

Activation of Auditory Centers during Freezing Response Depends on Visual Afferentation in Course of the Development of Pied Flycatcher Nestlings

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Abstract

The study of defense behavior development in pied flycatcher altricial nestlings has shown that preceding visual deprivation decreased the capability to freeze in the young. At the same time, auditory thresholds of deprived nestlings were lower than those of the nestlings developing in unaltered visual environment. Our goal in the present work was to study effects of visual input on the functional development of neural circuitry for flycatcher's acoustically-guided defensive behavior. We compared the immunohistochemical response of transcription factors ZENK and c-Fos in the nestlings' auditory telencephalic structures in visually deprived and non-deprived flycatcher nestlings to alarm calls emitted by adult birds. We have demonstrated that the development of flycatcher nestlings under the conditions of limited visual afferentation results in the decrease of induction of the immediate-early genes c-Fos and ZENK in auditory structures of 9-day-old nestlings performing auditory-guided defense behavior. The most marked decrease of induction of both genes is observed in Field L. Our data suggest that visual afferentation affects neuronal activations in higher auditory structures of nestlings' brain that, in turn, may increase the efficiency of species-typical alarm call. Thus, the work demonstrated for the first time that visual afferentation facilitates the growth of efficiency of alarm call affecting the activity of neurons in auditory integrative structures.

Keywords

Defense Behavior, Freezing, Alarm Call, Visual Deprivation, Flycatcher Nestlings, ZENK, c-Fos

1. Introduction

Defense behavior develops in the ontogeny of pied flycatcher nestlings after the feeding behavior. From post-hatching day 5 nestlings respond with freezing to species-specific alarm call—rhythmic sounds with energy maximum near 5 kHz [1] [2] [3] [4] [5]. During the nesting period, defense behavior is modified [1] [2] [6]. At the first stage, it is manifested in the decrease of motor and vocal activity, feeding behavior is noticeably suppressed. On post-hatching day 8, nestlings develop patterned vision while defense behavior includes the specific posture—the young freeze and press themselves into the bottom of the nest. In the same period the efficiency of alarm call (*i.e.* its ability to suppress vocal and motor activity) increases; also, the young learn to discriminate between the alarm call and sounds imitating it [7]. If visual afferentation is excluded from the process of defense behavior development (visual deprivation), the efficiency of alarm call remains low and nestlings fail to discriminate between species-specific signal and its imitations [8]. The study of thresholds of auditory evoked potentials from Field L (the higher avian auditory center) has demonstrated that thresholds in visually deprived nestlings were lower than in the control ones, including the frequency range of alarm call [9]. These results suggest that visual afferentation may facilitate auditory signals discrimination and involvement of species-specific alarm call into the sensory organization of defense behavior influencing the activity of neurons of higher auditory structures.

Our goal in the present work was to study further these effects of visual input on the functional development of neural circuitry for flycatcher's acoustically-guided defensive behavior. For this purpose, we compared neural responses of telencephalic auditory centers in visually deprived and non-deprived flycatcher nestlings to alarm calls emitted by the adults. Our study was carried out in pied flycatcher nestlings aged 9 days, the age when visual afferentation just starts to affect acoustically-triggered defense behavior [8]. Mapping of call-induced neuronal activity was performed by immunohistochemical detection of inducible transcription factors ZENK and c-Fos in the nestlings' auditory telencephalic structures—Field L, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM). Field L is the highest projection centre of the avian ascending auditory pathway [10] [11]. Neurons of Field L send axons into adjacent auditory nuclei—NCM and CMM [12]. NCM and CMM neurons are more selective for species-specific vocalization as compared to Field L neurons [13] [14] [15] [16]. Induction of ZENK and c-Fos was observed in these structures in response to acoustic stimulation in the adult songbirds [17] [18] [19] [20], and juvenile nestlings [21] [22] [23].

2. Method

The studies were carried out in 10 pied flycatcher nestlings (*Ficedula hypoleuca*) aged 9 days ($n = 5$ in each group). Throughout all time, nestlings stayed in their natural nests in the wild. Nestlings of the control group were kept in unaltered visual environment. Eyes of the nestlings from the visual deprivation group were

covered with nontransparent cups starting from the 1st post-hatching day. To counterbalance the effect of handling, the intact nestlings from the control group were handled. On day 9, nestlings from both groups were presented with 15-min playback of an adult flycatcher alarm call in their home nests (**Figure 1**); after the stimulation the nestlings remained in their nests. During the call exposure nestlings' behavior in the nests was video-monitored and recorded. 90 minutes after the onset of sound exposure, the nestlings were decapitated. The brains were frozen in liquid nitrogen and stored at -70°C . $20\ \mu\text{m}$ transverse brain sections were cut on a cryostat at -16°C and thaw-mounted on poly-L-lysine coated glass slides. Corresponding sections from all experimental groups were arranged on each slide in pseudorandom order. The sections were fixed in 4% paraformaldehyde for 10 min at 4°C and washed in phosphate buffer (PBS, $\text{pH} = 7.4$). The sections were then incubated with 2.5% normal horse serum for 20 min. Incubation with primary antibodies against c-Fos (K-25, rabbit polyclonal IgG; 1:1000; Santa Cruz Biotechnology) and ZENK (Egr-1, C-19, rabbit polyclonal IgG; 1:700; Santa Cruz Biotechnology) was carried out for 4 h at room temperature. After that, the sections were washed in PBS and then incubated for 30 min with secondary antibodies (anti-rabbit IgG; ImmPRESS reagent; Vector Laboratories). Following several washes in PBS the slides were stained with 3,3'-diaminobenzidine (Sigma). After dehydration in ethanol the sections were mounted using xylene-based mounting medium and coverslipped. Sections were digitized using Olympus BX-50 microscope equipped with Surveyor/Turboscan mosaic imaging system (Objective Imaging Ltd). Immunopositive cells were counted using Image Pro Plus 3.0 image analysis software (Media Cybernetics). For each brain, at least 9 transverse sections were analyzed. The cell counting was performed for the auditory structures associated with processing of species-specific vocalization (**Figure 2** CMM (A), Field L (B) and NCM (C)).

For each structure, at least 3 frames were counted and the average value was used for further statistical analysis. The density of immunopositive cells was calculated as the ratio of the number of labeled cells within a structure to its area.

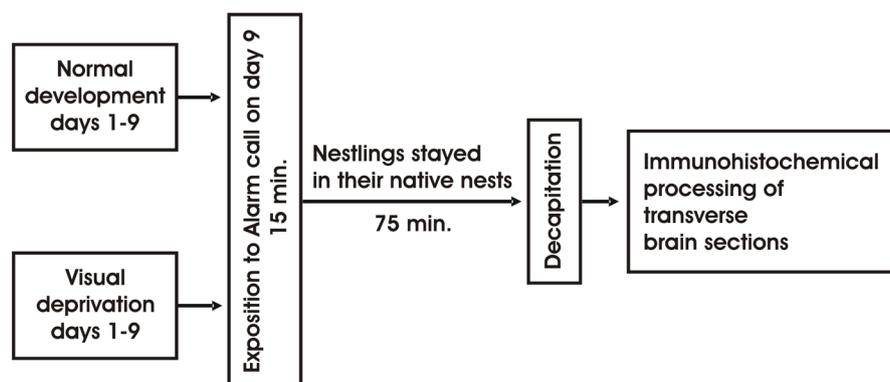


Figure 1. The study design. 9-day-old nestlings from the control ($n = 5$) and visual deprivation group ($n = 5$) were presented with 15-min playback of an adult flycatcher alarm call, then they remained in their nests for 75 min. After that they were sacrificed, and the brains were cut in transverse sections and treated immunohistochemically.

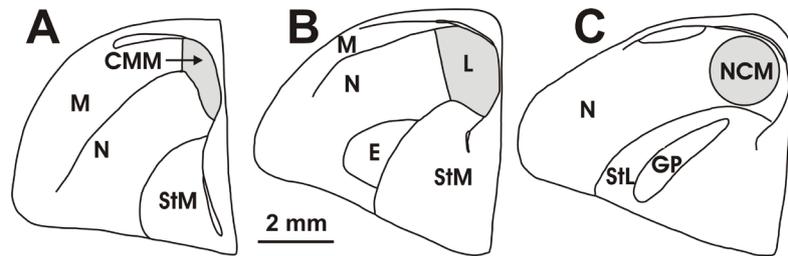


Figure 2. (A-C) Camera lucida drawings of coronal sections of the right hemisphere of the 9-day-old flycatcher brain. The gray regions indicate the areas where ZENK- and c-Fos-labeled cells were counted. Abbreviations: M—*mesopallium*, CMM—*caudomedial mesopallium*, N—*nidopallium*, E—*entopallium*, L—*Field L*, NCM—*caudomedial nidopallium*, StM—*medial striatum*, StL—*lateral striatum*, GP—*globus pallidus*. Scale bar—2 mm.

Differences were analyzed with the help of Statistica 8.0 program. Expression differences between control and experimental groups were accessed with Mann-Witney criterion for two independent samples. Interhemispheric differences were accessed with Wilcoxon test for dependent samples. Differences were considered significant at $p < 0.05$.

3. Results

Analysis of the nestlings' behavior during the acoustic stimulation has shown that the subjects from the control group demonstrated typical freezing posture at the first sounds of the alarm call and remained virtually motionless until the end of the stimulation. In the visual deprivation group, the nestlings in response to alarm call stopped moving without assuming the typical posture. They demonstrated more motor activity during the stimulation as compared to the control group, which manifested as periodic shudders.

The quantitative analysis revealed asymmetric character of c-Fos induction in the CMM. The density of c-Fos-positive neurons in the left CMM was comparable in both groups (**Figure 3(A)**)— 139.5 ± 31.2 for the visual deprivation group and 161 ± 24.4 for the control one. In contrast, the level of expression in the right CMM was significantly lower in the visual deprivation ($p = 0.047$) group (111.0 ± 15.9 and 162.1 ± 14.3).

In Field L, the density of c-Fos positive cells was lower than in the CMM. The level of expression in the visual deprivation group was significantly lower than in the control (17.9 ± 2.3 and 26.8 ± 3.6 , $p = 0.047$ for left hemisphere and 16.9 ± 2.6 and 31.0 ± 4.5 , $p = 0.043$ for right hemisphere) (**Figure 3(B)**).

In the NCM, c-Fos expression was equal in the right and left hemispheres, the level of induction did not differ between the groups and varied from 30.7 ± 4.5 to 39.6 ± 3.4 (**Figure 3(C)**).

Quantitative analysis of ZENK expression in the CMM has demonstrated comparable density of ZENK-positive cells in both groups. The mean density of labeled cells varied from 29.1 ± 6.7 to 35.6 ± 11.7 ; no interhemispheric differences were found (**Figure 3(D)**).

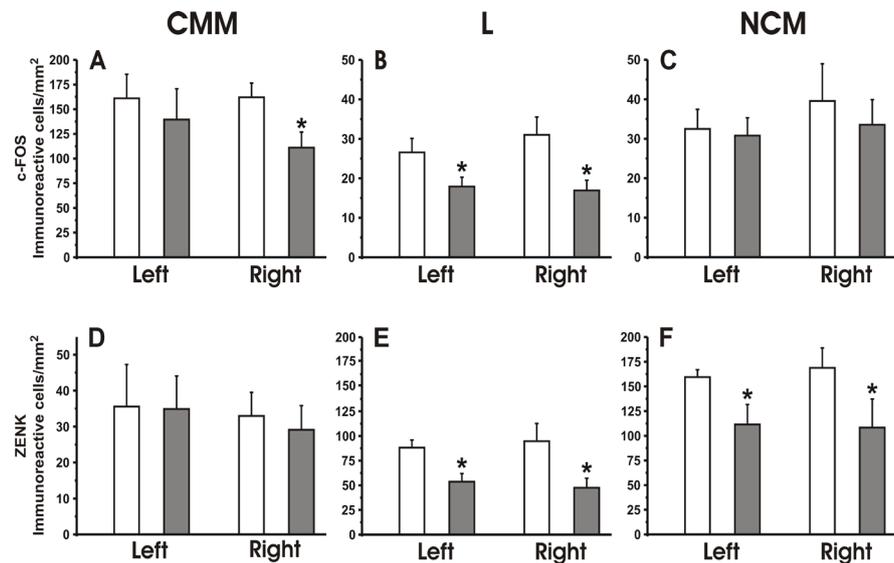


Figure 3. Density of c-Fos—(top panels) and ZENK—(bottom panels) immunopositive neurons in the left (Left) and right (Right) *caudomedial mesopallium* (CMM), Field L (L) and *caudomedial nidopallium* (NCM) of 9-day-old flycatcher nestlings following the presentations of species-specific alarm call. *—significant difference between groups ($p < 0.05$). Open bars—control group, grey bars—visually deprived group. $n = 5$ for each group.

The mean density of ZENK-positive neurons in Field L was higher than in the CMM (**Figure 3(E)**). In both left and right hemispheres of the control group the density of labeled cells was comparable and varied from 88.1 ± 7.9 to 94.6 ± 18.0 , respectively. In the visual deprivation group, the level of ZENK expression was significantly lower: 53.6 ± 8.3 in the left Field L and 47.5 ± 9.6 in the right one ($p = 0.028$).

Density of ZENK-positive neurons in the left and right NCM in the control group was highest (159.4 ± 7.5 and 168.7 ± 20.3 , respectively). Significantly lowest density was in visual deprivation group; it was 111.4 ± 20.2 (left hemisphere, $p = 0.016$) and 108.2 ± 20.0 (right hemisphere, $p = 0.047203$) (**Figure 3(F)**).

4. Discussion

Defense behavior developing under the conditions of limited visual afferentation differs from that in nestlings developing in the normal visual environment. Starting from post-hatching 9 this deviation defined by capability of alarm call to suppress feeding behavior becomes evident [8].

We observed dramatic divergence in both c-Fos and ZENK expression in the visually deprived nestlings in this study. Brain of these nestlings had been formed with lack of visual afferentation, which probably resulted in establishing of altered connections. In mammals, it was widely demonstrated that lack of visual experience from birth not only affected development of visual cortex but altered areas of other sensory modalities and resulted in a different cross-modal interaction as well [24] [25]. Particularly, early visual deprivation resulted in higher au-

ditory neurons specificity [26] and better sound localization abilities [27] [28]. Previously we found similar effect in flycatcher nestlings visually deprived from hatching. However, defense behavior in flycatchers developing under limited vision conditions differed from normal. Species-specific alarm call that almost completely suppressed feeding behavior in intact nestlings was significantly less effective in the visually deprived birds [8]. In the present work, we show that deviation in freezing behavior of visually deprived nestlings was accompanied by altered expression of *c-Fos* and *ZENK* in auditory telencephalic structures. Expression of at least one of these genes was reduced in all studied structures in the visual deprivation group as compared with the normally developing nestlings.

The most dramatic reduction of the immediate early genes expression following long-term visual deprivation was observed in Field L—the higher center of the ascending auditory pathway in birds in which the primary processing of acoustic signals showed that this area was involved not only into simple frequency analysis of sounds but also participated in the complex processing of species-specific acoustic information [29]. This area is also known to be important for maintaining of the species-specific song stability in adult birds [30] [31]. We found previously that capability to discriminate between species-specific alarm call and its artificial imitations (rhythmic tone pips of various frequencies) was considerably reduced in the nestlings visually deprived from the day of hatching, in spite of their lower thresholds of auditory evoked potentials from Field L [9]. Based on the results of the present study, we suggest that the insufficient number of transcriptionally activated neurons in Field L may be responsible for this altered discrimination capability.

5. Conclusion

Our study has demonstrated that the development of pied flycatcher young without adequate visual afferentation while performing acoustically-guided defense behavior results in the decrease of induction of immediate early genes *c-Fos* и *ZENK* in auditory structures of 9-day-old nestlings. The most marked decrease of both genes induction was observed in Field L. Our data suggest that visual afferentation affects activation of neurons in higher acoustical structures of young's brains thus increasing the efficiency of species-specific alarm call.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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