTOR in the Auditory Forebrain of Posthatch Day 30 Males. We then tested if hearing song activated mTOR signaling in juvenile auditory forebrain. We were particularly interested in examining conditions that would inform about the ability to learn from tutor experience. In males, tutor song experience exclusively before posthatch day 30 (P30) does not support tutor song copying, whereas experience with a tutor starting at P30 does (26). Hence, we examined two developmental points, one a week before the onset of demonstrable song copying (P23) and the other at the beginning of demonstrable song copying (P30). Further, because tutor song experience contributes to the end of the sensitive period for tutor song memorization, we were interested in examining if prior song exposure affected cascade responsibility at P30 (29, 30). Additionally, we included males and females because both learn to discriminate songs; sex differences indicate possible mechanisms that support tutor song memorization separately from discrimination. We therefore compared males and females raised normally in the aviaries (P23 and P30) or housed individually with an adult female (i.e., isolated from hearing song) in a sound-attenuating chamber for 1 wk before P30 (P30i).

We assessed mTOR cascade activation after a short (75 s) playback of novel conspecific song (Song). We used phosphorylation of S6 (pS6) as a functional readout of mTOR activation because its kinase (S6K) is itself directly phosphorylated by mTOR complex 1 kinase activity (3, 31, 32) (Fig. 1C). We first verified that this paradigm, initially used for immediate early gene induction in adults, also induces pS6 above Silence in adult auditory forebrain but not in an adjacent region used to control for technical variation in staining (33) (P > 0.52; Fig. 1 C and D and SI Appendix, SI Materials and Methods). We then used this paradigm for the juvenile playback experiment. We calculated a normalized measure of pS6/total S6 cell density (pS6/total S6) for each bird, and compared Song pS6/total S6 densities with pS6/total S6 densities of birds left in Silence. Because pS6 staining was essentially absent from the primary auditory cortex, Field L (Figs. 1C and 2C and SI Appendix, Fig. S1), we quantified normalized pS6 levels in the two secondary auditory processing areas, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM).

Novel conspecific song playbacks significantly induced mTOR signaling in P30 males, but not in females or P23 males (Fig. 2A). In both NCM and CMM, we found a significant main effect of Sex [NCM: F(1,18) = 20.82, P = 0.002; CMM: F(1,18) = 35.16, P = 0.0001], and Age/Rearing Condition [P23, P30, and P30i; NCM: [0.002, 0.0001]. For example, for the adult validation group (male auditory forebrain), NCM contained bands of the expected size for each protein (Fig. 1B).
In Vivo, Bidirectional Manipulation of mTOR Diminishes Tutor Song Copying. Given that hearing song playbacks selectively activates the mTOR cascade in males at an age when tutor experience affects later song structure, we hypothesized that experience-dependent mTOR signaling in the auditory forebrain contributes to tutor song copying. To test this hypothesis, we combined in vivo manipulation of mTOR activation with controlled tutor experiences. We used an established paradigm that supports normal levels of tutor song copying when molecular mechanisms are not disrupted (27) (SI Appendix, SI Materials and Methods). Briefly, juvenile males were removed from the aviary at P21, and were thereafter socially housed with an adult female. The only exposure to song the juveniles experienced was eight daily, 1.5-h tutor sessions starting on P42. Here, we used two tutor males (Tutor A and Tutor B); each juvenile experienced only one (Fig. 3A).

Molecular and cellular data indicate that balanced mTOR signaling is required for function (4). Although mTOR signaling has been activated and inhibited in various adult learning paradigms, we found no studies that directly compared the effect of bidirectional mTOR manipulation within the same design. We therefore infused either SC79, which constitutively activates mTOR signaling, or rapamycin (Rapa), which selectively inhibits mTOR signaling, via a bilateral cannula targeted to the auditory forebrain (Fig. 1D and SI Appendix, SI Materials and Methods). Before combining in vivo mTOR manipulations with the controlled tutor experience paradigm, we needed to (i) verify that drug infused 30 min before each of the 1.5-h tutor sessions would be effective throughout the experience and (ii) consider the effective half-lives of the drugs to schedule the temporally offset infusions for tutor song memorization control (discussed below) appropriately. We found no previous reports of in vivo effective half-lives for either Rapa or SC79, although there is one record of an in vitro Rapa half-life (35). We therefore performed time-course experiments infusing drugs into the auditory forebrain before initiating the tutoring experiment (SI Appendix, SI Materials and Methods). Results indicated that 200 ng/μL SC79 and 1 μg/μL Rapa constitutively activated or inhibited, respectively, mTOR signaling appropriate for our two objectives (Fig. 1D).

We performed quantitative song analysis comparing crystallized songs from experimental birds with their tutor’s song (36) (SI Appendix, SI Materials and Methods). Our three main groups tested the effect of Rapa, SC79, or vehicle (Veh) infusions 30 min before each tutor session. A fourth group included songs from juveniles raised without hearing any tutor song (Isolate), which permits assessment of song similarity between two zebra finch songs that have no relation to each other.

Rapa and SC79 infusions just before tutor sessions significantly lowered the fidelity of tutor song copying [F(6,34) = 19.68;
of syllables is related to the duration of a bout \( [F(1,4) = 0.18, P = 0.7] \), Rapa \([F(1,5) = 1.9, P = 0.22]\), or SC79 \([F(1,4) = 0.22, P = 0.66]\) groups, and Isolate scores did not differ when compared with Tutor A or Tutor B \((P = 0.065)\).

The Rapa, SC79, and Isolate birds sang significantly fewer syllables than the Tutors \([F(6,62) = 5.18, P = 0.0011; \text{Rapa: } P = 0.004; \text{SC79: } P = 0.014; \text{Isolate: } P = 0.0066; \text{Fig. 3C}]\). The number of syllables is related to the duration of a bout \([R^2 = 0.59; F(1,25) = 37.16; P = 2.26E^{-4}]\), and there was also a significant difference in song bout duration \([F(7,33) = 3.42, P = 0.007]\); post hoc tests show Rapa songs are shorter than Tutor songs \((A: P = 0.02; B: P = 0.02)\). We did not detect a significant effect of Drug treatment on Accuracy, a fine-grained \((10 \text{ ms})\) measure of how faithfully each song element that met similarity criteria was reproduced \([F(4,20) = 0.58; P = 0.68; \text{Fig. 3D}]\), or Sequential Match, a measure of the ordering of copied elements \([F(4,20) = 2.49, P = 0.08; \text{Fig. 3E}]\) across tutored groups. We also did not find a significant difference between the songs of our experimental birds and the Tutors on measures of Pitch \([F(5,21) = 1.43, P = 0.25]\), Frequency Modulation \([\text{FM; } F(5,21) = 1.96, P = 0.13]\), or Goodness of pitch \([F(5,21) = 0.61, P = 0.69; \text{SI Appendix, Table S1}]\). There was a significant main effect on Wiener entropy \([F(5,21) = 3.07, P = 0.03]\), but no significant pairwise differences \((\text{SI Appendix, Table S1})\), and a significant main effect of Amplitude Modulation \([\text{AM; } F(5,21) = 3.74, P = 0.01]; \text{Rapa and SC79 birds’ songs had significantly lower AM than the Tutors’} (\text{Rapa: } P = 0.049; \text{SC79: } P = 0.013; \text{SI Appendix, Table S1})\).

**Cannula Analysis.** After completion of behavioral analysis, we verified that all cannula tips were located within the auditory forebrain \((\text{SI Appendix, SI Materials and Methods})\). There were no significant differences in dorsal-ventral [left hemisphere: \(F(4,20) = 0.84, P = 0.52\); right hemisphere: \(F(4,20) = 0.45, P = 0.77]\), rostral-caudal [left hemisphere: \(F(4,20) = 1.14, P = 0.37\); right hemisphere: \(F(4,20) = 2.83, P = 0.052\)], and medial-lateral [left hemisphere: \(F(4,20) = 0.35, P = 0.84\); right hemisphere: \(F(4,20) = 0.64, P = 0.62]\) cannula tip coordinates across groups. The only statistically significant correlation between cannula tip position and Song Similarity score was in the left hemisphere rostral-caudal dimension in the SC79 group \([R^2 = 0.71, \text{SI Figure 4}, P = 0.036; \text{Fig. 3G}]\), although both Rapa and SC79 groups tended to show lower Song Similarity scores with more rostral cannula positions \((\text{Fig. 3G})\).

**Controls for Sensory Song Learning.** We assessed four possible alternative explanations for effects of drug during tutor sessions. One is that Rapa or SC79 prevents auditory perception. To check that the birds could still hear, we performed a sharp noise check that the birds could still hear, we performed a sharp noise test on separate sets of birds to test for effects of Rapa and SC79 in the auditory forebrain at times other than the tutor sessions, when the birds could be rehearsing and therefore performing sensorimotor processing. We infused this independent set of birds with either Rapa or SC79 temporarily offset from the tutor sessions so that drugs were not effective during tutor experiences \((\text{SI Appendix, SI Materials and Methods})\). We measured the extent of tutor song copying as for the other conditions; both control groups were included in the tutor song copying statistics above. Song Similarity score post hoc analysis revealed significant differences between the Rapa and SC79 groups and their respective control groups \((\text{Rapa- CTL: } P = 0.00016; \text{SC79- CTL: } P = 0.0016)\), but no significant differences in the level of tutor song copying between the Veh and the offset temporal control groups \((\text{Rapa- CTL: } P = 0.97; \text{SC79- CTL: } P = 0.99; \text{Fig. 3B})\).

**Increasing mTOR Activation Decreases a Behavioral Measure of Social Engagement.** Increased neural mTOR activity correlates with lower levels of social interactions \((6, 11, 37, 38)\). We therefore predicted that SC79 birds would display diminished social interactions during tutor sessions. Because the juvenile and the Tutor were in separate cages, we quantified the proportion of time that the juveniles spent in the vicinity of the tutor bird’s cage, which we termed the Hot Zone (HZ), and facing the tutor, as a proxy for intent to interact \((\text{SI Appendix, SI Materials and Methods})\). We found a significant main effect of Drug \([F(2,30) = 3.91, P = 0.03\]; \text{Fig. 3H}) on the proportion of time juveniles spent in the HZ facing the tutor’s cage, but not in how long the juveniles were in the HZ but facing away from the tutor’s cage \([F(2,30) = 0.06, P = 0.94; \text{Fig. 3H}]\). Post hoc analysis revealed a significant difference in HZ time facing toward the tutor between Rapa- and SC79-treated birds \((P = 0.009)\); there were no differences between the Veh group and either the Rapa \((P = 0.13)\) or SC79 \((P = 0.39)\) group. Both behaviors were unaffected by Session [facing toward: \(F(2,30) = 0.12, P = 0.88\); facing away: \(F(2,30) = 0.28, P = 0.76\)] or Session * Drug interaction [facing toward: \(F(4,30) = 0.65, P = 0.63\); facing away: \(F(4,30) = 0.40, P = 0.81\)]. Examination of linear correlations between the proportion of time each juvenile spent in the HZ suggested no relationship with the Song Similarity score for Rapa or SC79 birds. In addition, cannula tip positions largely do not affect HZ behaviors; of the 54 possible relationships (3D coordinates, two hemispheres, three drug treatment conditions, and three sessions), only three reached significance.

**Discussion.** Zebra finches are a powerful system in which to examine the long-term neural and behavioral legacies of developmental experiences. The mTOR cascade is implicated in neurodevelopmental disorders that affect complex social and communication behaviors but is little studied in juveniles. We causally established the functional relevance of experience-dependent mTOR cascade activation in the socially mediated learning required for juveniles to acquire song. Our use of temporally restricted, bidirectional, and localized drug administration provided high specificity that emphasizes the importance of precise experience-dependent signaling for behavioral learning.

Inhibition and constitutive activation of mTOR signaling in the auditory forebrain during tutor experiences significantly lowered the fidelity of tutor song copying. Nonlinear relationships between...
levels of signaling factors and learning outcomes do occur, and balanced mTOR signaling is a feature of learning and memory in other models (e.g., refs. 2, 4, 39, 40). For example, diminished learning is observed in rodents after manipulations that either increase or decrease mTOR function, perhaps because the proper complement of proteins required for long-term memory formation cannot be synthesized (e.g., refs. 41, 42). Additionally, Rapa and SC79 affect the number of pS6+ cells in the auditory forebrain. It is possible that high-fidelity tutor song copying requires synaptic remodeling across a select set of cells; modifying the scale of the cellular network activated during tutor experiences may thus disrupt tutor song memorization (43, 44).

Indeed, the drugs do not need to affect the entire auditory forebrain directly to reduce tutor song copying. Rapa and SC79 songs were no more similar to Tutor song than to Isolate song, and like Isolate songs, included fewer syllables than the Tutor songs. Accuracy and Sequential Match scores indicated that the tutor song elements that Rapa and SC79 birds did copy were as faithfully perceived, processed, and produced as in tutored control birds. This finding may reflect distinct neural control or preserved functionality in unaffected portions of the auditory forebrain. Just as for humans, deficits in learned vocal communication are meaningful for zebra finches. Simpler songs evoke distinct molecular and behavioral responses in females, and males who produce them are less preferred in mate choice paradigms (45–50).

Social interactions enhance vocal learning in juvenile zebra finches and children (24, 25, 51–53). Although our tutor sessions do not allow physical contact between juveniles and Tutors, they do permit social interaction. During tutor sessions, SC79 birds spent less time in close proximity facing the Tutor than Rapa birds, consistent with correlations between increased mTOR signaling and ASD social and communication difficulties (10, 11, 54). This observation has mechanistic implications, because we do not yet understand how social interactions improve vocal learning. Notably, mTOR influences on social interactions are partially dissociable from the mTOR influences that regulate tutor song copying: Rapa diminishes tutor song copying without altering our measure of social behavior, but SC79 disrupts both behaviors. A combination of molecular and circuit properties likely explains this pattern. For example, Rapa and SC79 may create different cellular perturbations, and invoke compensatory or feedback processes that affect distinct neural networks for each behavior (e.g., refs. 55, 56). Additionally, it may be that the functional relationship between the two behaviors is not serial; perhaps an attentional or motivational component of social engagement is separable from tutor song copying.

Baseline and experience-dependent patterns of gene expression change as the role of the auditory forebrain shifts from juvenile tutor song memorization to adult song recognition learning, indicating that molecular mechanisms can provide insight into function (27, 34, 57, 58). Indeed, song-induced mTOR activation and tutor song memorization appear to emerge at the same age in males, and song playback-induced pS6 levels are twice as high in the CMM compared with NCM, mirroring the trend between rostral cannula position and lower fidelity of tutor song copying (26). Further, we failed to find song induction in juvenile females, which can form discriminatory auditory memories but cannot sing (59, 60). mTOR activation in the auditory forebrain may therefore be a useful marker for broader cellular processes that contribute specifically to tutor song memorization.

Additionally, the mTOR cascade may be among the first to come “online” for encoding tutor experience. ERK is the other signaling cascade known to be required for tutor song memorization (27). ERK and mTOR cascades can intersect, with ERK typically positioned upstream of mTOR (61). It is intriguing to consider that the song-copying effects of ERK were a consequence of disrupted mTOR signaling. However, in P30 males, novel song playbacks induce mTOR activation but not ZENK (zif268, egr-1, ngn1-a, krox24) transcription, a readout of ERK activation (34, 62). Further investigation into age- and experience-dependent regulation of these two molecular cascades will inform about acquisition of complex behaviors.

This study tests the role of mTOR signaling in developmental learning. Our results have immediate relevance to developmental disruptions in sensory processing of social interactions that have persistent consequences for behaviors, such as vocal communication. They also open the possibility that mTOR could regulate other components of developmentally acquired behaviors. For example, production of meaningful song depends on dynamic integration of sensory and motor functions across a distributed neural circuit; mTOR could mediate plasticity in several of these other brain areas. More broadly, knowledge gained here provides a platform for multiple lines of inquiry into the fundamental question of how early life experiences are encoded to affect brain function and behavior.

Materials and Methods

Detailed procedures are provided in SI Appendix, SI Materials and Methods. All procedures were conducted in accordance with the NIH guidelines for the care and use of animals for experimentation, and were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP no. 72220).

Song Playback-Induced mTOR Activation in Juveniles. After ~16 h alone in acoustic chambers, juveniles were either exposed to a 75-s playback of triple song (Song) or left in silence [Silence; n = 4 for all Sex, Age/Rearing (P23, P30, P30i), and playback combinations]. Immediately after playbacks, or within 10 min in the case of the Silence birds, the right hemisphere was fixed in 4% paraformaldehyde in 0.025 M PBS in preparation for pS6 and S6 immunohistochemistry. The left hemisphere was used for another experiment.

Tutor Song Memorization in Juvenile Males. We followed a previously established paradigm (27). On P40, we surgically implanted a bilateral guide cannula into the auditory forebrain of males as previously described (27, 57, 62). Thirty minutes before each 1.5-h tutor session, experimental groups received 0.5-μL bilateral infusions of either Rapa (1 μg/μL; n = 7) or SC79 (200 ng/μL; n = 6) in DMSO. The Veh control group (n = 6) received 0.5-μL infusions of undiluted DMSO as in the study by London and Clayton (27). Drug infusions and tutor sessions were conducted once daily for eight consecutive days, from P42 to P49. All juveniles experienced four tutor sessions in the first 7 h of lights-on (except for the first hour after the lights are on: AM), and four in the second 7 h of lights-on (except for the last hour of the day: PM). Males continued to live with their companion female within a sound-attenuating chamber until their songs were crystallized. We performed several controls (SI Appendix, SI Materials and Methods), including groups that received temporally offset drug [Rapa-Control, SC79-Control (n = 3 per group), final group numbers were determined by statistical power analysis using data acquired from preliminary studies] infusions. Based on results from the time-course experiment, SC79-Control birds were infused 2 h after the completion of an AM tutor session and 2 h before the start of a PM tutor session, and Rapa-Control birds received drug infusions 4 h after the conclusion of each tutoring session. All aspects of housing, tutoring, data acquisition, and analysis were consistent across all experimental groups, except Isolates, which were not exposed to tutor sessions.

Song Similarity Analysis. Experimental birds were recorded every 10 d, beginning at P90, until their songs were crystallized. Acoustic analysis and similarity scoring were conducted using Sound Analysis Pro2011 (SAP2011), excluding songs recorded during the first 3 h after lights-on (36).

Tutor Session Behavioral Scoring. A perch placed ~3 inches from the end of the juvenile’s cage adjacent to the tutor cage delineated an HZ. Reviewers blind to condition used JWatcher (63) to quantify how long the juvenile spent in the HZ either not facing the tutor’s cage or facing the tutor’s cage and not engaged in any other behaviors. We scored the entire 90 min for tutor sessions 1, 4, and 8 (first, middle, and last). Due to technical difficulties, this dataset contains a subset of birds included for the song similarity scoring (n = 3 for Veh and n = 5 each for the Rapa and SC79 groups).
Cannula Placement Analysis, Immunobots, Immunhistochemistry, Imaging, and Quantification. Details are provided in SI Appendix, SI Materials and Methods.

Statistics. StatPlus software (AnalytSoft) was used to run all statistical tests (α = 0.05), including the Student’s t-test, one- and two-way ANOVAs, and linear regressions. In the instance of significant main effects or interactions, statistical analyses were conducted using StatPlus software (AnalystSoft) to run all statistical tests. The significance of differences was determined using the Student’s t-test, one- and two-way ANOVAs, and post hoc Tukey’s honest significant difference test.

Supplementary Information for Ahmadiantehrani & London “Bidirectional manipulation of mTOR signaling disrupts socially-mediated vocal learning in juvenile songbirds”

SI Methods and Materials.

*Experimental animals.* All zebra finches were housed on a 14 hr:10 hr light:dark cycle, with seed and water provided *ad libitum*. The juveniles used in this study hatched in flight aviaries, amongst males and females of all ages. Experimental adult females were raised normally in flight aviaries; to avoid using adults in different breeding or parenting statuses, they were segregated into single-sex cages in rooms housing both sexes at least two weeks prior to experiments.

*Bilateral cannulation surgery.* To neuroanatomically target drug and vehicle infusions, bilateral guide cannula (1mm intercannula distance, cut to 2mm length (cat # C235G-1.0/SPC), Plastics One, Roanoke, VA) were implanted into the auditory forebrain (coordinates 700µm anterior to \( Y_0 \) (the anterior-most midline cerebellar boundary), 500µm lateral to midline, 45° head angle) as previously described (1-3).

*Drug timecourse.* To empirically determine the half-life of SC79 and Rapa in the brain, adult females were surgically implanted with bilateral cannula targeted to auditory forebrain. After several days of recovery, each bird was placed individually into a sound-attenuating chamber, per Mello et al. (4). The next day, after ~16 hr of sound isolation, they were infused and exposed to song playbacks or left in silence. We used a “triple song” playback stimulus, a composite of single bouts from three different birds strung together, repeated twice for a total of 75 sec of song exposure (1, 5). All songs in the triple song stimulus were recorded from birds in Dr. Susan Volman’s laboratory at Ohio State University years ago, thus were unknown to all subjects in this study. The triple song stimulus robustly induces phosphorylation of the
extracellular signal–regulated kinase (ERK) molecular cascade and expression of the immediate early gene ZENK (\textit{zif268, egr-1, ngfi-a, krox24}; (1, 5, 6)). Here, we assessed a normalized measure of phosphorylated S6 (pS6+/S6+ cell density, details described below) in the auditory forebrain. S6 is downstream of mTOR and its requisite kinase (S6K) is a well-studied readout of cascade activation because its phosphorylation in turn requires mTOR activation (7-9).

We included two control conditions. One set of control birds was infused with undiluted DMSO vehicle 30 min prior to sacrifice without experiencing song playbacks (n = 3 adult females). pS6+/S6+ cell density in this “Silence” group indicates baseline, uninduced levels. Birds in the other control condition received undiluted DMSO infusions 2 hr before novel song playbacks (n = 3 females). The pS6+/S6+ cell density in this group indicates high song-induced levels. We anticipated that Rapamycin (Rapa) would reduce experience-dependent phosphorylation of the mTOR cascade (10). To assess this, we infused Rapa (1 µg/µl in DMSO; LC Labs, Woburn, MA) 2, 5, 10, or 15 hr prior to song playbacks (n = 2 adult females at each timepoint). We did not expose birds to song playbacks to test the duration of SC79 effectiveness; SC79 constitutively activates mTOR (11, 12). For the SC79 timecourse, we infused birds with 200 ng/µl of SC79 in DMSO 0.5, 1, 2, or 3 hr before sacrifice (n = 2 adult females for each time point). All infusions were a total volume of 0.5µl, and were performed through a 33 gauge internal cannula at 0.2µl/min (1-3). All brains were prepared for pS6 and S6 immunohistochemical analysis (below).

We also verified that both Rapa and SC79 affected mTOR activation equivalently in juvenile males. We performed the same cannulation and infusion procedures as above in P45 males to capture an age in the middle of the series of tutor sessions. We assayed Silence to control for the possibility of shifting baseline levels of pS6 across development, and the 2 hr timepoint to
confirm drug effectiveness across the duration of the tutor sessions (tutor session details below; n= 1 for Silence + DMSO, n= 2 for Rapa + Song, n = 2 SC79 + Silence).

Novel song playback-induced mTOR activation in juveniles. The evening before song playbacks, juveniles were placed individually into an acoustic chamber. Approximately 16 hr later, birds were either exposed to a single playback of the triple song (Song) or left in silence (Silence; n=4 for all Sex, Age/Rearing (P23, P30, P30i), playback combinations). Immediately after playbacks, or within 10 min in case of the Silence birds, we dropped the right hemisphere into fixative (4% paraformaldehyde in 0.025M phosphate buffered saline; PBS) in preparation for pS6 and S6 immunohistochemistry. The left hemisphere was used for another experiment. We first confirmed that neither pS6+ nor S6+ densities in the hippocampus (HP) changed across experimental conditions, and used this measure to normalize for inter-section staining variability (details below). For baseline (Silence) pS6+/S6+ cell density, we ran a two-way ANOVA to test for significant main effect and interactions between the factors of Sex and Age/Rearing condition. In NCM, we found no significant effects of Sex ($F_{(1,18)}=1.928, p=0.18$), Age/Rearing condition ($F_{(2,18)}=0.849, p=0.44$), or Sex * Age/Rearing condition interaction ($F_{(2,23)}=1.05, p=0.37$). In CMM, there are no effects of Age/Rearing condition ($F_{(2,18)}=0.114, p=0.89$), or the Sex * Age/Rearing condition interaction ($F_{(2,23)}=0.66, p=0.53$). To assess playback-induced pS6+/S6+ cell density (Song/ Silence), we first obtained a Fold-change-over-Silence measure by normalizing each experimental group’s pS6+/S6+ cell density to that of their Sex- and Age/Rearing-matched Silence controls. We then ran a two-way ANOVA to test for significant main effects and interactions between the factors of Sex and Age/Rearing condition.

Tutor song sessions. We followed a previously-established paradigm (3). At P21, juvenile males (females cannot sing) were removed from the aviary and placed with a companion female within a sound attenuating chamber, preventing them from hearing song other than the experimental
tutor bird’s. On P40, we surgically implanted bilateral guide cannula into the auditory forebrain of males as previously described (1-3). 30 min prior to each tutor session, experimental groups received 0.5µl bilateral infusions of either Rapa (1µg/µl; n=7) or SC79 (200ng/µl; n=6) in DMSO. The vehicle control group (Veh; n=6) received 0.5µl infusions of undiluted DMSO. 1.5 hr tutor sessions were set up as in London and Clayton (3). Drug infusions and tutor sessions were conducted once daily for eight consecutive days, from P42-P49. To check that the birds could still hear, we performed a sharp noise outside of the bird’s visual field after infusion and prior to tutor sessions, to ensure that all birds responded to this sound. All juveniles experienced four tutor sessions in the first 7 hr of lights-on (except the first hour after the lights are on; “am”), and four in the second 7 hr of lights on (excepting the last hour of the day; “pm”). All other aspects of housing, tutoring, data acquisition and analysis were conducted in the same manner across experimental groups, except Isolates, who did not experience tutor sessions. For birds that received temporally-offset drug infusions, birds were infused either 2 hr after the completion of an “am” tutor session or 2 hr before the start of a “pm” tutor session (SC79-Ctrl), or 4 hr after the conclusion of each tutoring session (Rapa-Ctrl). Males continued to live with their companion female within a sound attenuating chamber until their songs were crystallized.

**Song similarity analysis.** Experimental birds were recorded once every 10 days, beginning at P90, until their songs were crystallized, when global self-similarity scores (from symmetric analysis of mean values) were greater than 90 over the course of 24 hr and did not change more than 0.5% between recording sessions 10 days apart. Self- and tutor-similarity scoring was conducted using Sound Analysis Pro (SAP2011), excluding songs recorded during the first 3 hr after lights-on (13, 14).

Tutor song similarity (asymmetric analysis of mean values) was analyzed using recordings of crystallized songs. We used the same bout of the appropriate Tutor’s song as the template for
song comparisons. Eight song bouts separated by at least 45 min were analyzed for each experimental bird. The SAP2011 Similarity Score is a combination of the Accuracy (song element-by-element similarity) and Sequential Match (song element order) between two songs. The acoustic features Pitch (Fundamental Frequency-based estimate; (15)), Goodness of Pitch, Wiener entropy, and Amplitude and Frequency Modulations (AM, FM), were used to assess non-structural acoustic similarity. We also included adult songs from a set of Isolates, males raised from P21 through adulthood in an acoustic chamber with a companion female exactly as in our other groups, but with no exposure to a tutor. These birds were raised previously and their songs, recorded at P120, were reanalyzed to specifically compare each Isolate song with Tutor A and Tutor B. We conducted pairwise statistical comparisons on Similarity Score between birds tutored by Tutor A and Tutor B to verify that the individual tutor did not bias the data. One-way ANOVAs were used to ascertain differences in the level of tutor song copying across experimental groups.

_Tutor session behavioral scoring._ We designated a “Hot Zone” (HZ) that was bounded by the perch inside the juvenile’s cage that was closest to the tutor’s cage. In all cages, this perch was placed ~3 inches from the end of the cage. The HZ extended from the perch all the way across the width of the cage, included the space between the perch and the cage wall, and the floor space below it. Reviewers blind to condition used JWatcher (16) to quantify how long the juvenile spent in the HZ either not facing the tutor’s cage, or facing the tutor’s cage and not engaged in any other behaviors, which could indicate willingness to socially interact. We scored the entire 90 min for tutor sessions 1, 4, and 8 (First, Middle and Last). Frequency of calls can be informative of relationships and reproductive status, as well as social interaction, but we could not reliably discriminate between the juvenile and adult calls with our recording setup, so we did not analyze call data (17). We tested for significant main effects and interactions
between the Drug treatment (Veh, Rapa, and SC79) and Session (First, Middle and Last) factors with two-way ANOVA.

_Cannula placement analysis_. For birds from the tutoring experiment, we waited until song crystallization occurred. Birds were intracardially perfused and brains were treated and sectioned as described for immunohistochemistry (below). A series of sections was mounted onto Superfrost Plus slides (Fisher Scientific), dried overnight, and then Nissl-stained. Sections containing cannula tracks were imaged and the medial-lateral, rostral-caudal, and dorsal-ventral positions of the cannula tip were calculated from the midline, or measured from the caudal boundary of the telencephalon or lateral ventricle, respectively (FIJI; (18)). We obtained coordinates for both the right and left hemispheres. We also used these coordinates along with the tutor song similarity scores to run linear regression analysis and determine if there was any correlation between the location of drug infusion and the fidelity of tutor song copying.

_Immunoblots_. The auditory forebrain was bilaterally dissected as described previously (1) then immediately flash frozen in tubes on dry ice, and stored at -80°C until use. Ice-cold RIPA buffer (50mM Tris-HCl, pH 7.4, 5mM EDTA, 120mM NaCl, 1% NP-40, 0.1% deoxycholate, and 0.5% SDS) containing protease inhibitors was added directly to frozen tissues for mechanical homogenization and subsequent sonication. Lysates were allowed to rest on ice for 30 min before protein concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Fifteen µg of protein from each sample was resolved on a 4-20% SDS-PAGE gel (Tris-Glycine, BioRad), and transferred onto a PVDF membrane. To assay multiple proteins from the same biological samples and immunoblot, we cut the membrane horizontally, between the 150 and 100kDa, and between the 50 and 37kDa, molecular weight standards before proceeding with blocking (5% nonfat dry milk (NFDM) in phosphate buffered saline (PBS) containing 0.1% Tween-20; PBST) for 30 min at room temperature, and primary antibody
incubation prepared in 1% NFDM in PBST, performed overnight at 4°C. After three 10 min
washes in PBST, membranes were incubated in HRP-conjugated secondary antibodies (anti-
rabbit IgG, anti-goat IgG, and anti-mouse IgG, all at 1:1000; Vector Laboratories, Burlingame,
CA, USA) for 2 hr at room temperature. Immunoreactivity was detected via an enhanced
chemiluminescent reaction (Pierce ECL, ThermoFisher Scientific, Waltham, MA), and
developed on autoradiography film (Amersham Hyperfilm ECL, GE Healthcare Life Sciences,
Pittsburgh, PA USA).

Primary antibodies used for immunoblotting: rabbit IgG anti-mTOR (1:2000, cat #05-1592; EMD
Millipore, Billerica, MA, USA), rabbit IgG anti-S6K1 (1:2000, cat #2708; Cell Signaling
Technology), goat IgG anti-S6 (1:5000, cat #E-13; Santa Cruz Biotechnology, Santa Cruz, CA,
USA). Mouse IgG anti-NeuN (1:10000, cat #MAB377; EMB Millipore) was used for loading
control on all blots.

**Immunohistochemistry.** Birds were transcardially perfused with ice-cold 0.1M PBS, followed by
4% paraformaldehyde in 0.025M PBS. Brains were dissected and post-fixed overnight at 4°C.
They were then embedded in gelatin (8% in 0.1M PBS) and fixed overnight at 4°C. Gelatin-
embedded brains were cryoprotected first in 15% and then 30% sucrose in 0.1M PBS. Brains
were sectioned into 55µm sagittal sections on a cryostat, in a series of three.

For each protein, we performed immunohistochemistry with all sections from a single series
from midline to ~990µm lateral for each bird to capture the extent of auditory forebrain, using
both hemispheres except for the juvenile playback experiment (above). After permeabilization
with 0.3% Triton-X in 0.1M PBS (30 min), endogenous peroxidases were exhausted with 2%
H₂O₂ in 0.1M PBS containing 0.05% Tween-20 (PBST) for 15 min. After extensive washes in
PBST, sections were blocked with 3% normal serum for 60 min at room temperature. Sections
were incubated with primary antibodies overnight at 4°C, followed by PBST washes and a 60 min room-temperature incubation with biotinylated secondary antibodies. After washing with PBST, sections were incubated in avidin-biotin complex (Vectastain Elite ABC Kit; Vector Laboratories) for 30 min at room temperature. The peroxidase complex was visualized with DAB (Sigma, St. Louis, MO, USA) containing 0.003% H₂O₂ in 0.1M PBS. Sections were then mounted, dehydrated, cleared, and coverslipped with Permount (Fisher Scientific).

Antibodies and serum were as follows. pS6: rabbit anti-pS6 primary antibody (1:500 in 1% NGS-PBST; Cell Signaling Technologies #2211) blocked in normal goat serum (NGS), with a biotinylated goat anti-rabbit IgG secondary (1:500; Vector Laboratories); S6: goat anti-S6 primary antibody (1:2000 in 1% NHS-PBST; Santa Cruz Biotechnology) blocked in normal horse serum (NHS), with a horse anti-goat IgG secondary antibody (1:500; Vector Laboratories).

**Immunohistochemistry imaging and quantification.** To assess the density of phosphorylated S6-positive (pS6+), total S6-positive (S6+) immuno-stained cells, we captured images using the microscopes at the University of Chicago Integrated Light Microscopy Core Facility. For all brain sections, we obtained images that contain the homologous secondary auditory forebrain regions (caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) and primary auditory forebrain (Field L) plus the adjacent hippocampus (HP) with a 4X objective. We used two microscopy systems: a Zeiss Axiovert 200m microscope with a Zeiss Axiocam digital color CCD camera (Carl Zeiss Microscopy, Thornwood, NY, USA) running Slidebook 5.5 software (Intelligent Imaging Innovations, Denver, CO, USA), and an Olympus IX81 microscope (Olympus Corporation of the Americas, Center Valley, PA) with a Hamamatsu Orca Flash 4.0 sCMOS camera (Hamamatsu Photonics, Skokie, IL) running Slidebook 5.0 software (Intelligent Imaging Innovations). For each experiment, all images used for quantification were captured with the same system across proteins.
For all images, we applied a threshold to exclude background staining in FIJI (18). For all immunohistochemistry except the drug timecourse experiment (see below), we acquired particle count data (i.e., positively-stained cells) for NCM, CMM, and HP; we observed very little staining in Field L (Fig. 1C, 2C, and S1). Neuroanatomical landmarks to identify each of these brain regions are visible; specific boundaries used to consistently quantify NCM, CMM, and HP across brain sections and individuals were informed by the Histological Atlas on the Zebra Finch Expression Brain Atlas (ZEBrA, Oregon Health and Science University, Portland, OR, USA: zebrafinchatlas.org). Positively-stained cell counts were divided by the region of interest area to calculate a cell density measure.

The HP does not display immediate early gene induction after song playbacks ((6); but see (19)), and thus can be useful to control for inter-section variation in immunostaining intensity. In both juvenile males and females, we found no significant main effects and no interaction between Age/Rearing condition and Playback on cell densities in the HP. For pS6: Playback (males: F(1,18)=0.03, p=0.87; females: F(1,18)=3.04e-6, p=0.99), Age/Rearing condition (males: F(2,18)=0.68, p=0.52; females: F(2,18)=0.04, p=0.96), and Playback * Age/Rearing condition interaction (males: F(2,23)=0.31, p=0.74; females: F(2,23)=0.09, p=0.91). S6+ cell density also shows no significant effects of Playback (males: F(1,18)=0.08, p=0.78; females: F(1,18)=0.23, p=0.64), Age/Rearing condition (males: F(2,18)=0.63, p=0.54; females: F(2,18)=0.07, p=0.94), or Playback * Age/Rearing interaction (males: F(2,23)=0.06, p=0.94; females: F(2,23)=0.09, p=0.91). We therefore normalized CMM and NCM pS6+ and S6+ cell density measures to that of the HP from the same brain section to account for any technical variation in staining intensity. We calculated the ratio of pS6+/S6+ cell densities for each section and then calculated a mean ratio for each bird. We used this bird average to represent the normalized level of S6 phosphorylation, and thus mTOR cascade activation, in NCM and CMM. For the drug
timecourse experiment, we used this process to quantify pS6+ and S6+ cell densities in the entire auditory forebrain.
SI Figure 1. Representative brightfield images of phosphorylated S6-positive (pS6+) and total S6+ cells in the auditory forebrain of females from each experimental group (P23, P30, P30i). Boxed insets of auditory forebrain are shown at a higher magnification. Dashed oval indicates Field L. Brightness and contrast were adjusted for figure clarity. Scale bars = 500µm (auditory forebrain) and 250µm (insets).
SI Table 1. Analysis of song acoustic features. Acoustic features, including pitch, frequency modulation, Wiener entropy, goodness of pitch, and amplitude modulation, of songs produced by all birds were analyzed using SAP. Middle columns list the mean and SEM (or in the case of the two tutors, standard deviation (s.d.)) for each Group. Main effect F statistics and p-values are reported for each acoustic feature, with significant post-hoc comparisons listed in the Post-hoc column.