Androgens and Isolation From Adult Tutors Differentially Affect the Development of Songbird Neurons Critical to Vocal Plasticity

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Livingston, Frederick S. and Richard Mooney. Androgens and isolation from adult tutors differentially affect the development of songbird neurons critical to vocal plasticity. *J Neurophysiol* 85: 34–42, 2001. Song learning in oscine birds occurs during a juvenile sensitive period. One idea is that this sensitive period is regulated by changes in the electrophysiological properties of neurons in the telencephalic song nucleus lateral magnocellular nucleus of the anterior neostriatum (LMAN), a structure critical for song development but not adult singing. A corollary of this idea is that manipulations affecting the pace and quality of song learning will concomitantly affect the development of LMAN′s electrophysiological properties. Manipulations known to affect song development include treating juvenile male zebra finches with exogenous androgens, which results in abnormally truncated adult songs, and isolation of the juvenile from adult tutors and their songs, which extends the sensitive period for song learning. Previously, we showed that synaptic transmission in LMAN changes over normal song development and that these changes are accelerated or retarded, respectively, by androgen treatment and isolation from an adult tutor. The intrinsic properties of LMAN neurons afford another potential target for regulation by steroid hormones and experience of adult tutors. Indeed previous studies showed that the capacity for LMAN neurons to fire action potentials in bursts, due to a low-threshold calcium spike, and the width of single action potentials in LMAN, wane over development. Here we analyzed these and other intrinsic electrophysiological features of LMAN neurons over normal development, then tested whether either early androgen treatment or isolating juveniles from adult tutors affected the timing of these changes. The present study shows that androgen but not isolation treatment alters the developmental time at which LMAN neurons progress from the bursting to nonbursting phenotype. In addition, other intrinsic properties, including the half-height spike width and the magnitude of the spike afterhyperpolarization (AHP), were found to change markedly over development but only changes to the AHP were androgen sensitive. Interestingly of all of the synaptic and intrinsic electrophysiological properties in LMAN studied to date, only the half-height spike width continues to change in the late juvenile stages of song learning. Furthermore raising juveniles in isolation from an adult tutor transiently delays the maturation of this property. The present results underscore that beyond their effects on LMAN′s synaptic properties, both androgens and adult tutor experience are potent and selective regulators of the intrinsic properties of LMAN neurons.

INTRODUCTION

The sensitive period for the acquisition of learned song in oscine songbirds comprises three distinct phases. First, during sensory acquisition, young male zebra finches (*Taeniopygia guttata*) memorize the song of an adult male tutor. Next, during sensorimotor learning, the plastic song of juveniles is matched to the memorized song model. Song learning ends with crystallization, when the song becomes highly stereotyped. A major goal is to understand the neuronal properties that change during development to limit sensitive periods for song learning.

One advantage of using songbirds to study the neuronal regulation of sensitive periods for learning is that the timing and quality of song learning can be experimentally manipulated and used to separate general developmental neuronal changes from those that might be specific to song learning. Young zebra finches implanted with testosterone have shorter songs and a reduced number of syllables as adults (Korsia and Bottjer 1991), while similar treatment will cause white-crowned sparrows to prematurely and abnormally crystallize their song (Whaling et al. 1995). In contrast, zebra finches raised in auditory and visual isolation from an adult tutor have an extended period of sensory acquisition, past the normal endpoint of PHD 65 (Aamodt et al. 1995; Eales 1985; Jones et al. 1996; Livingston et al. 2000; Morrison and Nottebohm 1993). Here we examine whether these two behavioral manipulations, which are known to alter the pace of song learning, also alter the development of electrophysiological properties in brain regions critical to song learning. Establishing this correlation would strengthen the link between candidate electrophysiological changes and sensitive-period regulation.

Although many brain areas are important to singing and thus potentially important to song learning, one brain region that may be especially important to regulating sensitive periods for song learning is the lateral magnocellular nucleus of the anterior neostriatum (LMAN). Lesion studies reveal that this telencephalic nucleus is essential for juvenile song development but not for adult singing (Bottjer et al. 1984; Scharff and Nottebohm 1991). LMAN is likely to affect vocal plasticity via its monosynaptic connections with the robust nucleus of the archistriatum (RA), a vocal motor area critical to song production throughout life. Beyond affecting juvenile vocal quality, LMAN also is likely to be important to the memorization of tutor songs, because blocking *N*-methyl-D-aspartate (NMDA) receptors in LMAN during tutoring can interfere with song development (Basham et al. 1996). One hypothesis is that the electrophysiological properties of LMAN projection neurons change over song development, gradually altering the capacity

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TABLE 1. *Effects of androgens and isolation from adult tutors on LMAN neuronal development*

Parameter	Fledgling	Juvenile	Late Juvenile	Adult	Fledgling + DHT	Juvenile + DHT	Juvenile + Isolation	Late Juvenile + Isolation
Mean age by cell (PHD)	26.8	45.2	66.3	110.2	25.3	44.9	45.1	66.1
n: cells, animals	29, 12	20, 4	18, 4	19, 4	44, 9	18, 5	25, 5	19, 5
V_m	−77 ± 2*	−72 ± 2	−73 ± 2	−71 ± 2	−73 ± 1	−73 ± 2	−75 ± 1	−71 ± 2
R_i	84 ± 6	98 ± 6	90 ± 7	91 ± 7	84 ± 5	107 ± 7	112 ± 6	109 ± 7*
IF-CV	0.58 ± 0.03	**0.27 ± 0.03**	**0.16 ± 0.01**	0.15 ± 0.01	***0.41 ± 0.03***	***0.16 ± 0.01***	0.28 ± 0.03	0.16 ± 0.02
Percent bursting cells (IF-CV > 0.4)	90	30	0	0	68	11	28	11
½-height spike width, ms	1.07 ± 0.03	**0.62 ± 0.01**	**0.56 ± 0.01**	**0.48 ± 0.01**	1.04 ± 0.03	0.64 ± 0.01	***0.70 ± 0.02***	0.61 ± 0.03
AHP, mV	18.4 ± 0.5	**22.2 ± 0.5**	**24.3 ± 0.6**	23.3 ± 0.5	***20.6 ± 0.4***	***25.1 ± 0.6***	23.2 ± 0.4	22.6 ± 0.5

All statistics are in text and figure legends for instantaneous frequency coefficient of variations (IF-CV), ½-height spike widths, and afterhyperpolarizations (AHPs). Bold indicates significant difference from next youngest age group. Bold italics indicates significant difference from age-matched controls. LMAN, lateral magnocellular nucleus of the anterior neostriatum; PHD, posthatch day. * Significantly more negative than juvenile values, $P < 0.05$.

of LMAN to influence either song memorization or song quality (Livingston and Mooney 1997). A corollary of this idea is that manipulations affecting the pace of song learning should also alter the developmental time course of any changes in LMAN′s electrical properties. Specifically, if LMAN is a crucial site for sensitive-period regulation, then exogenous androgens, which disrupt song learning and cause premature song crystallization, should also hasten electrophysiological changes in LMAN that are important for limiting sensitive periods (White et al. 1999). Similarly, raising birds in visual and auditory isolation from adult tutors, which extends sensitive periods for song learning, should delay these electrophysiological changes (Livingston et al. 2000). Searching for these correlations can help identify electrophysiological mechanisms governing sensitive periods for song learning.

Indeed, there are major developmental changes in the electrophysiological properties of LMAN neurons. At synapses between thalamic axons and LMAN neurons, the decay times of NMDA receptor-mediated currents become faster over development (Livingston and Mooney 1997), and the time course of these changes can be accelerated by exogenous testosterone (White et al. 1999) or transiently delayed by isolation (Livingston et al. 2000). However, it is not known whether other electrophysiological properties of LMAN neurons, including their intrinsic properties, are also sensitive to these types of manipulations. Normal developmental changes in these intrinsic properties include the disappearance of a low-threshold Ca^{2+} spike (LTS): when injected with positive currents, fledgling LMAN projection neurons [~25 posthatch day (PHD)] fire in part with bursts of action potentials, due to the LTS, yet adult neurons only fire regular spike trains (Livingston and Mooney 1997). This juvenile bursting could be important for sensory acquisition perhaps by enhancing transmitter release (Lisman 1997) or by otherwise altering the transfer function of LMAN neurons. In addition, the spike width of LMAN neurons changes between juvenile and adult times (Bottjer et al. 1998). Acute changes in action potential width can have profound effects on neurotransmitter release (Sabatini and Regehr 1997), while persistent changes in spike width underlie certain forms of behavioral sensitization in *Aplysia* (Siegelbaum et al. 1982). Thus changes in either synaptic or intrinsic electrophysiological properties can influence neuronal and behavioral plasticity, making them both attractive candidates for sensitive period regulation. However, while androgens and isolation from adult tutors can alter the development of synaptic properties in LMAN neurons (Livingston et al. 2000; White et al. 1999), it remains unclear whether these manipulations also modulate the development of their intrinsic properties. Here we examine this issue and provide evidence that exogenous androgens and the quality of the juvenile bird's early auditory and visual experience of an adult tutor influence when LMAN neurons' intrinsic properties change during development.

METHODS

These experiments use behavioral and electrophysiological techniques that have been extensively described in previous published studies (Livingston and Mooney 1997; Livingston et al. 2000; Mooney 1992; White et al. 1999). Therefore only a brief description of these techniques is provided here.

Subjects

Brain slices were made from 48 male zebra finches, in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. The four age groups studied here were fledglings (~25 PHD), when young male zebra finches are near the onset of sensory acquisition; juveniles (~45 PHD), during the early stages of sensorimotor learning and prior to the end of sensory acquisition; late juveniles (~65 PHD), when sensory acquisition ends in birds with prior exposure to tutors but before the end of sensorimotor learning; and adults (~110 PHD), when song has become crystallized (see Table 1 for the numbers of cells and animals used for each group). Some of the data from recordings of fledgling neurons have been used in a previous study (Livingston and Mooney 1997); otherwise, data have not been previously reported. Finches were raised in our breeding colony on a 14-h light:10-h dark cycle.

Isolation protocol and androgen manipulations

At 25 PHD, fledglings were removed from breeding cages and housed alone in small stainless steel cages (22 × 22 × 25 cm) until either 45 or 65 PHD; siblings could only hear and not see one another during this isolation period and were completely deprived of auditory, visual, or other forms of contact with an adult tutor. We have previously documented that this treatment extends sensory acquisition beyond 65 PHD and also transiently depresses serum testosterone levels ~45 PHD (Livingston et al. 2000). Serum androgen levels in other birds were augmented using silastic implants placed subcutaneously in the chest region; implants were made at either 15 or 35 PHD, and birds were killed for brain slice recordings ~10 days later (25

PHD, fledgling; 45 PHD, juvenile—see Table 1). The implants contained ~50 μg dihydrotestosterone (a nonaromatizable form of testosterone; similar implants of testosterone significantly elevate serum androgen titers for 7–10 days post implant; see White et al. 1999).

Brain slices

Briefly, sagittal brain slices that included LMAN were cut at 400 μm and transferred to a holding chamber (room temperature); intracellular recordings were made using an interface-type chamber (30°C; Medical Systems). Artificial cerebrospinal fluid (ACSF) consisted of (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose; equilibrated with 95% O_2-5% CO_2. Equiosmolar sucrose was substituted for NaCl during the tissue preparation stage.

Electrophysiological recordings

Sharp intracellular recordings were made with borosilicate glass pipettes (Sutter Instrument) pulled to a final resistance of 80–200 MΩ when filled with 2 M potassium acetate. Intracellular potentials were amplified with an Axoclamp 2B amplifier (Axon Instruments) in bridge mode, low-pass filtered at 1–3 kHz, and digitized at 10 kHz.

Data acquisition and analysis

Data acquisition and analysis for intracellular recordings were performed using a National Instruments data acquisition board (AT-MIO-16E2), controlled by custom Labview software developed by F. Livingston and R. Neummann. Suprathreshold responses from each neuron were measured in response to two different depolarizing current pulses (1-s duration, +400, and +600 pA), injected from the resting potential. Previous work has shown that prior hyperpolarization of the membrane potential can either induce or enhance bursting (Livingston and Mooney 1997). Thus we also elicited suprathreshold responses beginning from a hyperpolarized state (tonic hyperpolarizing current, 400 pA; depolarizing pulses were 1-s duration, net +400 and +600 pA), and also included them in the analysis. In total, four spike trains were collected and analyzed from each neuron (i.e., responses to 1-s duration, +400-, +600-pA current pulses in both normal resting and hyperpolarized states). A software threshold-event detector was used to measure instantaneous spike rates throughout the 1-s period of positive current injection and the coefficient of variation in the instantaneous rates was calculated as the standard deviation in the instantaneous firing rate divided by the mean firing rate. Spike widths were measured at one half the spike amplitude, which was determined as the point midway between the sharp positive inflection in membrane potential immediately prior to the action potential and the spike peak; the amplitude of the spike afterhyperpolarization (AHP) was the voltage difference between the inflection point and the membrane potential minimum following the spike peak. Input resistance measurements were calculated by measuring the steady-state voltage caused by injecting small (−200 pA) hyperpolarizing current pulses. Two-way ANOVAs (group vs. current injection) were used to assess statistical significance, unless otherwise noted. In all cases, the minimum significance level was set at $P < 0.05$ using two-tailed comparisons. Averages are reported with the standard error of the mean (±SE).

RESULTS

Developmental changes in burst firing

Intracellular sharp-electrode recordings previously revealed that the regularity of action potential firing in LMAN projection neurons in response to depolarizing current injection could be quite varied (Fig. 1) (also see Livingston and Mooney 1997). We originally classified neurons as either "bursting" or "nonbursting" based solely on visual inspection of spike trains, which could be insufficient to describe more subtle differences that may occur over development. Therefore we wanted to use a *quantitative* measure that did not rely on visual inspection of the traces to assess whether a neuron fired in a bursting or nonbursting mode.

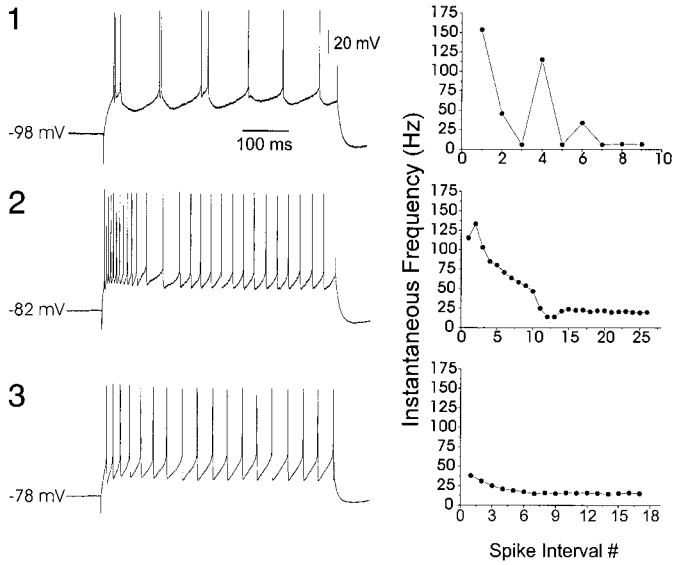

FIG. 1. Examples of DC-evoked burst firing and regular firing in fledgling lateral magnocellular nucleus of the anterior neostriatum (LMAN) neurons. *Left*: action potential trains from 3 different fledgling neurons, evoked by a 1-s-long, +400-pA DC current pulse, show the range of firing behaviors encountered here. *Top trace*: robust burst firing; *bottom trace*: a highly regular spiking pattern; *middle trace*: an intermediate firing behavior. *Trace 1* was depolarized from a tonically hyperpolarized state whereas *traces 2* and *3* were depolarized from rest. *Right*: corresponding plots of the instantaneous frequencies plotted as a function of spike interval number. See Fig. 2*A*, *top*, for where the coefficient of variation of the instantaneous firing frequencies (IF-CVs) calculated from each of these 3 examples lies in the entire population of IF-CVs collected from fledgling LMAN neurons (IF-CVs: *neuron 1*, 1.23; *neuron 2*, 0.85; *neuron 3*, 0.12).

We quantified the variety of DC-evoked action potential trains by measuring the coefficient of variation of the instantaneous spike frequencies (IF-CV), which estimates how much the instantaneous frequencies vary around the mean value for a given spike train. One characteristic of fledgling LMAN neurons is that they often exhibited both very high and low instantaneous spike frequencies in a single spike train, due to the LTS, thus yielding a very high IF-CV. In contrast, neurons that varied little in their instantaneous spike frequencies throughout the train, or simply underwent a gradual monotonic decay of instantaneous firing frequency, yielded low IF-CVs. Individual current-clamp records of spike trains and plots of instantaneous firing frequencies obtained from three different fledgling neurons illustrate differences in firing behavior and IF-CV (Fig. 1). The neuron in *trace 1* quickly alternated between high and low instantaneous firing frequencies, while the neuron from which *trace 2* was collected exhibited a less abrupt decline from high to low instantaneous firing frequencies; both neurons had relatively high IF-CVs (Fig. 2*A*, *top*). The other fledgling neuron, from which *trace 3* was collected, declined gradually in its instantaneous firing frequency over the duration of the depolarizing current injection and had a low IF-CV (Fig. 2*A*, *top*).

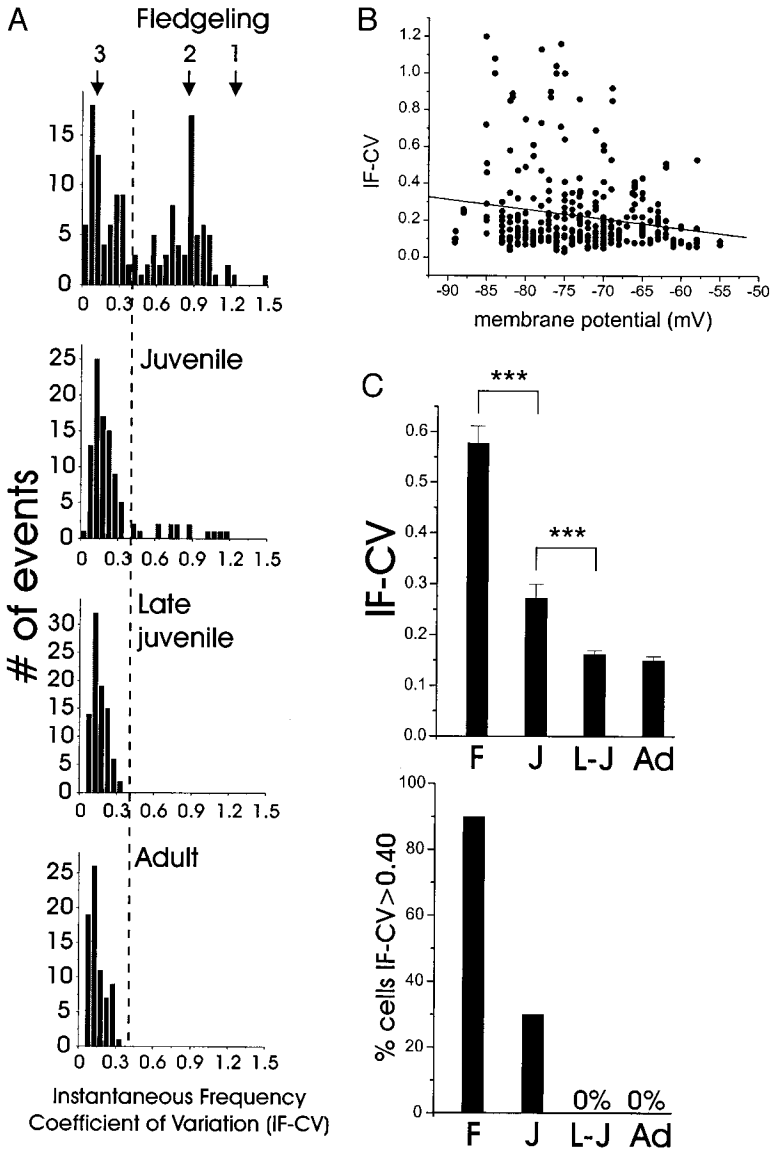

FIG. 2. Burst firing in LMAN disappears over development. *A*: histograms show the distribution of instantaneous firing frequency coefficient of variations (IF-CVs) from the 4 different developmental periods studied here. Note the bimodal distribution in fledglings, with the 2 separate regions of high and low IF-CVs; in contrast, distributions from late juveniles and adults were unimodal, having only low IF-CVs. - - -, at IF-CV = 0.4, lies in between the 2 fledgling regions and was used to classify cells at all ages as either bursting or nonbursting. The numbers and ↓ in the fledgling histogram refer to the IF-CVs calculated from the traces shown in Fig. 1*A*. *B*: IF-CVs measured from all cells (in all age groups) plotted vs. their native resting membrane potentials revealed a negative correlation ($R = -0.147$, $P < 0.01$); a similar comparison using only IF-CVs measured at artificially hyperpolarized membrane potentials did not show a significant correlation ($R = 0.0163$, $P = 0.77$; not shown). These 2 comparisons suggest that tonic hyperpolarization alleviated voltage-dependent variations in IF-CVs and thus could control for cell-to-cell differences in resting membrane potential. *C, top*: bar graphs of the mean (±SE) IF-CVs show a significant decline over development, specifically from fledgling to juvenile and from juvenile to late juvenile periods. *Bottom*: bar graphs showing the percentage of cells that had at least 1 action potential train IF-CV > 0.4. ***$P < 0.0005$. (F), fledgling; (J), juvenile; (L-J), late juvenile; (Ad), adult.

Using this quantitative measure, we found that the distribution of fledgling IF-CVs is bimodal (Fig. 2), with a distinct notch at 0.4 separating the two peaks (note - - - in Fig. 2). We used this value as a cutoff, classifying a cell as bursting only if at least one of its DC-evoked action potential trains yielded an IF-CV > 0.4. The three fledgling traces shown in Fig. 1 illustrate the method: *traces 1* (IF-CV = 1.23) and *2* (IF-CV = 0.85) are from different parts of the region representing bursting neurons, while *trace 3* (IF-CV = 0.12) is an example from the nonbursting or regular spiking region. Ultimately, statistical significance was assessed using two-way ANOVAs on the IF-CV (group vs. level of current injected; see METHODS).

This quantitative measure allowed us to determine that the number of bursting cells in a given age group declined over development from 90 to 0% (i.e., 90% of fledgling neurons displayed at least 1 spike train with an IF-CV > 0.4, while no adult neurons had an IF-CV > 0.4; Table 1; Fig. 2). As shown in Fig. 2*A*, this cutoff value was never exceeded by cells in the late juvenile and adult groups even though their IF-CVs were determined both at their native resting potentials and in a tonically hyperpolarized state. This tonic hyperpolarization of membrane potential, used to relieve deinactivation of any latent LTS, ensured that slight group to group differences in resting potential (see Table 1) did not inadvertently bias our estimates of bursting (Fig. 2*B*). Most (90%; 26/29 cells) fledgling neurons displayed an IF-CV 0.4, while only 30% (6/20 cells) of juvenile neurons showed this behavior (mean IF-CV, fledgling: 0.58 ± 0.03 vs. juvenile: 0.27 ± 0.03, $P < 0.0001$). Late juvenile LMAN neurons did not display any bursting, and had a mean IF-CV less than that of juveniles (0.27 ± 0.03 vs. 0.16 ± 0.01, $P < 0.0002$) and equivalent to that of adults (0.16 ± 0.01 vs. 0.15 ± 0.01, $P = 0.22$). Thus to summarize, there are major developmental changes in the way LMAN projection neurons fire action potentials trains in response to depolarizing currents, progressing from a bursting to a nonbursting phenotype (Fig. 2*C*).

Effects of dihydrotestosterone and isolation on bursting

To directly test whether the developmental decline in bursting behavior contributed to sensitive periods for song learning,

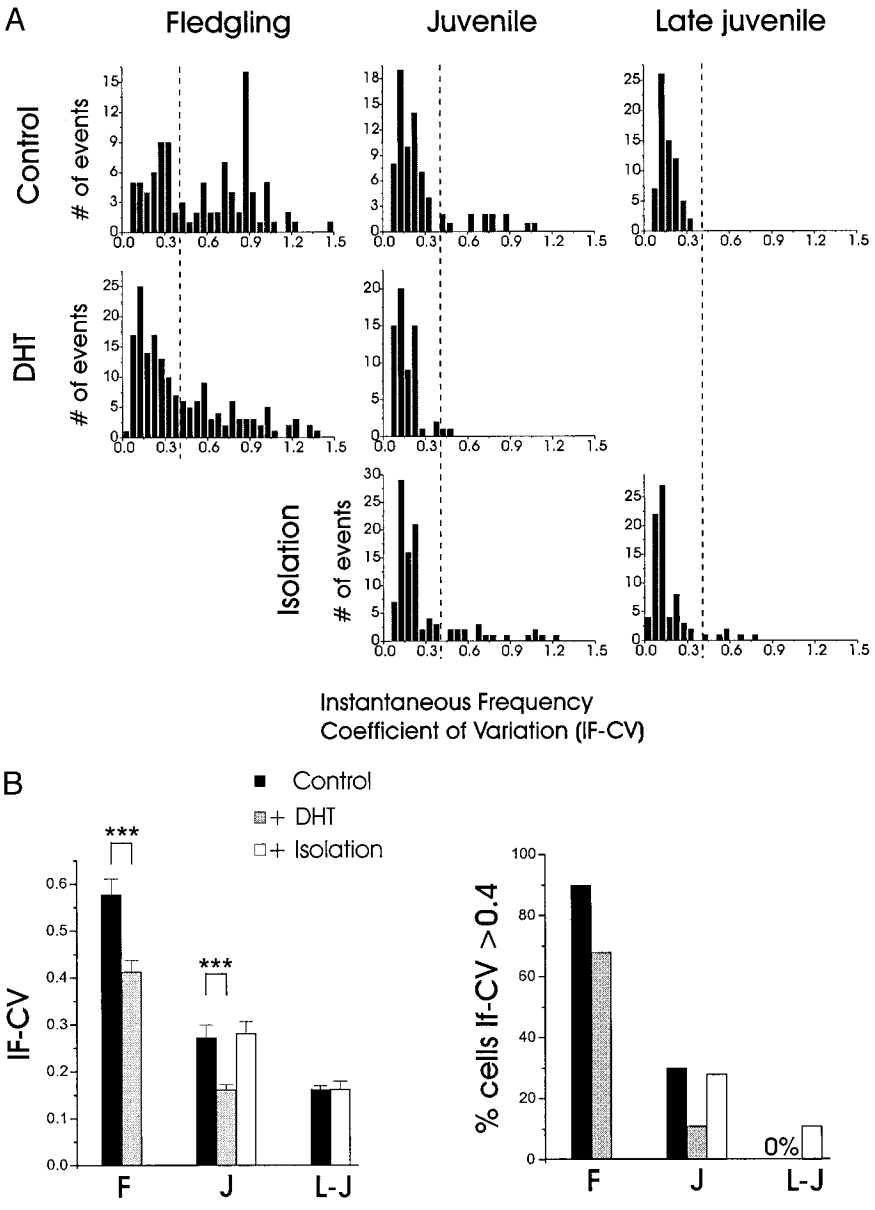

FIG. 3. The effects of androgens and isolation on the incidence of burst firing of LMAN neurons and on the mean IF-CV. *A*: androgen treatment decreases the incidence of burst firing in LMAN neurons at fledgling and juvenile times relative to age-matched controls. Histograms show the distribution of instantaneous frequency coefficient of variations (IF-CVs) from controls, androgen-treated birds, or isolates. - - -, IF-CV = 0.4, used to classify cells as either bursting or nonbursting. *B, left*: bar graphs of the mean IF-CVs. Androgen treatment reduced the mean IF-CV at fledgling and juvenile times (***$P < 0.0005$). *Right*: bar graphs showing the percentage of cells that had at least 1 action potential train IF-CV > 0.4. (F), fledgling; (J), juvenile; (L-J), late juvenile; (Ad), adult.

we examined whether factors that are known to alter the pace of song learning also alter the progression from bursting to nonbursting firing patterns. Androgens are one such factor that can perturb song development. To test whether it concomitantly alters the intrinsic properties of LMAN neurons, either fledgling or juvenile male zebra finches were implanted with dihydrotestosterone ~10 days prior to intracellular recording (see METHODS). Androgen treatment reduced bursting in both fledgling and juvenile LMAN neurons (Table 1; Fig. 3) compared with age-matched controls. At the fledgling age, dihydrotestosterone lowered the number of bursting neurons from 90 to 68% (26/29 vs. 30/44 cells), with the mean IF-CV lying between those of fledglings and juveniles (mean IF-CV, fledgling: 0.58 ± 0.03 vs. juvenile: 0.41 ± 0.03, $P < 0.0001$). In juveniles, dihydrotestosterone lowered the number of bursting cells from 30 to 11% (6/20 vs. 2/18 cells; mean IF-CV: 0.27 ± 0.03 vs. 0.16 ± 0.01, $P < 0.001$), and decreased the mean IF-CV to a value equivalent to those of both late juvenile and adult neurons.

Auditory and visual isolation from and adult tutor is another factor that alters the pace of song development: birds raised as isolates retain their ability to learn song until after 65 PHD, a time when control birds do not copy new songs. We tested for delayed developmental changes in the firing properties from juvenile finches that were isolated from tutor song beginning at ~25 PHD. In contrast to the effects of androgens on bursting in both fledgling and juvenile neurons, isolation did not affect the developmental progression from bursting to nonbursting phenotypes. Isolation of juveniles from tutor song had no effect on the number of their LMAN neurons displaying bursting properties [Table 1; 30% (6/20) juvenile cells vs. 28% (7/25) isolate juvenile cells; mean IF-CV 0.27 ± 0.03 vs. 0.28 ± 0.03; Fig. 3]. In contrast, brain slices from late juvenile isolates did contain a small number of bursting cells (2/19 cells or 11%), suggestive of slightly delayed neuronal maturation, although the mean IF-CVs for the late juvenile isolates and their age-matched controls were equivalent (0.16 ± 0.01 vs. 0.16 ± 0.02; note the widened data distribution revealed by the 2-fold

increase in the SE in Fig. 3*B*). In summary, dihydrotestosterone markedly accelerated the transition from the bursting to the nonbursting state, while isolation had little or no effect on the developmental time at which this property disappeared.

Spike widths and AHPs

We also examined the half-height width of single action potentials (the half-height spike width) and the amplitude of spike AHPs of LMAN neurons, two features that could be regulated independently of their bursting properties. We first measured how these features change across development and then examined the effects that androgens or raising juvenile birds in isolation from adult tutors had on the developmental changes of these two features. Aside from describing how features of spike shape may relate to sensitive periods for learning, these analyses can reveal the specificity of the actions of androgens and isolation on the electrophysiological properties of LMAN neurons (Livingston et al. 2000; White et al. 1999).

The shape of action potentials changed dramatically over development; at the fledgling age action potentials have both a slower rise and a slower decay than seen in adults, making them much wider (Fig. 4; Table 1). The spike width previously has been examined in LMAN from juvenile to adult times (Bottjer et al. 1998). Here we have both confirmed and extended this analysis to the fledgling age, a time when zebra finches can memorize song but prior to the time they start to sing. The present study reveals dramatic differences in shape and half-height spike width over development and illuminates how androgens and isolation affect these developmental changes.

Differences in the half-height spike width were seen throughout development (Table 1; Fig. 4; $P < 0.0001$). The largest change was between the fledgling and juvenile periods (1.07 ± 0.03 vs. 0.62 ± 0.01 ms, $P < 0.0001$), but changes also occurred among juveniles, late juveniles, and adults (0.62 ± 0.01 vs. 0.56 ± 0.01 vs. 0.48 ± 0.01 ms; all comparisons $P < 0.0001$); these later measurements are similar to those in Bottjer et al. (1998).

We also measured spike AHPs, the amplitude of which increased over development ($P < 0.0001$), specifically among fledglings, juveniles, and late juveniles (Fig. 5; 18.4 ± 0.5 vs. 22.2 ± 0.5 vs. 24.3 ± 0.6 mV; $P < 0.0001$ and $P < 0.01$, respectively). Representative examples of fledgling and adult AHPs reflective of their respective group means are provided in Fig. 5. In contrast to the changes in half-height spike width, these changes in AHPs were complete by the late juvenile period with no difference between late juveniles and adults. To determine whether a change in driving force or input resistance could underlie the change in AHP amplitude, we measured these features over development (Table 1). This analysis revealed that the resting membrane potential but not the input resistance changed slightly but significantly in the positive direction between fledgling and later time points, suggesting that early decreases in the AHP might reflect in part a diminution in driving force over development.

Although the half-height spike width and AHPs both changed over development, androgens and isolation had distinct effects on these parameters (Table 1; Figs. 4 and 5). The half-spike width was not affected by androgen treatment at any

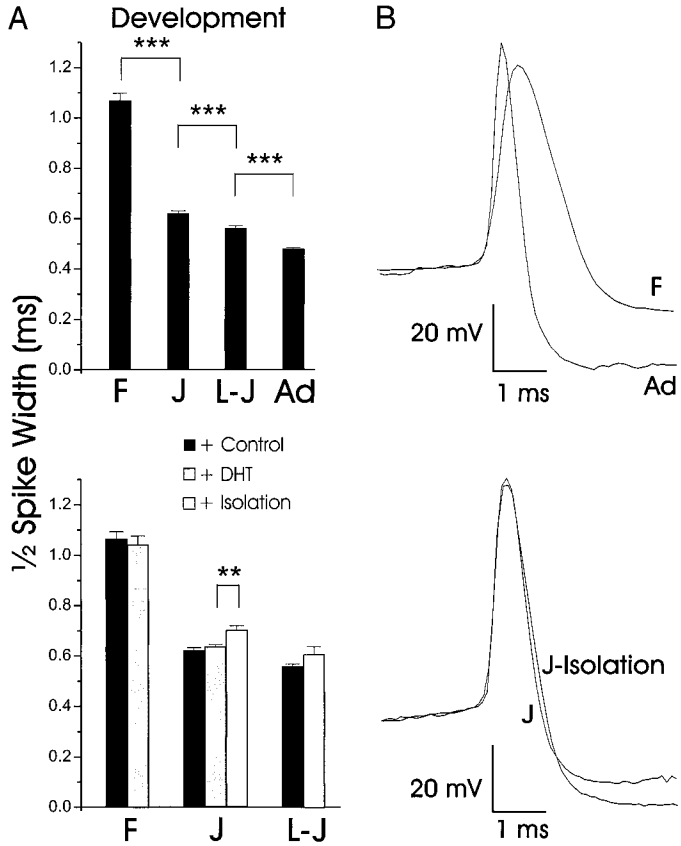

FIG. 4. The spike widths of LMAN neurons changes over development and are affected by isolation, but not androgen-treatment. *A, top*: bar graphs showing the mean half-height spike width from the different developmental periods. ***$P < 0.0001$. *Bottom*: bar graph shows the effects of either androgens or isolation on the mean half-height spike width. Note that of these 2 manipulations only isolation had an (albeit small) effect. **$P < 0.01$. *B, top*: superimposed fledgling and adult action potential traces illustrate the differences in half-height spike width that occur over development in LMAN. *Bottom*: superimposed isolate and control juvenile action potential traces illustrate the small effect of isolation on the half-height spike width. (F), fledgling; (J), juvenile; (L-J), late juvenile; (Ad), adult.

age, but isolation did slightly increase this measurement in juveniles (Fig. 4; 0.62 ± 0.01 vs. 0.70 ± 0.01 ms, $P < 0.0003$); there was a similar but non-significant trend in late juveniles (0.56 ± 0.01 vs. 0.61 ± 0.03 ms, $P = 0.1635$). In contrast, dihydrotestosterone did increase the AHPs of fledglings (Fig. 5; 18.4 ± 0.5 vs. 20.6 ± 0.4 mV, $P < 0.0001$) and juveniles (22.2 ± 0.5 vs. 25.1 ± 0.6 mV, $P < 0.0001$), but this feature was entirely unaffected by isolation. Although resting membrane potentials and input resistances were not significantly affected by either dihydrotestosterone or isolation, the slightly more positive mean membrane potential we saw in DHT-treated fledges may account for the change in AHP amplitude (Table 1). In summary, while dihydrotestosterone did not affect the half-height spike width, isolation did have small but significant effects. In contrast, dihydrotestosterone did affect the development of AHPs, but isolation did not.

DISCUSSION

Along with prior studies examining the androgen-sensitivity of NMDA receptor-mediated currents in LMAN, the present studies show that androgens are a potent regulator of a major

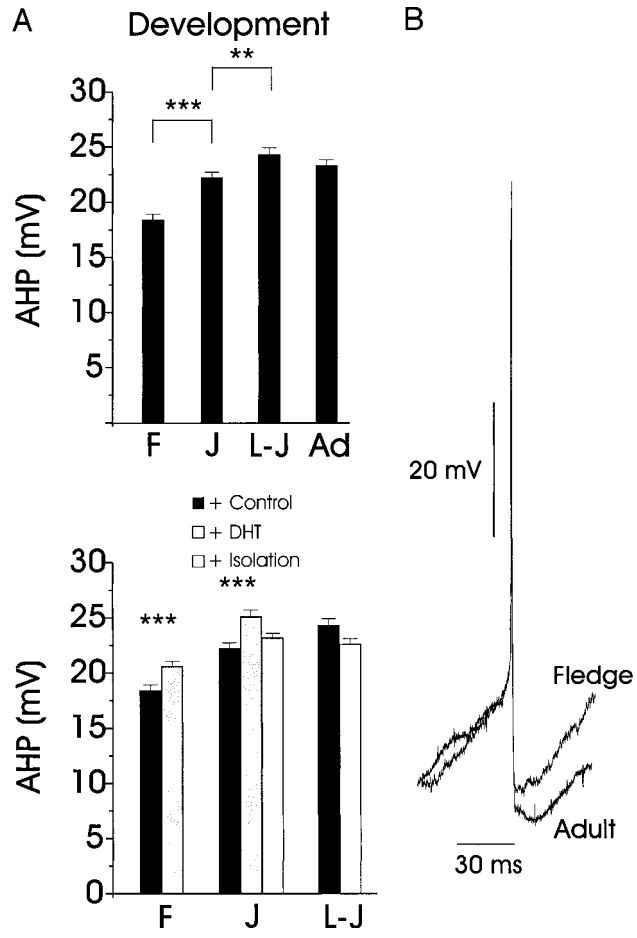

FIG. 5. Androgens increase the amplitude of the spike afterhyperpolarization (AHP). *A*, *top*: bar graphs showing the mean spike AHPs from the different developmental periods. The amplitude of the AHP increased between fledgling and juvenile and from juvenile to late juvenile times. $***P < 0.0001$, $**P < 0.01$. *Bottom*: bar graphs showing the effects of either androgens or isolation on the mean AHPs. Note that only androgens had an effect and acted to increase the AHP amplitude. $***P < 0.0001$. *B*: superimposed fledgling and adult action potential traces illustrate the differences in AHPs. (F), fledgling; (J), juvenile; (L-J), late juvenile; (Ad), adult.

subset of electrophysiological properties of LMAN neurons. Androgens have been previously shown to regulate the decay times of NMDA receptor-mediated excitatory postsynaptic currents (NMDA-EPSCs) and the ratio of the overall glutamate receptor-mediated EPSC to the AMPA receptor-mediated EPSC (White et al. 1999). Beyond these synaptic effects, the present study shows that androgens act ether directly or indirectly to also modulate specific intrinsic electrophysiological properties of LMAN neurons, including the progression from burst to nonburst action potential firing and the magnitude of AHPs. Furthermore androgens do not merely act as a nonspecific maturational factor since exogenous androgen treatment failed to alter the development of half-height spike widths, a property that changes markedly over normal development. The present results underscore the potent effect of sex steroids on the electrophysiology of LMAN, a nucleus critical to the development of song, a steroid-sensitive learned vocal behavior.

In contrast to the potent effects of exogenous androgens, raising juveniles birds in auditory and visual isolation from adult tutors had little or no effect on the progression from burst to nonburst firing or on the magnitude of spike AHPs, suggesting that these parameters can develop without exposure to an adult tutor. The two bursting cells we saw in late juvenile isolates indicate a very slight retention of this neuronal feature in this group, possibly indicative of slightly slower neuronal maturation. Ultimately, because isolates older than 65 PHD can learn song even though their LMAN neurons largely lack the capacity for burst firing, this feature does not seem to be essential for song learning. Furthermore because juvenile isolates have abnormally low testosterone levels (Livingston et al. 2000), it is unlikely that high testosterone levels are essential for either the disappearance of the burst firing or changes in the magnitude of the AHP even though androgens can affect these features. Therefore other factors must be involved to explain the continued development in these properties that are seen in juvenile isolates with depressed androgen levels. One key factor could be the amount of singing, which is known to affect auditory and/or vocal-related activity in LMAN (Doupe and Konishi 1991; Hessler and Doupe 1999). In this view, where singing-related activity is the proximal cue driving changes in LMAN, the systemic androgen treatment used here could act primarily through other androgen-sensitive song or nonsong areas to augment singing rather than acting directly via LMAN neuronal androgen receptors to affect changes in excitability (see White et al. 1999 for a broader treatment of possible mechanisms of steroid action).

A major question is the functional role of burst firing in LMAN neurons. Bursting could alter the transfer function of LMAN because it induces a highly nonlinear function into a neuron's current-voltage conversion, allowing increased spiking due to a given amount of current input compared with neurons that do not have this property. This amplification could enable LMAN neurons to fire more action potentials in response to the weak excitatory synaptic inputs that may predominate during early juvenile development. In addition, because the bursting is enhanced and sometimes even induced by prior hyperpolarization of the membrane (data not shown) (but see Livingston and Mooney 1997), this amplification could act in concert with inhibitory inputs as a gate for information throughput. Indeed $GABA_A$ receptor-mediated inhibition generated within LMAN, driven by local interneurons, could serve to prime excitatory throughput by enhancing the LTS (Boettiger and Doupe 1998; Livingston and Mooney 1997). In other brain regions, such as the mammalian lateral geniculate nucleus (LGN), the voltage-dependence of the LTS allows LGN relay neurons to fire in either a "relay" or a "burst" mode (McCormick et al. 1995; Steriade and Llinas 1988). Neuromodulators, including norepinephrine, can modulate inhibitory input onto LGN relay cells and thus determine their firing mode (McCormick 1992a,b). In LMAN, there are several candidate modulators, including catecholamines (Ball 1994; Bottjer 1993; Soha et al. 1996), which could act similarly to influence the firing mode of juvenile LMAN neurons, ultimately determining their ability to influence vocal change.

One feature of bursting that makes it an attractive candidate for enhancing neuronal plasticity is the high instantaneous frequencies achieved in the beginning of spike trains. These high firing rates could enhance the probability of synaptic release (Lisman 1997; Miles and Wong 1986) at sites where LMAN axons form synapses, including locally within LMAN (Boettiger and Doupe 1998) and in basal ganglia homologue area X and nucleus RA (Vates and Nottebohm 1995). Although

the relative lack of bursting in isolate neurons suggests this feature is not essential for extended song learning, it may nonetheless be important to early song learning, especially if the probability of transmitter release is diminished at these earlier ages. Specifically, developmental increases in the probability of release could obviate the need for bursting in late juveniles, although this feature may still be needed earlier in development, when individual synapses may be less potent. In essence, bursting could boost initially weak afferent synapses from the thalamus as well as enhance the ability of LMAN to excite its postsynaptic targets.

The present results confirm and extend a prior study (Bottjer et al. 1998) by showing that there are major developmental changes in the spike width of LMAN neurons from fledgling through adult ages. One noteworthy feature of the developmental decrease in spike width is that it is the only electrophysiological feature of LMAN neurons identified to date, including synaptic properties (White et al. 1999), that continues to change after 65 PHD. Although small relative to changes in spike width occurring earlier in development, these late changes do correlate with the period of song crystallization. Interestingly this feature was unaffected by exogenous dihydrotestosterone, suggesting that the isolation effects may stem directly from deprivation from the adult tutor and its song, rather than the abnormally depressed testosterone levels seen in isolates at 45 PHD (Livingston et al. 2000). The role of the spike width changes in LMAN is unclear, but spike width changes underlie behavioral sensitization in *Aplysia*, where presentation of noxious stimuli can induce sensitization, which requires changes in neurotransmitter release at specific synapses. Sensitization is due to a reduction of a K^+ current, resulting in action potential broadening, and increased Ca^{2+} entry into the presynaptic terminal, ultimately enhancing synaptic transmission (Siegelbaum et al. 1982). While isolation did induce a significant widening of the action potential in LMAN neurons, it is a small difference of 80 μs ($\sim$10% widening). However, at the cerebellar granule cell to Purkinje cell synapse, increases in neurotransmitter release due to a modest 23% widening of the action potential can *double* the magnitude of postsynaptic currents (Sabatini and Regehr 1997), suggesting that the subtle changes seen here could also exert profound effects on synaptic strength. Ultimately resolving the significance of different spike durations in LMAN neurons will require an understanding of their consequences for downstream events, including the resultant postsynaptic potentials they may evoke.

Both synaptic and intrinsic electrophysiological properties can be important in regulating neuronal plasticity. These and earlier studies (Livingston and Mooney 1997; Livingston et al. 2000; White et al. 1999) show that both sex steroids and exposure to an adult tutor, which strongly affect the pace and quality of song learning, impact both the intrinsic and synaptic properties of LMAN neurons. Although these results suggest that neuronal maturation in LMAN may be influenced by both of these factors, the dissociation of the timing of these changes relative to late learning in isolates points away from them as major neuronal regulators of sensitive periods for song learning. Future studies can address whether changing single factors within one or even many song nuclei is sufficient to regulate the closure of sensitive periods for song learning and whether the mechanisms underlying extended learning in isolates are similar or distinct from those that enable song learning earlier in life.

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REFERENCES

AAMODT SM, NORDEEN EJ, AND NORDEEN KW. Early isolation from conspecific song does not affect the normal developmental decline of *N*-methyl-D-aspartate receptor binding in an avian song nucleus. *J Neurobiol* 27: 76–84, 1995.

BALL GF. Neurochemical specializations associated with vocal learning and production in songbirds and budgerigars. *Brain Beh Evol* 44: 234–246, 1994.

BASHAM ME, NORDEEN EJ, AND NORDEEN KW. Blockade of NMDA receptors in the anterior forebrain impairs sensory acquisition in the zebra finch (*Poephila guttata*). *Neurobiol Learn Mem* 66: 295–304, 1996.

BOETTIGER CA AND DOUPE AJ. Intrinsic and thalamic excitatory inputs onto songbird LMAN neurons differ in their pharmacological and temporal properties. *J Neurophysiol* 79: 2615–2628, 1998.

BOTTJER SW. The distribution of tyrosine hydroxylase immunoreactivity in the brains of male and female zebra finches. *J Neurobiol* 24: 51–69, 1993.

BOTTJER SW, BRADY JD, AND WALSH JP. Intrinsic and synaptic properties of neurons in the vocal-control nucleus IMAN from in vitro slice preparations of juvenile and adult zebra finches. *J Neurobiol* 37: 642–658, 1998.

BOTTJER SW, MIESNER EA, AND ARNOLD AP. Forebrain lesions disrupt development but not maintenance of song in passerine birds. *Science* 224: 901–903, 1984.

DOUPE AJ AND KONISHI M. Song-selective auditory circuits in the vocal control system of the zebra finch. *Proc Natl Acad Sci USA* 88: 11339–11343, 1991.

EALES LA. Song learning in zebra finches: some effects of song model availability on what is learnt and when. *Anim Behav* 33: 1293–1300, 1985.

HESSLER NA AND DOUPE AJ. Singing-related neural activity in a dorsal forebrain-basal ganglia circuit of adult zebra finches. *J Neurosci* 19: 10461–10481, 1999.

JONES AE, TEN CATE C, AND SLATER PJB. Early experience and plasticity of song in adult male zebra finches (*Taeniopygia guttata*). *J Comp Psychol* 110: 354–369, 1996.

KORSIA S AND BOTTJER SW. Chronic testosterone treatment impairs vocal learning in male zebra finches during a restricted period of development. *J Neurosci* 11: 2362–2371, 1991.

LISMAN JE. Bursts as a untit of neural information: making unreliable synapses reliable. *Trends Neurosci* 20: 38–43, 1997.

LIVINGSTON FS AND MOONEY R. Development of intrinsic and synaptic properties in a forebrain nucleus essential to avian song learning. *J Neurosci* 17: 8997–9009, 1997.

LIVINGSTON FS, WHITE SA, AND MOONEY R. Slow NMDA-EPSCs at synapses critical for song development are not required for song learning in zebra finches. *Nat Neurosci* 3: 482–488, 2000.

MCCORMICK DA. Cellular mechanisms underlying cholinergic and noradrenergic modulation of neuronal firing mode in the cat and guinea pig dorsal lateral geniculate nucleus. *J Neurosci* 12: 278–289, 1992a.

MCCORMICK DA. Neurotransmitter actions in the thalamus and cerebral cortex. *J Clin Neurophysiol* 9: 212–223, 1992b.

MCCORMICK DA, TRENT F, AND RAMOA AS. Postnatal development of synchronized network oscillations in the ferret dorsal lateral geniculate and perigeniculate nuclei. *J Neurosci* 15: 5739–5752, 1995.

MILES R AND WONG RK. Excitatory synaptic interactions between CA3 neurones in the guinea-pig hippocampus. *J Physiol (Lond)* 373: 397–418, 1986.

MOONEY R. Synaptic basis for developmental plasticity in a birdsong nucleus. *J Neurosci* 12: 2464–2477, 1992.

MORRISON RG AND NOTTEBOHM F. Role of a telencephalic nucleus in the delayed song learning of socially isolated zebra finches. *J Neurobiol* 24: 1045–1064, 1993.

SABATINI BL AND REGEHR WG. Control of neurotransmitter release by presynaptic waveform at the granule cell to Purkinje cell synapse. *J Neurosci* 17: 3425–3435, 1997.

SCHARFF C AND NOTTEBOHM F. A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. *J Neurosci* 11: 2896–2913, 1991.

SIEGELBAUM SA, CAMARDO JS, AND KANDEL ES. Serotonin and cyclic AMP close single K^+ channels in Aplysia sensory neurons. *Nature* 299: 413–417, 1982.

SOHA JA, SHIMIZU T, AND DOUPE AJ. Development of the catecholaminergic innervation of the song system of the male zebra finch. *J Neurobiol* 29: 473–489, 1996.

STERIADE M AND LLINAS RR. The functional states of the thalamus and the associated neuronal interplay. *Physiol Rev* 68: 649–742, 1988.

VATES GE AND NOTTEBOHM F. Feedback circuitry within a song learning pathway. *Proc Natl Acad Sci USA* 92: 5139–5143, 1995.

WHALING CS, NELSON DA, AND MARLER P. Testosterone-induced shortening of the storage phase of song development in birds interferes with vocal learning. *Dev Psychobiol* 28: 367–376, 1995.

WHITE SA, LIVINGSTON FS, AND MOONEY R. Androgens modulate NMDA receptor-mediated EPSCs in the zebra finch song system. *J Neurophysiol* 82: 2221–2234, 1999.