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RESEARCH ARTICLE

Sensory Processing

Auditory discrimination learning and acoustic cue weighing in female zebra finches with localized FoxP1 knockdowns

Fabian Heim,[1,2,3] Constance Scharff,[3] Simon E. Fisher,[2,4] Katharina Riebel,[1] and Carel ten Cate[1,5]

[1]*Institute of Biology, Leiden University, Leiden, The Netherlands;* [2]*Language and Genetics Department, Max Planck Institute for Psycholinguistics,Nijmegen, The Netherlands;* [3]*Institute of Biology, Freie Universität Berlin, Berlin, Germany;* [4]*Donders Institute for Brain, Cognition and Behaviour, Radboud University, Nijmegen, The Netherlands; and* [5]*Leiden Institute for Brain and Cognition, Leiden, The Netherlands*

Abstract

Rare disruptions of the transcription factor FOXP1 are implicated in a human neurodevelopmental disorder characterized by autism and/or intellectual disability with prominent problems in speech and language abilities. Avian orthologues of this transcription factor are evolutionarily conserved and highly expressed in specific regions of songbird brains, including areas associated with vocal production learning and auditory perception. Here, we investigated possible contributions of FoxP1 to song discrimination and auditory perception in juvenile and adult female zebra finches. They received lentiviral knockdowns of FoxP1 in one of two brain areas involved in auditory stimulus processing, HVC (proper name) or CMM (caudomedial mesopallium). Ninety-six females, distributed over different experimental and control groups were trained to discriminate between two stimulus songs in an operant Go/Nogo paradigm and subsequently tested with an array of stimuli. This made it possible to assess how well they recognized and categorized altered versions of training stimuli and whether localized FoxP1 knockdowns affected the role of different features during discrimination and categorization of song. Although FoxP1 expression was significantly reduced by the knockdowns, neither discrimination of the stimulus songs nor categorization of songs modified in pitch, sequential order of syllables or by reversed playback were affected. Subsequently, we analyzed the full dataset to assess the impact of the different stimulus manipulations for cue weighing in song discrimination. Our findings show that zebra finches rely on multiple parameters for song discrimination, but with relatively more prominent roles for spectral parameters and syllable sequencing as cues for song discrimination.

NEW & NOTEWORTHY In humans, mutations of the transcription factor *FoxP1* are implicated in speech and language problems. In songbirds, FoxP1 has been linked to male song learning and female preference strength. We found that FoxP1 knockdowns in female HVC and caudomedial mesopallium (CMM) did not alter song discrimination or categorization based on spectral and temporal information. However, this large dataset allowed to validate different cue weights for spectral over temporal information for song recognition.

categorization; cognition; Go/Nogo; songbird; vocal learning

INTRODUCTION

Human spoken language or any form of vocal communication relies on the ability to correctly perceive and categorize auditory stimuli to respond appropriately. Genetic factors contribute to the complex processes underlying human speech and language (1). Rare mutations of human *FOXP1* cause a neurodevelopmental syndrome including speech problems and language-related issues (2) next to more prominent forms of autism spectrum disorder and/or intellectual disability (3–6). Putative perceptual impairments have been reported in some humans carrying heterozygous FOXP1 disruptions (e.g., see Ref. 7), but exactly how relevant genes contribute to perception, development, and production of speech remains difficult to examine in humans (1, 8, 9). However, suitable animal models

Correspondence: F. Heim (fabian.heim@bi.mpg.de).
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such as songbirds, allow the examination of the role of various genes for vocal perception, production, and learning. Like humans, songbirds learn, imitate, and modulate vocalizations (10–12), processes that all require auditory feedback (13, 14). The pallial songbird brain regions involved in the production and perception of song are functionally similar to mammalian, and especially human, cortical brain areas involved in production and perception of vocalizations, despite considerable neuroanatomical differences (15–18). Furthermore, some of these functionally similar regions in songbirds and humans show convergent gene expression profiles (19) for example the high prevalence of *FoxP1* expression in brain areas involved in vocal perception and production. The behavioral, neural, and genetic parallels between human speech and birdsong make songbirds a suitable animal model to study the neurobiological mechanisms underlying vocal learning and auditory perception (e.g., see Refs. 10, 20–22), and to investigate the impacts of FoxP1 disruptions on vocal processing (23–25). Note however that despite the similarities, conclusions drawn from evolutionarily distant songbirds can only be applied with certain limitations to human speech and language as well as cognition, and thus songbirds with disruptions of avian FoxP1 do not represent a direct biomedical model of the FOXP1-related syndrome itself. Moreover, in this and other studies, roles of songbird FoxP1 are investigated through targeted knockdowns localized to particular brain regions, in contrast to the situation for human patients with naturally occurring *FOXP1* mutations, where all cells in the body carry the disruption, potentially leading to wider systemic effects.

In songbirds, *FoxP1* is highly expressed in the basal ganglia (including the striatal nucleus Area X, which is essential for song learning), the robust nucleus of the arcopallium (RA), the caudomedial mesopallium (CMM), and HVC (proper name), throughout development and into adulthood of zebra finches and other songbirds (26–28). CMM has been shown to be involved in song learning and song perception of adult male and female zebra finches (29–31), auditory discrimination learning in adult male zebra finches (32), tutor song memory (33, 34), and the perception of frequency as well as amplitude and their modulation within songs by adult female zebra finches (35). CMM neurons are also active during deviating call sequences in adult female (36) and syllable discrimination in adult male zebra finches (37). Neuronal activity in CMM is also increased during song playbacks as shown by immediate early gene expression in juvenile (38) and adult male zebra finches (34, 39–42) and BOLD imaging in adult female zebra finches (43). HVC is hypothesized to be involved in auditory motor integration in adult male zebra finches (44) and adults of other songbirds (45, 46). Specifically, lesions of HVC in adult female canaries alter perception of conspecific songs (47) and lead to decreased immediate early gene expression in auditory areas such as CMM (48) that provides direct auditory input to HVC (49–52). FoxP1 knockdowns in HVC of adult female zebra finches resulted in lower song preference strength (amount of times a female initiates the playback of one stimulus over another) for familiar songs and overall fewer requested auditory stimuli (24). The localized *FoxP1* expression patterns in the aforementioned areas involved in auditory processing are suggestive of potential contributions of FoxP1 to auditory processing.

In this study, we therefore used localized FoxP1 knockdowns to examine the contribution of FoxP1 to auditory perception and processing of conspecific vocalizations in female zebra finches. Female zebra finches do not sing (53, 54) but memorize songs heard early in life and prefer those later on to unfamiliar songs (55–58) thus providing an excellent opportunity to study how development and maintenance of auditory processing affect adult song discrimination as, unlike in singing males, all song input can be controlled experimentally. Note that as adults, independent of the pronounced sex differences in song production learning, both sexes learn equally well to discriminate between auditory stimuli of different kinds (59). Interestingly, the neural expression patterns of *FoxP1* are similar in both sexes across development (27, 28) further supporting the idea that FoxP1 might be involved in the development of auditory processing rather than singing. Given that HVC and CMM are implicated in perception and processing of auditory stimuli in both sexes, and show higher *FoxP1* expression than the surrounding tissue [although the volume of HVC is significantly smaller in females than in males (60, 61)], *FoxP1* expression in these areas may be related to perception and discrimination of song and other vocalizations in zebra finches.

The primary aim of this study was to investigate this idea by testing whether *FoxP1* expression in HVC and CMM contributes to the ability of female zebra finches to discriminate songs and to the perceptual processing of different song parameters. The females of this study had received lentiviral FoxP1 knockdowns in HVC or CMM, either as adults or juveniles. These localized knockdowns did not affect their preferences for their tutor song over unfamiliar songs, suggesting that FoxP1 is not involved in tutor song memorization and recall (24). However, recognizing songs heard early in life is but one form of auditory learning; adult zebra finches continue learning to recognize and distinguish various songs by attending to often very detailed and specific acoustic features, such as spectral structure, pitch, or sequences (62–68).

The earlier study by Heim et al. (24) only compared preferences for a familiar song resulting from exposure at a young age to the preference for novel, unfamiliar songs, without examining whether knockdowns affected the processing of particular sound parameters. In the current experiment, we examined whether learning to discriminate between two unfamiliar songs in adult females is affected by the knockdowns, and whether the FoxP1 knockdown affected the processing of more specific acoustic features involved in the discrimination by using an established operant Go/Nogo discrimination paradigm (e.g., see Refs. 59, 69, 70). Moreover, such a paradigm allows to compare both the speed of song discrimination learning and the performance during discriminatory tasks in experimental and control groups of females. We tested whether these groups differed in their responses to songs that were modified in parameters that are known to be used by adult zebra finches to discriminate auditory stimuli: pitch (67, females), the overall spectral structure of syllables (62, both sexes; 66, 71, 72, both sexes) and the sequence of syllables (68, 73–77, both sexes; 78, females).

If localized high levels of *FoxP1* expression are required to develop or maintain auditory discrimination (distinguish between Go and Nogo type stimuli) and stimulus categorization (the use of specific acoustic parameters for such discrimination), experimentally reduced *FoxP1* expression levels should result in slower discrimination learning, stimulus discrimination, and/or differences in the females' responses to modified songs compared with matched controls without knockdowns. As the experiments involved several groups that combined resulted in a large sample size of females ($n = 96$), that all had been tested individually for their abilities to discriminate songs by different features, the complete data was also used for a detailed analysis of the relative importance of pitch, spectral structure, and syllable sequence for the identification and categorization of different songs.

MATERIALS AND METHODS

Subjects, Virus Preparation, and Injections

All subjects ($n = 96$ females) were the offspring of domesticated zebra finches from the breeding colony at the Freie Universität in Berlin. All individuals had received local FoxP1 knockdowns as part of an experiment that had tested whether these knockdowns affected the development and learning of song preferences (24). Briefly, all subjects had been raised and housed with their siblings and parents ($n = 79$) or foster parents (a subset of birds were cross fostered at 15 dph, $n = 17$) in steel-wire breeding cages (180 × 42 × 33 cm) until 90 dph. Subjects had then been assigned to four different treatment groups that were defined by where (HVC or CMM) and when (as juveniles: 23 ± 2 dph or as adults: 210 ± 124 dph) they received viral injections: HVC adult, HVC juvenile, CMM adult, CMM juvenile. Each knockdown group was also assigned a matched control group. To keep variation within treatment-matched comparisons low, young females were pseudo-randomly assigned one by one to each treatment and a matched control group (assigning sisters to a matched treatment and control group wherever possible) until a sample size of $n = 12$ was reached for each of the four particular treatments and the four matching control groups.

For delivery of the knockdown, viral constructs were injected bilaterally in a stereotaxic setup (for coordinates see Supplemental Table S1 and Ref. 24). Three different viral constructs with a green fluorescent protein (GFP) marker sequence were used (24, 25): control (only the GFP marker), and two constructs that additionally contained shRNA sequences with target sites in FoxP1 transcripts, to reduce local *FoxP1* expression ("knockdown"). The rationale for using two different shRNA constructs was to reduce the probability of undetected off-target effects (79). The construct that only led to GFP expression was used to control for the effects of surgery, injection, virus infection, and protein expression in the control birds. Even though bird ID and the injected viral construct had to be known to the experimenter during the initial surgery, the birds' further treatment was conducted without any reference to group identity. The group to which each bird belonged was unblinded after the birds were euthanized to allow for unbiased treatment during the behavioral experiments.

After the injections, adult birds were moved to same sex aviaries (200 × 200 × 300 cm) whereas juveniles were returned to their home cages where they remained until 90 dph when they were also transferred to same sex aviaries. Birds were always housed in their home cages for at least 14 days after the procedure before they were transferred to Leiden (The Netherlands) for behavioral testing. At Leiden University, before and after behavioral testing, 2–6 subjects were housed in cages of 120 × 90 × 90 cm. All experimental procedures were approved by the veterinary department of the Freie Universität Berlin and the ethics committee of the Regional Office for Health and Social Affairs Berlin, Germany (LAGeSo) under REG 0019/15. All experiments conducted at Leiden University were approved by the Animal Experimentation Committee at Leiden University (DEC License 14234) and by a license of the Ministry of Infrastructure and Environment (GGO License 14-097) in accordance with Dutch laws.

Go/Nogo Training and Tests

All birds were trained as adults (mean ± standard deviation, juvenile groups: 135 ± 7 dph, adult groups: 255 ± 132 dph) in one of 12 sound attenuated chambers (at least 2.4 × 1.4 × 2.3 m) at Leiden University. The birds could be observed via a one-way mirror and all chambers were identically equipped with an experimental cage (Skinner box, 70 × 30 × 45 cm, made of mesh wire with a solid back panel and floor) placed on a trolley at the long end of the chamber. The Skinner box contained a food hatch in the back wall of the cage. Left and right of the hatch were piezoelectric sensors with red LED light indicators. A perch was placed in front of the hatch as well as the sensors, and additional perches were located at either side of the cage. Each cage was illuminated by a fluorescent tube emitting light at daylight spectrum on a 13:11 light:dark schedule with dawn and dusk phases of 15 min each. The food hatch, lights, and LED indicators were controlled by a custom-built steering unit (electronics workshop, Leiden University) connected to a laptop (Sony Vaio E series, Sony, Minato) containing a sound chip (MSM6388, Oki, Tokyo) that could be accessed from outside the experimental chamber.

In the first phase of the Go/Nogo training (Fig. 1), birds were moved from their home cage into individual Skinner boxes. Water and grit were provided ad libitum with food being freely available behind an open hatch. During this stage, birds pecking one of the sensors would elicit a playback in the form of two song motifs from an unfamiliar zebra finch. The keys remained active and the food hatch open until 9:00 AM the following day when the hatch was closed to begin training. From this time onward, a bird had to peck at either one of the two buttons next to the food hatch to elicit a playback (same sound as before, for both buttons) and gain food access from the hatch for 20 s. If the bird did not regularly feed after the food hatch was opened by a key peck, the hatch was opened in the evening at least one hour before lights off, and closed again the following morning until each bird had learned to peck the keys for access to food. Once the birds had learned how to acquire food, access could be achieved any time during daylight. After a bird was observed to peck regularly (feeding after the hatch opened in >7 of 10 trials), the next shaping phase was introduced.

During this next phase, birds were required to first peck the key to the left side of the hatch that would elicit the same

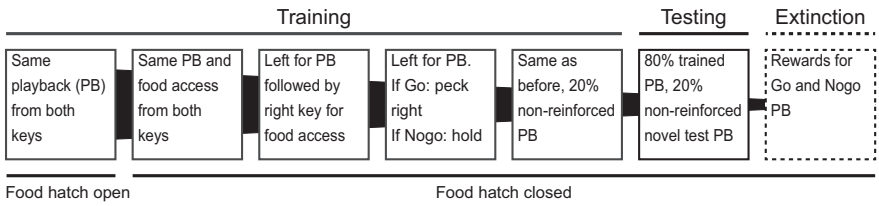

Figure 1. Flow chart of the training and testing procedures during the experiments. Initially, the food hatch was open to allow unlimited food access while the birds could already elicit playback via the pecking keys. Over the course of the training, birds were conditioned to open the food hatch and respond in a sequence (first left to initiate the playback, then right to indicate a response and obtain a food reward) until novel and nonreinforced stimuli were tested. During the extinction period, the food hatch always opened after pecking the second key, irrespective of stimulus category (Go or Nogo).

playback as during previous shaping phases. To gain access to food for 20 s, birds had to peck at the right key within 10 s after a playback was initiated. As soon as a bird gained access to food in >75% of the initiated trials during three consecutive days, the Go and Nogo training stimuli replaced the initial stimulus. Both stimuli were now played back randomly. When a bird initiated a trial by pecking the left key and a Go stimulus was played, it was required to peck the right key within 5 s to be rewarded with 10 s of food access. In case a Nogo stimulus was played, the birds had to refrain from pecking the right key until they could initiate a new trial via the left key after 5 s. If a bird pecked the right key within 5 s after a Nogo stimulus the lights were turned off for 12 s before a new trial could be initiated.

To determine the birds' progress, the discrimination rate (DR) was calculated as follows:

$$\frac{\text{Proportion correct Go}}{(\text{Proportion correct Go} + \text{Proportion false positive Nogo})} = \text{DR}.$$

Once birds discriminated between Go and Nogo stimuli in more than 75% of trials over three consecutive days, the amount of trials required to reach this stage was used as an estimate for learning speed and the next training phase began during which 20% of the initiated trials were not reinforced. This accustomed the birds to unrewarded stimuli before the actual test phase began. After a subject had performed for another three consecutive days at a discrimination rate >75%, the testing phase began.

During this phase, the test stimuli were played back at a rate of 20% among the trained Go and Nogo sounds. This testing phase lasted for six consecutive days. Responses to test stimuli were never reinforced. Playback of test stimuli was organized in a pseudo random fashion that played stimuli randomly but ensured that all stimuli had comparable playback rates. The number of responses per stimulus was measured and the response rate for each individual test stimulus was calculated by dividing the number of Go responses by the number of presentations of that particular test stimulus.

This testing period was followed by an extinction phase during which both Go and Nogo stimuli from the training phase were always rewarded with 10 s access to food. As soon as the discrimination rate between the two stimuli reached chance level (50%) during one entire day, the extinction phase was concluded and subjects were moved back to their home cages.

Stimulus Songs

All stimuli consisted of unfamiliar, undirected song recordings of birds from the breeding colony in Leiden. Song was previously recorded under standardized conditions in sound attenuated chambers with Ishmael software (v.1.0.2, https://ishmael.software.informer.com/1.0/) directly onto the hard disk of a computer (CDX-01 soundcard, Digital Audio Labs, Chanhassen, MN) with a microphone (MKH40, Sennheiser, Wedemark-Wennebostel, Germany) at 75 cm distance above the cage and a sampling rate of 44.1 kHz at 16 bits. No bandwidth filters were applied. All playbacks were adjusted to a SPL of 70 dB re 20 μPa (peak amplitude, continuous fast measurements over 5 s, NL 15, RION, Shanghai, PR China) at the location where the bird would initiate the playback. Files were played back as .wav files from speakers (Vifa 10BGS119/8, Viborg) connected to custom-made Skinner box devices, which were individually controlled via software written by the Leiden University electronics department. Stimuli consisted of two repetitions of the same motif without introductory notes and were matched according to duration (1.6–1.9 s, mean = 1.71 s) and number of syllables (4–7, mean = 5.4). A motif was defined as the longest most common sequence within five song bouts. Go and Nogo training stimuli were selected from natural motif repetitions of multiple males and compiled into six different stimulus sets (see Fig. 2*B* for one exemplary training stimulus). The same stimulus sets were assigned to 3–10 matched pairs of knockdown and control females with similar occurrence of each stimulus during the different training periods. Experimental stimuli were modified versions of the training stimuli and are further referred to as test stimuli. Depending on whether the original version was a "Go" or a "Nogo" stimulus, they are referred to as "TestGo" and "TestNogo" stimuli. Stimulus modifications consisted of pitch manipulations, reversals of the element sequence, and stimuli played back in reverse (Fig. 2). For pitch manipulations, all frequencies of a stimulus song were increased or decreased by 8% (Fig. 2, *C* and *D*) using Praat v5.4 (80, Praat Vocal Toolkit by Ramon Corretge). This level of change is close to the threshold necessary to correctly discriminate song in zebra finches (67). For the sequence reversal, the syllable sequence within the trained songs was reversed (Fig. 2*E*, ABCD > DCBA, indicated as "sequence reverse"). Furthermore, backward played songs (Fig. 2*F*, "reversed playback") were used to determine whether knockdowns alter the ability of female zebra finches to recognize and discriminate the spectral structure of song syllables (62, 66, 81, 82) while syllable number and their pitch remain unaltered.

Brain Extractions and Knockdown Validation

Two to three weeks after tests were completed, each bird was placed individually in a sound attenuated room (between

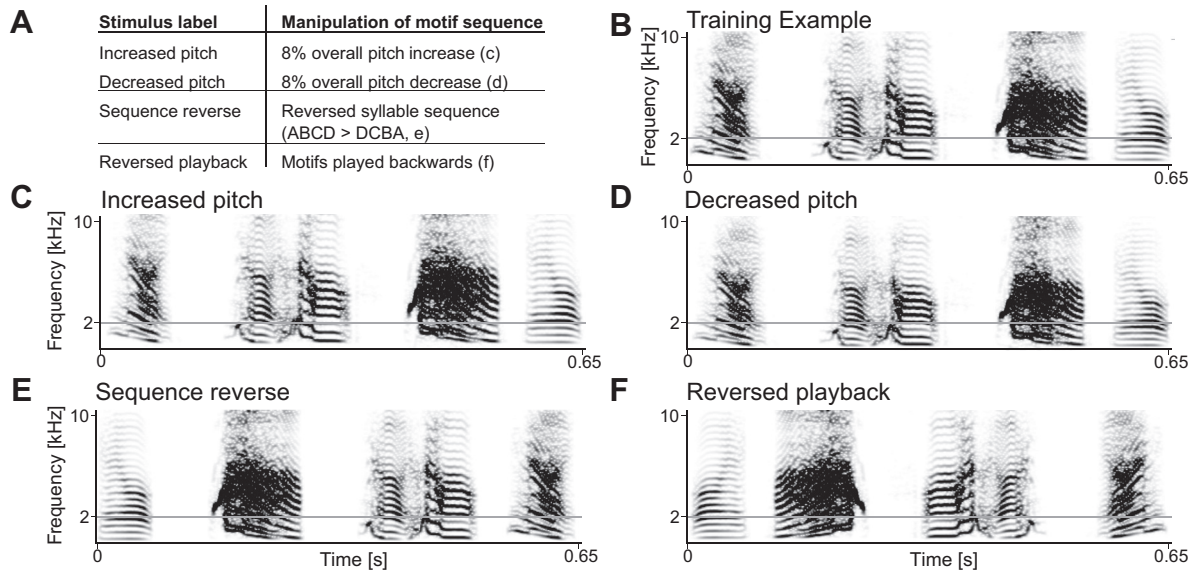

Figure 2. Examples and explanations for the test stimuli. *A:* list of the different stimulus manipulations. *B*: spectrogram of an unmanipulated song used as Go-stimulus. *C* and *D*: manipulations of the Go-stimulus used as test stimuli, as follows: 8% Pitch increase (*C*), 4 8% Pitch decrease (*D*), "Sequence reverse" with unchanged inter syllable intervals (*E*), "Reversed playback" (reversal of the entire original Go stimulus) (*F*). The gray horizontal line marks 2,000 Hz for better illustration of pitch changes.

3:00 and 5:00 PM, 8 to 10 h after lights on) and euthanized by an overdose of isoflurane for brain extraction the next morning before light onset at 7:00 AM. Brains were transported for RNA extraction to the Max Planck Institute for Psycholinguistics in Nijmegen. The abundance of FoxP1 mRNA transcripts was determined via qPCR. Samples generated from HVC and CMM punches showed significantly lower *FoxP1* expression across both hemispheres of all knockdown groups compared with their respective matched controls (see Supplemental Fig. S1, see also Ref. 24).

Statistical Analyses

All analyses were performed in R v4.2.3 (83). Normal distribution of data was tested and confirmed with Shapiro–Wilk tests. General linear model (GLM) analyses were performed assuming a Poisson distribution of the response and discrimination rates. Post hoc analyses comparing the different groups were conducted with the emmeans package (84). Multiple tests were corrected for false discovery rate (85).

To test if the knockdowns affected learning, we created GLMs with the number of necessary trials or days during the training phase until a sufficient DR was reached as response variables. We added the injected virus batch as random effects and treatment, region, age, and their interactions as fixed factors. Once the birds had reached criterion, a similar model was used to determine if the birds' training performance was affected by the knockdowns. Therefore, the DR toward training stimuli was used as response variable and the same factor configuration as for the previous models was used. To identify differential behaviors toward trained Go or Nogo stimuli, respectively, we created another model. This GLM used response rates toward trained Go or Nogo stimuli as response variable and included bird ID as a random effect. It further included stimulus type (Go or Nogo) and its interaction with all other factors as a fixed effect.

To identify treatment-related effects on the birds' performance toward the test stimuli, we designed three different models. The first one included the DR as response variable, bird ID and the batch of the injected virus as random factors and treatment (control or knockdown), region (HVC or CMM) and age (juvenile or adult) as fixed factors.

Next, data were modeled separately for the birds' responses toward TestGo stimuli (correct response) or TestNogo stimuli (false positive) as response variables to prevent potential masking effects of very low response rates toward Nogo-stimuli. Both of these models were initially set up from a null-model that contained only random factors. Fixed factors were added one by one and the best fitting model was subsequently determined by the Akaike information criterion (AIC) and the models' weights.

After initial consideration of treatment status, neither area and age group nor their interactions yielded treatment-related effects and all birds responded within previously reported margins during comparable tasks (59). Thus, data were pooled irrespective of the treatment to analyze female responses to the different types of stimulus modifications.

We designed two separate models with the DR and the response rates toward the stimuli as response variables, respectively. The model on the birds' DR contained bird ID as random and the different test stimuli as fixed factor. To model the birds' response rates, we added bird ID as random factor and added the different test stimuli as well as the stimulus type and their interaction as fixed factors. Depending on the design of the model, stimulus type is included as a single factor consisting of multiple levels for each manipulation.

RESULTS

Training Performance

Training performance was measured both in terms of the number of days and the total number of trials needed to

reach criterion. During the training phase, all birds ($n = 95$) but one (juvenile HVC control) reached a discrimination rate (DR) > 0.75 one to five days after introducing the training Go and Nogo stimuli (mean ± standard error = 2.01 ± 0.21 days). Once a bird reached a DR > 0.75, the DR remained high or increased during the following training days. The number of days to reach DR > 0.75 did not differ between the knockdown groups and their controls and was not influenced by age, region, or their interactions with the treatment (Supplemental Table S2). The number of trials needed to reach DR > 0.75 differed between groups (Fig. 3*A*) as identified by the significant interaction between region and age (Table 1). Post hoc analyses revealed a significant effect of juveniles injected into HVC with both the control [$P = 0.021$, False Discovery Rate (FDR) adjusted] and knockdown constructs ($P = 0.001$, FDR adjusted). Those groups required more trials to reach DR > 0.75 (Fig. 3*A*). Once birds had reached the training criterion, the DR between the Go and Nogo stimuli did not differ between any of the tested groups (Fig. 3*B*, Supplemental Table S3) for the unrewarded 20% of playback stimuli. There was also no group-based effect on the response rates toward the unrewarded trained Go (Fig. 3*C*) or Nogo stimuli (Fig. 3*D*, Supplemental Table S4) during the three days prior to the onset of test stimulus playbacks. Learning-related behaviors such as required days or trials to reach the training criterion were also not correlated to measurements taken during the previously published preference tasks (Supplemental Fig. S2).

Responses to Test Stimuli

The discrimination rate of test stimuli did not differ between treatments, areas, or age at injection (GLM, see Supplemental Table S5). To circumvent potential effect-masking (e.g., simultaneous reduction of Go and Nogo responses and thus no change in the discrimination rate), the birds' response rates toward TestGo and TestNogo stimuli during the testing phase were analyzed separately. Response rates differed to the same degree in all groups between TestGo and TestNogo stimuli with increased (Fig. 4*A*) or decreased pitch (Fig. 4*B*), sequence reverse (Fig. 4*C*), and reversed playback versions of the training stimuli (Fig. 4*D*). Thus, none of the stimulus manipulations resulted in a loss of discrimination. To analyze whether different stimuli

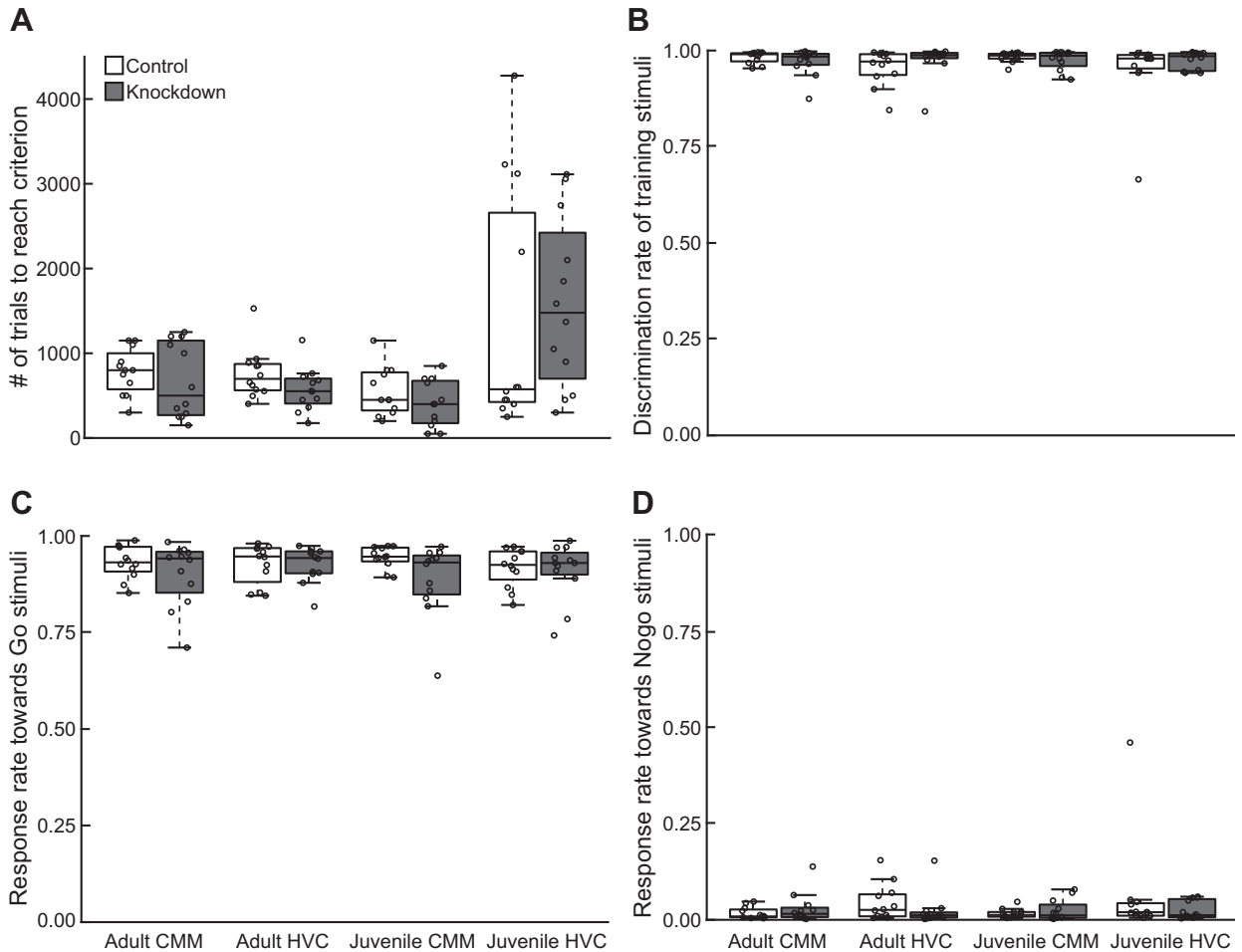

Figure 3. Performance of all groups ($n = 12$ per group = 96 females) during the Go/Nogo task training period. Boxplots: box = range between 1st and 3rd quartile; line within the box = median; whiskers = 1.5 interquartile range; data points depict individual birds. *A*: number of trials required to reach training criterion was higher in birds receiving injections (control or knockdown) as juveniles than as adults but there was no systematic effect of area [HVC or caudomedial mesopallium (CMM)] or treatment (control or knockdown). *B*: discrimination rates during the three days prior to testing showing all groups discriminated equally well between the trained Go and Nogo stimuli. Response rates to Go (*C*) and to Nogo stimuli during training (*D*).

Table 1. *GLM-based analyses of the number of trials needed until the training criterion of a discrimination rate > 0.75 was reached in the different experimental groups*

Trials to Training Criterion	Estimate	Std. Error	*t* Value	*P* Value
Model: 1|Virus Batch + Treatment + Region + Age + Treatment × Region × Age[1]				
Intercept	787.5	194.26	4.054	**0.001**
Treatment	−117.5	274.72	−0.428	0.676
Region	−28.92	274.72	−0.105	0.918
Age	−237.5	274.72	−0.865	0.403
Treatment × Region	−72.17	388.52	−0.186	0.855
Treatment × Age	−28.33	388.52	−0.073	0.943
Region × Age	851.5	388.52	2.192	**0.047**
Treatment × Region × Age	430.58	549.45	0.784	0.447

Significant *P* values are marked in bold. GLM, general linear model. [1]The model assumes Poisson distributed data and contains injected virus batch as a random factor. All other parameters are equally weighted as full factorials and tested for interaction.

gave rise to behavioral differences between any of the tested groups, separate GLM analyses were conducted for response rates to Go (see Table 2, and Supplemental Table S6) and Nogo stimuli (see Table 3, Supplemental Table S7, Supplemental Fig. S3). The birds' response rate to the respective stimulus category was set as the dependent variable. Based on the AIC and its corresponding weight, *model B* was identified as the best fit for both the models of Go and those of Nogo responses, indicating that test stimulus had the largest impact on the birds' performance, whereas Treatment, Region (targeted brain area), or the birds' Age during the injection did not contribute significantly to behavioral variation. A significant intercept

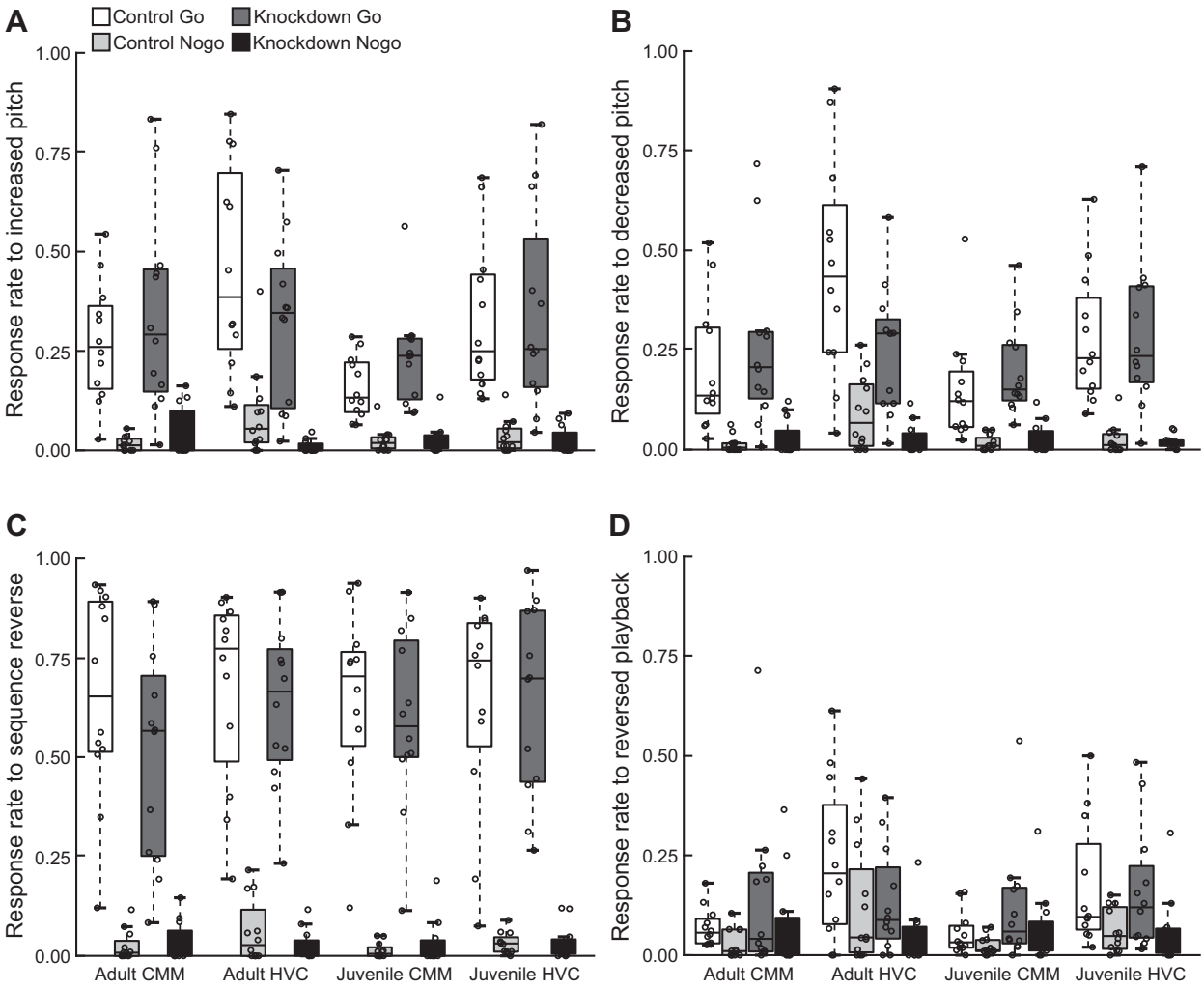

Figure 4. The proportion of Go responses (response rate) of all groups ($n = 12$ per group = 96 females) to TestGo and TestNogo stimuli. Data points depict individual birds. Birds responded in a similar manner to stimuli that were increased (*A*) or decreased in pitch (*B*). They showed the highest Go-response rates to TestGo stimuli with reversed syllables (*C*) and the lowest Go-response rates to TestGo stimuli played back in reverse (*D*). Boxes show range between first and third quartile, horizontal lines indicate median values while whiskers show 1.5 interquartile range. CMM, caudomedial mesopallium.

Table 2. *GLM-based analyses of the Go-response rate of birds to TestGo stimuli during Go/Nogo-tasks*

Go Response to Go	Estimate	Std. Error	*t* Value	*P* Value
	Model B[1]*: Test Stimulus*			
Intercept	0.251	0.024	10.566	**1.026×10^{-17}**
Test stimulus	0.049	0.021	2.312	**0.023**

Different test types are included as different levels (one for each manipulation of pitch, sequence or reversal) of a single factor. Only the best model [based on the Akaike information criterion (AIC) and its weight] is shown. Significant *P* values are marked in bold. Complete GLM results are shown in Supplemental Table S6. GLM, general linear model. [1]The model assumes Poisson distributed data and includes individual bird identification number and injected virus batch as random factors. All other parameters were added as equally weighted fixed effects one after another.

throughout all models indicates that the tested birds behaved differently in response to the various test stimuli, but not according to the experimental manipulations. Hence, there was no impact of the knockdown on female responses to the stimuli. FDR-corrected post hoc analyses revealed that test stimuli of the Go-type significantly affected the amount of birds' responses. Although increased pitch had no significant effect ($P = 0.727$), all remaining stimulus types significantly affected the birds' response rates (decreased pitch $P = 5.683 \times 10^{-8}$; reversed syllables $P = 1.434 \times 10^{-52}$; reverse playback $P = 1.140 \times 10^{-38}$). Although test stimuli of the Nogo-type in general did not significantly affect the birds' response rates (Table 3), post hoc analyses revealed significant effects on the birds' false positive responses for stimuli with decreased pitch ($P = 0.024$) and reversed playbacks. Nogo stimuli with increased pitch ($P = 0.115$) did not affect the birds' false positive responses and reversed syllables showed a trend to affect the birds' behavior ($P = 0.0498$).

Extinction of Trained Discrimination

To assess whether birds from the different treatments differed in how quickly they could learn that the previously learnt rules would not result in rewards anymore (extinction learning), Go and Nogo stimuli were both positively reinforced after completion of the testing phase. The amount of trials that the birds required to respond equally often both to Go and Nogo stimuli again was used as response variable for further analyses. There were no differences between controls and knockdowns in the number of trials needed until the birds responded equally often toward the former Go and Nogo stimuli (mean ± standard deviation; juveniles: HVC control = 1,319 ± 929, HVC knockdown = 1,313 ± 928, $P = 0.92$; CMM control = 1,130 ± 739, CMM knockdown = 1,299 ± 1,328, $P = 0.66$; adults: HVC control = 630 ± 668, HVC knockdown = 1,757 ± 1,552, $P = 0.13$; CMM control = 2,738 ± 2,001, CMM knockdown = 2,018 ± 1,392, $P = 0.4$; Wilcoxon rank sum tests).

Table 3. *GLM-based analyses of the Go-response rate (false positive) of birds to TestNogo stimuli during Go/Nogo-tasks*

Go Response to Nogo	Estimate	Std. Error	*t* Value	*P* Value
	Model B[1]*: Test Stimulus*			
Intercept	0.031	0.007	4.506	**1.881×10^{-5}**
Test stimulus	0.003	0.005	0.576	0.566

Only the best model (based on the AIC and its weight) is shown. Significant *P* values are marked in bold. Complete general linear model (GLM) results are shown in Supplemental Table S7. [1]The model assumes Poisson distributed data and includes individual bird ID and injected virus batch as random factors. All other parameters are added as equally weighted fixed effects one after another. Different test types are included as different levels (one for each stimulus manipulation) of a single factor.

Comparison of Response to Different Test Stimuli

No overall effects of treatment, area of injection, or age on any of the tested groups were evident from the results of the statistical analyses (Fig. 4, GLMs in Tables 1, 2 and 3 and Supplemental Tables S2, S3, S4, S5, S6, and S7). Therefore, all experimental groups were merged, thus creating a large sample size that we used to examine how female zebra finches differentiate among the test stimuli, and to assess their discriminatory abilities with respect to detecting and weighting the different types of changes to unrewarded training stimuli (Fig. 5).

Across all groups, the birds discriminated between all sets of TestGo and TestNogo stimuli (Fig. 5*A*). The females discriminated stimuli with altered pitch, independent of the direction (8% increase: $t_{95} = 13.05$, $P < 0.0001$; 8% decrease $t_{95} = 12.31$, $P < 0.0001$), sequence reverse ($t_{95} = 24.39$, $P < 0.0001$, paired *t* Tests) or reversed playbacks ($t_{95} = 6.9$, $P < 0.0001$). The DR of training stimuli remained the highest compared with test stimuli. Although test stimuli in general had an effect on the birds' discrimination rate of different stimuli (Table 4), post hoc analyses revealed that this effect was driven by high DRs of training stimuli ($P = 1.306 \times 10^{-11}$) and the birds' discriminatory abilities of test stimuli with reversed syllables ($P = 2.024 \times 10^{-8}$) or those played back in reverse ($P = 9.837 \times 10^{-45}$). Manipulated pitch, either increased ($P = 0.1029$) or decreased ($P = 0.1061$), did not significantly affect the DR.

In addition to the differences among the birds' DRs for TestGo and TestNogo stimuli, birds' response rates, upon which the DR scores are based, differed as well. Response rates toward TestGo stimuli varied between stimulus types (Fig. 5*B*, Table 5). In contrast, response rates toward all TestNogo stimuli remained comparably low. Responses to Nogo stimuli occurred in less than 4% of all trials. Birds responded with lower variance to different TestNogo than to TestGo stimuli (mean variance = 0.004 vs. 0.042, $P = 0.0025$, $t = 4.985$, paired *t* Test). Both stimulus type (Training or Test) and the manipulation of the Test stimuli as well as their interaction, significantly affected the birds' response rates (Table 5). Post hoc analyses revealed that response rates to all versions of TestGo stimuli were significantly reduced compared with the trained Go stimuli ($P < 0.001 \times 10^{-60}$). Response rates between increased ($P = 1.575 \times 10^{-20}$) or decreased pitch ($P = 3.181 \times 10^{-35}$) differed as well and the birds responded most to sequence reversed TestGo stimuli ($P = 5.173 \times 10^{-27}$) and least to reversed playbacks ($P < 0.001 \times 10^{-60}$).

DISCUSSION

We had hypothesized that local FoxP1 knockdown in HVC or CMM could impair auditory discrimination in female zebra finches because of prior studies that had shown associations between disrupted *FoxP1* expression or function and

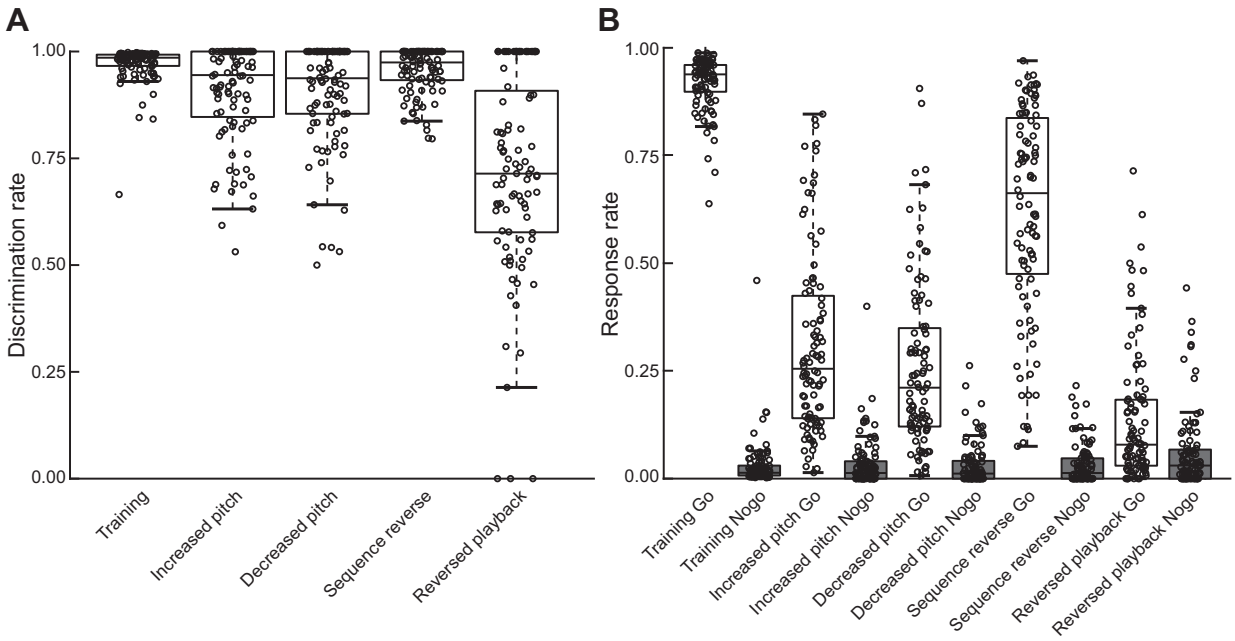

Figure 5. Responses to the different stimuli during the testing phase (pooled data of all birds across all treatment groups, $n = 96$ females). *A*: discrimination rate between TestGo and TestNogo stimuli remained high except for the much lower discrimination rate of reversed compared with the other stimuli. *B*: response rates to the TestGo stimuli are lower than to the training Go stimulus. Responses toward TestNogo stimuli showed no change in response. Data points depict individual birds. Boxes show range between first and third quartile, horizontal lines indicate median values while whiskers show 1.5 interquartile range.

auditory perception and cognition in human patients (4, 6), knockout mice (86–88), and zebra finches (24). In the present study, we tested whether female zebra finches with local FoxP1 knockdowns in the forebrain areas HVC or CMM (both implicated in auditory perception and cognition) would show an impaired ability to discriminate songs and to categorize different song modifications. Our hypotheses were also based on the observation that the same female birds with experimentally reduced *FoxP1* expression levels in HVC had previously shown reduced preference for familiar (tutor) song and reduced motivation to actively trigger playback of these songs compared with controls without knockdowns or with knockdowns in other areas (24). Moreover, in juvenile males, FoxP1 knockdowns in Area X (25) or HVC (23) resulted in impaired song learning, which is dependent on auditory feedback.

However, contrary to our expectations there were no systematic effects of the knockdown regarding how fast and how well birds learnt during the discrimination tasks (Fig. 3). Learning speed, measured by the number of trials to reach a DR > 75%, was reduced in birds that received injections in HVC as juveniles but intriguingly this was the case for both controls and knockdowns. However, once they had reached the criterion, they discriminated Go from Nogo training stimuli as well as control birds and also did not differ from them in response to the various test sounds (Fig. 4) nor during extinction of the trained discrimination. Even though tissue damage above or within HVC was not evident from histological analysis, juvenile HVC and/or the overlying (para)hippocampus could have been disturbed that might have subsequently resulted in effects on spatial learning (89, 90) during the initial learning period until the birds mastered the task.

Besides the required trials to reach a discrimination ratio (DR) > 75%, learning and discrimination as well as extinction were unaffected by FoxP1 knockdown in HVC and CMM. This shows that even though both brain regions strongly express *FoxP1* in juvenile and adult zebra finches (26–28), the experimental reduction of FoxP1 levels in HVC or CMM does not impair processes underlying auditory discrimination at the times tested. Projections to and from transduced neurons might have been disrupted by the knockdown construct. However, the absence of group-specific effects opposes this explanation. A lack of group-specific effects is also unlikely to have resulted from a misplaced or dysfunctional viral construct as several virus batches were used that resulted in a significant decrease of *FoxP1* expression in HVC of all knockdown groups (see Supplemental Fig. S1). FoxP1 knockdowns were expected to have behavioral effects on some groups, given that in a previous study with the same

Table 4. *GLM-based analyses of the discrimination rate between all stimuli of all birds*

Discrimination Rate All Birds	Estimate	Std. Error	*t* Value	*P* Value
	Model: 1|Bird ID + Test Stimulus[1]			
Intercept	0.904	0.015	60.853	**6.759×10^{-78}**
Test stimulus	0.067	0.02	3.391	**0.001**

Significant *P* values are marked in bold. GLM, general linear model. [1]The model assumes Poisson distributed data and contains bird ID as a random factor. Stimulus type is included as a fixed factor with multiple levels (one for each stimulus manipulation of pitch, sequence or reversal). The test stimulus category includes all stimuli that were played back to the birds, including the training stimuli.

Table 5. *GLM-based analyses of the response rates towards all stimuli of all birds*

Response Rate All Birds	Estimate	Std. Error	*t* Value	*P* Value
	Model: 1|Bird ID + Stimulus Type × Test Stimulus[1]			
Intercept	0.27	0.014	18.678	**1.447×10^{-33}**
Stimulus type	−0.228	0.017	−13.54	**6.69×10^{-24}**
Test stimulus	0.659	0.017	39.077	**2.449×10^{-60}**
Stimulus type × Test stimulus	−0.66	0.024	−27.695	**2.765×10^{-47}**

The model further includes interactions between stimulus type (Go/Nogo) and both training and test stimuli. Significant *P* values are marked in bold. GLM, general linear model. [1]The model assumes Poisson distributed data and contains bird ID as a random factor. Stimulus type and test stimuli are included as fixed factors, each containing multiple levels (one for each stimulus manipulation).

birds, adult females that received a FoxP1 knockdown in HVC had weaker learned song preferences than matched controls (24). During these previous experiments, which focused on preference tests in which song playback was the reward for the operant key pecking, the adult HVC knockdown group also showed a lower activity than the other groups. These behaviors showed correlations with the measured *FoxP1* expression levels in the target areas in relation to their respective controls (24). However, in the present study neither virus treatment, nor injected area or age affected the birds' responses to stimuli. This means that either FoxP1 levels were not reduced sufficiently for the birds to lose the ability to discriminate between the stimuli and their different parameters or that the presence of FoxP1 in HVC and CMM is neither required for auditory learning and discrimination nor affects the weighting of auditory parameters birds rely on during stimulus identification. As the knockdowns were on target (see also Ref. 24) and significant but partial rather than complete knockdowns (Supplemental Fig. S1, ∼50% expression related to the matched controls), the reduced expression levels might have been sufficient to induce subtle downstream effects leading to differential preference responses but not an inability to compare and distinguish different sounds. In addition, learning-related behaviors during the Go/Nogo experiments were not correlated to preference measurements (Supplemental Fig. S2) taken during the previously published preference tests (24). The analyses did not provide any evidence of a direct link across both studies regarding the training or sensitivity to particular song parameters. This might be partly due to the fact that the preference tests were centered around familiar songs whereas the songs used in the current study were all unfamiliar ones. Also, the motivational context differs between the studies. The discrimination training uses a food reward whereas in the preference tests exposure to the song acts as reinforcement. Furthermore, in the previously published preference experiments, birds were not tested for their responses toward specific parameters as was the case for the current study.

We next focused the analyses on the pooled data of all birds as our large data set allowed us to examine the significance of different manipulated spectral and sequential parameters for identifying and discriminating the training songs (Fig. 5).

Previous studies on cue weighing in song discrimination (e.g., see Refs. 62, 66, and 67) found spectral changes to have stronger effects on song discrimination than changes in syllable sequences. The comparison of the DR between the TestGo and TestNogo versions of training stimuli in our study showed that birds discriminated and categorized stimuli that differed from the original Go stimulus in pitch or element sequence equally well as the initially trained stimuli. The DR of reversed playback stimuli was poorer than that of training stimuli (although still significant), indicating that similar to previous studies, the birds paid more attention to the spectral structure of syllables than to syllable order or pitch changes of a motif. However, pitch change is a quantitative measure and its impact depends on the magnitude of change (67) and hence the use of smaller or larger pitch changes might have resulted in smaller or larger impact on discrimination in our birds, respectively.

In addition to the DR, subjects' response rates to the different TestGo and TestNogo stimuli were analyzed separately to control for potential changes in strategies, such as to avoid negative reinforcement by reduced responses to both TestGo and TestNogo stimuli. Subjects reduced their responses to the TestGo stimuli compared with the trained Go stimuli, despite maintaining a high DR suggesting that subjects did distinguish all test stimuli from the training stimuli. Simultaneously, responses to TestNogo stimuli remained low. Taken together, this suggests that birds behaved more cautiously toward the test stimuli than toward familiar training sounds. In general, response rates varied more than the DR among all stimuli. The highest response rate was maintained to test stimuli in which the syllable sequence was reversed compared with the training stimulus while maintaining pitch and acoustic fine structure of syllables. This manipulation had a much smaller effect than reversed playback of songs, again indicating that syllable sequence is less important for categorization than the acoustic fine structure of song syllables. This finding corresponds with earlier studies (62, 66, 75, 91, but see Ref. 68) and is consistent with variable positioning of syllables during sung motifs (92) and prior preference tests suggesting that zebra finches recognize songs by their syllable composition not sequence (93). Nevertheless, our data do show that syllable sequence reversal was noticed, as it did affect the response rate compared with the original stimuli.

In line with the importance of spectral over sequential features, subjects reduced their responses more strongly to stimuli with increased or decreased pitch than to the training stimuli or the reversed syllable sequence. Response rates differed between stimuli with increased and decreased pitch where subjects responded more to stimuli with increased than decreased pitch—a discrimination absent in female zebra finches tested with comparable stimuli in another study (67). This difference might be explained by the smaller sample size in the previous study and the large overlap between

the responses toward both pitch-shifted stimuli in the current experiment. In addition, whereas Nagel et al. (67) also found that pitch-changed stimuli were less well categorized, they did not report a reduced response rate to changed stimuli. This difference from our results may be related to different experimental conditions. We used a Go/Nogo paradigm whereas the prior study used a two-alternative forced choice (2AFC) design, which may reduce the tendency to refrain from responding during a trial as birds need to respond to avoid negative reinforcement during 2AFC. Although this is an advantage of the 2AFC over Go/Nogo tasks, the latter is an easier and faster task to learn, which was important for this experiment to diminish potential weakening of effects of the lentiviral constructs over time.

Reversing the individual syllables changes the acoustic fine structure of songs the most compared with the other experimental stimuli, and also led to the lowest response rate during the experiments. Similar to our study, two previous experiments (62, 66) also found that zebra finches perceive stimuli that are played back in reverse as very different from nonreversed versions. Lawson et al. (66) suggested that zebra finches fail to recognize stimuli played back in reverse. However, our results show that, even though response to reversed playbacks is greatly reduced, some cues in reversed stimuli remained that enabled correct categorization. Likewise, in a set of preference tests, female zebra finches did not discriminate against reversed playbacks in phonotaxis experiments (94).

Taken together, our findings show that syllable sequence is differently weighed with regard to song identification than overall pitch (at least with the 8% of pitch change during this study) or the structure of individual syllables. This difference may be related to the way juvenile male zebra finches modify their song when their tutor song changes. They first adjust the pitch of an already learned syllable followed by a rearrangement of its sequence (95). Furthermore, rearrangement of an already learned sequence of syllables in juvenile male zebra finches requires more time than they need to integrate an entirely new syllable (96) hinting at different mechanisms for learning syllable structure and sequences that might be comparable to human language acquisition in infants where learning of phonemes occurs before words and grammar emerges (10, 11, 97, 98). If females are sensitive to the same features to categorize songs as young males are during song learning, our findings might point to potentially similar mechanisms in both sexes to recover sequential information from auditory stimuli, as was also observed by Ning et al. (68). Different recognition mechanisms for pitch and sequence have also been suggested by neurophysiological research where single units in auditory cortex of starlings either respond to the type of a motif or its pitch where neurons in CMM appeared to be of low selectivity (99).

In summary, auditory discrimination was not influenced by endogenous *FoxP1* expression in HVC or CMM. Despite the high and developmentally stable expression of *FoxP1* in these brain regions, both of which are involved in perception and processing of song, a local knockdown in these areas did not affect discriminatory performance or any of the related features tested in the present study. This preserved function might result from individually variable expression, buffering, or redundancy mechanisms around critical transcription factor families such as FoxPs, which may have masked local knockdown effects. Further experiments will be needed to identify the molecular underpinnings of this fine-tuned auditory discrimination and why reduced *FoxP1* expression and/or functionality are associated with auditory and cognitive tasks in humans (2, 4, 6, 7, 100–103) and mice (86, 104). Although the exact role of *FoxP1* in developing songbirds (23, 24) needs to be studied in further detail, FoxP1 knockdowns in both juvenile and adult females did not affect song discrimination learning. The extensive auditory discrimination tasks in the present study also confirmed that zebra finches rely more on spectral than sequential features for song identification, although we showed that they are still capable of recognizing songs with changed syllable sequences and may categorize reversed playbacks correctly and identify relevant features (94).

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Tables S1–S7 and Supplemental Figs. S1–S3: https://doi.org/10.6084/m9.figshare.25140683.

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Present address of F. Heim: Max Planck-Institute for Biological Intelligence, Eberhard-Gwinner-Straße, 82319 Seewiesen, Germany.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

F.H., C.S., S.E.F., K.R., and C.t.C. conceived and designed research; F.H. performed experiments; F.H. analyzed data; F.H., C.S., S.E.F., K.R., and C.t.C. interpreted results of experiments; F.H. prepared figures; F.H. drafted manuscript; F.H., C.S., S.E.F., K.R., and C.t.C. edited and revised manuscript; F.H., C.S., S.E.F., K.R., and C.t.C. approved final version of manuscript.

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