Seasonal-Like Plasticity of Spontaneous Firing Rate in a Songbird Pre-Motor Nucleus

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Received 27 September 2004; accepted 13 January 2005

ABSTRACT: **Many animals exhibit seasonal changes in behavior and its underlying neural substrates. In seasonally breeding songbirds, the brain nuclei that control song learning and production undergo substantial structural changes at the onset of each breeding season, in association with changes in song behavior. These changes are largely mediated by photoperiod-dependent changes in circulating concentrations of gonadal steroid hormones. Little is known, however, about whether changes in the electrophysiological activity of neurons accompany the dramatic morphological changes in the song nuclei. Here we induced seasonal-like changes in the song systems of adult white-crowned sparrows and used extracellular recording in acute brain slices from those individuals to study physiological properties of neurons in the robust nucleus of the arcopallium (RA), a pre-motor nucleus necessary for song production. We report that: RA neurons from birds in breeding condition show a more than twofold increase in spontaneous firing rate compared to those from nonbreeding condition; this change appears to require both androgenic and estrogenic actions; and this change is intrinsic to the RA neurons. Thus, neurons in the song circuit exhibit both morphological and physiological adult seasonal plasticity.**
J Neurobiol 00: 000–000, 2005

Keywords: **androgen; estrogen; testosterone; electrophysiology; birdsong**

INTRODUCTION

Seasonal plasticity of neural structure and function is a common feature of vertebrate brain organization, and provides an excellent model for studies of plasticity of adult brains in general (Tramontin and Brenowitz, 2000). The leading model of seasonal plasticity is the song control system of songbirds. Seasonal plasticity in this and other model systems, however,

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Contract grant sponsor: NIH; contract grant numbers: DC000018 (K.H.J.P.); MH56646 and MH066128 (D.J.P.); MH53032 (E.A.B.); MH065974 (J.W.); P30 Core Grant DC004661.

Contract grant sponsor: NSF Graduate Research Fellowship (J.M.).

Published online in Wiley InterScience (www. interscience.wiley.com).

DOI 10.1002/neu.20145

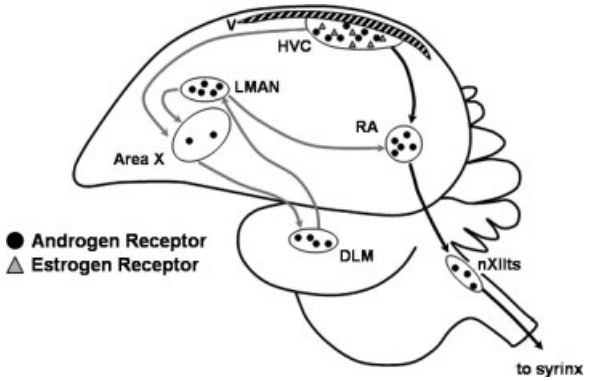

Figure 1 Schematic diagram of the song circuit. The new nomenclature used here follows Reiner et al. (2004). The motor pathway is necessary for song production (Nottebohm et al., 1976). It includes nucleus HVC (used as proper name), which projects to the robust nucleus of the arcopallium (RA), whose axons synapse upon the tracheosyringeal portion of the hypoglossal motor nucleus (nXIIts) and respiratory motoneurons. The anterior forebrain pathway (AFP) is necessary for song learning but not production (Bottjer et al., 1984; Scharff and Nottebohm, 1991). The AFP traditionally includes Area X (to which HVC projects), the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM), and the lateral magnocellular nucleus of anterior nidopallium (LMAN), which projects to RA. Androgen receptors are present in HVC, RA, LMAN, and nXIIts; androgen receptor mRNA is expressed in area X. Estrogen receptors are present only in HVC.

has largely been studied from the perspective of morphological changes in brain regions. We hypothesized that seasonal changes in neural morphology are accompanied by changes in neurophysiology. In the present study, we take a first step toward identifying seasonal electrophysiological changes in the song system.

The avian song system is well suited for studies of seasonal plasticity. Song is a learned behavior that is regulated by discrete neural circuits (Fig. 1). There are pronounced changes in the volume and neural attributes of the song nuclei (reviewed by Brenowitz, 2004). Seasonal changes in neural morphology are accompanied by changes in the stereotypy and duration of song (Brenowitz et al., 1998; Smith et al., 1995), and in the metabolic capacity of song nuclei (Wennstrom et al., 2001).

Seasonal changes in the song system are primarily regulated by changes in the circulating levels of testosterone (T), and its metabolites in the brain (Bernard et al., 1997; Gulledge and Deviche, 1997; Smith et al., 1997a,b). Each breeding season, as day length increases, the testes grow and secrete higher levels of T. Most of the song nuclei express steroid receptors (Fig. 1). Although the circulating T that reaches the brain could act directly via androgen receptors present in most of the song nuclei, enzymatic activity by aromatase and 5α-reductase in the songbird brain locally converts circulating T into estradiol (E_2) and 5α-dihydrotestosterone (DHT), respectively (Schlinger, 1997). These metabolites, in turn, could act via estrogen receptors (E_2) known to be present only in HVC (Bernard et al., 1999; Metzdorf et al., 1999) and/or androgen receptors present in all of the song nuclei, with the possible exception of area X (Arnold et al., 1976; Bernard et al., 1999; Kim et al., 2004; Metzdorf et al., 1999; Nastiuk and Clayton, 1995). In castrated adult white-crowned sparrows, a systemic implant of DHT induced a high singing rate and seasonal-like growth of the song nuclei. An E_2 implant alone also stimulated growth of the song nuclei, but these birds sang infrequently. The combination of E_2 and DHT induced full growth and a high singing rate (Tramontin et al., 2003). Thus, both estrogens and androgens derived from T contribute to seasonal plasticity. This observation is consistent with studies from other species that show that each of these two classes of steroid hormones has activational effects (McEwen, 1991; Sisneros and Tricas, 2000; Sisneros et al., 2004; Yamaguchi and Kelley, 2002; Zakon, 1998).

We designed our study to answer two questions. First, are the known seasonal changes in the vocal behavior and cellular morphology in a songbird accompanied by changes in neurophysiology as well? And second, can these changes be manipulated by changing the circulating levels of different steroid hormones? To answer these questions, we used the white-crowned sparrow, *Zonotrichia leucophrys*, a well-studied seasonally breeding songbird, and focused on the pre-motor nucleus RA. Nucleus RA provides the telencephalic pre-motor output of the motor pathway for song (Fig. 1). It projects directly to the motor neurons in the tracheosyringeal portion of the hypoglossal vocal motor nucleus (nXIIts) and other brainstem nuclei that control the muscles involved in respiratory control for song production (Wild, 1993). Just prior to the breeding season, somata in RA grow larger, neuron spacing increases, and dendrites lengthen and have a higher density of spines (reviewed in Tramontin and Brenowitz, 2000). These changes in neuronal morphology underlie RA's substantial change in volume during the breeding season. Also, RA receives projections from the anterior forebrain pathway, which is necessary for learning and adult plasticity in other species of songbirds. Finally, RA neurons are known to be spontaneously active both *in vivo* and *in vitro* (Mooney, 1992; Spiro et al., 1999; Yu and Margoliash, 1996). For all of these reasons, RA is a logical place to begin the

investigation into the neurophysiology of seasonal plasticity of vocal production. We report in this study that the *in vitro* spontaneous firing rate of RA neurons is higher in breeding condition than in nonbreeding condition sparrows. Furthermore, this increase appears to require both estrogenic and androgenic actions.

METHODS

Animals and Seasonal-like Manipulation

All procedures used in this study were approved by the Institutional Animal Care and Use Committee at the University of Washington. We collected 32 adult male Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*) in eastern Washington during their autumnal migration in 2001 and 2002. These birds were housed in outdoor aviaries prior to being placed in indoor aviaries, where they were maintained on a short-day photoperiod (8 h light:16 h dark) for at least 10 weeks to ensure that they were photosensitive and therefore sensitive to the effects of steroids and long-day photoperiod. Food and water were available *ad libitum* during the duration of the experiment.

Steroid implants were made using Silastic tubing segments (i.d. 1.0 mm; o.d. 2.0 mm; length,12 mm) that were filled with crystalline T, DHT, or E_2, as in Tramontin et al. (2003). The capsules were rinsed with ethanol and soaked overnight in 0.1M phosphate-buffered saline (PBS) prior to implantation. Silastic capsules release hormones in a temporally stable manner (Moore, 1982, 1983, 1984). Birds were implanted subcutaneously with either a single capsule of T, DHT, or E_2, or the combination of one DHT and one E_2 capsule. After implantation, some T implanted birds were shifted to a long-day photoperiod (LD+T) to mimic conditions typical of their Alaskan breeding grounds (20 h light:4 h dark). All other groups were maintained on SD, to determine whether hormone stimulation alone might contribute to neurophysiological change. Birds were individually housed in indoor cages and could see and hear the other birds housed in the same room. Birds housed in LD were implanted with T because exposure of wild-caught birds to LD alone in the laboratory does not elevate circulating T levels into the physiological breeding range of 4–25 ng/mL observed in wild white-crowned sparrows (Wingfield and Farner, 1978; Wingfield and Moore, 1987; Smith et al., 1995; J. Wingfield, personal communication). It should be noted that circulating steroid hormone levels are not necessarily identical to those in the brain parenchyma, due to local neurosteroid synthesis (Schlinger and Arnold, 1992, 1993). This study was designed to ask which steroid receptors activate physiological change in RA, rather than to determine precisely what level of steroid is necessary to induce such changes. The use of DHT and E_2 is necessary to answer this question, as T can be converted in the brain to both androgenic and estrogenic metabolites. DHT is a potent and nonaromatizable androgen that binds to the androgen receptor, while E_2 binds to the estrogen receptor (Yamaguchi and Kelley, 2002). As in a previous anatomical study (Tramontin et al., 2003), we used DHT and E_2 to probe which family of steroid receptors can affect electrophysiological properties of RA neurons. Birds maintained on SD were not castrated prior to implantation because they have regressed testes that have been shown not to secrete significant levels of T (Smith et al., 1995; Tramontin et al., 2000). Electrophysiological recordings were made from brain slices of birds that had been exposed to hormone implants for 20 to 22 days, which is adequate for full seasonal-like anatomical growth of the song circuit (Smith et al., 1997b; Tramontin et al., 2000).

Electrophysiology

Preparation of Brain Slices. Methods for preparing slices have been described elsewhere (Farries and Perkel, 2000). Briefly, each animal was anesthetized with isoflurane, euthanized by decapitation, and the brain rapidly dissected into ice-cold, oxygenated artificial cerebral spinal fluid (ACSF), containing (in mM) 119 NaCl, 2.5 KCL, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 16.2 $NaHCO_3$, 11 D-glucose, and 10 HEPES, osmolarity adjusted to 310–320 mOsm with sucrose. Parasagittal brain slices (300–400-μm thick) were prepared using a Vibratome, and slices were stored at room temperature submerged in bubbled ACSF in which HEPES was replaced with equiosmolar $NaHCO_3$. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). We used an acute brain slice preparation because it allows the pharmacological manipulations necessary to determine whether the neuron's spontaneous firing rate is an intrinsic property, and it ensures that recordings were made from RA neurons.

Electrophysiological Recording. Recordings were carried out at least 60 min after slices were collected. For recording, a slice was submerged in a small chamber perfused with ACSF maintained at 30°C and containing 150 μM picrotoxin (Sigma) to block inhibitory $GABA_A$ receptors. Single-unit extracellular recordings were obtained from neurons within a region that could be reliably identified as RA using trans-illumination. Only well-isolated spikes with high signal-to-noise ratios were studied. Recording electrodes were made from pulled borosilicate glass pipettes (WPI, Sarasota, FL) with tips broken to a resistance of 6–15 MΩ and filled with 0.9% NaCl. Extracellular potentials were amplified using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) and further amplified using a Brownlee model 410 amplifier (Brownlee Precision, Santa Clara, CA). The filtered signals (low-pass filtered at 3 kHz) were digitized at 20 kHz with a National Instruments digitizing board (Austin, TX) and stored on a PC using a custom data acquisition program written in LabView (National Instruments) by Michael A. Farries and David J. Perkel. We used extracellular single-unit recording for the following reasons: (1) Extracellular recording is less invasive than

intracellular recording; and (2) extracellular recording often allows for a larger number of recordings per experiment. Yield is an important consideration for this first experiment, as this is the first demonstration of the phenomenon, and because white-crowned sparrows must be collected in the wild.

Data Analysis. Spontaneous spike trains were analyzed off-line using a custom written program in IGOR (Wavemetrics, Lake Oswego, OR) by Michele M. Solis. To ensure that spike-events were single units, we analyzed the spike amplitude, waveform, and time derivative. The spontaneous activity was observed for at least 5 min and the mean firing rate was obtained by dividing the number of spikes observed by the duration of the recording. One-way ANOVA was used to assess the significance of differences in the firing rate measured for the six treatment groups using Prism 3.0 (GraphPad Software, San Diego, CA). A power test with an alpha level of 0.05 was run. Pairwise comparisons were made using Tukey's post-hoc test unless otherwise specified. Additionally, the nonparametric Kolmogorov-Smirnov two-sample test (K-S test) was used for pairwise comparison of cumulative frequency distributions between selected groups. An alpha level of 0.05 was used.

Brain Histology and Morphometry

At the end of the recording, we fixed the slices overnight in 4% paraformaldehyde solution in 0.1 M PBS at 4°C. The slices were then cryoprotected in 30% sucrose in 0.1 M PBS, and re-sectioned in the parasagittal plane to a thickness of 50 μm using a freezing microtome. We mounted sections on slides and stained them with cresyl violet. We then measured the area of somata in RA of each treatment group, using the random systematic sampling method described by Tramontin et al. (1998). Measurements were made blind to treatment group. Neurons were distinguished from glia by having one round nucleolus, a well-defined nuclear envelope, nongranular cytoplasm, and/or an obvious axon hillock (Goldman and Nottebohm, 1983; Smith et al., 1995, 1997a,b; Tramontin et al., 1998). One-way ANOVA was used to assess the significance of differences in the average soma sizes measured for the six treatment groups using Prism 3.0 (GraphPad Software, San Diego, CA). Pairwise comparisons were made using Tukey's post-hoc test. In all cases, we performed a power test with an alpha level of 0.05. This study was not initially designed to include morphological measurements, and we therefore collected sections for morphological analysis from 2 SD, 4 LD+T, 4 SD+DHT+E_2, 4 SD+E_2, and 5 SD+DHT treated birds.

Hormone Assay

On the day of each recording, we collected blood following decapitation of each subject into a heparinized microhematocrit tube and stored the blood on ice until centrifugation (within 1 h). Plasma was harvested and stored at -20°C for subsequent steroid radioimmunoassay (RIA). To measure circulating T, we followed the RIA protocol of Tramontin et al. (2001) using a Coat-a-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA). For DHT, T, and E_2, blood samples were analyzed in duplicate following the procedures of Wingfield et al. (1991). DHT, T, and E_2 samples were purified by column chromatography, and plasma hormone concentrations were corrected for individual extraction efficiency. Detection limits for the assay depended on the plasma volume used and the individual extraction efficiency (DHT: $\sim$0.12 ng/ml; T: $\sim$0.03 ng/ml; E_2: $\sim$0.15 ng/ml). The samples were run in single assays with the following intra-assay variations: DHT: 4.5%, T: 4.5%, E_2: 2.2%.

RESULTS

We recorded 167 single units from 32 birds. RA neurons showed spontaneous activity *in vitro*, as reported in zebra finches, *Taeniopygia guttata* [Fig. 2(A)] (Mooney, 1992). The observed spontaneous activity was likely an intrinsic property of recorded neurons, as the bath contained picrotoxin (150 μM), and application of the glutamate receptor antagonists CNQX (10 μM) and AP-5 (50 μM) did not alter the firing rate ($n = 6$, pre: 3.58 $\pm$ 1.51 Hz; during: 3.67 $\pm$ 1.23 Hz; post: 3.85 $\pm$ 1.20 Hz; $p > 0.05$).

Plasma Hormone Levels

Silastic steroid implants elevated plasma steroid levels (Table 1). T levels were basal in the nonimplanted birds exposed to SD photoperiod, while T-implanted birds had elevated plasma T levels regardless of photoperiod. These levels are consistent with those observed in previously studied T-treated and wild Gambel's white-crowned sparrows (Smith et al., 1995, 1997a,b; Tramontin et al., 2003; Wennstrom et al., 2001; J. Wingfield, personal communication; Wingfield and Farner, 1978; Wingfield and Moore, 1987). Animals treated with DHT, E_2 or DHT and E_2 showed an elevated level of the implanted hormone(s), but not of any other, which is consistent with previous studies (Soma et al., 2004; Tramontin et al., 2003). The circulating plasma levels of DHT in the SD+DHT and SD+DHT+E_2 groups were higher than those observed in wild white-crowned sparrows (less than 1 ng/ml; Wingfield and Farner, 1978). It is also likely that the circulating plasma E_2 levels in the SD+E_2 and SD+DHT+E_2 groups were superphysiological. It

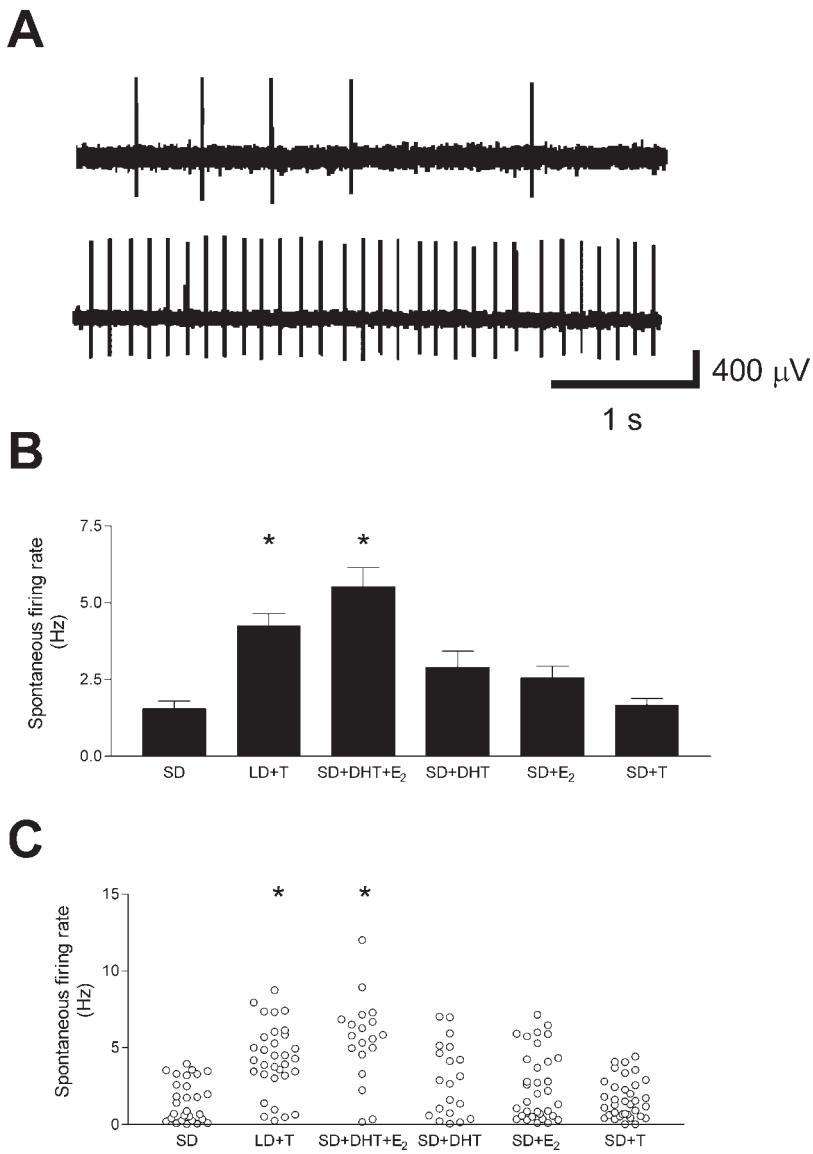

Figure 2 Seasonal-like changes increase the spontaneous firing rates of RA neurons. (A) Representative extracellular recordings obtained from SD (top) and LD+T (bottom) groups. Breeding condition-like LD+T treatment results in a higher spontaneous firing rate. (B) Overall mean and S.E.M. of spontaneous firing rates from each treatment group. Asterisks indicate that the LD+T and SD+DHT+E_2 groups significantly differ from all other treatment groups, but not each other. The influence of SD+DHT or SD+E_2 alone does not induce the magnitude of change seen in the LD+T or SD+DHT+E_2 groups. (C) Distribution of spontaneous firing rates from each treatment group. We include this figure to emphasize the changes in distributions between the treatment groups. Asterisks indicate that the LD+T and SD+DHT+E_2 groups significantly differ from all other treatment groups, but not each other.

should be noted, however, that steroid levels measured from the peripheral circulation do not necessarily reflect local levels in the brain (Schlinger and Arnold, 1992, 1993). These two measures might differ for several reasons including local steroid binding, production, and/or metabolism by brain enzymes. Thus, this study asks which steroid receptors, when activated, can induce neurophysiological changes in RA, not what concentration of steroid is necessary to induce the change (see Methods).

Table 1 Plasma Hormone Levels[1]

	Hormone Measured		
Treatment Group	T	DHT	E_2
SD	0.15 ± 0.26[a]	N/A	N/A
LD+T	12.6 ± 1.97[b]	N/A	N/A
SD+DHT+E_2	0.47 ± 0.13[a]	10.44 ± 4.85[c]	1.02 ± 0.17[e]
SD+DHT	0.47 ± 0.12[a]	11.57 ± 4.25[c]	0.13 ± 0.03[f]
SD+E_2	0.08 ± 0.03[a]	0.16 ± 0.06[d]	1.16 ± 0.74[e]
SD+T	9.62 ± 1.75[b]	N/A	N/A

[1] Values are mean ng/mL ± S.E.M. N/A indicates values not available due to limited serum volume. Within rows, values with different superscripts differ significantly from each other (Tukey's post-hoc comparison, $p < 0.05$).

Effects of Systemic Hormone Manipulation on Spontaneous Firing Rate

Hormone treatments significantly affected the spontaneous firing rate of RA neurons (one-way ANOVA, $F_{5,139} = 13.96$, $p < 0.0001$, power = 1.00) [Fig. 2(B)]. The mean firing rate of RA neurons in LD+T birds was approximately 2.5 times higher than that of unimplanted SD birds (Tukey's post hoc test, $p < 0.001$; Table 2). Firing rates from SD+DHT+E_2 birds were also significantly elevated compared to the unimplanted SD group ($p < 0.001$), and not significantly different from the LD+T group ($p > 0.05$, power = 1.00).

Exposure to the combination of androgens and estrogens increased the spontaneous firing rate of RA neurons. Birds exposed to SD and implanted with either DHT or E_2 alone showed mean firing rates that did not differ significantly from those of the SD or SD +T groups ($p > 0.05$, power = 1.000 [Fig. 2(B)]; Table 2). For both the SD+DHT and the SD+E_2 groups, the mean firing rate was significantly lower than for the SD+DHT+E_2 ($p < 0.01$) and LD+T ($p < 0.05$) groups. Birds implanted with T and exposed to SD had low spontaneous firing rates that did not differ significantly from the SD group. The SD+T group's mean firing rate was significantly lower than that of the SD+DHT+E_2 and LD+T groups ($p < 0.001$), but did not differ significantly from the SD+DHT or SD+E_2 groups ($p > 0.05$, power = 1.000).

Exposure to either DHT or E_2 alone did not significantly increase the firing rate of RA neurons. An apparent partial effect of DHT or E_2 alone was further explored by comparing the cumulative distribution functions (CDF) of firing rates corresponding to each treatment group [Fig. 3(A)]. The SD+DHT group's CDF significantly differed from that of LD+T birds (K-S test, $p < 0.005$), but not from that of SD birds. The SD+E_2 group's CDF was not significantly different from either the SD or the LD+T groups. The SD+DHT+E_2 group, however, significantly differed from the SD group ($p < 0.05$), but not the LD+T group. This analysis provides further evidence that E_2 or DHT alone is not sufficient to significantly elevate firing rate, but that the combination of E_2 and DHT is effective.

Plasma Testosterone Level and Spontaneous Firing Rate Significantly Correlate

The rate at which RA neurons spontaneously discharge is related to plasma T levels. To explore further the relationship between spontaneous firing rate and reproductive condition, we plotted the spontaneous firing rate of units in the SD and LD+T treatment groups against the plasma testosterone level [Fig. 3(B)]. We found that plasma testosterone level significantly correlated with spontaneous firing rate (Pearson's Correlation Test, $r^2 = 0.3445$, $p < 0.0001$).

Table 2 Spontaneous Firing Rates of RA Neurons[1]

SD[a] $n = 28$ (7)	LD+T[b] $n = 32$ (5)	SD+DHT+E_2[b] $n = 19$ (5)	SD+DHT[a] $n = 20$ (5)	SD+E_2[a] $n = 33$ (5)	SD+T[a] $n = 35$ (5)
1.550 ± 0.2553	4.245 ± 0.4002	5.516 ± 0.6331	2.899 ± 0.5274	2.553 ± 0.3888	1.669 ± 0.2153

[1] Values are mean firing rate in Hz ± S.E.M. Values in parentheses are numbers of animals. Groups with different superscripts differ significantly from each other (Tukey's post-hoc comparison, $p < 0.05$).

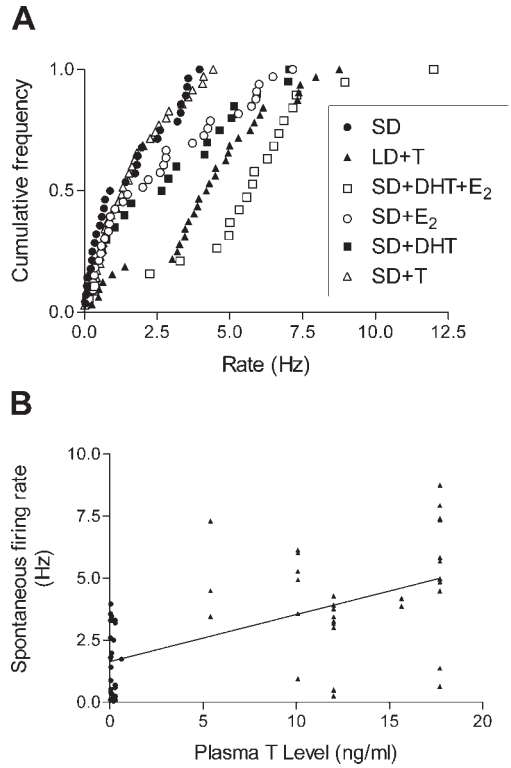

Figure 3 Spontaneous firing rate is dependent upon both androgens and estrogens. (A) Cumulative frequency distribution of the average firing frequencies observed from the six groups studied. The SD+DHT and SD+E_2 distributions are intermediate between the SD and LD+T. SD+E_2 is not significantly different from either the SD or LD+T. SD+DHT is significantly different from LD+T (K-S test, $p < 0.005$), but not SD. SD+DHT+E_2 is not significantly different from LD+T. (B) Plasma testosterone level significantly correlates with spontaneous firing rates of neurons from the SD and LD+T groups, with an r^2 of 0.3445 and a p-value of less than 0.0001. Testosterone can be modified in the brain to create other androgens or estrogens.

Spontaneous firing rate and plasma T levels were not correlated within either the SD or LD+T treatment groups alone, due to an insufficient statistical power of 0.213, which likely is affected by low variance in T levels within each treatment group.

Effects of Systemic Hormone Manipulation on Soma Size

Exposure to a systemic testosterone implant and long-day photoperiod significantly increased the soma size of neurons in RA (one-way ANOVA, $F_{5,18} = 2.278$, Tukey's post hoc test, $p < 0.05$; Table 3). Post-hoc comparisons among all treatment groups revealed that the average soma size of neurons in the LD+T treatment group was significantly larger than for the SD group ($p < 0.05$). The SD+DHT, SD+E_2, SD+DHT+E_2, and SD+T groups had somata that were not significantly different from SD, from LD+T, or from one another ($p > 0.05$). The lack of difference between these groups should be interpreted cautiously, however, because of insufficient statistical power (0.344), which likely is affected by the low number of birds in the SD group.

DISCUSSION

This study is the first demonstration that an electrophysiological property of neurons of adult songbirds changes in association with seasonal-like hormonal and morphological changes. Increased concentrations of plasma T in conjunction with LD led to an increase in the rate of spontaneous firing of RA neurons *in vitro* when compared to control birds maintained on a short-day photoperiod. This change, furthermore, could be mimicked by a combination of androgen and estrogen treatments, suggesting that both receptor types must be activated to produce this effect.

Plasticity in RA Spontaneous Firing Rate

The average *in vitro* spontaneous firing rate dramatically increased between the breeding-like and non-breeding condition, extending the effects of known adult seasonal plasticity. The spontaneous firing reported here is likely an intrinsic property of the recorded RA neurons, the majority of which were probably projection neurons. In the zebra finch, RA projection neurons fire spontaneously while the interneurons tend to be silent or fire few spontaneous action potentials in brain slice preparations (Spiro et al., 1999). RA projection neurons receive glutamatergic inputs from RA-projecting cells in HVC, LMAN (Herrmann and Arnold, 1991; Mooney and Konishi, 1991; Mooney, 1992), and from axon collaterals of other RA projection neurons (Perkel, 1995). They also receive GABAergic inputs from interneurons (Spiro et al., 1999). In the parasagittal slices used in our experiment, all of these connections may be preserved, with the exception of LMAN inputs, whose cell bodies are not present in the slice. Although the inhibitory interneurons are thought to be silent or fire few spontaneous action potentials *in vitro*, our recordings were carried out in the presence of the $GABA_A$ receptor antagonist picrotoxin, to guard against inhibitory influence. Furthermore, addition of ionotropic glutamate receptor blockers CNQX and AP-5 did not alter the spontaneous firing rate, suggesting that ongoing firing is not driven

Table 3 Average Soma Area of RA Neurons[1]

SD[a,c] $n = 2$	LD+T[b,d] $n = 4$	SD+DHT+E_2[c,d] $n = 4$	SD+DHT[c,d] $n = 5$	SD+E_2[c,d] $n = 4$	SD+T[c,d] $n = 5$
67.06 ± 3.160	109.5 ± 6.086	86.29 ± 5.008	92.64 ± 5.648	94.38 ± 12.62	91.57 ± 6.419

[1] Values are mean soma area in μm^2 ± S.E.M. Groups with different superscripts differ significantly from each other (Tukey's post-hoc comparison, $p < 0.05$).

by recurrent excitation among RA projection neurons, but rather reflects the intrinsic properties of the neuron.

Mechanisms for RA Plasticity

The full LD+T-mediated increase in the rate and regularity of intrinsic firing was reproduced in SD birds only when they were simultaneously treated with a combination of E_2 and DHT implants. Treatment with E_2 or DHT alone was not sufficient to increase the firing rate significantly. This observation supports the idea that both androgen and estrogen receptor activation is necessary for increasing the spontaneous firing rate. In the avian telencephalon, endogenous T is converted to DHT and E_2 by the activity of aromatase and 5α-reductase, respectively (Schlinger, 1997). This is likely how LD+T increased firing rates. E_2 mediates its effects via estrogen receptors, which are present in HVC but not in RA (Bernard et al., 1999, Metzdorf et al., 1999). DHT effects are mediated via androgen receptors, which are present in HVC, RA, and LMAN (Arnold et al., 1976; Nastiuk and Clayton, 1995; Bernard et al., 1999; Metzdorf et al., 1999).

Our results, which implicate both estrogens and androgens, suggest that activation of the estrogen receptor in HVC and the androgen receptors in HVC, LMAN and/or RA are necessary for increased spontaneous activity in RA. One possibility is that the neurophysiological effects require that estrogen-sensitive neurons in HVC provide some sort of trans-synaptic signal to targeted RA neurons. This idea draws some support from anatomical studies, which show that afferent input from HVC is critical in mediating the seasonal-like growth of RA; lesions of HVC prevent the seasonal-like growth of RA in adult white-crowned sparrows implanted systemically with T (Brenowitz and Lent, 2001). Other studies, however, highlight a potential difference between the mechanisms underlying T-mediated changes in morphology and electrophysiology. While E_2 or DHT alone does not significantly elevate RA spontaneous firing rate, an implant of either steroid is alone is sufficient to elicit significant seasonal-like growth of RA (Tramontin et al., 2003). Furthermore, androgen receptors in RA do not seem to play a direct role in mediating the seasonal-like growth of RA, given that an intracerebral T implant near HVC induced growth of RA (and HVC), whereas a local T implant near RA failed to induce growth of RA (Brenowitz and Lent, 2002). Thus, it is possible that the seasonal-like growth of RA may depend primarily on trans-synaptic signals from HVC, while physiological changes might also require direct activation of androgen receptors in RA.

Possible Contribution of Photoperiod

It is noteworthy that the combination of SD and T for 3 weeks failed to induce an increase in spontaneous firing rate. Given that SD+T birds had plasma T levels that did not differ significantly from the LD+T group, it appears that the difference between SD and LD animals may not be due only to plasma hormone levels. One possible explanation for the lack of a physiological effect in the SD+T birds lies in the fact that both aromatase and 5α-reductase (Riters et al., 2001; Soma et al., 2003), as well as estrogen and androgen receptors (Bernard et al., 1999; Soma et al., 1999) are regulated seasonally. If, for example, the expression or activity of aromatase was low in SD birds, T released by the implants might not have been converted to estrogen in sufficient quantity to activate estrogen receptors over the 3 weeks of our study. This could have delayed the effect of T on spontaneous firing rate. Supporting this suggestion, Smith et al. (1997b) found that if SD birds were given T for 6 weeks, neuronal morphology and nucleus volume were enlarged compared to SD birds. Perhaps the spontaneous firing rate of RA neurons in SD birds exposed longer to T would not differ from LD+T as well.

RA Soma Morphometry

Our observation that hormone treatments did not significantly increase soma size in RA in any of the SD groups should be interpreted cautiously for two reasons. First, our statistical test showed insufficient

power. Thus, the present data set is too small to adequately test whether significant differences exist between these groups. Second, the conditions necessary for the brain slice physiology experiments are not ideal for measuring neuronal morphology using the Nissl stain. For example, we were unable to perfuse the brain, since fixative is necessarily toxic. This could have led to increased variability in our measurements. Nevertheless, the *relative* change between the SD and LD+T groups, if not the absolute numbers that we report, is largely consistent with earlier studies in this species (Smith et al., 1997b), which supports our observations. The fact that soma size did increase in the LD+T birds shows that the implants did release T.

Functional Significance

Some evidence suggests that increased spontaneous firing from RA projection neurons is related to the production of more stereotyped song. In the zebra finch, spontaneous RA firing increases and becomes more regular as the bird matures (Adret and Margoliash, 2002), and song becomes more stereotyped with age. Furthermore, the increase in spontaneous firing that we observed in white-crowned sparrows coincides with the more stereotyped song characteristic of birds in breeding condition (Brenowitz et al., 1998; Smith et al., 1995). RA neurons, however, are not tonically active during song production, but instead fire stereotyped sequences of action potential bursts (Hahnloser et al., 2002; McCasland, 1987;Yu and Margoliash, 1996). Also, the influence of RA on nXIIts motoneurons when the bird is not singing is hypothesized to be negligible (Sturdy et al., 2003). This evidence argues against a direct effect of spontaneously active RA neurons upon the syringeal motoneurons when the bird is not singing. Spontaneous firing along with other intrinsic properties, however, affects how a neuron responds to synaptic inputs from other cells (Kandel and Siegelbaum, 2000). A higher rate of spontaneous firing could be indicative of and interact with other changes in the intrinsic physiology of RA projection neurons to make them more sensitive to inputs from HVC, LMAN, or GABAergic interneurons. During the nonbreeding season, when producing stereotyped song is not as essential because song is not used for mate attraction, the intrinsic excitability of the projection neurons could be down-regulated, resulting in a lower *in vitro* spontaneous firing rate. While this might make the projection neurons less sensitive to synaptic inputs (possibly manifested as a decrease in song stereotypy), it might be offset by the reduction in metabolic demand imposed by neurons in RA (see Wennstrom et al., 2001).

Concluding Remarks

Since the discovery of seasonal plasticity in the song control system (Nottebohm, 1981), research has focused primarily on structural changes. Our study takes a first step towards understanding functional changes, a complementary aspect of plasticity. These seasonal electrophysiological changes in the song system are probably not limited to intrinsic changes in spontaneous firing rate of RA neurons. Increases in dendritic spine density and number accompany the growth of RA neurons (Canady et al., 1988; DeVoogd et al., 1985), suggesting formation of new synapses and the likelihood of associated electrophysiological changes. Moreover, with the addition of new RA-projecting HVC neurons in adults (Paton and Nottebohm, 1984), additional synaptogenesis and synaptic plasticity in HVC and RA are expected. Downstream, as well, hypoglossal motoneurons could also undergo seasonal functional changes. We hypothesize that such plasticity will reflect adaptive changes crucial for seasonal modulation of song behavior.

We thank Karin Lent, Annegret Faulkner, and Hawkeye King for expert technical assistance. We are grateful to Dr. John Wingfield for allowing us to perform the hormone assays in his laboratory.

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