Involvement of *Wulst* Neurons in Hiding Reaction of Pied Flycatcher Nestlings with Different Visual Afferentation

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We studied transcriptional activity in the higher avian center visual system (Wulst area) in acoustically guided defensive behavior in visually deprived and non-deprived nestlings to evaluate the effects of visual afferentation on functional involvement of visual structures in acoustically guided defensive behavior. Exclusion of visual afferentation from already formed defensive behavior did not significantly change immunoreactivity of *Wulst* neurons, which attests to substantial contribution of other, non-visual, activating influences. Limitation of visual afferentation during the formation of defensive behavior decreased immunoreactivity of *Wulst* neurons. Dendritic sprouting in *Wulst* neurons of visually deprived nestlings unable to promote the formation of complex interneuronic interactions.

Key Words: *visual deprivation; defensive behavior; Wulst; birds; c-Fos*

Visual afferentation is an important component of behavior organization in pied flycatcher nestlings. After eye opening on days 5-6 it become involved in sensory support of feeding behavior [5], and starting from day 9 in support of defensive behavior [5,2]. Limitation of visual afferentation results in substantial disturbances in defensive behavior organization of nestlings [1].

Changes in visual afferentation induce plastic reorganisations in the neurons [6,15]. Thus, architectonic changes in neurons, dendritic sprouting, were found in *Wulst* of 10-day-old pied flycatcher nestlings visually deprived from the birth. These changes may appear as an adaptation that increases efficiency of incoming signal under conditions of impaired visual afferentation [4].

The objective of the study was to investigate the effect of visual afferentation on functional involvement of visual structures in acoustically-guided defensive behavior. We also studied whether plastic changes that increase efficiency of incoming signal under conditions of impaired visual afferentation promote neuron activation in visually deprived nestlings in defensive behavior.

Birds have well-developed vision and two main visual pathways in the brain: thalamofugal with central projections in the *Wulst* area [7] and tectofugal with central projections in the entopallium [8]. *Wulst* is a complex polyfunctional structure that besides visual information processing [9,14] is also characterized by associative functions [11]. This structure has been chosen for the investigation in the context of this polyfunctionality.

We studied transcriptional activity in *Wulst* in acoustically guided defensive behavior in visually deprived and non-deprived nestlings. In order to map neuronal activity in visual structures, we detected expression of transcriptional factor c-Fos. The age of 9 days has been chosen, since vision starts to influence manifestations of defensive behavior at this age [2].

MATERIALS AND METHODS

Experiments were conducted in accordance with international rules for work with laboratory animals. The experiments were carried out on 4 groups of 9 day old pied flycatcher nestlings (*Ficedula hypoleuca*), 5

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nestlings in each group. All the time nestlings spent in natural surrounding. The groups were formed by pooling the nestlings from different clutches 1 day before the experiment. Nestlings from 3 groups were fostered under normal visual conditions: passive control group (no stimulation during the experiment), group of sighted nestlings (stimulation during the experiment, adequate visual afferentation is preserved) and group of blind nestlings (eyes were plastered with lightproof cups 3 h before the experiment). In nestlings from visual deprivation group, the eyes were plastered with lightproof caps starting from day 1. On day 9, nestlings from sighted, blind, and visual deprivation groups in standard nest boxes were exposed to species-specific alarm signal for 15 min; after the end of exposure, the nestlings continued to stay in the nests. Video registration of nestling behavior was carried out during the experiment. Ninety minutes after start of the experiment, the nestlings were decapitated; passive control group was decapitated without preliminary exposure at the dark time of the day in order to exclude both visual and acoustic inputs. Nestling brains were frozen in liquid nitrogen and stored at -70°C. Immunohistochemical detection of c-Fos protein was carried out on serial 20-μ cryostat frontal sections. The corresponding brain sections of nestlings from different groups were arranged on one slide in a random order. Sections were fixed in 4% paraformaldehyde, washed in phosphate buffer and incubated with polyclonal rabbit anti-c-Fos antibodies (1:1000; Santa Cruz). Thereafter, secondary horse anti-rabbit antibodies, conjugated with horseradish peroxidase were applied (ImmPress KIT, Vector Labs), and diaminobenzidine detection was carried out (Sigma). Sections were dehydrated and embedded under coverslips. Their panoramic scanning was conducted at ×4using a TurboScan system (Objective Imaging) based upon Olympus BX-50 microscope. Expression patterns in higher representative center of thalamofugal visual projection, *Wulst* area, were quantitatively analyzed using Image Pro Plus 3.0 software (Fig. 1). Statistical treatment was performed using Mann–Whitney *U* test; the differences were significant at $p<0.05$.

RESULTS

With the first tones of species-specific alarm signal, the nestlings from sighted and blind groups took specific hiding pose (typical forcing in the nest surface) and remained almost completely immobile up to the end of the experiment. In nestlings from visual deprivation group, species-specific alarm signal induced freezing without hiding pose; increased motor activity was noted in these nestlings (startles).

Visual analysis revealed two zones with marked c-Fos immunoreactivity within the *Wulst* area: dorsomedial part, *hyperpallium apicale* (HA), and ventral part, *hyperpallium densocellulare* (HD). Intermediate part of HA contained no c-Fos-positive neurons (Fig. 1). Quantitative analysis was carried out separately for two *Wulst* areas in each hemisphere.

Quantitative analysis within HA demonstrated that sighted nestlings following acoustically guided defensive behavior had the highest density of c-Fos-positive neurons on both left hemisphere (343±28) and right hemisphere (316±52), which significantly differed from the corresponding parameters in passive control groups ($p=0.03671$ and $p=0.03734$, respectively; Fig. 2, *a*). Values in blind nestlings (276±23 for the left hemisphere and 204±43 for the right hemisphere) were

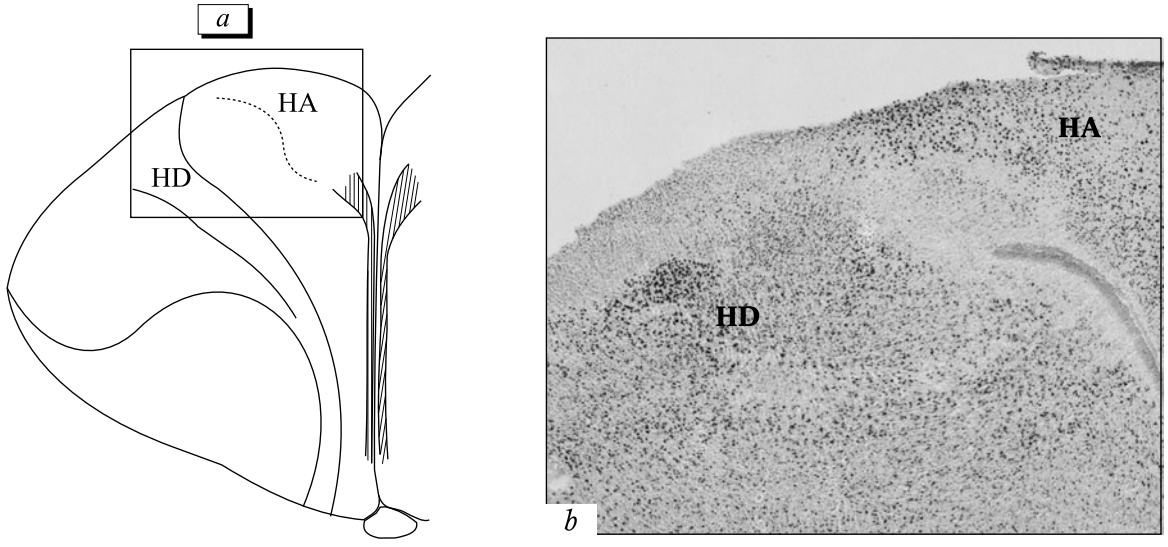

Fig. 1. Frontal brain section of 9 day old pied flycatcher nestling trough the *Wulst* area. *a*) schematic representation of the section in *Wulst* area, *b*) microphotograph of the marked part of the section. HA: *Hyperpallium Apicale*, HD: *Hyperpallium Densocellulare*.

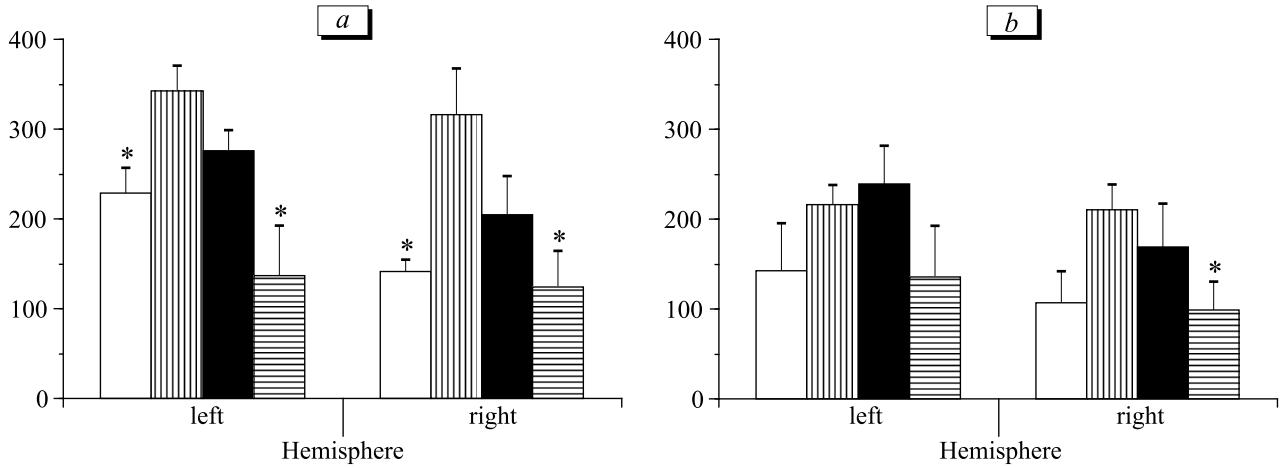

Fig. 2. Expression of transcriptional factor c-Fos in Wulst*:* density of c-Fos-positive neurons in HA (*a*) and HD (*b*). Ordinate: number of c-Fos-positive neurons per 1 mm^2. Light bars: passive control group; vertical shading: group of sighted nestlings; dark bars: group of blind nestlings; horizontal shading: group of deprived nestlings. *p<0.05 in comparison with group of sighted nestlings.

lower than in sighted nestlings, but higher than in passive control group (229±28 and 142±13, respectively); however, no significant differences from these groups were found. Visually deprived nestlings had the lowest density of c-Fos-positive neurons in both the left and right HA (137±56 and 124±40, respectively) and significantly lower in comparison with the group of sighted nestlings (p=0.02157 for left hemisphere and p=0.03671 for right hemisphere). In addition, in the left hemisphere, there was a trend toward reduction in comparison with passive control group and blind nestlings (p=0.09469 and p=0.0601, respectively).

Quantitative analysis of c-Fos-immunoreactivity in HD area showed that densities of c-Fos-positive neurons in sighted and blind nestlings in the left (216±22 and 239±43, respectively) and right hemispheres (210±28 and 169±48, respectively) were higher than in passive control group (153±52 and 107±35, respectively), however, these differences were insignificant (Fig. 2, *b*). Nevertheless, a trend toward increased density of c-Fos-positive cells in the right hemisphere was noted for sighted nestling group (p=0.06619). The density values in visually deprived nestlings were similar to that in passive control group (136±57 and 99±32, respectively). Significant differences were detected in the right HD between sighted and deprived nestlings (p=0.03671).

Thus, acoustically guided defensive behavior in 9-day-old pied flycatcher nestlings forested in normal visual surrounding led to an increase in *Wulst* neurons immunoreactivity, more pronounced in HA. Exclusion of visual afferentation from already formed defensive reaction results in altered immunoreactivity of *Wulst* neurons, which attests to additional activation of these neurons without direct association with the visual influences. Limitation of visual afferentation results in reduced immunoreactivity of *Wulst* neurons. Dedritic sprouting that develops in *Wulst* neurons of visually deprived nestlings cannot promote the formation of complex interneuronic interaction and appear to be non-adaptive in this case.

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