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Castration Modulates Singing Patterns and Electrophysiological Properties of RA Projection Neurons in Adult Male Zebra Finches

Castration can change levels of plasma testosterone. Androgens such as testosterone play an important role in stabilizing birdsong. The robust nucleus of the arcopallium (RA) is an important premotor nucleus critical for singing. In this study, we investigated the effect of castration on singing patterns and electrophysiological properties of projection neurons (PNs) in the RA of adult male zebra finches.Adult male zebra finches were castrated and the changes in bird song assessed. We also recorded the electrophysiological changes from RA PNs using patch clamp recording. We found that the plasma levels of testosterone were significantly decreased, song syllable's entropy was increased and the similarity of motif was decreased after castration. Spontaneous and evoked firing rates, membrane time constants, and membrane capacitance of RA PNs in the castration group were lower than those of the control and the sham groups. Afterhyperpolarization AHP time to peak of spontaneous action potential (AP) was prolonged after castration.These findings suggest that castration decreases song stereotypy and excitability of RA PNs in male zebra finches.

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Background

Steroid sex hormones change adult avian song behavior and modulate the underlying neural substrates. Androgens, particularly testosterone, play an important role. For example, testosterone can increase the stability of song , the size of song nuclei, the expression of androgen receptor mRNA and the excitability in song-control neurons in seasonally breeding songbirds.

The robust nucleus of the arcopallium (RA) is a crucial nucleus in the song control system, receiving synaptic input from both the HVC (used as a proper name) and lateral magnocellular nucleus of the anterior nidopallium (LMAN) . The HVC-RA pathway produces a stereotyped pattern contributing to stable song, and while the LMAN-RA pathway fires when male birds sing to female birds, LMAN neurons exhibit reliable firing of single spikes precisely locked to song. Thus, the LMAN may act as a source of variability . The axons of projection neurons (PNs) in the ventral RA project topographically onto the hypoglossal motor nucleus (nXIIts) that innervates the syrinx (the avian song organ), and the axons of PNs in the dorsal RA project to the areas in the lateral medulla that control respiration. Lesion of the RA causes severe song deficits. Moreover, RA activity is significantly correlated with variations in the spectral entropy of

22 syllables, and RA shows accurately timed and structured bursts of activity that are associated with
23 specific syllables.
24 Recent studies have shown that testosterone and photoperiod affect the excitability of RA PNs in
25 seasonally breeding songbirds that undergo major hormonal shifts as a result of photoperiod, but
26 have no effect on the electrophysiological properties of HVC neurons. Zebra finches are
27 opportunistic breeders rather than seasonal breeders. Castration of adult male zebra finches
28 reduces testosterone levels in plasma and their singing rate. High levels of testosterone decrease
29 the frequency of syllable in song and reduce the potential for vocal plasticity . However, the
30 neural mechanism that androgen influence singing pattern is less well understood. Thus, the aim
31 of the present study was to examine the effect of castration on singing patterns and
32 electrophysiological properties of RA PNs in adult male zebra finches, to further understand the
33 neural mechanism of androgens in adult songbirds.

Materials and Methods

Animals and experimental treatments

A total of 27 adult male zebra finches (*Taeniopygia guttata*) (> 120 days old) obtained from a commercial breeder were used in this study. All experiments were carried out in accordance with the University and China animal guidelines. The care and use of animals for this study was approved by the Institutional Animal Care and Use Committee at South China Normal University and in accordance with National Institutes of Health guidelines (scnu20070033). Birds were housed in stainless steel cages (23.5 × 22.5 × 27.5 cm), and each of the cages contained a pair of male and female birds, which were provided with ad libitum food and water and were kept in 14:10 h light/dark cycles. All birds were divided into three main experimental groups: castration group (n = 11 birds), control group (n = 12 birds), and sham group (n = 4 birds).

Before castration, the songs of all birds were recorded in the presence of adult female birds. Birds were then anesthetized with 10% chloral hydrate (0.02 mL/10 g). A small incision was made on the lateral wall of the body cavity between the last two ribs just ventral to the ventral margin of the kidney. The testicles were removed with ophthalmic forceps. The sham group underwent the same surgery without removing the testicles. The control group did not receive surgery.

Song recording

The song recording room (2.5 × 2 × 2.5 m) contained TAKSTAR directional microphones (Guangdong Victory Electronics Co.Ltd., Guangzhou, China; frequency range: 50–20000 Hz) and a glass window (85 × 65 cm). Birds in the song recording room could see the other birds from the glass window. When the songs were recorded, the male bird was placed in a cage in the

song recording room near the glass window, while the female bird was placed in a cage near the glass window outside of the song recording room, so that the male bird could observe the female bird through the window. On each recording day, every bird was recorded for 90–120 min. Songs were recorded between 8:00 a.m and 11:00 a.m. Songs were recorded before the castration and sham operation. When birds produce a stable song, the date defined as 'pre'. The songs were then recorded again at the 30th day after castration and sham operation. The songs of birds in control group also were recorded at 'pre' and 30th day. Song recording was performed using Cool Edit 2000 (Adobe Systems Co., SAN Jose, CA, USA; sampling rate: 44100 Hz; channels: stereo; resolution: 16-bit).

Stereotypy of song

We analyzed song stereotypy by calculating entropy (a measure of randomness, entropy is high when the waveform is random, and low when the waveform is of pure tone) of the longest syllable (the distance-call element, whose structure matched that of distance call and is derived from distance call) in the motifs within a record using Sound Analysis Pro 2011 (contrast: 0, frequency range: 0–11025 Hz, FFT data window: 10 ms, advance window: 1 ms, contour thresh: 10). On each recording day the entropy of 30 syllables in 30 motifs was analyzed. Sixty motifs were used to analyze the percentage similarity (% similarity) of the motif in the song. Higher entropy indicates less stereotypy, while higher (% similarity) indicates more stereotypy.

Slice preparation

At the 30th day after castration, the birds were anesthetized with 10% chloral hydrate and then rapidly decapitated. Brains were dissected into ice-cold, oxygenated (95% O_2 and 5% CO_2) slice solution. Slice solution consisted of KCl 5 mM, $NaH_2PO_4 \cdot H_2O$ 1.26 mM, $MgSO_4 \cdot 7H_2O$ 1.3 mM,

77 $NaHCO_3$ 28 mM, glucose 10 mM, sucrose 248 mM, and NaCl 62.5 mM. Coronal brain slices
78 (250–300 μm thick) containing the RA were cut with a vibrating microtome (World Precision
79 Instruments Inc., Sarasota, FL, USA) and collected in artificial cerebrospinal fluid (ACSF) that
80 was warmed to 37°C. After 30 min the ACSF was cooled to 35°C, and the slices were allowed to
81 recover in the holding chamber for 1–1.5 h. Standard ACSF consisted of NaCl 125mM, KCl 2.5
82 mM, $NaH_2PO_4 \cdot H_2O$ 1.27 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 25 mM and
83 $CaCl_2$ 2.0 mM, and the osmolality was adjusted with sucrose to 350 mOsm.
84 **Patch-clamp recording**
85 During the experiments, slices were transferred to a recording chamber where they were
86 continuously perfused with ACSF, saturated with 95% O_2 and 5% CO_2 at room temperature (23–
87 28°C). RA and the surrounding tissues were observed at low magnification (50×) under a
88 BX51WI microscope connected with a DIC-IR video camera (Olympus, Tokyo, Japan). At high
89 magnification (400×), RA neurons were visualized and the recordings were made from RA PNs.
90 Recording pipettes were fabricated from borosilicate glass (Sutter Instrument Co., Novato, CA,
91 USA) using a Flaming-Brown puller (Micropipette Puller P-97; Sutter Instrument Co.), and were
92 filled with the solution containing $KMeSO_4$ 120 mM, NaCl 5 mM, HEPES 10 mM, EGTA 2 mM,
93 Mg-ATP 2 mM, and Na-GTP 0.3 mM (pH 7.3-7.4). Osmolality was adjusted with sucrose to 340
94 mOsm. The recording pipettes, which had resistances ranging from 4 to 7 MΩ, were positioned
95 using an integrated motorized control system (Sutter Instrument Co.). Signals were amplified
96 with a MultiClamp 700B (Axon Instruments, Sunnyvale, CA, USA). Signals were low-pass
97 filtered at 5 kHz, digitized at 10 kHz with DIGIDATA 1322A (Axon Instruments) and acquired
98 using Clampfit 9.2 (Axon Instruments). Tight-seal and whole-cell recordings were obtained using

99 standard techniques. The baseline membrane potential was held at −70 mV during the stimulation
100 protocols. RA PNs were identified by their distinct intrinsic properties as described previously.

101 **Electrophysiological data analysis**

102 Clampfit 9.2 and Origin Pro 8.0 (Origin Lab, Northampton, MA, USA) were used for analysis. In
103 measuring spontaneous firing rates in the cell-attached configuration, we analyzed the spike
104 amplitude, waveform, and time derivative to ensure that spike events were single units. We
105 measured spontaneous activity for at least 5 min, and calculated the firing rate by dividing the
106 number of spikes observed by the duration of the recording as reported . Action potentials (AP)
107 were detected using the event detection package of the Clampfit 9.2. Spontaneous firing rates
108 were calculated at the beginning of the recording as soon as it stabilized following patch rupture.
109 The AP threshold was detected using a custom algorithm described previously by Baufreton ; the
110 afterhyperpolarization (AHP) peak amplitude was the difference between the AP threshold and
111 the most negative voltage reached during the AHP. The AHP time to peak was the time of this
112 minimum minus the time when the membrane potential crossed the AP threshold on descent from
113 the AP peak . For each neuron, the measurements of five APs were averaged to produce the final
114 AP measurements for that neuron. Evoked firing rates were measured after patch rupture. The
115 evoked firing rate was defined as the number of AP evoked over the duration of the current
116 injection. The slope of the F-I relationship was estimated by linear fitting. Slope parameters were
117 estimated separately for individual neurons and mean slope values were averaged for the whole
118 groups of neurons. Input resistance was estimated by applying small hyperpolarizing current
119 pulses. The membrane time constant was calculated by fitting a single exponential curve to the
120 membrane potential change in response to −200 pA hyperpolarizing pulses. Membrane

121 capacitance was calculated using the following equation: capacitance = membrane time
122 constant/input resistance.

123 **Hormone assay**

124 On the day of each electrophysiological recording, carotid artery blood was rapidly collected
125 from each subject before they were decapitated into a heparinized microhematocrit tube and
126 stored on ice until centrifugation (within 1 h). The plasma was harvested and stored it at −80°C.
127 To measure circulating testosterone levels, enzyme-linked immunosorbant was used in a bird
128 testosterone ELISA kit (IBL, Hamburg, Germany), which contained a substrate standard. The
129 minimum detectable plasma testosterone concentration was 0.12 ng/mL, and the maximum was
130 7.20 ng/mL. All samples were tested in one single assay.

131 **Statistical analysis**

132 All values are reported as mean ± SEM. We used two-way repeated measures ANOVA to
133 compare the song data at the 30th day after castration and sham operation with the song data at
134 'pre'(see the part of song recording), and injected current on the evoked firing rate of RA PNs in
135 the castration group with sham and control groups. We used one-way ANOVA to compare the
136 song data at the 30th day with the song data at 'pre' in the control group. We also used one-way
137 ANOVA to compare all plasma testosterone levels and other electrophysiological data of RA PNs
138 in the castration group with sham and control groups. *P* values < 0.05 were considered
139 significant.

Results

We analyzed plasma testosterone levels, stereotypy of the song, and electrophysiological properties of RA PNs of the experimental groups in adult male zebra finches.

Plasma testosterone levels

In the castration group (n=11), plasma testosterone levels were lower (3.91±0.08 ng/mL) compared with the control group (n=12) (5.15±0.08 ng/mL, $F_{(1,22)}$=150.49, P<0.01) and the sham group (n=4) (5.27±0.09 ng/mL, $F_{(1,13)}$=98.32, P<0.01).

Stereotypy of the song before and after castration

We randomly selected five birds in each of the castration and control groups, respectively, to analyze the stereotypes of their songs, while four birds were analyzed in the sham group. Zebra finch song usually contains motifs. Every motif includes two to eight syllables. In our experiment, we recorded song motifs from castration (Figure 1A_1, A_2), control and sham (Figure 1B_1, B_2) groups, and analyzed the entropy of syllable and % similarity of the motif in each group (Figure 2).

The longest syllable of motif was first analyzed. In the castration group, the entropy was altered gradually, and entropy was significantly increased from −3.71±0.31 to −3.31±0.33 ($F_{(1,58)}$=33.61, P<0.01) at the 30th day after castration (Figure 2A). To test the effect of castration on all syllables in the motif, we analyzed other syllables, as shown in Figure 1 A_1, A_2. The entropy of syllable ‘a’ in ‘pre’ was −2.76±0.06, while at the 30th day after castration the entropy changed to −2.48±0.05. Castration increased the entropy of syllable ‘a’ ($F_{(1,58)}$=10.77, P<0.01). Castration also increased the entropy of other syllables (Figure 1A, B).

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Next, we analyzed the % similarity of the motif. Castration significantly decreased the % similarity of the motif from 93.83±0.80 to 87.7±1.04 ($F_{(1,58)}$=201.32, P<0.01) (Figure 2B). However, in the sham group the entropy of syllables and % similarity of the motif did not change before and after operation, and were similar to the control group (Figure 2A, B).

Electrophysiological properties of RA PNs

Twenty one RA PNs from 11 birds of the castrated group, 23 RA PNs from 12 birds of the control group, and 8 RA PNs from 4 birds of the sham group were recorded.

Castration decreased spontaneous firing rates in the cell-attached configuration

When cells were sealed, many RA PNs were spontaneously active *in vitro*, as described previously in wild song sparrows. Castration significantly affected the spontaneous firing rate of RA PNs compared with the control group ($F_{(1,42)}$=7.85, P<0.01) and the sham group ($F_{(1,19)}$=8.41, P<0.01) (Table 1, Figure 3A–C). The mean firing rate of RA PNs was approximately 1.5 times higher in the control group and sham group than that in the castrated group.

Castration decreased spontaneous firing rates in the whole-cell configuration

In seasonally breeding songbirds, breeding conditions increase spontaneous firing rates in the whole-cell configuration. In our experiment, castration significantly decreased spontaneous firing rate of RA PNs compared with the control group ($F_{(1,31)}$=6.65, P=0.015) and the sham group ($F_{(1,22)}$=8.26, $P<0.01$) (Table 1, Figure 3D–F).

Castration decreased evoked firing rates

AP firing rates evoked by depolarizing current injection were significantly decreased in the castrated group (Figure 4A, B). At the current of 100 pA for 500 ms, the mean number of evoked firing was 13.63±1.12 (n=15) in the castrated group, 18.00±0.72 (n=16) in the control group and

17.88±0.88 (n=8) in the sham group. Castration significantly decreased the evoked firing rates compared with the control group ($F_{(1,31)}$=10.43, P<0.01) (Figure 4C) and the sham group ($F_{(1,22)}$=5.76, P=0.048). When the currents were set from 0 to 200 pA for 500 ms at 50 pA steps and 10 s intervals, castration decreased the mean number of evoked firing, particularly at 50 pA, 100 pA, 150 pA, and 200 pA (P<0.01). F-I curves were linearized. Castration significantly decreased the slope of F-I curve compared with the control group ($F_{(1,31)}$=21.87, P<0.01) (Figure 4D) and sham group ($F_{(1,22)}$=27.89, P<0.01) (Table 1).

Castration decreased the membrane time constant and capacitance, but increased AHP time to peak

To compare input resistance, currents from −200 to 20 pA for 500 ms at 10 pA steps and 10 s intervals were injected. The slope of the I–V curve by linear fit was the input resistance. There was no significant difference between the castrated and control groups. Castration significantly decreased the membrane time constant compared with the control group ($F_{(1,29)}$=10.62, P<0.01) (Figure 5A) and the sham group ($F_{(1,21)}$=12.17, $P<0.01$). Castration also decreased membrane capacitance compared with the control group ($F_{(1,29)}$=6.93, P=0.013) (Figure 5B) and the sham group ($F_{(1.21)}$=8.35, $P<0.01$). The intrinsic properties of spontaneous AP were also analyzed. AP threshold, AHP peak amplitude, half-width, and peak amplitude of the castration group were similar to the control and sham groups. However, castration significantly prolonged the AHP time to peak compared with the control group ($F_{(1,29)}$=12.76, P<0.01) (Figure 5C, D) and the sham group ($F_{(1,21)}$=13.32, P<0.01) (Table 1).

Discussion

In the present study, castration decreased plasma testosterone levels in adult male zebra finches, although the decrease was not as marked as those previously reported . This difference may be due to residual testicular tissue in our study, or due to differences between breeding and non-breeding zebra finches.

High testosterone levels are associated with song stability, which reduces the potential for vocal plasticity. We found that castration also increased the entropy of syllable and decreased the % similarity of motif in the songs, suggesting that castration decreased the stability of syllables and songs. Like seasonally breeding songbirds, when the testosterone levels in adult male zebra finches are high, the song nuclei and the syringes are fully grown . Therefore, male zebra finches have the ability to produce highly stereotyped song. In our castrated birds, lower testosterone levels resulted in less stereotyped songs.

It was previously reported that testosterone regulates the electrophysiological properties of RA PNs . RA is an important premotor nucleus, and these changes in its intrinsic properties may directly modify the motor control of song production, resulting in changes in song stereotypy. In this study, castration decreased spontaneous and evoked firing rates, and the membrane time constant and capacitance, but increased AHP time to peak. These results indicate that castration decreased the excitability of the RA PNs, as previously reported . Castration also decreased the spontaneous firing rates and evoked firing rates, which reduce the ability of RA PNs to produce AP in response to synaptic input, particularly from HVC. Castration decreased the membrane time constant, which might reduce the time to integrate synaptic input. As such, RA PNs slowly

integrate relatively sparse inputs from the HVC to produce patterned firing that is closely correlated with song production . The decrease in membrane capacitance induced by castration may be related to the decrease of the size of RA PNs. Castration also increased the AHP time to peak, which may be associated with the suppression of large conductance calcium-activated potassium channels .

The HVC-RA pathway contributes to stable song, while the LMAN-RA pathway contributes to variable song . Testosterone-stimulated growth of the HVC is sufficient to induce growth of RA. High testosterone levels increase axonal density in the HVC-RA pathway. In our castrated birds, low testosterone levels may decrease axonal density in the HVC-RA pathway. High testosterone levels decrease levels of NR2B mRNA, which is modulatory subunits of N-methyl-D-aspartic acid receptor (NMDAR), within the LMAN and the RA . The LMAN-RA input is largely mediated by the NMDAR. In our castrated birds, low testosterone levels may increase the modulation of the LMAN-RA input by the NMDAR. These effects may produce unstable songs. Finally, the castration-induced decrease in testosterone levels and excitability of the RA PNs may decrease the size of the song nuclei, the mass of the syrinx, and the synapses from the HVC to the RA, and increase the input from the LMAN to the RA. All of these effects would produce less stereotyped songs.

In conclusion, our study revealed that castration decreased song stereotypy and the excitability of RA PNs. The results provide a further understand of how androgens, rather than the photoperiod, modulate electrophysiological properties to change song behavior.

Competing interests

The authors have declared that they have no conflicts of interests.

Author contributions

WSH, LCS, LFL, LSY, and MW performed research and analyzed data. LDF designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 The electrophysiological properties of RA PNs

Property	Castration	Control	Sham
Spontaneous firing rate (Hz) (cell-attached)	8.13±1.00 (n=21)	12.49±1.17** (n=23)	11.32±0.74 ** (n=10)
Resting membrane potential (mV)	-64.27±1.52 (n=16)	-65.46±1.90 (n=17)	-66.48±1.67 (n=8)
Spontaneous firing rate (Hz) (Whole-cell)	6.58±0.89 (n=15)	10.53±1.23* (n=16)	10.97±1.26** (n=8)
Membrane time constant (ms)	25.44±3.58 (n=16)	43.98±4.42** (n=16)	46.28±4.46** (n=8)
Input resistance (MΩ)	197.75±10.25 (n=16)	228.11±14.58 (n=17)	226.98±17.33 (n=8)
Capacitance (pF)	126.65±14.71 (n=16)	194.08±20.97* (n=16)	201.49±21.88** (n=8)
FI slope (Hz/pA)	0.186±0.014 (n=15)	0.296±0.017** (n=13)	0.289±0.016** (n=6)
AP threshold (mV)	-50.12±1.73 (n=15)	-49.59±1.74 (n=16)	-50.14±1.63 (n=8)
AHP peak amplitude (mV)	-17.30±1.16 (n=15)	-17.57±1.64 (n=16)	-16.05±1.70 (n=8)
	21.38±1.53 (n=15)	15.44±1.85** (n=16)	15.03±2.03** (n=8)
AHP time to peak (ms)	1.10±0.10 (n=15)	1.26±0.34 (n=16)	0.99±0.13 (n=8)
Half-width (ms)	42.32±2.44 (n=15)	49.17±2.69 (n=16)	48.99±2.50 (n=8)
Peak amplitude (mV)			

373 Numbers in parentheses indicate sample size. *P<0.05; **P<0.01.

374 **Figure legends**

375

376 **Figure 1 Song sonograms and entropy curves (white line) of castration and sham groups in**
377 **adult male zebra finches.** **A_1,A_2**. The motifs of two birds in the castration group at "pre"
378 operation and the 30th day after castration, respectively. **B_1,B_2.** The motifs of two birds in the
379 sham group at "pre" operation and the 30th day after sham operation, respectively. When white
380 line became lower, the entropy of syllable was smaller.

381

382 **Figure 2 The entropy of syllable and % similarity of motif in castration, control, and sham**
383 **groups. A.** Castration group increased the entropy of syllables. Control and sham groups did not
384 change. **B.** Castration group exhibited decreased % similarity of motif. Control and sham groups
385 showed no change.

386

387 **Figure 3 The spontaneous firing of RA PNs in the cell-attached and whole-cell**
388 **configuration. A, B.** Example traces of spontaneous firing in RA PNs of the castration and
389 control groups, respectively. **C.** Castration significantly decreased spontaneous firing rates. **D, E.**
390 Example traces of spontaneous firing in RA PNs of the castration and control groups,
391 respectively. **F.** Castration significantly decreased spontaneous firing rates.

392

393 **Figure 4 The evoked firing of RA PNs in the whole-cell configuration. A, B.** Example traces
394 of AP firing in RA PNs of the castration and control groups in response to injecting a current of
395 100 pA for 500 ms, respectively. **C.** Castration significantly decreased evoked firing rates when

injecting a current of 100 pA for 500 ms. **D.** F–I curve of the castration and control groups. The slope of the F–I curve in the castration group was lower than that of the control group.

Figure 5 The differences in membrane time constant, capacitance and AHP time to peak between the castration and control groups. A. Castration decreased the membrane time constant. **B.** Castration decreased the membrane capacitance. **C.** Example traces of AP in the castration and control groups, showing differences in the AHP time to peak. **D.** Castration prolonged the AHP time to peak.

Figure 1

Figure 5 The differences in membrane time constant, capacitance and AHP time to peak between the castration and control groups.

A. Castration decreased the membrane time constant. B. Castration decreased the membrane capacitance. C. Example traces of AP in the castration and control groups, showing differences in the AHP time to peak. D. Castration prolonged the AHP time to peak.

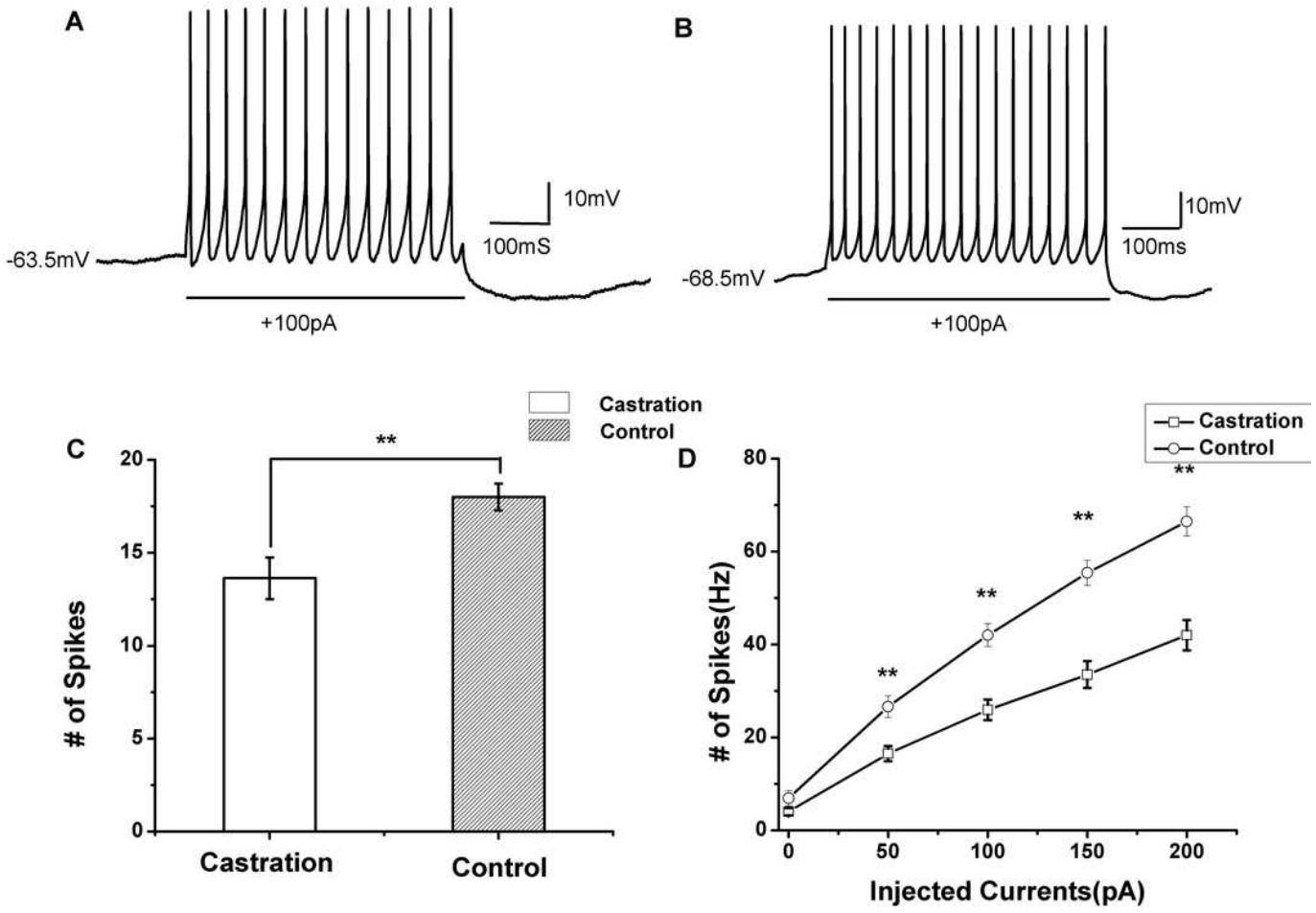

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Figure 2

Figure 4 The evoked firing of RA PNs in the whole-cell configuration. A, B.

Example traces of AP firing in RA PNs of the castration and control groups in response to injecting a current of 100 pA for 500 ms, respectively. C. Castration significantly decreased evoked firing rates when injecting a current of 100 pA for 500 ms. D. F–I curve of the castration and control groups. The slope of the F–I curve in the castration group was lower than that of the control group.

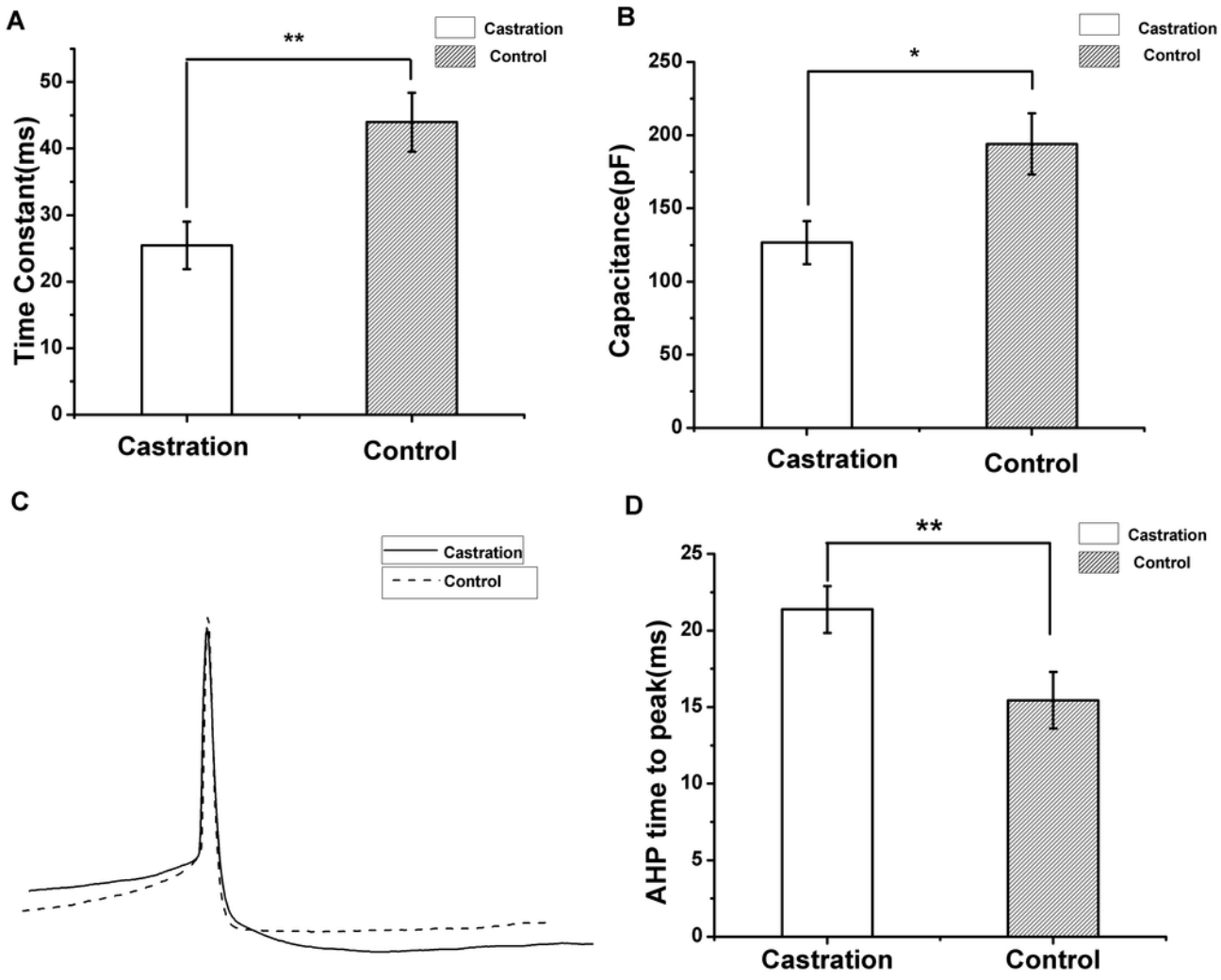

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Figure 3

Figure 3 The spontaneous firing of RA PNs in the cell-attached and whole-cell configuration.

A, B. Example traces of spontaneous firing in RA PNs of the castration and control groups, respectively. C. Castration significantly decreased spontaneous firing rates. D, E. Example traces of spontaneous firing in RA PNs of the castration and control groups, respectively. F. Castration significantly decreased spontaneous firing rates.

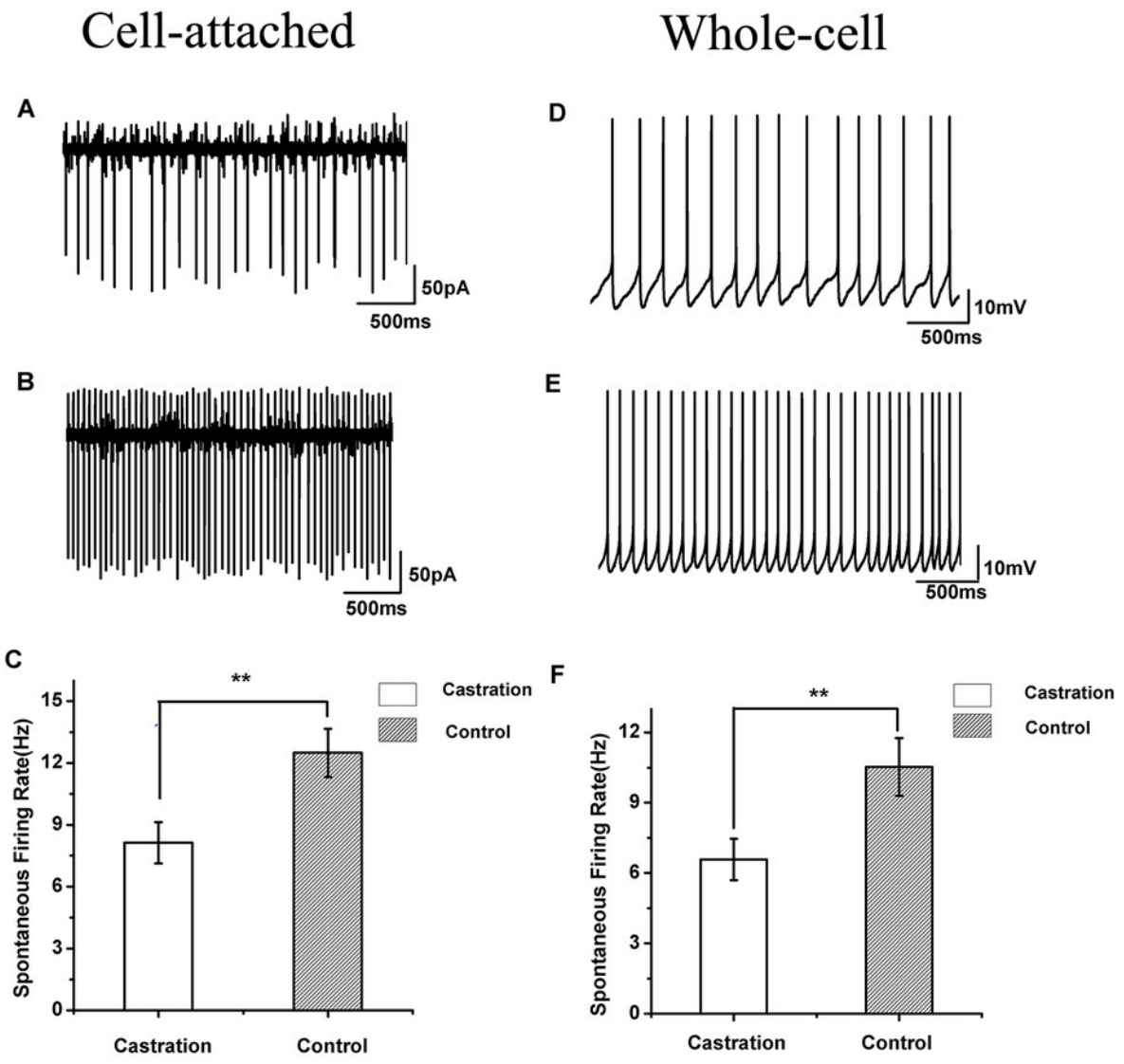


Figure 4

Figure 2 The entropy of syllable and % similarity of motif in castration, control, and sham groups.

A. Castration group increased the entropy of syllables. Control and sham groups did not change. B. Castration group exhibited decreased % similarity of motif. Control and sham groups showed no change.

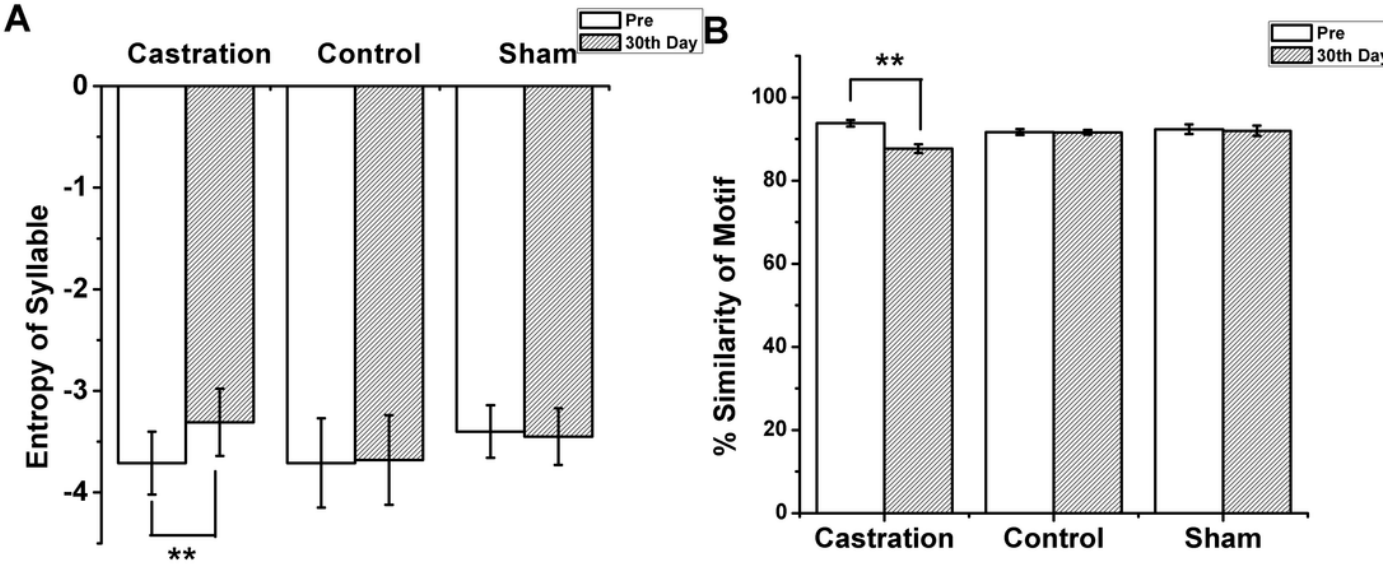
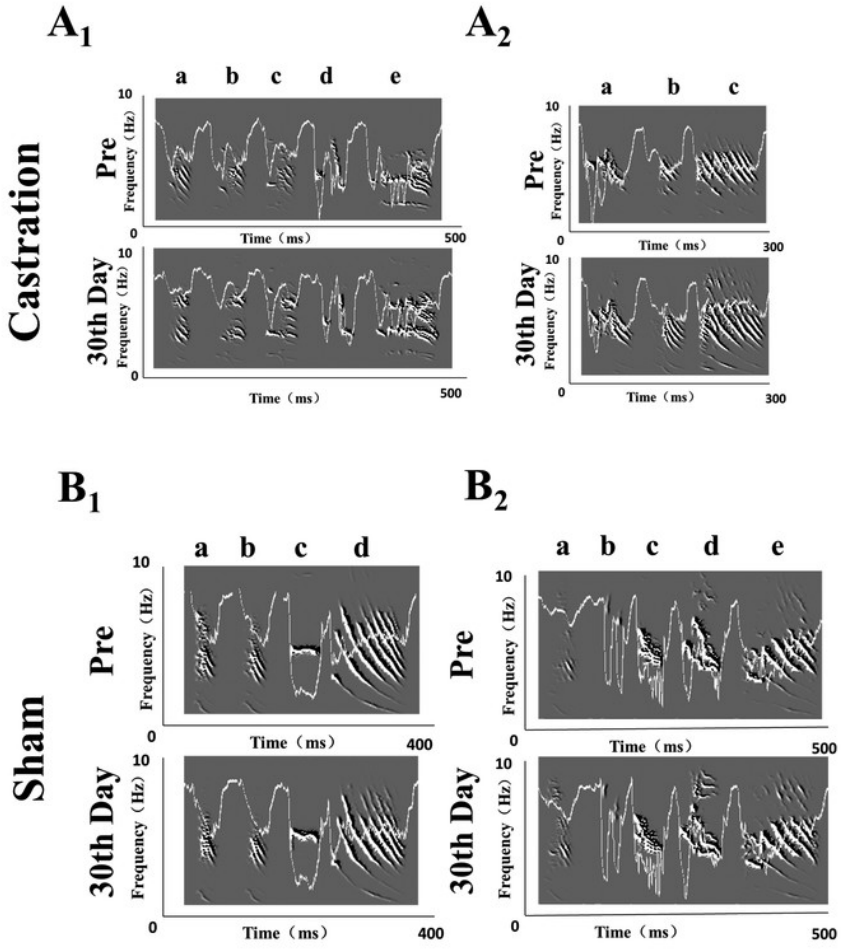


Figure 5

Figure 1 Song sonograms and entropy curves (white line) of castration and sham groups in adult male zebra finches.

A1,A2. The motifs of two birds in the castration group at “pre” operation and the 30th day after castration, respectively. B1,B2. The motifs of two birds in the sham group at “pre” operation and the 30th day after sham operation, respectively. When white line became lower, the entropy of syllable was smaller.



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