Supplemental Information

Diminished FoxP2 levels affect dopaminergic modulation of corticostriatal signaling important to song variability

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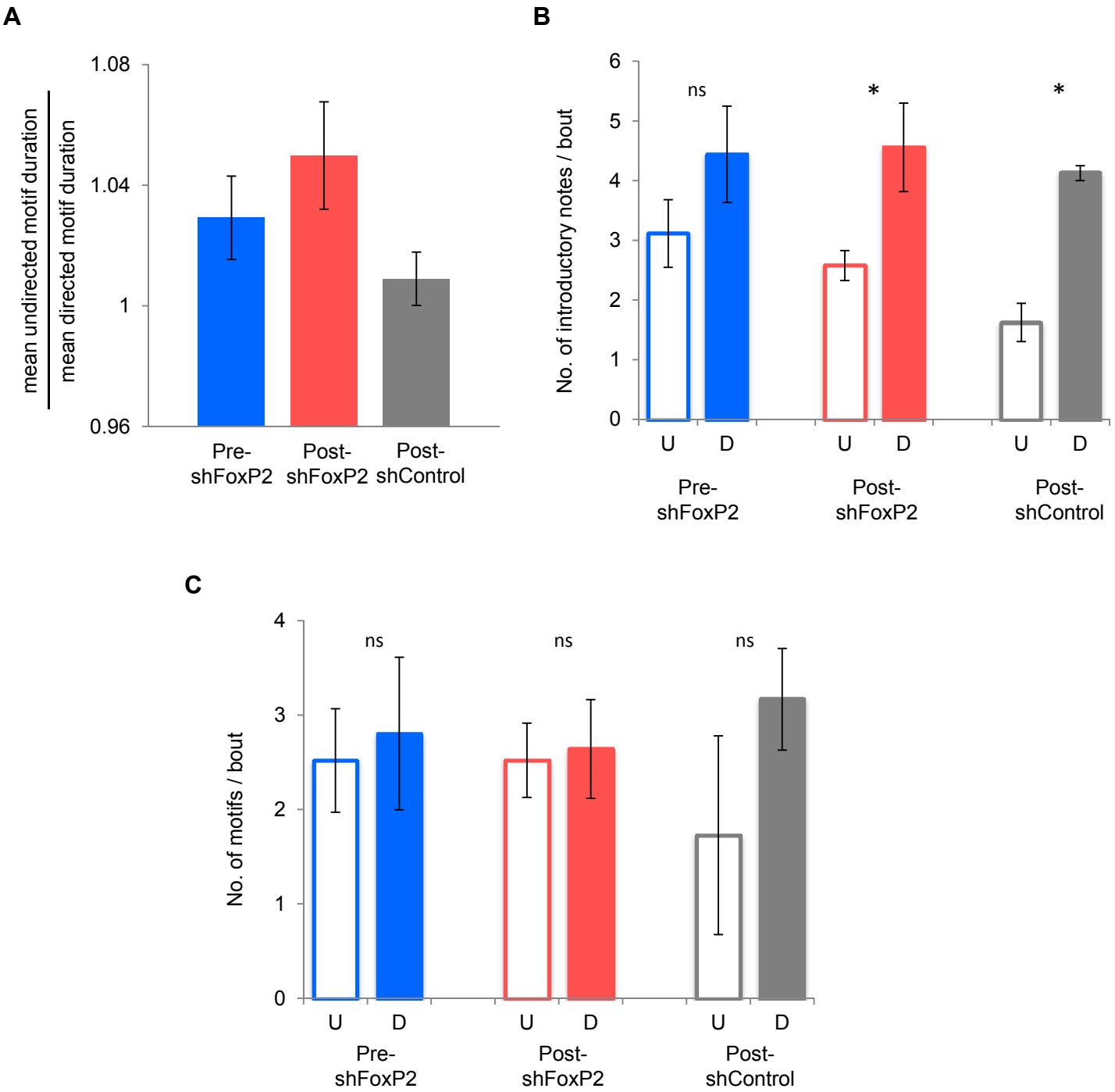

Figure S1, related to Figure 2

Murugan et al.

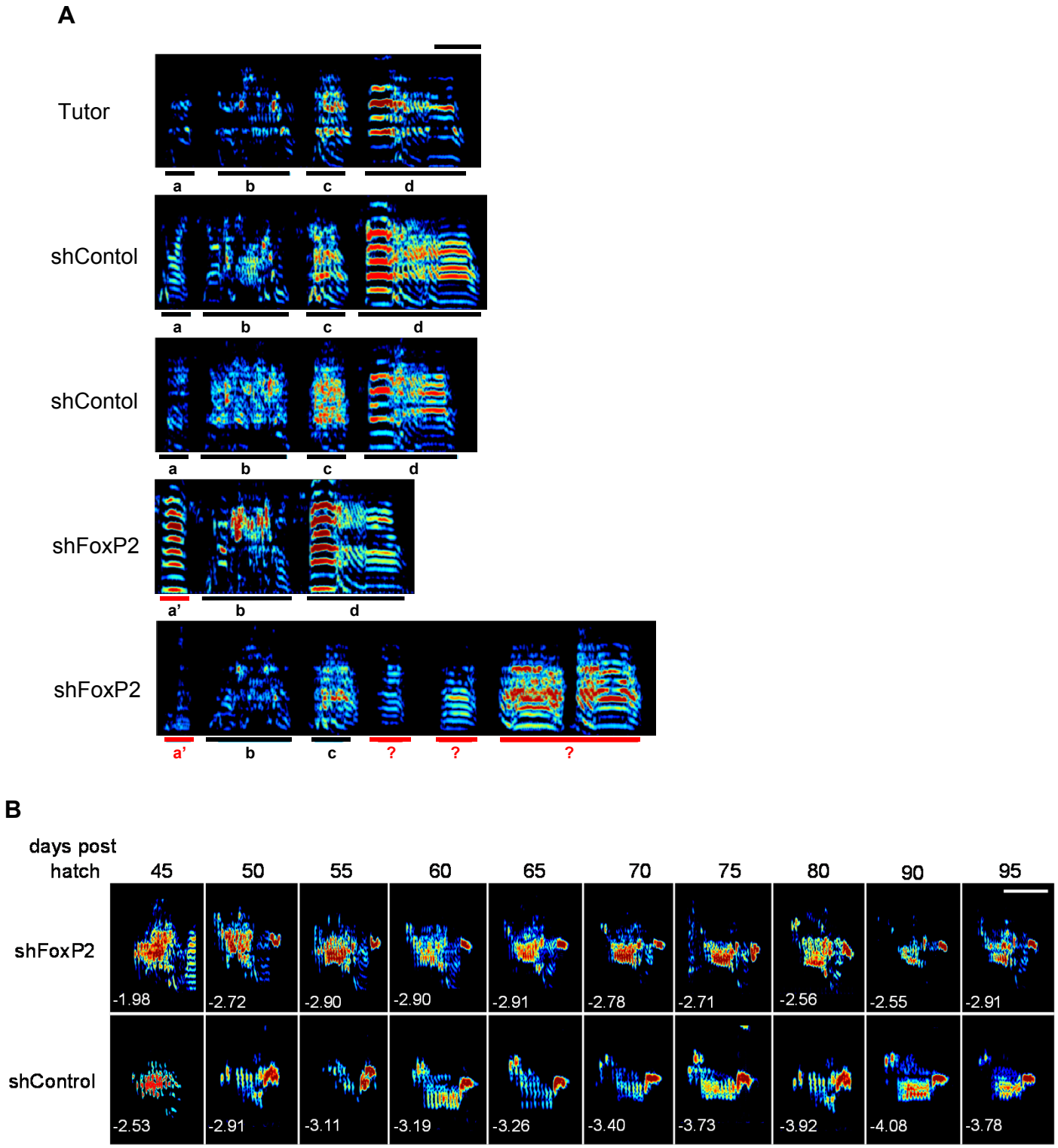

Figure S2, related to Figure 3

Murugan et al.

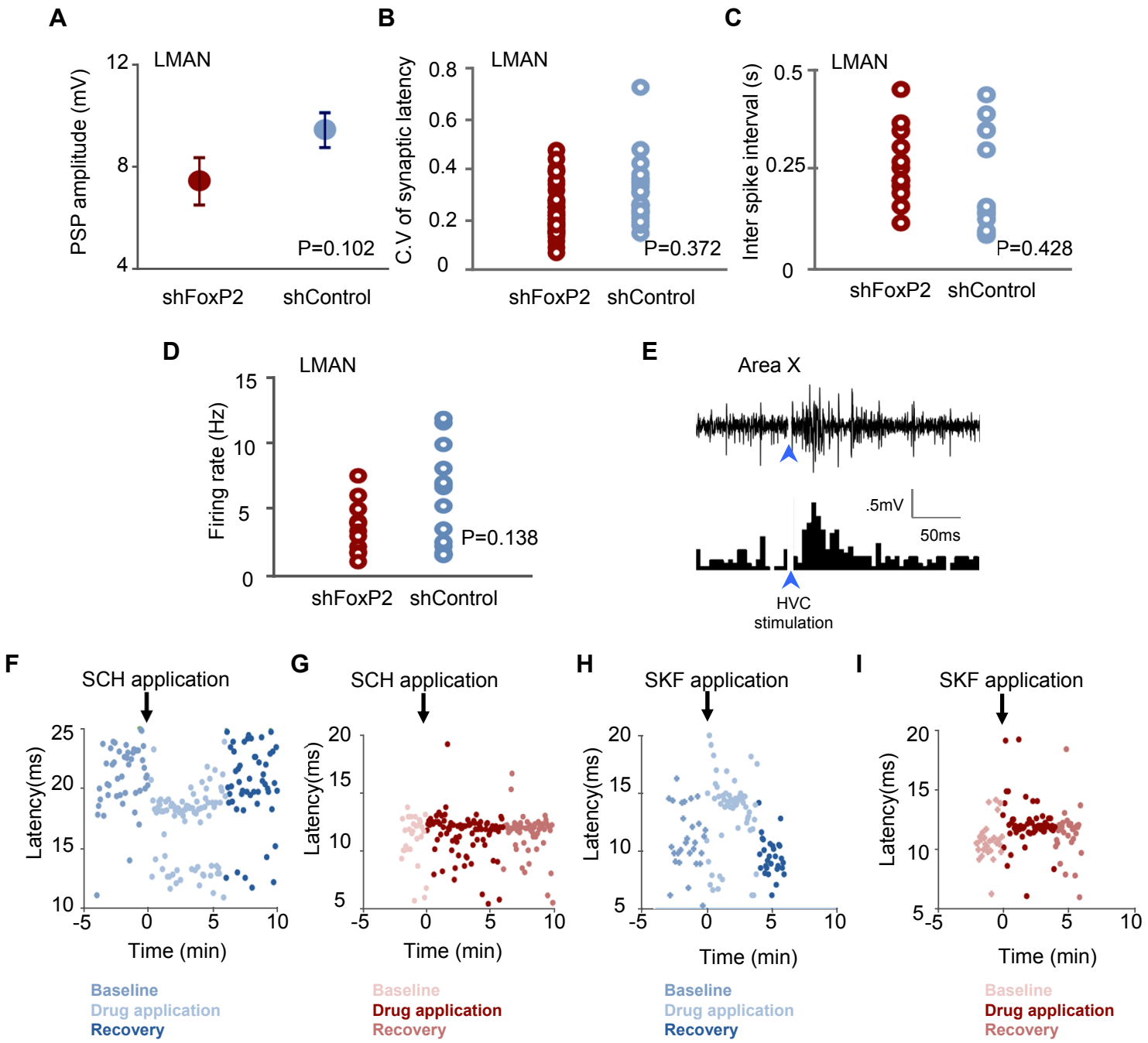

Figure S3, related to Figure 4

Murugan et al.

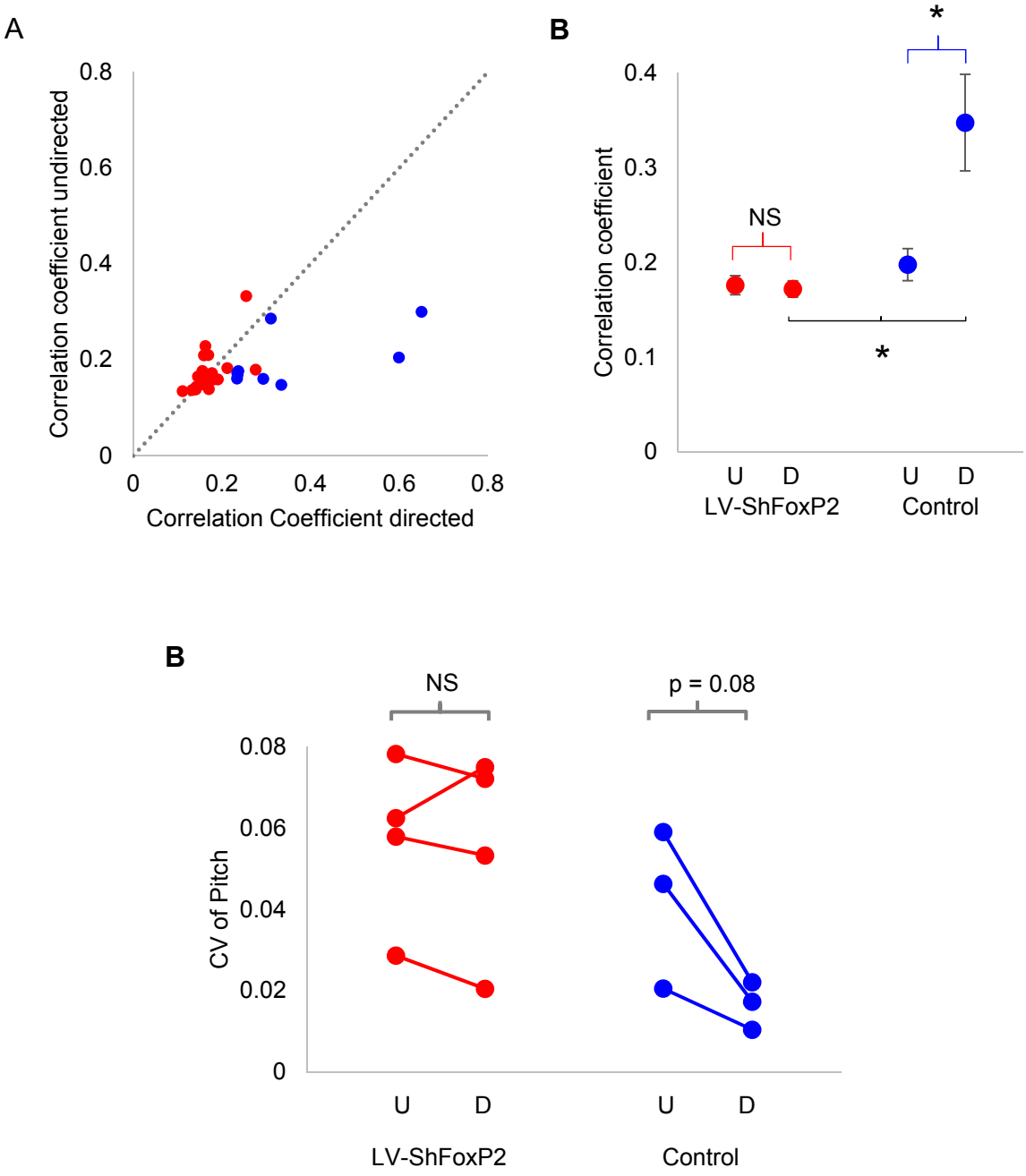

Figure S4, related to Figure 6

Murugan et al.

Supplemental figure legends

Figure S1, related to figure 2. Knockdown of FoxP2 in Area X of adult zebra finches has no effect on context-dependent differences in higher-level features of song. A) Reducing FoxP2 levels has no effect on context dependent differences in motif duration ($p = 0.3$, ANOVA, followed by *t*-test). B) Number of introductory notes in a bout is higher in the directed state (shaded bars) compared to the undirected state (open bars) and reduction in the levels of FoxP2 has no effect on this measure. Bars indicate group means; error bars indicate s.e.m, asterisk denotes $p < 0.05$. C) The number of motifs in a bout is not significantly different in the directed state compared to the undirected state and reduction in the levels of FoxP2 does not alter the number of motifs a bird sings in a bout in either state.

Figure S2, related to figure 3. Knockdown of FoxP2 in Area X of juvenile zebra finches impairs song development. A) Representative spectrograms showing a tutor's song and four of his pupils' adult (95 dph) songs, including two juveniles that received injections of LV-ShC and two juveniles that received injections of LV-ShFoxP2. Black bars underline individual syllables and red bars represent inaccurately copied syllables (scale bar indicates 100 ms). B) Representative spectrograms of a syllable that undergoes a decrease in entropy across development resulting in less noisy syllables following the injection of LV-ShC (bottom panel). In contrast the same syllable produced by a sibling that received injections of LV-ShFoxP2 (top panel) shows significantly higher entropy values over the entire course of development. Values in the panels are mean values for a given day (scale bar indicates 100 ms).

Figure S3, related to figure 4. Reducing FOXP2 levels in Area X alters signal propagation through the AFP. A) Knockdown of FOXP2 (red) has no effect on the amplitude of evoked post synaptic potential in LMAN neurons in response to HVC stimulation compared to the control (blue) animals (p = 0.10, *t*-test). B) Reduction in the levels of FoxP2 has no effect on the coefficient of variation of synaptic latency from HVC to LMAN (p = 0.37, *t*-test). C) Reduction in the levels of FoxP2 has no effect on the inter spike interval of LMAN neurons (p = 0.43). D) Reduction in the levels of FoxP2 has no effect on the firing rate of LMAN neurons (p = 0.14). E) Raw trace and histogram of an extracellular recording from Area X showing spikes evoked by brief electrical stimulation in HVC (40 μA, 400 μs, bipolar electrodes). Histogram binned every 2.5 ms. F-G) Representative cells from a control and FOXP2 knockdown animal respectively showing the changes in synaptic latency from HVC to LMAN following the application of a D1R antagonist, SCH (drug application indicated by an arrow, sampling rate: 0.25Hz). H-I) Representative cells from a control and knockdown animal respectively showing the changes in synaptic latency from HVC to LMAN following the application of a D1R agonist, SKF.

Figure S4, related to figure 7. Reducing FOXP2 levels in Area X abolishes context dependent changes in both song variability and LMAN activity. A,B) In control animals, LMAN neurons (n = 3 animals, 9 units, blue) exhibit lower trial-by-trial variability (correlation coefficient across trials) in firing patterns during directed singing relative to undirected singing (blue; p = 0.01). In contrast, FoxP2 knockdown abolishes context-dependent difference in trial-by-trial variability (red, p = 0.65). LMAN neurons recorded in the FoxP2 knockdown birds had higher trial-by-trial variability during directed singing compared to the control animals (p = 0.01). C) FoxP2 knockdown in Area X of the adult abolishes context-dependent differences in syllable variability (red, n = 4 syllables, 3

birds; p = 0.626). In the control conditions (blue), the fundamental frequencies of syllables are more variable in the undirected state and become more stereotyped in the directed state (n = 3 syllables, 3 birds; p = 0.08).

Supplementary Table 1. Summary of results of *t*-tests comparing fundamental frequency of syllables in different social contexts and injection conditions.

Comparison	P-value
Pre ShFoxP2 directed versus Post ShFoxP2 directed	p=0.048
Post ShFoxP2 directed versus Post ShControl directed	p=0.023
Pre ShFoxP2 undirected versus Pre ShFoxP2 directed	p=0.033
Post ShFoxP2 undirected versus Post ShFoxP2 directed	p=0.548
Pre ShFoxP2 undirected versus Post ShFoxP2 undirected	p=0.874
Post ShControl undirected versus Post ShControl directed	p=0.031
Pre ShFoxP2 undirected versus Post ShControl undirected	p=0.890
Pre ShFoxP2 directed versus Post ShControl directed	p=0.798

Shaded boxes represent $p < 0.05$.

Table S1, related to Figure 2

Murugan et al.

Supplementary Table 2. Application of D1R agonist or antagonist had no effect on the resting membrane potentials and spontaneous firing rates of LMAN neurons in both control and FoxP2 knockdown birds

Resting membrane potential (mV)					
Group	**Drug**	**Baseline**	**Drug**	**Recovery**	**P-value**
Control	SKF	-70.9 ± 4.05	-71.5 ± 6.26	-73.9 ± 8.61	0.94
ShFoxP2	SKF	-73.4 ± 3.19	-75.5 ± 3.03	-73.1 ± 3.66	0.99
Control	SCH	-73.4 ± 1.31	-72.7 ± 3.61	-69.2 ± 6.33	0.71
ShFoxP2	SCH	-79.4 ± 4.84	-72.9 ± 2.80	-79.3 ± 2.10	0.37
Spontaneous firing rates (Hz)					
Control	SKF	3.88 ± 1.39	2.44 ± 1.59	3.01 ± 2.03	0.59
ShFoxP2	SKF	2.98 ± 1.14	2.55 ± 0.99	3.97 ± 1.68	.61
Control	SCH	3.42 ± 1.25	2.56 ± 1.14	2.93 ± 1.47	0.70
ShFoxP2	SCH	2.38 ± 1.58	2.35 ± 1.54	2.61 ± 2.14	0.59
ANOVA, p values for all conditions and groups were > 0.05					

Table S2, related to Figure 4

Murugan et al.

Supplemental Experimental Procedures:

Virus injections: Birds were anaesthetized with 1-2% isoflurane and placed in a stereotaxic setup. Lidocaine (Fougera) was used as a local anesthetic and the scalp was dissected along the midline. The coordinates for Area X (5.3 mm rostral, 1.6 mm lateral and 2.7-3 mm in depth) were measured from the midsagittal sinus bifurcation at a 45° head angle. Multiunit recordings were performed using Carbostar-1 (Kation Scientific) to map out the extent of Area X. A glass pipette attached to a Nanoject-II (Drummond Scientific) was used to deliver an overall volume of ~1 µL in each hemisphere. An average of 4-6 sites were targeted per hemisphere in order to span the entirety of Area X, with each site receiving no more than 200 nl of the virus at a rate of 32.2 nL every 30 s. The shFoxP2 constructs were packed into lentivirus particles to yield a final titer of $1x10^7$ to 10^9 particles/ mL (Marguerita Klein, Duke University; Roberts et al., 2008). The lentivirus particles expressing either U6-shFoxP2-h-ubiquitin-gfp or the U6-shFoxP2-f-ubiquitin-gfp were used interchangeably to knockdown FoxP2 expression in Area X (Haesler et al., 2007). Control lentiviral shRNA (shControl) particles (Santa Cruz Biotechnology, sc-108080) were used for the control experiments. All behavioral and electrophysiological experiments were carried out > 15 days post injection.

Immunohistochemistry: Birds were anesthetized with 0.08 ml equithesin (i.m. injection) and transcardially perfused with 0.025 M phosphate-buffered saline (PBS), followed by fixation with 4% paraformaldehyde in PBS (PFA). Brains were dissected out and post-fixed in 4% PFA with 30% sucrose overnight at 4° C. Parasagittal sections were cut on a freezing microtome (Reichert) at 50-75 µm. A GFP antibody staining was run to enhance visualization of the knockdown. The primary antibody used was a mouse monoclonal anti-GFP (Invitrogen, 1:1000 dilution) followed by a goat anti-mouse secondary antibody coupled to Alexa 488 (Invitrogen, 1:500 dilution).

Western Blots:

All birds were placed in a dark chamber without light for 2 hours (~ noon). The birds had received injections of either LV-ShFoxP2 or LV-ShControl ~ 90 days prior to retrieval of brain samples. The birds were anesthetized with 5% isoflurane and decapitated. Their brains were quickly removed from the skull and were mounted on a vibratome (Leica, VT 1000s) stage. 100 µm sections were cut until Area X was visible (~ 1200 µm from the rostral tip of the brain; Miller et al., 2008). GFP fluorescence from the viral infection was used to confirm the location of Area X, there is a possibility that this may have biased us towards sampling from regions of higher labeling density. A 0.8mm tissue punch was used to remove much of the tissue encompassing Area X. Only samples from the left hemisphere were used for the western blot experiments. The samples were quickly frozen in liquid N2 and stored at -80° C until later use. Frozen tissue was homogenized in modified RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate), centrifuged at 16000g for 10 min, and supernatant collected. This supernatant was defined as tissue homogenate and was resolved with SDS-Page. The blots were incubated with FoxP2 (1:500, Sigma-Aldrich), D1R (1:1000, Abcam), DARPP-32 (1:1000, Abcam) and actin (1:10000, Sigma-Aldrich) primary antibodies for at least 12 hours at 4°C followed by incubation with secondary antibodies (1:5000, Jackson Labs) for at least 1 hour at room temperature. Only samples from the left hemisphere were used for the western blot experiments. The blots were incubated with FoxP2 (1:500, Sigma-Aldrich), D1R (1:1000, Abcam), DARPP-32 (1:1000, Abcam) and actin (1:10000, Sigma-Aldrich) primary antibodies for at least 12 hours at 4°C followed by incubation with secondary antibodies (1:5000, Jackson Labs) for at least 1 hour at room temperature. For quantitative analysis, immunoblots were scanned with a digital scanner and the optical band density was quantified using ImageJ analysis software. Optical

densities for FoxP2, D1R, and DARPP-32 were normalized to corresponding actin levels. Data are presented as mean ± SEM. Statistical significance was assessed with the Student's t-test.

Behavioral experiments: All songs were recorded in sound attenuation chambers with microphones (Shure SM 93), pre-amplified and saved to a computer using Sound Analysis Pro (Tchernichovski et al., 2000).

Adult behavioral experiments: For experiments examining the effect of knockdown of FoxP2 on context-dependent singing, adult male zebra finches (> 120 dph) were placed in recording chambers.

Recordings of each bird were obtained both 2 ± 1 days pre and 27 ± 1 day post-injection of either LV-ShFoxP2 or LV-ShC. Undirected songs were classified as those songs recorded when male birds were isolated in the recording chambers. In order to obtain directed songs, one or two female birds were introduced into the chamber in a separate cage. A webcam was used to monitor the behavior of the male birds. Those songs in which the male bird faced the female bird(s) were classified as directed songs. Directed singing was restricted to a 2 min period after the introduction of the female bird and was terminated by her removal. The process was repeated at 1-hour intervals until a minimum of 20 directed and undirected songs were acquired.

Juvenile behavioral experiments: For experiments examining the effect of knockdown of FoxP2 on juvenile song development, juvenile male zebra finches raised with their nesting group were injected with either LV-ShFoxP2 (n = 4 birds) or LV-ShC (n = 2 birds) at ~20 dph. Following the injection, juvenile birds were returned to a sound-attenuating chamber with an adult male tutor (their father) until 45 dph. The juvenile birds were then transferred to isolated sound-attenuating chambers and their songs were recorded

between 45-95dph (all songs were recorded in the undirected state). In a parallel set of experiments to measure the effect of knockdown on subsong, juvenile male zebra finches were isolated from adult male birds between 7-10 dph and injected with either LV-ShFoxP2 (n = 2 birds) or LV-ShC (n = 2 birds) at ~20 dph. The juvenile birds were introduced to an adult male zebra finch for five days of tutoring between 45-50dph. Following the tutor experience the juvenile birds were moved into isolated sound-attenuating chambers and their songs were recorded between 50-95dph. Birds raised in their nesting groups and those isolated between 10-40 dph from male birds showed comparable learning in their respective categories (ShFoxP2 groups, p = 0.4 and ShC groups, p = 0.2, t-test) and were therefore grouped together for data analysis.

Song analysis: Sound Analysis Pro (SAP) was used to analyze all behavioral data. All song files were bandpass filtered between 400 Hz and 10 kHz.

Adult behavioral experiments: For the adult experiments, only syllables with constant frequency components (i.e., harmonic stacks) were included in the analysis (Kao and Brainard, 2006). The syllables were manually selected using SAP. The fundamental frequency (FF) of harmonic stacks was measured for songs rendered in both directed and undirected conditions. To calculate the coefficient of variability, the FF of a minimum of 20 renditions of each syllable was used. Multifactor ANOVA was used to test for statistical significance followed by post-hoc t-tests.

For the tempo analysis, the duration of the complete motifs were measured in in both directed and undirected states for each bird and the mean duration calculated (Kao and Brainard, 2006). The number of introductory elements per bout was counted backwards from the first syllable of a motif till the presence of a 500ms silent period or a call (Kao and Brainard, 2006). A bout was defined as motifs separated by silent periods > 500 ms.

To count the number of motifs per bout, motifs that contained more than half the number of syllables of a complete motif were included in the analysis. For all the above analysis, a minimum of 20 bouts per condition (directed and undirected) per bird was used for analyses.

Juvenile behavioral experiments: For the juvenile experiments, 50 renditions of the motifs and syllables were chosen for any given day of analysis. To measure the amount of song learning at any given time point, 50 renditions of the pupil's motif from that day were compared to a single representative motif from his tutor. The percentage similarity score (asymmetric comparisons) was used to quantify the amount of song learning. The percentage similarity score (% similarity) takes into consideration multiple features of song such as pitch, amplitude modulation, frequency modulation, Wiener entropy and goodness of pitch over the course of a motif and determines how these features of the pupil's song compare to the song of the tutor. Multifactor ANOVA followed by post-hoc t-tests were used to determine if the amount of learning differed between the knockdown birds (n = 6) and control birds (n = 4) throughout development. For the syllable level analysis, the syllables were restricted to those that were copied by both the FoxP2 knockdown juveniles and their control siblings. The FF of harmonic stacks and Wiener entropy of syllables were calculated at multiple time points through development (Tchernichovski et al., 2001). For the entropy analysis, the entropy at all the different time points was normalized to the entropy of the same syllable at 45 dph. Therefore, a more positive value for fraction change in entropy indicates a noisier syllable.

Comparison to the bird's own song (BOS): The songs of juvenile male zebra finches at multiple points during development were compared to the BOS at 95 dph. The analysis is identical to the similarity comparisons to the tutor's song. Single or two-factor ANOVA was used to test statistical significance followed by post-hoc t-tests and reported errors

are s.e.m. Multifactor ANOVA followed by post-hoc t-tests were used to test for significance.

In vivo and in vitro sharp intracellular recordings:

All electrophysiological data were collected inside a sound-attenuating chamber (Industrial Acoustics) placed on an air table (Technical Manufacturing Corporation) and using a data acquisition board (National Instruments) controlled by custom Labview software. Borosilicate glass electrodes (80-150 MΩ, BF100-50-10, Sutter instruments) filled with 2M potassium acetate were used to obtain sharp intracellular recordings of LMAN and RA neurons. Membrane potential recordings were amplified with an Axoclamp 2B amplifier (Axon Instruments) in bridge mode, low-pass filtered at 3 kHz, and digitized at 11 kHz (10 kHz for slice data).

In vivo intracellular experiments: A day prior to the in vivo intracellular experiment, birds were anaesthetized with 2% isofluorane and a stainless steel post was mounted on the bird's skull with dental cement. On the day of the experiment, the birds were anaesthetized with either 20% urethane (30-40 µL doses every 30 minutes to final volume of 90-120µL) or diazepam (50 µl, 2.5 mg/mL) injected into their pectoral muscles. Urethane was used for all the pharmacological experiments to enable recording for a longer time. The average latencies obtained using both anesthetic agents were comparable (mean latency, ms: ShFoxP2diaz: 12.5 ± 0.3, ShFoxP2ure: 12.4 ± 0.4, shCdiaz: 15.4 ± 0.3, shCure: 16.4 ± 0.7 ms). The body temperature was maintained at ~37°C using a heat blanket (Harvard apparatus). The target nuclei (coordinates: LMAN- 5.3 mm rostral, 1.9 mm lateral, HVC – 0 mm caudal, 2. 4 mm lateral and Area X- 6.3 mm rostral, 1.6 mm lateral or 5.3 mm rostral and .5 mm lateral) were measured from the midsagittal sinus bifurcation and craniotomies (~200 µm by 200µm) were made over the

target nuclei (LMAN, HVC and Area X). Target regions were identified by their characteristic activity patterns; in addition, LMAN neurons were identified based on their characteristic spike shape and response to HVC stimulation. Sharp intracellular recordings of LMAN neurons were obtained while HVC was electrically stimulated using bipolar tungsten electrodes (0.1 MΩ, Microprobes) with the tips spaced ~300 μm. Biphasic stimulation for 400 μs was applied at a current strength of 40 μA. Synaptic latency and amplitude of postsynaptic potentials (PSPs) of LMAN responses to HVC stimulation were measured from median filtered traces using custom event detection software (Matlab, K.Hamaguchi). To quantify 'spikes' for intracellular and extracellular data, the threshold was manually assigned (and kept constant across conditions) to a value above the baseline and events that crossed the threshold were counted as spikes. The mean spontaneous firing rates and inter-spike intervals (ISIs) were measured from recordings of spontaneous activity during a 1s baseline period prior to HVC stimulation. Standardized two-tailed t-tests for independent samples were performed to test for statistical significance between the control and the knockdown groups.

Pharmacology experiments: For the pharmacology experiments, a glass pipette (tip diameter 15–20 μm) was attached to a tungsten electrode (0.5–1.0 MΩ, Microprobes). The glass pipette was filled with either 0.5 mM SKF 38393 hydrobromide (Tocris Bioscience) or 5 mM SCH 23390 (Sigma) in .9% saline and ~30-60 nl was injected using a Picospritzer II (General Valve) in 50–150 ms pulses at 30 psi (Leblois et al., 2010). The tungsten electrode was used to record multiunit neural activity in Area X and electrode placement was confirmed on the basis of robust responses in Area X to HVC stimulation. The glass pipette-tungsten electrode into Area X was lowered at a 25-30° to avoid passing through LMAN. In addition, previous work has shown that LMAN has very low expression levels of D1 receptors (Kubikova et al., 2010). In a subset of experiments

a red dye (Alexa Fluor 594 Cadaverine) was added to the drug to monitor the spread of the dye post hoc. Mean firing rate of Area X was calculated from multiunit recordings using custom written software (Matlab). Control birds used for pharmacological experiments received no viral injections. Sharp intracellular recordings of LMAN neurons with HVC stimulation were performed as described above. Each hemisphere was limited to a single instance of drug delivery. A two-factor ANOVA with repeated measures on one factor was used to test for statistical significance in latency measurements followed by post hoc two-tailed t-tests for correlated samples.

In vitro slice experiments: All birds used were older than 90 dph. For the in vitro slice experiments, birds were anaesthetized with isoflurane (5%) and decapitated. The brain was quickly removed and moved into a solution of ice-cold artificial cerebrospinal fluid (aCSF). 400 μm coronal brain slices including RA were cut using a vibratome (Leica, VT 1000s). Concentric tungsten bipolar stimulation electrodes (FHC) were placed ≤ 1mm lateral and dorsal to RA to stimulate either the LMAN or HVC axon tracts respectively (Mooney, 1992). Each axon tract was stimulated independently using A360 Stimulus Isolaters (World Precision Instruments) at 30-200 μA for a duration 100-200 μs while intracellular sharp recordings were obtained from RA neurons. Only cells (23 cells, 5 normal birds) that responded to both HVC and LMAN stimulation were included in the analysis. A subset of the 23 cells contributed to each stimulation parameter, all birds contributed to each parameter, and trial blocks were pseudo randomized to avoid bias. A Master-8 (A.M.P.I) controlled by custom LABVIEW software was used to allow either HVC or LMAN stimulation to lead the other by 0, 3 or 5ms. Spike latency was determined using custom software (MATLAB).

In vivo extracellular recordings in singing birds:

A custom built manually (threaded rod) operated microdrive carrying an insulated platinum electrode (1-5 MΩ) was stereotaxically implanted in LMAN in an anaesthetized adult male zebra finch. LMAN was located in the anaesthetized animal using stereotaxic coordinates and by its characteristic bursting activity, large units with narrow spike widths (~1ms). After LMAN was located the electrode was withdrawn to a position 200 µm dorsal to LMAN. A reference electrode, consisting of an uninsulated platinum wire, was positioned 1mm posterior to the recording electrode and a ground electrode, made of uninsulated silver wire, was placed over the cerebellum. The microdrive, electrodes and ground wire were secured to the bird's skull using dental cement. All recordings were limited to the right hemisphere. After the animal was fully recovered (~4 days post implant) the electrode was lowered slowly in ~25 µm steps every 1 hour until LMAN activity was detected. The electrode signal was amplified through a JFET on the cable attached to the bird's head and an op-amp followed by an instrumentation amplifier (Brownlee Precision, Model 440), filtered between 200 Hz and 10 kHz. All electrophysiological and song data were collected inside a sound-attenuating chamber (Industrial Acoustics) using a data acquisition board (National Instruments) controlled by custom MATLAB software (K.Hamaguchi). A video camera was used to observe the bird's behavior. Multinunit data were collected in interleaved trials of directed and undirected singing. The data were visually inspected to exclude trials that had movement artifacts. Spike sorting on the multiunit data was performed offline using Wave clus (Quiroga et al., 2004). The parameters on Wave Clus were set to identify spikes that were a minimum of 3 standard deviations from baseline and the detector dead time was set as 2ms. The waveforms of the sorted clusters were visually inspected and tested for refractory period violations (<1% of interspike intervals ≤ 1 ms; Control: n = 9 units from

3 animals; LV-ShFoxP2: n = 20 units from 3 animals). All sorted single unit data were aligned to the onset of the first syllable of the song motif without time warping for measuring firing rates and burst fractions. Bursts were defined as spikes separated by less than 5ms. Burst fraction was defined as the fraction of the total spikes that occur as bursts. Mean firing rate and burst fraction were calculated over the entire duration of the motif. Data were analyzed using custom software (MATLAB).

For analysis of trial-by-trial variability, first, the onset and offset times for each syllable in a motif were measured. A reference motif was selected for each bird and was kept constant for both conditions (directed and undirected). The data were time warped such that each syllable and the corresponding neural data was stretched or compressed to the reference syllable in the standardized motif (as described in detail by Kao et al., 2008). The instantaneous firing rates were generated for each time-warped unit by smoothing spike trains with a Gaussian filter (10 ms standard deviation). A linear pairwise correlation analysis was run on the instantaneous firing rates comparing all trials a given condition. This values generated was then used to calculate the average correlation coefficient for each unit for a given condition. All data are represented as mean ± ANOVA followed by post *t*-test were used to test for statistical significance.

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