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 (*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 and100kDa, 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% H_2O_2 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% H_2O_2 in 0.1M PBS. Sections were then mounted, dehydrated, cleared, and coverslipped with Permount (Fisher Scientific).

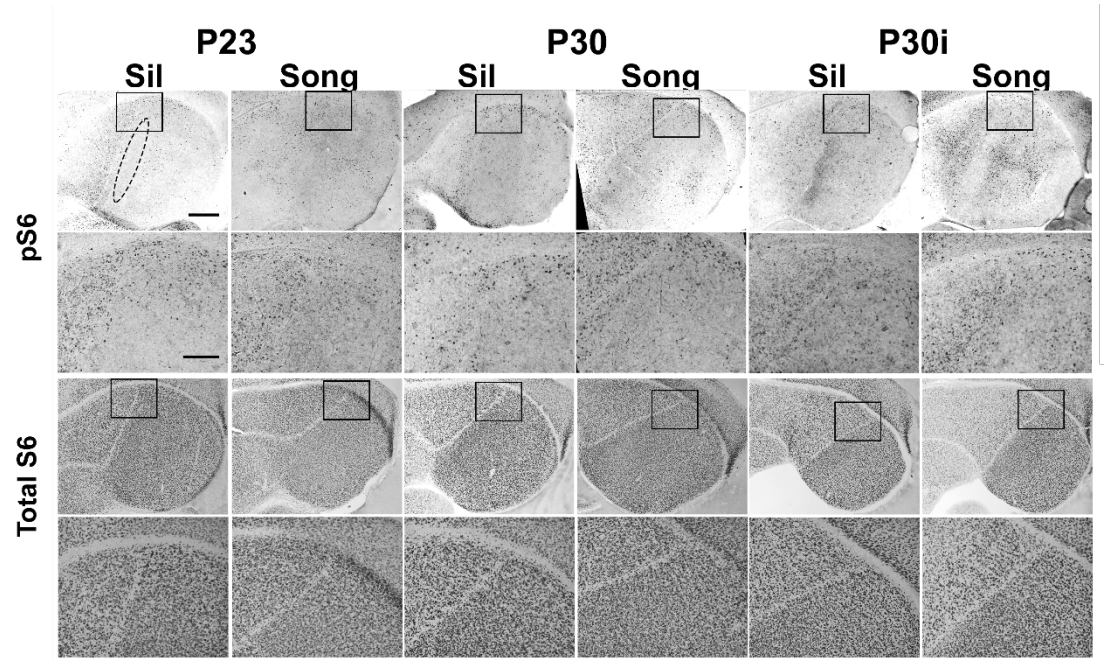
Antibodies and serum were as follows. pS6: rabbit anti-pS6 primary antibody (1:500 in 1% NGS-PBST; Cell Signaling Technologies #2211) blocked in normal goat serum (NGS), with a biotinylated goat anti-rabbit IgG secondary (1:500; Vector Laboratories); S6: goat anti-S6 primary antibody (1:2000 in 1% NHS-PBST;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

	Group	mean	s.e.m.	F(5,21)	p-value	Post-hoc
Pitch	Tutors	3576.50	413.66 (s.d.)	1.43	0.93	
	Veh	3435.90	146.95			
	Rapa	3103.64	476.64			
	SC79	3325.66	208.44			
	Rapa-Ctrl	3355.29	76.42			
	SC79-Ctrl	3675.31	24.24			
Frequency Modulation	Tutors	42.99	3.15 (s.d.)	1.96	0.13	
	Veh	38.80	1.68			
	Rapa	33.51	2.42			
	SC79	32.52	2.52			
	Rapa-Ctrl	41.19	2.12			
	SC79-Ctrl	28.27	7.43			
Wiener Entropy	Tutors	-5.45	0.19 (s.d.)	3.07	*0.02*	
	Veh	-4.21	0.37			
	Rapa	-4.64	0.16			
	SC79	-4.82	0.17			
	Rapa-Ctrl	-3.89	0.02			
	SC79-Ctrl	-4.13	0.25			
Goodness of Pitch	Tutors	70.53	4.50 (s.d.)	0.61	0.69	
	Veh	73.62	12.33			
	Rapa	87.79	18.73			
	SC79	59.96	7.43			
	Rapa-Ctrl	76.55	10.28			
	SC79-Ctrl	53.02	11.25			
Amplitude Modulation	Tutors	-0.02	0.003 (s.d.)	3.74	*0.01*	Rapa vs Tutors SC79 vs Tutors
	Veh	-0.01	0.001			
	Rapa	-0.01	0.002			
	SC79	-0.01	0.001			
	Rapa-Ctrl	-0.01	0.002			
	SC79-Ctrl	-0.01	0.004			

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.

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