Neural activity associated with rhythmicity of song in juvenile male and female zebra finches

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ABSTRACT

Rhythm is an important aspect of both human speech and birdsong. Adult zebra finches show increased neural activity following exposure to arrhythmic compared to rhythmic song in regions similar to the mammalian auditory association cortex and amygdala. This pattern may indicate that birds are detecting errors in the arrhythmic song relative to their learned song template or to more general expectations of song structure. Here we exposed juvenile zebra finches to natural conspecific song (rhythmic) or song with altered inter-syllable intervals (arrhythmic) prior to or during template formation, or afterward as males are matching vocal production to a memorized song template (sensorimotor integration). Before template formation, expression of the immediate early gene ZENK was increased in the caudomedial nidopallium (NCM) of birds exposed to rhythmic relative to arrhythmic song. During template formation, ZENK expression was increased in the caudomedial mesopallium (CMM) of birds exposed to arrhythmic relative to rhythmic song. These results suggest that the youngest birds may be predisposed to respond to a more natural stimulus, and a template may be required for arrhythmic song to elicit increased neural activity. It also appears that functional development across the brain regions investigated continues to maturity.

1. Introduction

Evidence is increasing for an association between rhythm skills and language development. For example, in typical speakers, there is a positive relation between language and literacy skills and the ability to analyze beat-based rhythmic sequences (Grube et al., 2013). Conversely, children with specific language impairment have difficulties processing and synchronizing tapping with a rhythmic stimulus (Corriveau et al., 2007; Corriveau and Goswami, 2009). People who stutter appear to have deficits in the internal generation of rhythm, and both adults and children who stutter can dramatically reduce the rate of disfluencies when they synchronize their speech with an external rhythmic stimulus such as a metronome (Hanna and Morris, 1977; Greenberg, 1970) or music (Johnson and Rosen, 1937). Children who stutter have a reduced ability to discriminate complex rhythms as compared to their age-matched peers (Wieland et al., 2015). Thus, the development of rhythmic processing abilities is important for typical speech and language development. A deeper understanding of how rhythm is processed in the brains of individuals at all stages of development should aid in understanding a range of developmental speech and language disorders, as well as other conditions that involve deficits in rhythm processing such as Parkinson's disease (Grahn and Brett, 2009).

An appropriate animal model allows for levels of investigation not possible in human subjects, and songbirds such as zebra finches can be particularly useful for the study of vocal development, reviewed in (Doupe and Kuhl, 1999). For example, in both species, vocalizations of adult tutors are memorized to form a template and then juveniles practice vocalizations to make modifications that increasingly match the template. This process includes babbling in humans as well as subsong and plastic song in birds such as zebra finches. Ultimately this process results in adult speech in humans and crystalized song in zebra finches. Vocal learning is limited to a critical period in both species.

It has been theorized that vocal learning is a necessary factor for the capacity to perceive and entrain to rhythmic auditory stimuli (Patel, 2006). A broad range of species has been identified as having this capacity, including elephants (Poole et al., 2005; Stoeger and Manger, 2014), cetaceans (Janik, 2014), bats (Esser 1994; Prat et al., 2015), parrots (Pepperberg, 2010), hummingbirds, and songbirds (Marler and Tamura, 1964; Bolhuis et al., 2010; Doupe and Kuhl, 1999). Recent
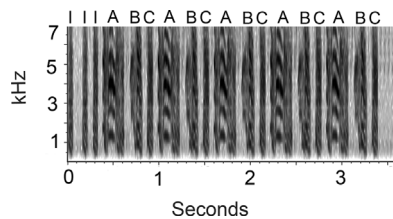

Fig. 1. A natural zebra finch song used to generate one of the stimuli from the present study is depicted. Introductory notes are labeled with I. A, B, and C labels indicate separate syllables in the song, with each repetition of the three syllables constituting a motif.
Modified from Fig. 1 in (Lampen et al., 2014).

studies have indicated that at least one species that does not learn vocalizations has the capacity to entrain to a beat (Cook et al., 2013; Wilson and Cook 2016). While the extent to which vocal learning is required for rhythmic entrainment is still unclear (Ten Cate et al., 2016), a far greater number of vocal-learning species than non-vocal learners have demonstrated a capacity to entrain to a beat (Schachner et al., 2009). As one of the few of these species amenable to breeding in laboratory conditions, the zebra finch is a good model to study the juvenile development of rhythm perception. Their songs, which are used for nest site defense and courtship, are highly rhythmic (Norton and Scharff, 2016; Zann, 1996). A zebra finch song bout consists of a sequence of repetitive introductory notes, followed by repetitions of an ordered set of syllables called a motif. The intervals between notes in these songs are very regular (Fig. 1). The natural rhythmic structure of zebra finch song adds to their value as a model species to improve our understanding of how rhythm perception develops in a vocal-learning species.

Whether zebra finches and other birds are capable of perceiving higher order rhythms or are simply focused on local timing elements is an issue still under debate (Benichov et al., 2016; Ten Cate et al., 2016). In a go/no go paradigm where birds were asked to discriminate between regular and irregular beat patterns, zebra finches seemed to default to making decisions based on local temporal structure, such as duration of notes or inter-onset intervals, but also appeared capable of detecting broader temporal structure, encompassing multiple shorter intervals (Ten Cate et al., 2016). A similar study judged birds' capacity to discriminate between isochronous and irregular tone sequences (van der Aa et al., 2015). While zebra finches could discriminate between the training stimuli, they were not able to generalize to isochronous rhythms at different tempi, which was interpreted to mean that they were only able to attend to local timing features (van der Aa et al., 2015). In addition, both of these studies utilize stimuli composed of pure tones to assess rhythmic discrimination capabilities in zebra finches. NCM and CMM do not respond as strongly to tones as conspecific vocalizations (Stripling et al., 2001; Mello et al., 1992; Bailey et al., 2002; Bailey and Wade, 2003, 2006), thus the capacity to process global rhythms may be different if the sound pattern is composed of natural zebra finch sounds which induce more activity in auditory processing areas of the brain. The overall capacity of zebra finches to attend to different levels of timing and rhythm requires further investigation. We used the immediate early gene ZENK with relatively natural song stimuli to assess differences in neural responses to ecologically relevant rhythmic and arrhythmic stimuli.

Expression of this gene and/or its protein product is frequently used to study neural activity in zebra finches. ZENK is an acronym for the multiple names that have been assigned to this evolutionarily conserved protein, zif-268 (Christy et al., 1988), egr-1 (Sukhatme et al., 1988), NGFI-A (Milbrandt, 1987), and Krox-24 (Lemaire et al., 1988). ZENK is involved in learning and synaptic plasticity (Mello et al., 2004), and can act through regulation of other genes through a DNA binding site (Christy and Nathans, 1989). Inhibition of ZENK expression in juvenile zebra finches during tutor song exposure prevents normal song learning

(London and Clayton, 2008).

A previous study in our lab focused on rhythm effects on ZENK expression in the adult zebra finch brain (Lampen et al., 2014). ZENK expression was assessed in the caudomedial nidopallium (NCM), the caudomedial mesopallium (CMM), which while anatomically distinct in the zebra finch brain, are both considered analogous to the auditory association cortex in humans (Bolhuis and Gahr, 2006), and nucleus taeniae (Tn) which is analogous to the mammalian amygdala (Riters et al., 2004). NCM is likely the location where the learned song template is stored in the brain (London and Clayton, 2008; Gobes et al., 2010; Yanagihara and Yazaki-Sugiyama, 2016). We hypothesize that this template may contain information about the proper timing of songs, allowing for discrimination of timing and rhythmicity. In our study on adults (Lampen et al., 2014), birds exposed to song that was modified to disrupt its natural rhythmic structure had significantly more ZENK expression in all three brain regions compared to birds exposed to song with the same syllables presented with the original (unmodified) rhythm. These different levels of activity in secondary auditory areas may suggest that birds perceive errors in the arrhythmic song relative to the learned template. The increased activity in Tn, which is involved in pair bonding and mate selection (Riters et al., 2004; Dios et al., 2013; Svec et al., 2009), may indicate an aversion to the disrupted rhythm and an assessment of poor quality as a potential mate.

The general timeline of vocal development in zebra finches is agreed upon, but the exact ages at which specific milestones occur is still debated to some degree (Doupe, 1993; Doupe and Solis, 1997; Mooney, 2009; Tomaszycki et al., 2009). Template formation occurs between approximately post-hatching days 20 and 60 (Tomaszycki et al., 2009; Mooney, 2009; Doupe and Solis, 1997). In males, vocalizations begin as food begging, then develop into subsong, an immature form of vocalization that is quiet and contains poorly formed notes with greatly variable structure and sequence (Zann, 1996). As the males mature, these songs are practiced and modified to relatively closely match the learned template. This phase of sensorimotor integration occurs between about post hatching day 25 and sexual maturity which occurs around 90 days of age (Doupe and Solis, 1997; Mooney, 2009; Tomaszycki et al., 2009). After sensorimotor integration, males have a crystalized song that will not undergo significant changes throughout the rest of their lives under normal circumstances (Doupe, 1993). In addition to learning to produce specific song syllables, juvenile males also learn a specific grammar to structure their song (Menyhart et al., 2015).

Understanding the developmental trajectory of neural responses to rhythm is useful in elucidating their relationship to function. We exposed birds to rhythmic or arrhythmic song prior to and during the template formation period, as well as during early sensorimotor integration. Differences in neural activity following stimulation with the two types of songs prior to acquisition of a template could indicate an innate capacity to perceive song-related rhythms. Discrimination during and after template formation, but not before, would suggest that characteristics of rhythm are learned. If rhythm discrimination emerges during sensorimotor integration in males, it would suggest that a motor component is required for distinct neural responses to rhythmicity. Differences between the sexes may be informative here as well, as both males and females appear to form templates (Lauay et al., 2004), even though only males engage in the production of song (Zann, 1996).

2. Material and methods

2.1. Subjects

Zebra finches hatched and were reared in large, walk-in aviaries containing 5–7 adult male and female pairs and their offspring. On day of hatching, toes were clipped as a means of unique identification. Tissue from the toes was used to identify the sex of the birds through

polymerase chain reaction (Agate et al., 2002). A total of 108 birds were used (18 males and 18 females at 15–17 days post hatching (d15), 25–27 days post hatching (d25), and 45–47 days post hatching (d45)). All animals were maintained on at 12:12 h light/dark cycle and had ad libitum access to seed (Kaytee Finch Feed; Chilton, WI, USA), water, cuttle bone and gravel. Their diet was supplemented weekly with hard boiled chicken eggs, bread, spinach, and oranges. All procedures were approved by the Institutional Animal Care and Use Committee of Michigan State University.

2.2. Stimulus exposure

Rhythmic and arrhythmic zebra finch song stimuli were used from our study on adults (Lampen et al., 2014). Details regarding their creation are reported in that paper. Briefly, rhythmic song stimuli were generated from recordings of natural zebra finch song, and arrhythmic stimuli used the same recordings with the silent intervals between notes altered to disrupt the temporal pattern of the song. Each interval was randomly changed to one of three durations: 1) 10 ms, 2) the average duration (based on all intervals in a song except those between introductory notes), or 3) double the average duration, minus 10 ms. Because the intervals added between notes were silence rather than background sound as present in the rhythmic song recordings, it is possible that the difference in contrast contributed to the perceptual distinction between stimulus types. Three different stimuli of each type were created, each of which contained three of nine different songs that were generated (rhythmic stimuli depicted in Supplementary Fig. S1, arrhythmic stimuli depicted in Supplementary Fig. S2). Each song was presented for 30 s followed by 30 s of silence, with the three songs repeating in random order for 30 min. Audio files of example rhythmic and arrhythmic songs are available as supplementary materials. All songs of each type were novel to all subjects.

Nine males and 9 females of each age group were exposed to each stimulus type (rhythmic or arrhythmic). One bird was exposed at a time between 9am and 3pm. Birds were placed into a sound isolation chamber (252-Mini Sound Shelter, IAC Acoustics, Bronx, New York, USA) to habituate for one hour. Following the habituation period, the stimulus was presented at approximately 70 dB. Birds remained in the chamber for one hour following song exposure to allow for peak ZENK protein expression. Brains were then immediately collected and flash frozen in methylbutane.

All song exposures were recorded using a Canon Vixia HF R300 camcorder. Recordings were reviewed to ensure birds were not producing subsong and no extraneous background noise existed that might influence the results.

2.3. Tissue processing

Brains were sectioned coronally at 20 μm and collected in 6 series. Sections were thaw mounted onto SuperFrost Plus slides (Fisher Scientific, Hampton, NH). One set of tissue from each bird was thionin stained to allow for clear identification of anatomy.

An alternate set of tissue from each bird was processed for immunohistochemistry for ZENK. Tissue was processed in five groups due to the large number of slides. All experimental groups were evenly represented in all immunohistochemistry runs. The protocol was conducted as described in (Lampen et al., 2014). Briefly, slides were warmed to room temperature, fixed 4% paraformaldehyde, and rinsed in 0.9% hydrogen peroxide in methanol. Slides were then incubated in 5% normal goat serum in 0.1 M phosphate buffered saline (PBS) with 0.3% Triton X-100. Slides were incubated overnight at 4 °C in ZENK (Egr-1) rabbit polyclonal antibody (0.5 μg/ml, sc-189, Santa Cruz Biotechnology, Inc., Dallas, TX) in 5% normal goat serum in PBS with 0.3% Triton X-100. The next day slides were incubated in biotin conjugated goat anti-rabbit polyclonal antibody (0.5 mg/ml; Vector Labs, Burlingame, CA) in PBS with 0.3% Triton X-100, followed by Elite ABC

reagents (Vector Labs, Burlingame, CA), and then treated with diaminobenzidine with 0.003% H_2O_2 to produce a color reaction.

An investigator blind to the stimulus exposure condition, sex, and age of the birds conducted analysis of all slides. Images of all areas of interest were captured using a 10X objective on an Olympus BX60 microscope (Olympus, Center Valley, PA), a MicroPublisher 5.0 RTV camera (QImaging, Surry, BC, Canada) and ImageJ software (National Institutes of Health, Bethesda MD). All regions of interest were analyzed bilaterally in two adjacent sections. Boxes were placed within NCM, CMM and Tn as described and depicted in (Lampen et al., 2014), and all cells containing a dark brown stained nucleus that was clearly distinguishable from the surrounding tissue were counted. For NCM, a 0.525 mm*0.393 mm box was placed with the medial corner under the hippocampus at the point where the ventricle begins to curve ventrally to run parallel with the midline. For CMM, a 0.496 mm*0.205 mm box was placed the ventricle just lateral to where it curves ventrally toward the midline between A 1.6 and A 1.2 from a songbird brain atlas (Stokes et al., 1974). For Tn, a 0.238 mm*0.244 mm box was placed near the ventral edge of the telencephalic lobe where a corner is formed by the ventral and medal edges of the lobe. Images depicting the location within each brain region where ZENK was analyzed were previously published (Lampen et al., 2014). The density of ZENK expressing cells (labeled nuclei per unit area) was calculated for each brain region. Due to high levels of baseline labeling apparent in these juvenile brains, values were also assessed in the control region nucleus rotundus (Rt), a thalamic visual area that should show no ZENK induction specifically from the auditory stimuli. A 0.268 mm*0.268 mm box was placed in the center of the region bilaterally in two adjacent sections and density of ZENK expressing cells was calculated. For each animal, average density values were calculated for each region of interest, collapsing across the hemispheres.

In order to confirm consistency of analysis by the investigator, the density of ZENK expression was assessed in replicates of a subset of the images in order to enable calculation of a coefficient of variance. For each combination of age, sex and rhythm condition, one image of NCM was randomly selected and copied in triplicate. The images were coded so the investigator was blind to the identity of the images (both the group each represented and which were replicates). The average coefficient of variance across replicates was 8.05%.

2.4. Data analysis

The brains in the final run of immunohistochemistry were excluded from analysis due to unusually high levels of background staining unique to this set of tissue, reducing the total number of animals in each group by one. Occasionally individual brain regions were not able to be analyzed in particular birds due to damage to the tissue sections. Final sample sizes are indicated in Table 1. Compared to our study in adults, constitutive levels of ZENK expression were increased across brain region and ages. Therefore, labeling in Rt was used as a covariate (Supplementary Fig. S3). The density of ZENK+ cells in Rt correlated significantly with the values obtained from NCM, CMM, and Tn (all $r > 0.642$, $p < 0.001$). Separate three-way ANCOVAs were run for NCM, CMM, and Tn, with age, sex, and stimulus type (rhythmicity) as factors. To provide pairwise comparisons probing main effects of age detected in NCM and CMM, separate one-way ANCOVAs, with rhythmicity as the factor, were conducted within each region. To investigate interactions of age and stimulus type detected in NCM and CMM, individual one-way ANCOVAs were conducted within each age group, as well as across all ages within each rhythm condition, with further oneway ANCOVAs used to provide pairwise comparisons between each combination of two ages within a rhythm condition. For all sets of pairwise comparisons, a Holm's Bonferroni correction was used (Holm, 1979), with adjusted α-levels indicated with each result. The Holm's correction provides α-values at which to assess the significance of multiple comparisons, with the most significant result analyzed at the

Table 1

Unadjusted ZENK + cells/mm^2 means and SEM for the caudomedial nidopallium (NCM), the caudomedial mesopallium (CMM), and nucleus taeniae (Tn). Values within each cell indicate the mean with the standard error in parentheses and n on the row below.

		d15		d25		d45	
		Male	Female	Male	Female	Male	Female
NCM	Rhythmic	671.8 (92.3) 9	791.7 (55.2) 9	387.6 (104.3) 9	334.6 (128.7) 9	430.6 (153.3) 8	273.2 (83.1) 9
	Arrhythmic	630.0(131.7) 9	629.5 (109.6) 9	481.7 (117.0) 8	394.9 (86.5) 8	351.6 (107.3) 9	352.8 (117.4) 9
CMM	Rhythmic	1063.6 (132.6) 9	944.4 (128.8) 9	560.1 (143.0) 9	485.0 (150.5) 9	430.0 (151.7) 9	428.9 (147.4) 9
	Arrhythmic	973.9 (158.0) 9	1020.3 (180.9) 9	561.3 (77.8) 9	765.8 (147.2) 9	538.4 (144.4) 9	513.9 (158.8) 9
Tn	Rhythmic	840.4 (158.1) 9	864.8 (84.8) 9	592.0(126.1) 9	532.9 (123.0) 9	558.7 (163.4) 8	535.6 (170.3) 9
	Arrhythmic	749.4 (169.6) 9	952.1 (168.0) 9	681.6 (112.2) 8	641.2 (99.0) 8	421.1 (97.9) 9	586.2 (126.0) 9

most strict α-value of 0.05/total number of comparisons, as in a traditional Bonferroni correction, and further comparisons assessed in rank order of their significance, reducing the value of the denominator in the α equation by one with each comparison. All statistics were calculated using SPSS (IBM, Armonk, NY).

3. Results

No main effects of sex were detected in NCM ($F_{1,79} = 1.079$, $p = 0.302$), CMM ($F_{1,82} = 0.288$, $p = 0.593$), or Tn ($F_{1,79} = 0.341$, $p = 0.561$). However, in NCM, there was a main effect of age ($F_{2,85} = 3.388$, $p = 0.039$; Fig. 2). D15 birds had a higher density of ZENK expressing cells than those at d25 ($F_{1,58} = 8.415$, $p = 0.005$, α = 0.0167), and a trend existed for greater ZENK expression at d15 compared to d45 ($F_{1,60} = 4.413$, $p = 0.040$, α = 0.025). ZENK expression did not differ significantly between d25 and d45 ($F_{1,57} = 0.960$, $p = 0.331$, α = 0.050). While a main effect of rhythm condition did not exist ($F_{1,85} = 0.458$, $p = 0.501$), age and stimulus type interacted ($F_{2,85} = 3.189$ $p = 0.047$; Fig. 2). At d15, a trend toward a greater density of ZENK+ cells was detected following exposure to rhythmic compared to arrhythmic song ($F_{1,29} = 6.038$, $p = 0.020$, α = 0.0167). However, no effect of the stimulus type was detected at d25 ($F_{1,29} = 1.383$, $p = 0.250$, α = 0.025) or d45 ($F_{1,29} = 0.300$, $p = 0.865$, α = 0.050). In addition, there was an effect of age within birds exposed to rhythmic ($F_{2,42} = 6.983$, $p = 0.002$, α = 0.025), but not arrhythmic ($F_{2,42} = 0.057$, $p = 0.945$, α = 0.050) song. In birds that heard the rhythmic song, a greater density of ZENK expressing cells was observed at d15 compared to both d25 ($F_{1,28} = 14.023$, $p = 0.001$, α = 0.0167) and d45 ($F_{1,28} = 5.784$, $p = 0.023$, α = 0.025), and in animals at d45 compared to d25 ($F_{1,27} = 4.817$, $p = 0.037$, α = 0.050).

Similarly, a main effect of age was detected in CMM ($F_{2,82} = 8.446$, $p < 0.001$; Fig. 3). D15 birds had greater ZENK expression than those at d25 ($F_{1,60} = 11.900$, $p = 0.001$, α = 0.025) and d45 ($F_{1,61} = 13.805$, $p < 0.001$, α = 0.0167), whereas no difference was detected between birds at d25 and d45 ($F_{1,60} = 0.544$, $p = 0.464$, α = 0.050). A main effect of rhythm condition was not seen ($F_{1,82} = 0.470$, $p = 0.495$), but age and stimulus type interacted ($F_{2,82} = 3.485$, $p = 0.035$; Fig. 3). At d25, a greater density of ZENK expressing cells was detected in birds that heard arrhythmic compared to rhythmic song ($F_{1,28} = 7.061$, $p = 0.013$, α = 0.0167). However, there was no effect of rhythm condition at d15 ($F_{1,29} = 1.671$, $p = 0.206$, α = 0.025) or d45 ($F_{1,29} = 0.001$, $p = 0.973$, α = 0.050). In addition, there was an effect of age in birds exposed to both rhythmic ($F_{2,43} = 7.788$, $p = 0.001$, α = 0.025) and arrhythmic ($F_{2,44} = 3.397$, $p = 0.042$, α = 0.050) song. In those hearing rhythmic song, an increased density of ZENK+ cells was observed in d15 compared to d25 ($F_{1,28} = 17.591$, $p < 0.001$, α = 0.0167), but no differences were seen between d15 and d45

($F_{1,29} = 2.928$, $p = 0.098$, α = 0.050), or in animals at d25 compared to d45 ($F_{1,28} = 3.678$, $p = 0.065$, α = 0.025). Within the birds exposed to arrhythmic song, a trend was detected toward greater ZENK expression in birds at d15 compared to d45 ($F_{1,29} = 6.016$, $p = 0.020$, α = 0.0167), but no differences between d15 and d25 ($F_{1,29} = 0.787$, $p = 0.382$, α = 0.050) or d25 and d45 ($F_{1,29} = 3.673$, $p = 0.065$, α = 0.025) were seen.

Main effects of rhythm and age were not detected in Tn ($F_{1,79} = 0.021$, $p = 0.884$; $F_{2,79} = 0.992$, $p = 0.376$ respectively), and no age by rhythm interaction existed ($F_{2,79} = 1.617$, $p = 0.205$) (Table 1).

4. Discussion

4.1. Summary of specific effects

Expression of the immediate early gene ZENK across the two sexes was affected by developmental stage and rhythmicity of song stimuli in two auditory regions of the zebra finch brain, NCM and CMM. In contrast, neither variable influenced the density of ZENK expressing cells in Tn, a homologue of the mammalian amygdala. Several results were parallel across the two auditory regions. For example, expression was greater at d15 compared to d25 in both NCM and CMM and at d15 compared to d45 in CMM; a trend for this age difference also existed in NCM. Similarly, within birds exposed to rhythmic stimuli, increased ZENK in both regions was detected at d15 compared to d25, effects that were not detected in either area in birds exposed to the arrhythmic songs. The magnitude of other effects differed between the NCM and CMM, although the patterns were quite similar across the two regions (compare Figs. 2B and 3B). Specifically, in NCM only, the density of ZENK+ cells in birds exposed to rhythmic song was greater at d15 than d45 and at d45 than d25, whereas in CMM ZENK was only increased in d15 compared to d25 in birds that heard the rhythmic stimuli. While significant differences between pairs of ages were not detected in either cortical region, a trend was detected for an increase at d15 compared to d45 in birds exposed to the arrhythmic stimulus. Finally, effects of stimulus type within specific ages differed across the two regions, such that at d25 arrhythmic song resulted in greater ZENK expression in CMM but not NCM, and a trend for an increase following rhythmic song existed at d15 in NCM but not CMM.

The increased ZENK expression generally detected in the youngest birds in this study parallels results from work documenting relatively high levels of constitutive expression of this protein at post-hatching day 20 in both NCM (Jin and Clayton, 1997; Stripling et al., 2001) and the broader auditory lobule (London et al., 2009). These earlier studies suggested that ZENK was not inducible with conspecific song in these regions. The present work cannot address this issue specifically, but
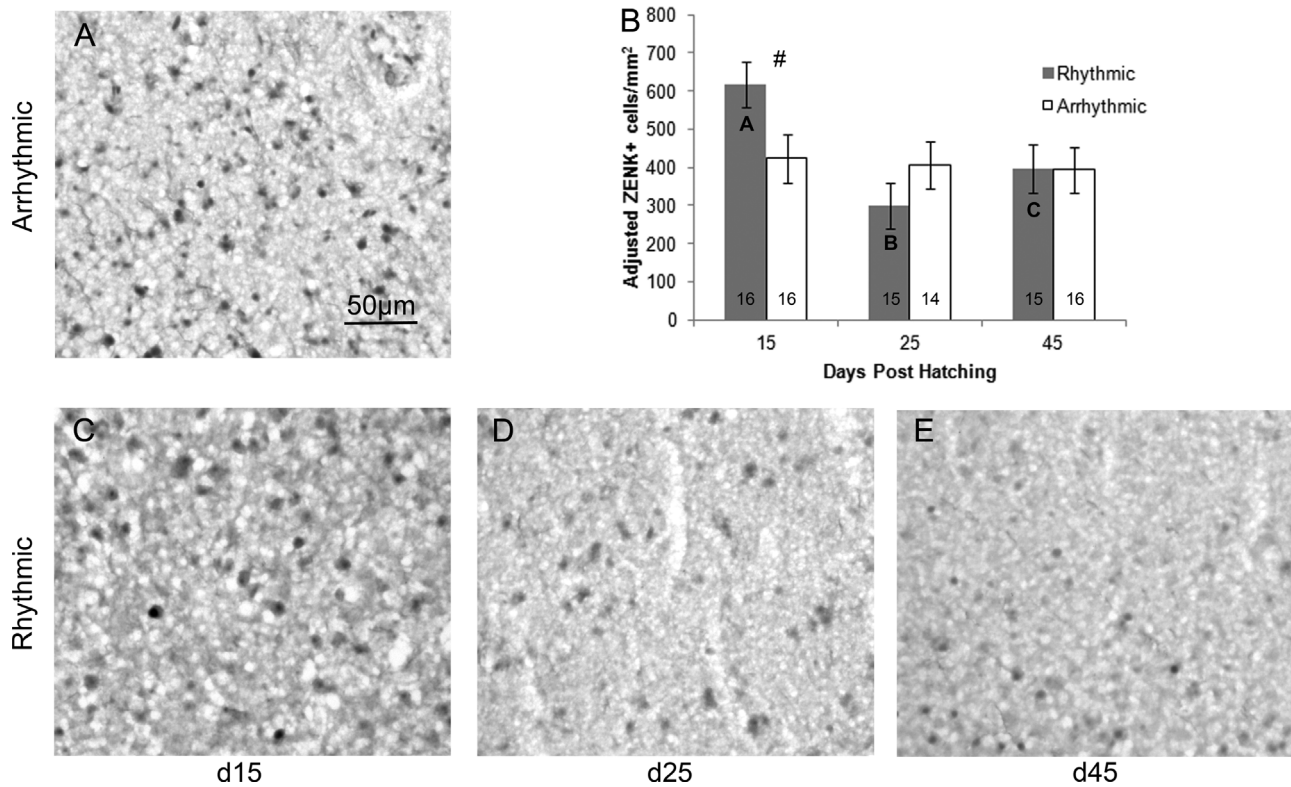
NCM

Fig. 2. Density of ZENK expressing cells in NCM. The photographs depict representative samples of ZENK expression in birds at A) d15 exposed to arrhythmic song, and birds exposed to rhythmic song at C) d15, D) d25, and E) d45. All photographs are of male brains, but are representative of both sexes, as no sex differences were detected. Panel B depicts the density of ZENK expressing cells across ages and rhythmic conditions. Marginal means from the overall ANCOVA were used to graph adjusted means $\pm$ SEM. Group sizes are listed within the bars. Data are collapsed across sexes, as no significant effects were detected. A main effect of age was detected, with greater density of ZENK expressing cells at d15 compared to d25 and a trend for greater expression at d15 compared to d45. Age and stimulus type (rhythmicity) also interacted. Bold letters within the bars indicate significant differences between all age groups of rhythmic song exposed birds, with the greatest density detected in d15 birds, and the least in d25. # = Trend toward an increase in ZENK expression following exposure to rhythmic song at d15.

using expression in Rt as a covariate suggests that ZENK is induced in NCM in response to typical song at d15 and to a greater degree than at later developmental stages. The lack of age and rhythm effects in Tn indicate that the effects detected in NCM and CMM are specific to these auditory areas, and not indications of global increases in activity or constitutive high ZENK expression in the early juvenile period.

While it remains possible that differences in baseline ZENK expression among the regions of interest contributed to the effects of age we detected, and this issue could be investigated directly in future studies, it seems unlikely because in both NCM and CMM, the overall effect of age appears to be driven by data from birds that heard rhythmic song. This suggests that developmental changes in auditory processing occur primarily in natural stimuli. The substantial reduction between d15 and d25 in ZENK expression following rhythmic song in both regions could be related to the birds' focus on memorizing song from tutors at the later age rather than reacting to the novel vocalizations presented in the current study. ZENK expression is associated with synaptic plasticity and learning (Mello et al., 2004), and during template formation substantial synaptic changes would be required for acquisition of the tutor song memory.

4.2. NCM

The trend we detected toward greater ZENK expression following rhythmic compared to arrhythmic song in NCM is consistent with other data collected during song development suggesting an innate preference toward more natural, species typical sounds. For example,

during the critical period for template memorization, birds reared in isolation show a behavioral preference for conspecific over heterospecific song by landing more frequently on a perch that elicits playback of conspecific song (Braaten and Reynolds, 1999). Additionally, juvenile zebra finches that have not been exposed to adult song prefer songs with more common elements, as shown by time spent near a speaker (ter Haar et al., 2014). Similar to the results of the present study, in the primary auditory area, field L, a group of neurons that code for the silent intervals between syllables seems to respond selectively to zebra finch songs, even in birds that have been cross fostered by Bengalese finches or raised in isolation from their fathers (Araki et al., 2016). Behavioral drift toward natural wild-type zebra finch song can be seen in colonies tutored by birds reared in isolation, again indicating a natural tendency toward the most species typical sounds (Feher et al., 2009).

The pattern of ZENK expression following exposure to the same auditory stimuli used in the present study differed in the NCM of adult zebra finches. In the older birds, greater ZENK expression was detected in individuals hearing arrhythmic compared to rhythmic song (Lampen et al., 2014). Such a difference across ages is not surprising, as substantial development occurs within NCM in the late juvenile period. For example, estrogens synthesized within NCM become sexually dimorphic between 46 and 80 days post hatching, with greater estrogen levels present in males compared to females (Chao et al., 2015). Estradiol facilitates improved discrimination of auditory stimuli within NCM (Remage-Healey et al., 2010; Remage-Healey et al., 2012; Maney et al., 2006; Maney et al., 2008). While effects of this hormone on
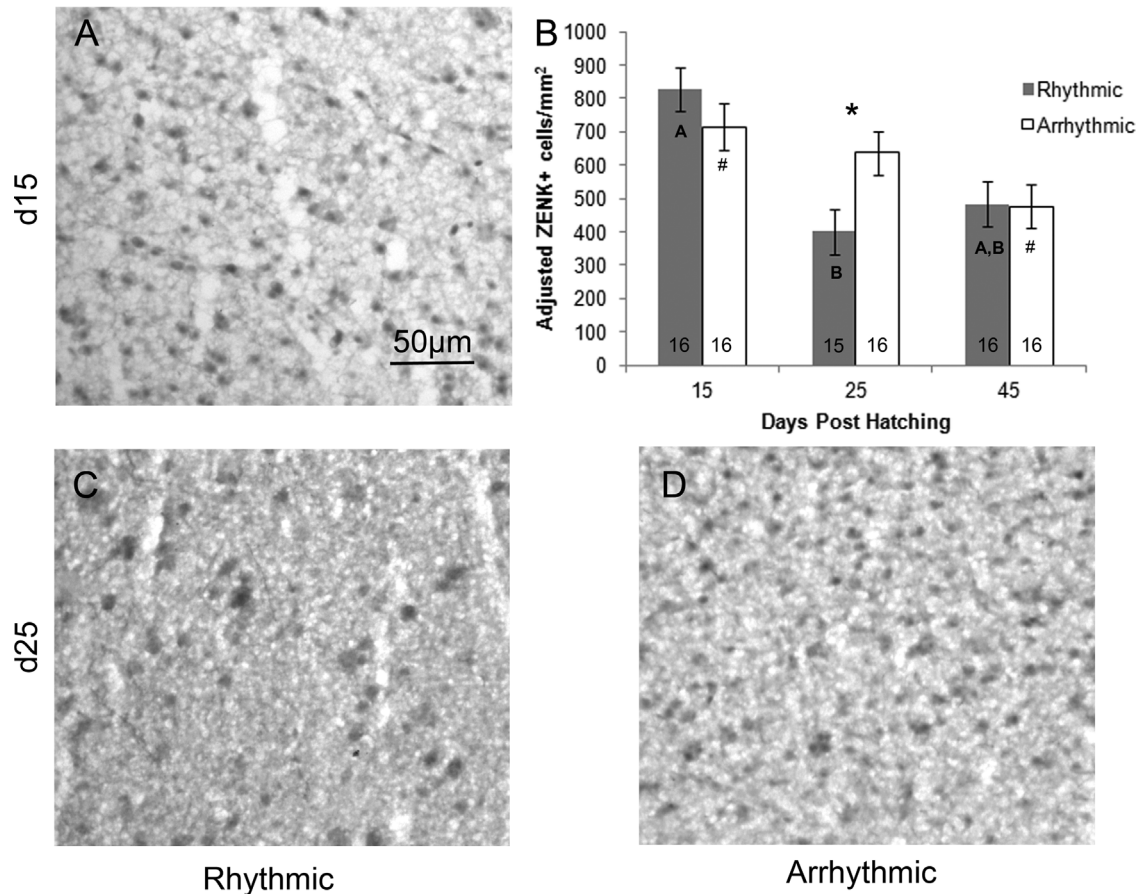
CMM

Fig. 3. Density of ZENK expressing cells in CMM. Photographs depict representative samples of ZENK expression in birds exposed to rhythmic song at A) d15, and C) d25, and D) a bird exposed to arrhythmic song at d25. All photographs are of male brains, but are representative of both sexes, as no sex differences were detected. Panel B indicates the density of ZENK expressing cells across ages and rhythmic conditions. Marginal means from the overall ANCOVA were used to graph adjusted means ± SEM. Group sizes are listed within the bars. Data are collapsed across sexes, as no significant effects were seen. A main effect of age was detected, such that a greater density of ZENK expressing cells was present a d15 compared to both d25 and d45. Effects of age and stimulus type (rhythmicity) also interacted. Bold letters within the bars indicate significant differences between ages of rhythmic song exposed birds, with greater ZENK expression in d15 compared to d25, but no differences between d45 and either of the other ages. # = a trend for an increase at d15 compared to d45 within birds exposed to arrhythmic stimuli. * = Significant increase in ZENK expression following exposure to arrhythmic compared to rhythmic song at d25.

rhythm perception in songbirds are unknown, it is possible that adult responses may require levels of estradiol availability not present in the juvenile birds in the present study.

It is also possible that relative levels of habituation influenced the ZENK responses in juveniles and adults. Faster habituation of electrophysiological responses to songs is seen in NCM in 35 day-old compared to adult zebra finches (Miller-Sims and Bottjer, 2014). Reduction of the ZENK response following repeated presentations of auditory stimuli also occurs in adult zebra finches (Mello et al., 1995). While the ZENK expression in juvenile birds exposed to repeated presentations of the same stimulus has not yet been assessed, it is possible that the pattern of increased habituation of neural responses during the juvenile period extends to ZENK expression. More rapid habituation of neural responses to auditory stimuli, including those in the present study, may result in reduced ZENK expression within the juvenile NCM relative to its potential peak level, which could result in diminished ability to detect differences in ZENK expression to the different stimuli.

A third possible explanation for the different pattern seen between d15 and adult birds (Lampen et al., 2014) relates to the phenotype of the cells. Many GABAergic neurons are present in NCM, and some of them express ZENK in response to song in adult birds (Pinaud et al.,

2004; Pinaud et al., 2008). The GABAergic neurons in NCM are involved in maintaining the selectivity of tutor song responsive neurons, and in the late juvenile period the inhibition of GABAergic cells can allow tutor song selective neurons to fire in response to a broader range of song stimuli (Yanagihara and Yazaki-Sugiyama, 2016). In zebra finches 51–83 days post-hatching, injection of GABA agonists into the auditory nucleus NIf is sufficient to disrupt the rhythm and stereotypy of plastic song (Naie and Hahnloser, 2011). Together these results indicate that by late in juvenile development GABAergic neurons are active within the auditory system and are important for song control and specificity of auditory responses. It is possible that in the present study the birds were too young for the inhibitory network of GABAergic neurons to be sufficiently developed to facilitate auditory discrimination.

4.3. CMM

The pattern of ZENK expression at d25 in CMM was the same as we detected in adults, with arrhythmic song producing an increase compared to rhythmic song (Lampen et al., 2014). That this pattern was detected at d25 during template formation as well as in adulthood, but

not at d45 during sensorimotor integration in males, leads to a challenge for interpretation. One possibility is that birds are focused around d25 on accurately perceiving external stimuli for creation of the highest fidelity template and this focus on auditory perception is important again in adulthood when the quality of a song is important for mate attraction and nest site defense. In contrast, during sensorimotor integration around d45, male zebra finches are more focused on the motor task of matching their own vocalizations to a preexisting template. The zebra finch brain may have a greater capacity to discriminate auditory stimuli during times when primary attention toward auditory signals is ecologically relevant, and both sexes may show a reduced capacity to discriminate rhythms at ages important for sensorimotor integration in males. If this explanation is valid, it is unclear why the CMM of females would not exhibit differential responses to the two song types at d45, as they do not practice vocalizations. One possibility is that while males at this age are focused on sensorimotor integration, females may undergo a different developmental process that may reduce their attention toward auditory discrimination. It is also possible that the developmental trajectories are parallel without obvious functional consequences for females.

Similar to the pattern we saw in CMM across d25 birds in the present experiment and our study on adults (Lampen et al., 2014), electrophysiological responses within NCM differed across a range of song stimuli at d20 and in adulthood, but not at d35 (Miller-Sims and Bottjer, 2014). While this effect was found in NCM rather than CMM, it is evidence that response patterns within the brain emerge during early stages of development, regress in later stages and are present again in adulthood.

In the present study, no effect of sex was seen in CMM in juvenile zebra finches which contrasts with adult zebra finches in which ZENK expression was enhanced in females compared to males across the same auditory stimuli (Lampen et al., 2014). The higher ZENK expression in CMM in adult females may be due to involvement of CMM in evaluating the quality of a male's song for the purpose of mate selection which would not occur prior to sexual maturity.

4.4. Tn

The differences in results in Tn compared to the adult study (Lampen et al., 2014) may also relate to sexual maturity. While no effects of age or rhythm were seen in this region in the present study on juveniles, a greater density of ZENK expressing cells was observed in the Tn of adult birds that heard arrhythmic compared to rhythmic song (Lampen et al., 2014). Populations of cells in this brain region in adult Bengalese finches are also responsive to particular types of vocalizations such as song or calls generated by only males or females (Fujii et al., 2016), suggesting its importance in processing socially relevant stimuli. Tn appears to be involved in mate selection and pair bonding; it responds in multiple avian species to acts of mating or the presence of a partner, and can indicate the strength of a pair bond (Svec et al., 2009; Dios et al., 2013; Riters et al., 2004). As for the other regions in this experiment, it will eventually be important to investigate responses to differences in the rhythmicity of song during the transition into adulthood.

4.5. Conclusions

Overall, the results of the present study, based on relative densities of cells expressing ZENK protein, suggest that the ability to discriminate temporal regularity in auditory stimuli fully develops between the stages of sensorimotor integration and adulthood. Some degree of innate rhythmic discrimination may exist in NCM, but the pattern of activity following rhythmic vs. arrhythmic stimulus exposure is reversed in adults compared to 15 day-old birds (Lampen et al., 2014), leaving the possibility that there is also a learned component, as error detection in arrhythmic song hearing adults could be facilitated by

memorization of rhythmic regularity as a component of the song template. In CMM, a learned component to rhythm discrimination is likely since differences in ZENK expression first appear during template formation. The development of rhythm discrimination is a complex process that develops at different rates across these brain regions. As in the present study, most research has focused on zebra finch neural development during the time periods surrounding major milestones in the development of vocal learning. Further work is necessary to understand how the auditory responsive brain nuclei mature during the late juvenile period in order to provide adult responses to varied stimuli.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.beproc.2017.12.003.

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