Neural activation in response to conspecific songs in zebra finch (*Taeniopygia guttata*) embryos and nestlings

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Classic studies on the effects of auditory stimulation in embryonic birds have largely been limited to precocial taxa. In altricial taxa, physiological responses of embryos and, subsequently, the behavioral responses of nestlings have begun to receive increasing attention, yet it remains unclear whether and to what specificity neural responses are generated *in ovo*. Using in-situ hybridization for an immediate early gene, *ZENK*, we detected significant neural activation in both the embryos and nestlings of an altricial songbird, the zebra finch (*Taeniopygia guttata*) when exposed to conspecific song playbacks relative to silence. In turn, embryonic *ZENK* responses to heterospecific songs were intermediate in strength. These results are consistent with physiological evidence for conspecific song selectivity in embryos of other altricial songbird taxa. *NeuroReport* **30:217–221**

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Introduction

A half-century of now classic research has documented the critical effects of prenatal auditory stimulation on both prenatal and postnatal neural and behavioral responses in precocial avian taxa [1]. New evidence from recent years increasingly suggests that similar effects may also be found in altricial avian taxa, including adaptive contexts regarding thermal biology [2,3], avian brood parasite–host recognition systems [1,3,4], and conspecific song learning [5,6]. Auditory studies in young altricial nestlings (<10 days posthatch) have been rare, likely because earlier evidence suggested under-developed auditory sensitivity in nestlings; but see studies by Aleksandrov and colleagues [7–9]. Emerging research in the zebra finch (*Taeniopygia guttata*), superb fairywren (*Malurus cyaneus*), and red-backed fairywren (*Malurus melanocephalus*) has provided evidence for the effects of prenatal auditory stimulation on prenatal sound learning and responsiveness to sound [4,10,11], as well as effects on postnatal characteristics including early-life body mass and begging behavior [3], postfledging vocal learning [5, 6], breeding behavior [3,6], and reproductive success [3].

Previous studies of adult songbirds have suggested a critical role of avian auditory forebrain regions in conspecific selectivity, specifically in Field L [12,13], the caudomedial nidopallium (NCM) [14,15], and the caudomedial mesopallium [15]. Several of these studies used neural activity-dependent immediate early gene expression as a transcriptional indication of increased cellular response to salient stimuli that may promote downstream processes [16]. These, in turn, may lead to tissue-level and organism-level changes, including neural plasticity and behavioral response [16–18]. Several immediate early genes have been implicated in differential neural activation of the avian auditory forebrain in response to conspecific vocalizations; these include *ZENK* (or *Egr-1*) [19,20], *c-FOS* [20], and *Arc* [21].

Our study investigates whether neural *ZENK* expression levels, following ontogenetic exposure to playbacks of conspecific or heterospecific songs, or no songs (silence), corroborate the results of recent embryonic auditory stimulation studies, which suggest detectable effects of prenatal auditory stimulation on songbird physiological auditory response selectivity and other diverse behavioral characteristics *in ovo* and beyond (see [3,6]). Specifically, we test for differential neural *ZENK* expression in the avian auditory forebrain following exposure to conspecific songs versus silence in both embryonic and nestling zebra finches.

Materials and methods

Song playback experiments

Playback trials with embryos

Zebra finches were bred and obtained from colonies at Rockefeller and Colgate Universities. Breeding pairs were established in single-family cages. Nests were checked daily and eggs were labeled to track genetic family and date laid; eggs were collected on the day of laying and transferred to an incubator (Grumbach S84; Grumbach,

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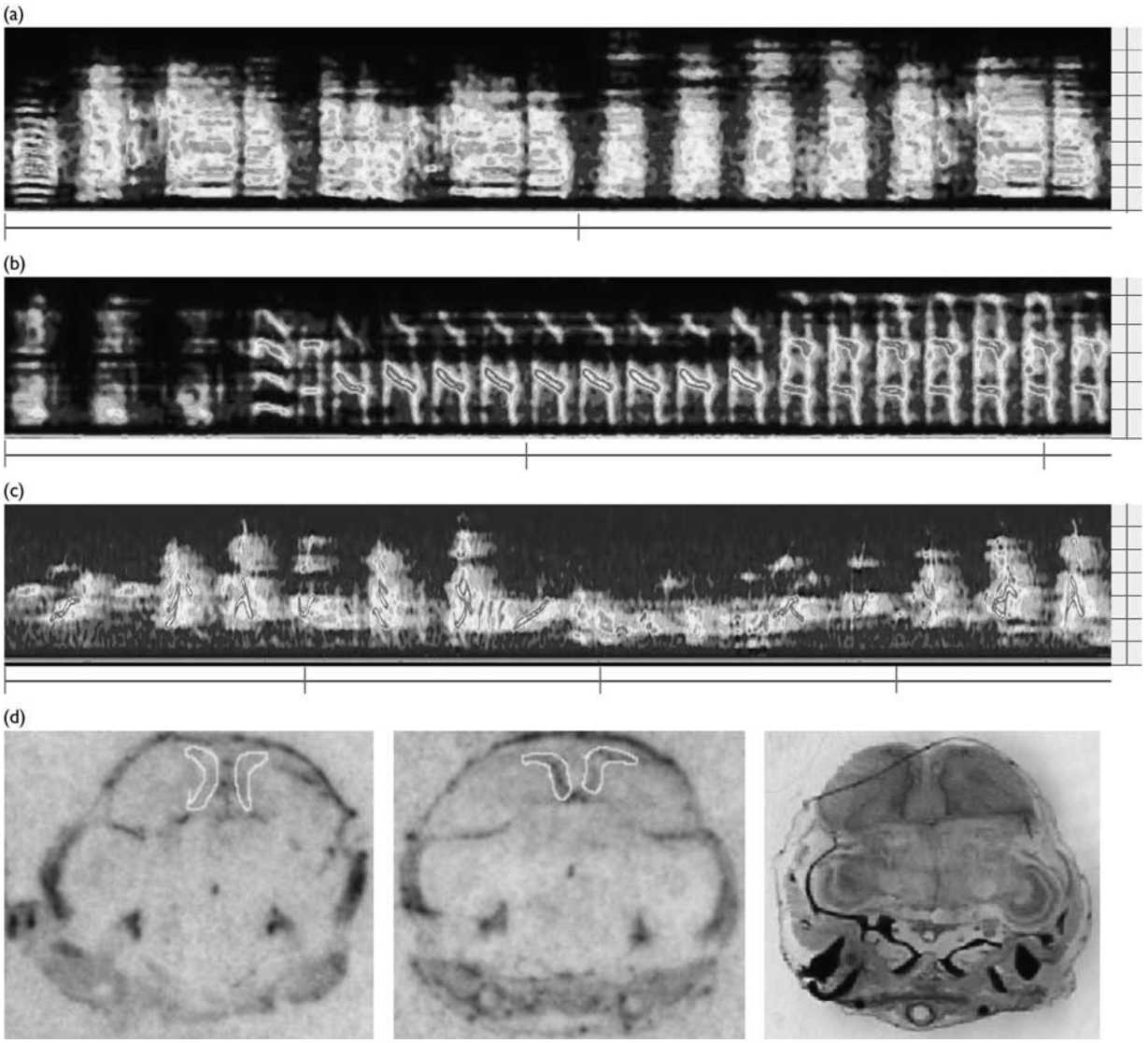
Fig. 1



Sample spectrograms of zebra finch (a), Bengalese finch (b), and pin-tailed whydah (c) songs. Axis under each spectrogram represents time with each tick mark indicating 1 s; axis to the right of each spectrogram represents frequency with each tick mark indicating 2 kHz. Also pictured are samples of neural *ZENK* activation (d) following silent (left) or zebra finch song (middle) playback, and Nissl-stained embryonic brain sections (right). Highlighted areas indicate auditory forebrain regions of interest (caudomedial nidopallium, Field L). All sections show the whole head, as the brain was not extracted.

Mücke, Germany). All embryos were incubated at 37.5° ± 0.5°C and 62.5 ± 2.5% humidity until day 10 of embryonic development (E10) out of the 14–16 days incubation period. In the afternoon of E10, embryos were begun to be exposed to their assigned experimental condition of zebra finch (conspecific) songs (ZF; Fig. 1a), Bengalese finch (*Lonchura striata*; heterospecific) songs (BF; Fig. 1b), pin-tailed whydah (*Vidua macroura*; heterospecific) songs (WH; Fig. 1c), or no songs (silence; SIL) *in ovo*. Playback stimuli were sourced through a previous study from previous studies using different colonies [13,22], edited with Raven Pro (v. 1.4, Ithaca, New York, USA), and normalized for duty cycle and amplitude at 15 dB with Audacity 2.1. The choice of heterospecific stimuli was made to parallel our labs' work on the neural basis of acoustic species-recognition in adult zebra finches [13] and pin-tailed whydahs [22]. Each of the final three 30 min tracks was produced by compiling single song motiffs from three individuals of the given species (ZF, BF, or WH) sequentially and repeatedly until obtaining a 30 min file.

Playback stimuli (i.e. ZF, BF, or WH) were broadcast to embryos in a second experimental incubator (Avery, Hugo, Colorado, USA) (playback incubator) equipped with two desktop PC speakers (Dell, Round Rock, Texas, USA).

Incubation temperature of 37.5°C was maintained throughout the experimental trials. Eggs were arranged equidistantly from the two speakers with their long axes parallel to the incubator floor on Styrofoam, which was modified to contain the eggs and prevent rolling. Eggs were placed in the playback incubator and exposed to 30 min of sound treatment; thereafter, eggs were returned to the home incubator for the rest of the day.

Playbacks were conducted daily from E10 until the expected day before hatching (ca. E14). Eggs were candled daily and embryonic development was noted to ensure accurate estimates for day of hatching. On the final day of playback, eggs were exposed to the assigned stimulus as described above. The same track used during the exposure days (E10–E13) was used on the final trial day (E14). Following this 30 min playback, eggs were collected and opened, and embryos were killed by rapid decapitation at 30 min following the final playback session. Embryo heads were preserved whole in tissue freezing medium (Tissue-tek O.C.T.; Sakura Finetek USA Inc., Torrance, California, USA) via rapid cooling with dry ice and stored in a −80°C freezer until sectioning. Tissue was sectioned at 20 µm using a cryostat and stored at −80°C until in-situ hybridization. We did not determine the sex of all embryos in this study using genetic or anatomical markers, and so we did not include sex in our statistical models.

Handling and tissue collection for embryos in the SIL treatment was as described for embryos in the sound treatments, except no sound was broadcast via the speakers in the second incubator.

Playback trials with nestlings

Nestling zebra finches (d6–d9 posthatch), from active nests of pairs housed in single-family cages in our colony room, were moved from the natal nest to a playback incubator at 37.5°C. All nestlings were initially maintained in silence for 3 h. For the sound treatment group, each nestling received 30 min exposure to ZF playback after the initial silence period. After playback, these birds were maintained in silence for 15 min before being killed by rapid decapitation; the brains were extracted, frozen, sectioned to 20 µm in a cryostat, and stored at −80°C until use as above. Silence treatment subjects were removed from the incubator and killed after the initial 3 h of silence.

All breeding, handling, and procedures were conducted under the approval of IACUC at Rockefeller and Colgate Universities.

In-situ hybridization

Embryonic and nestling brain sections were processed for in-situ hybridization by using ^{33}P-labeled *ZENK* antisense riboprobes. The cDNA probe of *ZENK* was made from an earlier study [23]. Frozen brain sections (20 µm) were fixed in 4% paraformaldehyde in PBS (pH 7.0), acetylated, dehydrated in an ascending ethanol series, and air dried. The hybridization solution was then placed on each slide. The hybridized slides were incubated at 62°C for 13–15 h under mineral oil. Excess probe was removed by washing in 2× saline–sodium citrate (SSC) at room temperature for 1 h, and then washed with 2×SSC for 1 h. The next wash was for 1 h at 65°C in a solution of 50% formamide with 2×SSC, and the last wash was in 0.1×SSC for 1 h at 65°C for 30 min Slides were dehydrated in an ascending ethanol series and exposed to radiographic film for 2–4 days before being developed.

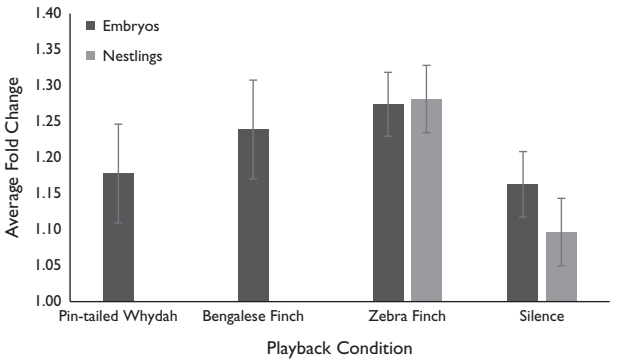
Film quantification/analysis

There is no atlas for embryonic and juvenile zebra finches, so the adult zebra finch atlas (*https://www.ncbi.nlm.nih.gov/books/NBK2354/*) was used as a guide to locate areas of the auditory forebrain (Field L, caudomedial mesopallium, and NCM). Nissl staining was performed on slides to aid identification (Fig. 1d). The films were scanned for digital analysis. The analysis of gene expression was done with the ImageJ program (*https://imagej.nih.gov/ij/*) from NIH. Auditory nuclei were outlined and mean pixel density was calculated with the histogram analysis function. Mean counts from control areas outside of the auditory forebrain regions in 20×20 boxes were also calculated using the histogram function. Expression levels were calculated by first finding the signal-to-background ratio (pixel density in the region of interest/pixel density in the control areas) in each hemisphere of a section, and then converting this value into its reciprocal for a fold change value. Average fold change values for the right and left hemisphere of each subject were calculated separately for all subjects in the respective conditions (ZF, BF, WH, SIL; Fig. 2) for statistical analyses.

Results

We found no statistically significant differences between *ZENK* expression level data from the left or right hemisphere in either the global embryonic (paired *t*-test:

Fig. 2



Average fold change for embryos and Nestlings across experimental conditions. Error bars represent ±1 SE.

$t = 1.28$, $d.f. = 24$, $P = 0.21$) or nestling ($t = -0.51$, $d.f. = 7$, $P = 0.63$) data sets; therefore, we averaged these values across hemispheres for each subject. We also found no statistically significant differences between our response data (i.e. expression levels) for the embryos for which *in situ* was conducted by two different researchers (unpaired *t*-test, $t = 0.95$, $d.f. = 24$, $P = 0.35$); therefore, all data were combined for additional analyses.

To compare directly whether our results showed parallel patterns between developmental stages, we assessed neural expression of *ZENK* (*Egr-1*) for conspecific (ZF) playbacks versus silence across either embryos or nestlings separately using an analysis of variance; we found that at each of these developmental stages, expression levels were significantly greater for zebra finch songs than for silence (embryos: $F = 4.95$, $d.f. = 1, 13$, $P = 0.044$; nestlings: $F = 7.78$, $d.f. = 1, 6$, $P = 0.032$, respectively; Fig. 2).

Finally, we compared the responses to heterospecific (BF and WH) songs relative to either conspecific (ZF) songs or silence using post-hoc Student's *t*-tests for the embryos only; the patterns showed intermediate responses to heterospecific songs with no statistically significant pairwise differences between heterospecific songs relative to conspecific songs or to silence (all $|t| < 1.2$, $P > 0.25$; Fig. 2).

Discussion

Recent work demonstrated response selectivity to conspecific versus heterospecific songs and provided evidence for learning in altricial songbird embryos through a nonassociative learning paradigm in response to conspecific acoustic playbacks [11]. Our study provides new and direct evidence for differential neural activation in the auditory forebrain regions of Field L and NCM of embryo and nestling zebra finches following prior exposure to conspecific songs. Specifically, *ZENK* levels in the auditory forebrain regions were higher for both embryos and nestlings following zebra finch song playback relative to silent control groups (Fig. 2). Our results thus identify potential neural substrates for the finding that physiological patterns of auditory selectivity to conspecific vocalizations in songbirds can be guided by acoustic playbacks *in ovo* [3,6]. Finally, these new data also suggest that at least some of the embryonic and nestling patterns in greater neural activation in response to conspecific songs relative to silence occurs in the same auditory forebrain regions as also implicated in conspecific song selectivity of adult zebra finches [12–15].

We also saw an increasing pattern in *ZENK* expression with WH playback eliciting the least, BF playback eliciting greater, and ZF playback activating the most *ZENK* expression; but the neural level of activation differences between these three song types, and between either WH or BF versus SIL, were not statistically significant and require larger sample sizes to establish any trends statistically. In contrast, our nestlings were all sourced from family cages in the zebra finch colony room, implying prior prolonged exposure to conspecific songs and calls. In future work, we may therefore parallel published neurophysiological [13] and RNA-sequencing [24] studies with cross-fostered adult zebra finches to assess whether prior experience with conspecific or heterospecific songs, respectively, critically influences subsequent neural selectivity for conspecific versus heterospecific songs within each of the exposure treatments; we did not conduct such a study here with embryos as all final playbacks paralleled the prior playback treatment.

Conclusion

Our study is the first to show how prenatal sound exposure could impact forebrain gene expression in perinatal altricial songbirds, with implications on the impact of prior experience on neural organization and function related to early onset and experience dependence of conspecific song recognition. Once neural templates for conspecific recognition are established, downstream processes may affect attentional behaviors, vocal tutor selection, tutor selectivity, and/or vocal learning when these templates are activated by conspecific song [9]. Identifying the effects of acoustic ontogenetic experience on neural development related to subsequent social behaviors also provides a mechanistic perspective to test evolutionary developmental pathways in different avian lineages. In turn, alterations in contemporary soundscape, including proximity and density of conspecifics versus heterospecifics, or noise pollution, may set populations on vastly different evolutionary trajectories as a consequence of divergence in prenatal neural organization and subsequent function.

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Conflicts of interest

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