Sexual dimorphism of the electrophysiological properties of the projection neurons in the robust nucleus of the arcopallium in adult zebra finches

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Abstract: Objective To observe the sexual differences in electrophysiological properties of neurons in the robust nucleus of the arcopallium (RA) in adult zebra finches, and to provide the direct electrophysiological evidence for the sexual dimorphism of birdsong. **Methods** Whole-cell recording was used to record the spontaneous action potential firing rates from RA projection neurons in acute brain slices. **Results** The projection neurons of RA in male birds fired spontaneously at 10 Hz or above, while in female birds, the frequency was significantly lower, and even no firings could be detected. **Conclusion** There is a sexual difference in electrophysiological properties of projection neurons in RA, which may result from the difference in the levels of steroid hormones in birds.

Keywords: adult zebra finches; the robust nucleus of the arcopallium; spontaneous action potential firing rates; electrophysiological properties; sexual dimorphism

1 Introduction

Singing is a highly complex learning behavior and an ideal model system for studying the mechanisms of learning and memory in songbirds. The neural substrate controlling song learning and vocal production consists of anterior forebrain pathway (AFP) and vocal motor pathway (VMP), both of which are referred to as the song control system^[1-3].

VMP is composed of the projection neurons from the high vocal center (HVC) and the robust nucleus of the arcopallium (RA), and RA ultimately projects to the midbrain and brainstem nuclei, thus controlling the muscles of the avian vocal organ and the muscles of respiration. VMP is required throughout life for the production of sound. AFP

starts from HVC, passing through area X, dorsolateral anterior thalamus nucleus (DLM) and the lateral magnocellular nucleus of the anterior neostriatum (LMAN), and finally signals to RA. The loop connecting area X, DLM and LMAN is also called the basal ganglia-forebrain circuit. RA is not only the nucleus responsible for the output of vocal information, but also the sole nucleus receiving input from AFP via LMAN. After integration of the input from AFP with motor commands from HVC, the premotor nucleus RA controls the syringeal muscles via tracheosyringeal part of hypoglossal nucleus (nXIIts), thus playing a crucial role in the song system^[4-8].

RA is made up of 2 types of neurons: projection neurons and interneurons, with different electrophysiological properties. Both types of neurons receive excitatory glutamatergic input from HVC and LMAN. The GABAergic interneurons make local inhibitory connections onto both cell types, and the glutamatergic projection neurons project to brain stem nuclei that control the muscles of the syrinx,

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Article ID: 1673-7067(2010)02-0147-06

Received date: 2009-10-10; Accepted date: 2009-12-25

the avian vocal organ^[9-11]. The projection neurons have larger input resistance and time constant, and a longer latency of afterhyperpolarization (AHP). When given hyperpolarizing current, their specific inward rectification can be induced, in a time-dependent way. In resting conditions, the projection neurons can display regular spontaneous action potential firings. On the other hand, the input resistance and time constant of interneurons are smaller, and the latency of afterhyperpolarization is shorter than that of projection neurons. Besides, in the interneurons, the inward rectification cannot be induced by hyperpolarizing current. In resting conditions, the interneurons display no spontaneous action potential firings. Moreover, the 2 types of neurons display distinct firing properties during depolarizing current injection. The projection neurons exhibit regular firing during the depolarizing current injection, with a smaller slope of firing frequency–injected current curve, while interneurons show a steep curve of firing frequency–injected current, a larger firing threshold and prominent irregular spikes after the depolarizing current injection^[12].

Oscine songbirds, such as the zebra finch, *Taeniopygia guttata*, have a clear sexual dimorphism in song. The male birds are good at singing complex songs, while the female birds can only make simple sounds^[13,14]. Morphological research has shown that the nuclei in male birds are bigger and more developed, while in female birds, the nuclei are smaller, some even undetectable^[15]. This suggests that the male birds have the inherent advantage in the development of the song system. Moreover, the volumes of RA in male and female zebra finches are obviously different, providing the morphological evidence for the vocal sexual dimorphism, since RA is a pivotal nucleus in the song system. However, the sexual differences in the electrophysiological properties of neurons in RA are still unclear. Here the frequency of spontaneous action potential firings of projection neurons in RA was recorded in both male and female zebra finches using whole-cell patch clamp technique *in vitro*, and the sexual differences in the electrophysiological properties of neurons in RA were analyzed.

2 Materials and methods

2.1 Animals A total number of 20 adult zebra finches (weighting 15-20 g) were supplied by the College of Life Sciences, South China Normal University. Birds were anesthetized with 10% chloral hydrate and sacrificed immediately.

2.2 Slice preparation Brains were dissected and put into oxygenated (95% O₂, 5% CO₂) ice-cold slice solution, containing 248 mmol/L sucrose, 5 mmol/L KCl, 28 mmol/L NaHCO₃, 10 mmol/L glucose, 1.3 mmol/L MgSO₄·7H₂O, and 1.26 mmol/L NaH₂PO₄·H₂O. Coronal slices (250-300-μm thick) containing RA were cut with a vibrating microtome and collected in artificial cerebrospinal fluid (ACSF) that had been warmed to 37 °C and subsequently cooled to room temperature. Slices were allowed to recover in the holding chamber for at least 1-1.5 h and equilibrate to room temperature before recordings were made. Standard ACSF consisted of 125 mmol/L NaCl, 25 mmol/L NaHCO₃, 1.27 mmol/L NaH₂PO₄·H₂O, 2.5 mmol/L KCl, 1.2 mmol/L MgSO₄·7H₂O, 2.0 mmol/L CaCl₂, and 25 mmol/L glucose, and adjusted with sucrose to a final osmolarity of 350 mOs.

2.3 Electrophysiological recording Recording pipettes with 1-2 μm tips were made by a Flaming/Brown four-stage puller (P-97, Sutter Instrument). The resistance of recording pipettes was 3-5 MΩ when the pipettes were filled with inner pipette solution (340 mOs). The pipette solution contained 120 mmol/L KMeSO₄, 5 mmol/L NaCl, 10 mmol/L HEPES, 2 mmol/L EGTA, 2 mmol/L Mg-ATP, and 0.3 mmol/L Na-GTP (pH 7.3-7.4). Brain slices were placed in a submerged-type chamber where they were continuously exposed to ACSF, and saturated with 95% O₂ and 5% CO₂ at a rate of 1-2 mL/min. Slices were immersed at 1.0-2.0 mm below the solution surface. RA and the surrounding tissues were observed under Olympus BX51 WI microscopy at a low magnification (5×). RA neurons were visualized and recordings were made from healthy cells at a high magnification (40×). Active cells were selected for the subsequent recordings. Whole-cell recordings were obtained using standard techniques. Giga-seal was obtained by a negative pressure. Each neuron was recorded for 15-20 min between 13:00-16:00.

2.4 Data analysis Whole-cell patch recordings were obtained using an Axopatch 700B amplifier and the output was digitized at 10 kHz (low-pass filtered at 1 kHz) with a Digidata

1332A converter. Data were expressed as mean $\pm$ SEM, and analyzed with pClamp 9.2 software and Student's *t*-test. $P < 0.05$ was indicated as significantly different.

3 Results

3.1 Morphological observation of RA In both male and female birds, RA could be clearly distinguished under a low magnification (5 $\times$). As shown in Fig. 1, RA in male zebra finch was much larger than that in female. Besides, in both male and female zebra finches, RA had distinct boundaries.

As being observed under a high magnification (40 $\times$), RA neurons were irregularly distributed throughout the nucleus and were interlaced with nerve fibers and capillary vessels. Most of the neurons had pyramidal or fusiform soma with several dendrites. The appearance of healthy neurons without mottled dark patches had a uniform refraction (Fig. 2).

3.2 The sexual difference in the spontaneous action potential firing rates of RA projection neurons The spontaneous

action potential firings of RA projection neurons in male and female zebra finches were recorded (Fig. 3). The rate of spontaneous action potential firing in projection neurons of RA in male birds was 10.24 Hz, while the rate in female birds was 6.49 Hz (Fig. 4A). Student's *t*-test revealed a significant difference between male and female birds ($P < 0.05$, $n = 28$).

The frequency distribution was also different (Fig. 4B). Firing rates of all the RA projection neurons in male birds were between 5-14 Hz, and in most of the neurons, the firing rates were 10 Hz or above. While in female birds, the firing rates were between 1-5 Hz, and some RA projection neurons even had no firings.

4 Discussion

Up to now, research in the song system has been focused on male birds due to the sexual dimorphism. In most species of songbirds, adult male birds can sing, while female birds seldom sing^[16,17]. The present study provides impor-

Fig. 1 Morphology of RA in male and female zebra finches, as observed in slice preparation at the 5 $\times$ magnification. A: RA in a male zebra finch. B: RA in a female zebra finch. Scale bar: 1.0 mm.

Fig. 2 The RA neuron with a recording electrode observed at the 40 $\times$ magnification. Scale bar: 30 μ m.

Fig. 3 The spontaneous action potential firings of RA projection neurons, as recorded by whole-cell patch clamp recording. A: The spontaneous firings in male zebra finch. B: The spontaneous firings in female zebra finch.

Fig. 4 Differences in the rates of spontaneous action potential firing of RA projection neurons, and in the frequency distribution between male and female zebra finches. **A:** The firing rates in male and female zebra finches. **B:** The frequency distribution in RA projection neurons in male and female zebra finches.

tant electrophysiological evidence to support the sexual dimorphism in songbirds.

The morphological observation shows that compared to that in female birds, the nucleus RA in male birds is larger, with a lower density of neurons. The neurons in RA in male birds are sparsely distributed, and each neuron occupies larger space. LI DF *et al.* have reported that the numbers of synapses per unit area, the Gray I type synapses and spiny synapses in RA are all significantly larger in male songbirds (*Fringilla montifringilla*) than in female^[18]. Therefore, RA neurons in male birds occupy larger space and form more synaptic connections, indicating that the RA neurons in male birds perform more complex functions. In both male and female birds, vocalizations are produced by syringeal muscles controlled by motoneurons within nXIIts. Roberts *et al.*^[19] have reported that the number of nXIIts neurons and the proportion of somata are not sexually dimorphic. The volume of nXIIts is larger in male than in female, partly because nXIIts neurons have larger somata in male. In contrast, female nXIIts motoneurons have a more complex dendritic structure than male neurons do, suggesting that the larger volume of male nXIIts is partly due to the increased number of afferents. Moreover, intracellular recordings in brain slices reveal that the intrinsic electrophysiological properties of female nXIIts

neurons are similar to those of male nXIIts motoneurons. These findings indicate that the disparate vocal repertoires of male and female zebra finches are produced by physiologically similar motoneurons. Thus, the upstream of the motoneurons in nXIIts may be the major determinant of sexually dimorphic vocal behaviors in this species. The nucleus RA is just at the upstream site of nXIIts.

Here we find that the electrophysiological properties of projection neurons in RA are sexually dimorphic, in that the spontaneous action potential firing rate in male birds was significantly higher than that in female. It is suggested that the different activities of neurons in RA may lead to the sexual differences in vocal ability. Furthermore, this difference may mostly result from the different levels of steroid hormones in birds. Recent findings^[20] show that the morphological and electrophysiological properties of RA projection neurons can be changed by modifying the photoperiod and influencing the level of systemic testosterone. In the conditions of long-day photoperiod, the level of testosterone in male birds is higher than that in the conditions of short-day photoperiod. Besides, the somata of RA projection neurons is bigger and the frequency of spontaneous action potential firings is higher. These findings provide a framework for integrating cellular physiological change modulated by steroid hormones,

to behavioral change. However, recent evidence suggests that the sexual differences in brain and behavior might result from the direct genetic effects, not just from the steroid hormones^[21]. Our data provide valuable evidence supporting the sexual differences in electrophysiological properties. However, our work on the vocal sexual dimorphism in songbirds is preliminary, and further studies are still needed.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (No. 30970363, 30570232) and the Natural Science Foundation of Guangdong Province, China (No. 05005910).

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成年斑胸草雀弓状皮质核投射神经元电生理特性的性别二态性

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摘要: **目的** 通过观察神经元在电生理特性方面的性别差异, 为鸣禽鸣唱行为性别二态性提供新证据。**方法** 采用脑片膜片钳全细胞模式, 记录成年斑胸草雀弓状皮质核(RA)投射神经元的动作电位自发放。**结果** 雄鸟RA核团投射神经元均有一定频率的自发放, 且多数神经元的自发放频率超过 10 Hz。而雌鸟RA核团投射神经元的自发放频率相对较低, 甚至无自发放。**结论** 成年斑胸草雀RA核团神经元的电生理特性存在较大的性别差异。导致这种发放活动差异的主要原因可能是雌雄鸣禽体内及相关脑区激素水平的差异。

关键词: 成年斑胸草雀; 弓状皮质核; 动作电位自发放频率; 电生理特性; 性别二态性