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Research report

Changes in ultra-structures and electrophysiological properties in HVC of untutored and deafened Bengalese finches relation to normally reared birds: Implications for song learning

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ABSTRACT

Songbirds are increasingly used as an experimentally tractable system to study the neurobiological underpinnings of vocal learning. To gain additional insights into how birdsongs are learned, we compared the size of HVC, the high vocal center for song production, and its ultrastructural or electrophysiological properties between the normally reared Bengalese finches, and the untutored or deafened ones before the onset of sensory learning (around post-hatching day 20). Our results showed that HVC had more synapses and concave synaptic curvature, but fewer perforated synapse, in the untutored or deafened birds in comparison with those in the normally reared birds. Although there was no significant difference of the ratio of straight or compound synapses, there was an increasing tendency for the untutored and deafened birds to possess more straight and compound synapses. These data revealed that synapses in the isolated or deafened birds had lower synapse activity in relation to those with normal hearing. This was confirmed by our electrophysiological results to show significant decreases in the firing rates of spike or burst in the isolated or deafened birds in the three types of HVC neurons i.e., putative X-projecting neurons, RA-projecting neurons and interneurons. In addition, low firing frequency (<10 Hz) occurred much more in the above three types of HVC neurons in the tutored or deafened birds than in the normally reared birds. These data suggest that all the three putative types of neurons in HVC might be involved in the activity of the production of adult normal songs.

1. Introduction

Despite widespread use of vocal communication in animals, vocal learning is quite rare in nonhuman species (bats, cetaceans and some birds). Of them oscine songbirds, a highly diverse group comprising roughly 4000 species (order: Passeriformes), are increasingly used as an experimentally tractable system to study the neurobiological underpinnings of vocal learning. Birdsong learning comprising two stages bears striking similarities to certain aspects of human vocal learning (Konishi, 1985; Marler, 1970). During the first stage of "sensory" learning, young birds must hear and memorize a parent tutor song or "template", which is stored in the neural circuitry. The memorized template is accessed during the ensuing phase of sensorimotor learning. In the second stage, the pupil relies on auditory feedback to gradually refine their initially variable and noisy vocalizations to the memorized model. Sensorimotor learning finishes with song "crystallization", a process wherein the learned song is highly stereotyped. Birds must be able to hear themselves for this vocal refinement; if they are deafened after exposure to tutor songs but before vocal practice, they develop abnormal songs that show no evidence of learning. After song learning, songbirds no longer need to hear tutors, but auditory feedback is necessary for maintaining songs (Catchpole and Slater, 1995; Nordeen and Nordeen, 1992; Okanoya and Yamaguchi, 1997; Watanabe and Aoki, 1998; Woolley and Rubel, 1997). In most songbirds, these two stages of learning are overlapped. The 'sensory' phase of learning is usually limited to a 'critical period' early in life, and usually appears from 20 to 60 days of age (Eales, 1985, 1987; Immelmann, 1969), while the sensorimotor learning starts around 35 days, and ends around 80–90 days, for example in zebra finch (Arnold, 1975; Immelmann, 1969).

Songbirds possess a unique system of interconnected brain nuclei which underlie song learning and production. It consists of two pathways (Nottebohm et al., 1976): the motor pathway

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which begins from the HVC, to the robust nucleus of the archopallium (RA), in turn to the tracheosyringeal portion of the hypogloss nucleus, and the anterior forebrain pathway which initiates from HVC, through area X, the medial nucleus of the dorsolateral thalamus (DLM) and the lateral magnocellular nucleus of the anterior nidopallium (LMAN), in turn to RA (Nottebohm, 2005). The motor pathway is essential for song production, as lesions in this pathway disrupt singing, whereas the anterior forebrain pathway is crucial for song learning (Brenowitz et al., 1997).

HVC is at an intersection of auditory and motor pathways. HVC neurons have highly selective auditory properties, responding strongly to complex acoustic stimuli, particularly the bird's own song (Margoliash, 1986). HVC forms a part of the forebrain central pattern generator for song production (Nottebohm et al., 1976; Vu et al., 1994). Before singing, HVC neurons show premotor firing, and during singing, the reliable changes of activity rates in HVC premotor neurons are uniquely associated with syllable identity (McCasland and Konishi, 1981; McCasland, 1987; Yu and Margoliash, 1996). HVC is thus a critically important station for sensorimotor integration of song learning and production. At least three major cell types in HVC are distinguished by a number of physiological, pharmacological, and anatomic criteria in the zebra finch (Hahnloser et al., 2003; Mooney, 2000). They include: (1) X-projecting neurons (HVC_X) which exhibit low spontaneous firing rates, and a slow inhibitory postsynaptic potential with an unusual hyperpolarizing response to metabotropic glutamate receptor activation; (2) RA-projecting neurons (HVC_{RA}) which show extremely low rates of spontaneous firing rates, and are not hyperpolarized by metabotropic glutamate receptor agonists; (3) interneurons (HVC_{INT}) which fire in a sustained fashion at very high spontaneous rates, and have divergent inhibitory connections onto both types of the projecting neurons (Dutar et al., 1998; Hahnloser et al., 2003; Mooney and Prather, 2005; Mooney, 2000).

Although the neural mechanism for birdsong learning has been known quite well, it is far more clear (Nordeen and Nordeen, 1997). To get insights into how song is learned, we first produced two types of abnormal song learning in Bengalese finch (*L. striata*): isolated and deafened by bilateral cochlear removing before the onset of sensory learning (at post-hatching day 20). Our behavioral study has shown that the isolated birds produce seriously degenerated songs, whereas the deafened birds cannot sing audible songs, much like the phenomenon in human deaf mute (Peng et al., 2012). Interestingly, the sizes of song nuclei remain unchanged significantly. These findings prompted us to continue comparing the coincident changes of ultrastructural structures and electrophysiological activity in HVC among these birds. Our results first revealed that most ultra-structures, including synapse density, the prominent thickening or postsynaptic membrane density (PSD), synaptic curvature and vesicular density changed largely in HVC among the studied groups, reflecting the decreasing efficiency of synaptic transmission in isolated or deafened birds. By recording extracellular single HVC unit in anesthetized finches in vivo, some electrophysiological measures, including instantaneous firing rate $R(t)$, spontaneous firing rate, and the number of the spikes per burst or the cluster interspike interval were all dramatically decreased for each of HVC cell types in the isolated or deafened birds with respect to those in the normal birds. This comprehensive study provides new data to address the neural mechanism underlying birdsong learning and production.

2. Methods

2.1. Animals and surgery

Bengalese finches were bred in our colony laboratory at Beijing Normal University or bought from a local supplier. The birds were housed three or four per cage (50 cm × 62 cm × 38 cm) in a room under a 14/10 h light/dark cycle. Fresh water and seed were available at any time, and green vegetable and cuttlebone were supplemented occasionally. To get isolation or song deprived birds, adult males were removed when the young hatched at post-hatching days (PD) 20. The hatchling birds were housed with their mother in a room isolated from conspecific songs until PD 120. However, these birds were kept acoustic and visual contact among one another. The deafened birds by bilateral cochlear removal were produced at PD 20 according to the procedure described by Konishi (1965a,b). Briefly, birds were anesthetized with intramuscular injections of 25 mg/kg Nembutal. By using a fine tungsten wire hook, both cochleae were removed through the oval window. After the skin incision was sealed with cyanoacrylic glue, the excised cochleae were examined microscopically for the presence of lagenae to confirm the complete removal of the cochleae. They were then back to their parents and raised till PD 120. The other thirty birds (20 males) served as manipulated controls were raised by their parents till adulthood (PD 120), keeping acoustic and visual contact with other conspecific birds in the same room. The total number of male birds (females were not counted) was 34 for normally reared birds, 23 for untutored birds and 31 for deafened birds, of which 6 for electron microscopic study in each of studied groups (normal, isolated, deafened), and 28, 17 and 25 for electrophysiological recordings in normally reared, untutored and deafened groups, respectively. All the birds were anesthetized for electron microscopy or for electrophysiological recordings at PD120.

2.2. Electron microscopy

2.2.1. Tissue preparation

The birds received a lethal dose of urethane at PD 120 and were then perfused through the left ventricle of the heart with 0.9% saline (NaCl), followed by a cold phosphate buffer (0.1 M, pH = 7.4) containing 4% paraformaldehyde and 1% glutaraldehyde. The reproductive glands were inspected to determine the sex. Well-developed testes confirmed that the birds were actually mature. The brains were stored in the same fixative overnight. Sagittal sections (100 μm) were cut with a vibratome (Leica CM1850) for both hemispheres. The sections containing HVC were dissected out with a sharp blade and they were then rinsed in phosphate buffer. After post-fixation in 1% osmium tetroxide for at least 1.5 h at 4 °C, and dehydration in a series of graded ethyl alcohols and acetone, they were immersed in propylene oxide overnight, and then embedded flat in Epon 812. Ultrathin sections (around 75 nm) were cut with a glass knife. They were mounted onto 200-mesh grids with formvar film, and counterstained with aqueous uranyl acetate and lead citrate. They were finally observed in an electron microscope.

For each studied birds, beyond 6 blocks were obtained randomly from either brain hemisphere, of which at least 3 blocks were chosen to cut for ultrathin sections and the others were ready for use. For each block, there were at least 5–6 copper grids on which at least 5 sections were obtained. To keep random, photos were taken along diagonal of the trapezoid section. For each bird, at least 30 microphotos were taken, to sure that our data undoubtedly represent a picture of the majority events in HVC. The microphotos were digitalized by scanning into a computer, and stored in tif format files.

2.2.2. Measurement of structural parameters

Synapse density. For the quantitative investigation of the number of synapses in HVC, micrographs intended for counting were randomly selected within HVC, excluding its borders. From each studied birds, 30–50 micrographs were taken at a magnification of 7000×. The total area for each micrograph was around 100 μm^2, yielding a magnification of 20,000× after photographic enlargement. The micrographs were coded and analyzed by a person who did not know the treatment condition of the bird. Synapses were characterized by some features, including the presence of a prominent thickening, a distinct synaptic cleft separating pre- and postsynaptic elements, and round synaptic vesicles. The sampling excluded the areas containing mostly non-neuropil elements: blood vessels, large myelinated axons and neural or glial somata. The proportion of micrographs ignored for each of the studied groups was 3.12 ± 0.54% ($n = 6$) for isolation, 4.11 ± 1.12% ($n = 6$) for deaf, and 3.76 ± 1.07% ($n = 6$) for control, and there was no significant difference among the studied groups ($F_{(2, 35)} = 0.39$, $p = 0.876$; one-way ANOVA, post hoc tests, SPSS for Windows 16.0). To get the number of synaptic contacts (synapses) per unit volume, we adopted the stereological formula of DeHoff and Rhines (1961): $N_a = N_v/d$, where N_a is the mean number of synapses per unit area, N_v represents the number of synapses per unit volume, and d is the mean length of profiles of synapses (PSDs). This formula provides accurate and consistent results on truly representative samples (Colonier and Beaulieu, 1985). The total number of synapses in HVC was calculated by N_v multiplying the volume of HVC, which has been obtained from the birds experienced the same process as described above (unpublished data).

Synaptic curvature and the number of perforated or compound synapses. The synaptic interface comprises several main types of curvature, including straight, convex and concave curvatures. The percentage of each type of synaptic curvature was counted in all the examined synapses. In addition, the incidence of synapses with perforated PSDs or compound synapses was counted.

Length of the PSD and vesicular density. The PSD was identified as an asymmetric thickening of the postsynaptic membrane, which are often associated with the accumulation of presynaptic vesicles (Cant and Morest, 1979; Fekete et al., 1984). With the aid of SPOT (Enhance 2e; Diagnostic Instruments), the PSD length was measured. For each brain, the mean value of the lengths of the PSDs was obtained from beyond