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Androgens Modulate NMDA Receptor–Mediated EPSCs in the Zebra Finch Song System

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White, Stephanie A., Frederick S. Livingston, and Richard Mooney. Androgens modulate NMDA receptor-mediated EPSCs in the zebra finch song system. *J. Neurophysiol.* 82: 2221–2234, 1999. Androgens potently regulate the development of learned vocalizations of songbirds. We sought to determine whether one action of androgens is to functionally modulate the development of synaptic transmission in two brain nuclei, the lateral part of the magnocellular nucleus of the anterior neostriatum (LMAN) and the robust nucleus of the archistriatum (RA), that are critical for song learning and production. We focused on *N*-methyl-D-aspartate–excitatory postsynaptic currents (NMDA-EPSCs), because NMDA receptor activity in LMAN is crucial to song learning, and because the LMAN synapses onto RA neurons are almost entirely mediated by NMDA receptors. Whole cell recordings from in vitro brain slice preparations revealed that the time course of NMDA-EPSCs was developmentally regulated in RA, as had been shown previously for LMAN. Specifically, in both nuclei, NMDA-EPSCs become faster over development. We found that this developmental transition can be modulated by androgens, because testosterone treatment of young animals caused NMDA-EPSCs in LMAN and RA to become prematurely fast. These androgen-induced effects were limited to fledgling and juvenile periods and were spatially restricted, in that androgens did not accelerate developmental changes in NMDA-EPSCs recorded in a nonsong area, the Wulst. To determine whether androgens had additional effects on LMAN or RA neurons, we examined several other physiological and morphological parameters. In LMAN, testosterone affected α-amino-3-hydroxy-5-methyl-4-isoxazoleproprianate–EPSC (AMPA-EPSC) decay times and the ratio of peak synaptic glutamate to AMPA currents, as well as dendritic length and spine density but did not alter soma size or dendritic complexity. In contrast, testosterone did not affect any of these parameters in RA, which demonstrates that exogenous androgens can have selective actions on different song system neurons. These data are the first evidence for any effect of sex steroids on synaptic transmission within the song system. Our results support the idea that endogenous androgens limit sensitive periods for song learning by functionally altering synaptic transmission in song nuclei.

INTRODUCTION

The learned vocalizations of zebra finches develop through the integration of auditory and vocal-motor experience during a sensitive period restricted to the first three months of life (Immelmann 1969). This sensitive period comprises sensory acquisition [*posthatch day* (*PHD*) *20–65*], when young birds memorize the song of an adult male tutor, and sensorimotor learning (*PHD 35–90*), when juvenile birds match their song to the memorized tutor song via auditory feedback. Song crystallization (∼*PHD 90*) marks the closure of sensorimotor learning, when the previously acoustically variable song becomes highly stereotyped.

A well-defined neural circuit mediates singing (Fig. 1). The vocal-motor part of this circuit controls learned song production and includes nucleus HVc (acronym now used as a proper name, in the convention of Fortune and Margoliash 1992), the robust nucleus of the archistriatum (RA), and brain stem motor areas involved in the control of syringeal and respiratory muscles (Nottebohm et al. 1976; Wild 1993). A second part of the circuit, known as the anterior forebrain pathway (AFP), indirectly connects HVc to RA via area X, the dorsolateral part of the medial thalamus (DLM), and the lateral part of the magnocellular nucleus of the anterior neostriatum (LMAN) (Bottjer et al. 1989; Nottebohm et al. 1982; Okuhata and Saito 1987). LMAN is an important locus for exploring the neural mechanisms that underlie song learning because it is essential to song development, but not to adult song production (Bottjer et al. 1984; Scharff and Nottebohm 1991). LMAN terminals innervate the same vocal premotor neurons in RA that receive HVc input, thus providing a site for the AFP to influence the vocal-motor pathway during song learning (Canady et al. 1988; Kubota and Saito 1991; Mooney and Konishi 1991).

N-methyl-D-aspartate (NMDA) receptors, which depend on both glutamate binding and depolarization to gate calcium flux into neurons, are crucial to several forms of synaptic plasticity (Bear 1996). NMDA receptors mediate synaptic transmission at several sites within the song system, including the DLM-LMAN and LMAN-RA synapses. In LMAN, blockade of NMDA receptors during tutoring decreases the number of learned notes (Basham et al. 1996b), which suggests that any developmental changes in NMDA receptors in LMAN could influence song learning. Indeed, NMDA receptor density in LMAN declines over song development, as indicated by antagonist binding, immunolabeling, and mRNA expression levels (Aamodt et al. 1992; Basham et al. 1996a; Carrillo and Doupe 1995). Changes in synaptic transmission also occur in LMAN, where NMDA receptor–mediated excitatory postsynaptic currents (NMDA-EPSCs) become faster over the same period of development (Livingston and Mooney 1997). In other systems, similar developmental changes in NMDA-EPSCs have been invoked to explain sensitive periods for synaptic reorganization (Carmignoto and Vicini 1992); the faster NMDA-EPSCs that emerge during development reduce postsynaptic calcium entry and could thereby diminish certain forms of calcium-dependent synaptic remodeling (Bear 1996).

As a first step in identifying cellular mechanisms that underlie song learning, we tested whether factors that disrupt song learning also alter the development of NMDA-EPSCs in

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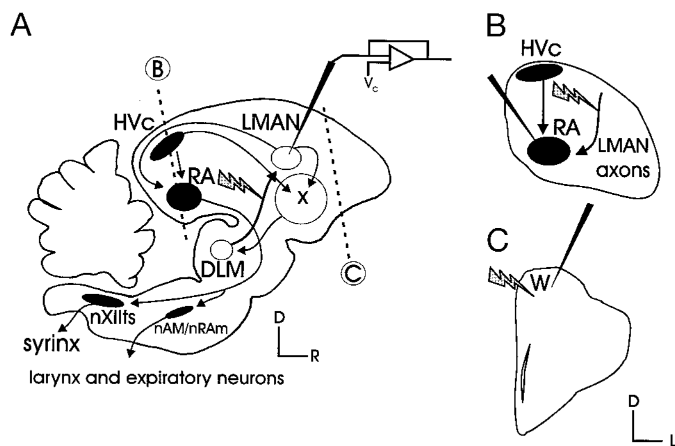

FIG. 1. Schematic of the song system and the brain slice preparations used to record *N*-methyl-D-aspartate–excitatory postsynaptic currents (NMDA-EPSCs) in lateral part of the magnocellular nucleus of the anterior neostriatum (LMAN), robust nucleus of the archistriatum (RA), and the Wulst (W). *A*: sagittal view of the zebra finch brain; the vocal motor pathway for learned song production (black) includes HVc, RA, the hypoglossal motoneurons (nXIIts), and respiratory areas [n. ambiguus and retroambigualis (nAm and nRAm)]. The anterior forebrain pathway (white) is implicated in song learning and includes area X, the thalamic nucleus dorsolateral part of the medial thalamus (DLM) and LMAN. NMDA-EPSCs were elicited in LMAN by electrically stimulating afferents from DLM at the site marked by the lightning bolt. *B* and *C*: coronal slices were made at the planes of section shown in *A* to record NMDA-EPSCs either from RA or from the Wulst; stimulation sites are shown by the lightning bolt. D, dorsal; R, rostral; L, lateral.

the song system. Testosterone is one factor that affects song learning, because young zebra finches treated with testosterone during early song learning have shorter songs and a reduced number of song syllables in adulthood (Korsia and Bottjer 1991). Further, endogenous androgen levels fluctuate during song development (Prove 1983), and LMAN and RA neurons contain androgen receptors, which provide a means for androgens to directly alter these neurons (Balthazart et al. 1992). Given that exogenous androgens disrupt early song learning, and that NMDA-EPSCs in LMAN are crucial for sensory acquisition (Basham et al. 1996b) and become faster during development (Livingston and Mooney 1997), we investigated whether NMDA-EPSCs are androgen sensitive. In addition to LMAN, we examined NMDA-EPSCs in RA; because it is the site of integration of activity from HVc and LMAN (Mooney and Konishi 1991; Nottebohm et al. 1982), it receives inputs from LMAN that are primarily mediated by NMDA receptors (Kubota and Saito 1991; Mooney and Konishi 1991; Stark and Perkel 1999), and its neurons contain androgen receptors (Balthazart et al. 1992).

A major finding is that exogenous androgens cause NMDA-EPSCs to become faster in LMAN and RA, but only during a developmental period that correlates with sensitive periods for song learning. These results extend previous work in the song system on sex steroid modulation of song behavior and neuronal morphology, by showing that androgens modulate NMDA receptor–mediated synaptic transmission in song nuclei. In addition, androgens have differential effects on glutamatergic synapses within the song system because they also altered AMPA-EPSCs, total dendritic length, and spine density in LMAN, but not in RA. These data provide the first evidence for an effect of sex steroids on synaptic transmission within the song system and suggest a potential mechanism for limiting sensitive periods for song learning.

METHODS

Subjects

Experiments were performed using brain slices made from male zebra finches, in accordance with a protocol approved by the Duke University Institutional Animal Care and Use Committee. Finches were obtained from our breeding colony where they were raised on a 14-h day:10-h night light cycle. We defined three age groups for these studies in LMAN and RA: fledglings (*PHD 21–32*), juveniles (*PHD 38–49*), and adults (>*PHD 90*). Briefly, these ages were chosen because ~*PHD 20* constitutes the onset of sensory acquisition in zebra finches, and birds between *PHD 38* and *49* in our colony are in the early stages of sensorimotor learning (see Livingston and Mooney 1997 for further details). By 90 days, male zebra finches have stereotyped songs. For developmental studies in the Wulst, data from juvenile and adult time points were combined (age range, *PHD 40–117;* mean, *PHD 73*) and were referred to as being from juveniles.

Hormonal manipulations

To increase androgen levels, birds were implanted with 2-mm pellets made of RTV sealant (Dow Corning, Midland, MI) containing ~50 μg of either testosterone (Steraloids, Wilton, NH) or 5α-dihydrotestosterone (DHT; Steraloids); control pellets contained RTV sealant alone. The pellets were placed subcutaneously over the pectoral muscle, and the incision was closed with cyanoacrylate (Elmer's Products, Columbus, OH) and dressed with antibiotic ointment (Neosporin, Warner-Lambert, Morris Plains, NJ).

To reduce steroid levels, young zebra finches (*PHD 12–17*) were castrated and chronically treated with 50 μg flutamide (Sigma, St. Louis, MO), an androgen receptor antagonist, delivered through RTV pellets prepared as described for the steroid treatments. For the gonadectomy, birds were anesthetized via intramuscular injection with 35 μl of Equithesin [3–5 μl/g body mass; 1.05% pentobarbitol sodium (Abbott Laboratories, Chicago, IL), 4.25% chloral hydrate (Sigma), 7% EtOH (AAPER, Shelbyville, KY), 36% propylene glycol (Sigma), 2.1% $MgSO_4$ (Mallinkrodt, Mundelein, IL)]. A small incision was made on the lateral wall of the body cavity, between the ribs that overlie the gonads. Testes were aspirated with a custom-fabricated glass pipette and the aid of a dissecting microscope (Zeiss, Germany). The incision was closed with cyanoacrylate and dressed with antibiotic ointment. Additionally, tetracycline (1 mg/ml) was supplied in the drinking water for 48 h postsurgery. Birds were reimplanted with flutamide pellets at 10-day intervals, following castration. Absence of gonadal tissue was visually confirmed on sacrifice with the aid of a dissecting scope, but was not histologically confirmed.

Testosterone radioimmunoassay

Blood was collected following decapitation between 10:00 and 11:00 a.m. Samples were briefly stored on ice, and then centrifuged to isolate plasma. Testosterone was measured directly by radioimmunoassay using HPLC-purified ^{3}H-Testosterone (Dupont-New England Nuclear, Wilmington, DE), as well as antiserum and HPLC-purified standards (ICN Biomedicals, Costa Mesa, CA). To facilitate the detection of low plasma levels of testosterone, all samples (50 μl) were spiked with 100 pg of testosterone, a value that was in the midrange of the standard curve. Standards and samples were extracted with ethyl acetate (Mallinkrodt), reconstituted in buffer (phosphate-buffered saline, 1g/l gelatin; Sigma), and incubated with antibody (1:56,000) and trace (10,000 cpm/test tube) for 1 h at 4°C for determination of free testosterone. Bound and free hormone was separated by the dextran-coated charcoal technique (see Nieschlag and Wick-

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ings 1978). Samples were counted by liquid scintillation spectrometry using scintillation fluid containing toluene (Mallinkrodt) and PPO-POPOP (Research Products International, Mount Prospect, IL). Reported values are the measured values minus the 100 pg spike. Pilot studies revealed that the testosterone implants augmented plasma testosterone levels over a 7- to 10-day period (data not shown).

Brain slices

The brain slice preparation procedure has been described previously in detail (Livingston and Mooney 1997; Mooney and Konishi 1991). Briefly, slices (400 μm) were cut from the same brain, in the sagittal orientation for LMAN and the coronal orientation for RA, and maintained on an interface-type holding chamber at room temperature. After ~2 h, slices were transferred to a superfusion chamber (24°C) for whole cell recordings. LMAN and RA were readily visualized under transillumination.

Electrophysiological recordings

Whole cell recordings were obtained in brain slices made from male zebra finches at fledgling, juvenile, and adult stages. Recording electrodes were positioned in either LMAN or RA with the aid of a dissecting scope (×40). Recording electrodes were made from 1.5-mm diameter borosilicate glass (VWR, West Chester, PA), pulled on a horizontal electrode puller (P-97, Sutter Instrument, Novato, CA) and filled with an internal solution consisting of (in mM) 3.19% (vol/vol) 50% D-gluconic acid (Sigma), 10 EGTA (Sigma), 5 $MgCl_2$ (Sigma), 40 HEPES (Fluka, Ronkonkoma, NY), 2 Na^+-ATP (Sigma), 0.3 Na^+-GTP (Boehringer-Mannheim, Indianapolis, IN), and 1 QX-314 (RBI/Sigma, Natick, MA), and the pH was adjusted to 7.25 with CsOH (50% g/ml H_2O; Aldrich Chemical Company, Milwaukee, WI). The final electrode impedances ranged from 2 to 5 MΩ. Whole cell currents were recorded with an Axopatch 1D (for LMAN) or an Axoclamp 2B (for RA) intracellular amplifier (Axon Instruments, Foster City, CA), and current traces were digitized at 10 kHz after low-pass filtering at 1–5 kHz. Glutamate EPSCs were recorded in 50 μM picrotoxin (Sigma); NMDA-EPSCs or AMPA-EPSCs were isolated with the addition of 5 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide disodium (NBQX, RBI/Sigma), or 100 μM D,L-2-amino-5-phosphonovaleric acid (D,L-APV, RBI/Sigma), respectively, accomplished by bath perfusion of the drug.

NMDA-EPSCs were electrically evoked while holding the membrane potential 20 mV more positive than the empirically determined EPSC reversal potential to remove the voltage-dependent blockade of the NMDA receptor by extracellular magnesium (Mayer et al. 1984; Nowak et al. 1984). AMPA-EPSCs from LMAN were recorded at the same membrane potential, but in RA, cells were held 80 mV negative of the reversal potential to increase the driving force, which was necessary to produce synaptic currents large enough for accurate quantification. Series resistance (<20 MΩ for LMAN, <30 MΩ for RA) was monitored throughout the recording by measuring the current transients resulting from small (2 mV) hyperpolarizing voltage pulses. For experiments that measured the ratio of the peak glutamate-to peak AMPA-EPSCs, only cells where the series resistance changed <20% during the course of drug application were used. Input resistance measurements were calculated by measuring the steady-state current resulting from the application of a small (2 mV) hyperpolarizing voltage pulse. Holding potentials were not corrected for the liquid junction potential. Electrode solutions used in recordings from a majority of animals contained neurobiotin (0.5%; Vector Laboratories, Burlingame, CA) in the internal solution to ensure that recorded cells were located within the appropriate nucleus (see below for histological methods).

Bipolar stimulating electrodes were either made from individual tungsten microelectrodes (Micro Probe, Clarksburg, MD), or were prefabricated concentric bipolar electrodes (FHC, Brunswick, ME). For LMAN, stimulating electrodes were placed in the thalamic fiber tract, which contains DLM axons that innervate LMAN (Livingston and Mooney 1997). For RA, which receives afferent input from both LMAN and HVc, we specifically activated LMAN-RA synapses by placing stimulating electrodes dorsolateral of RA in the LMAN fiber tract (see Mooney 1992). Synaptic responses were elicited at 0.1–0.3 Hz by applying a brief (100 μs) electrical stimulus (5–750 μA) to the thalamic axons that innervate LMAN or the LMAN axons that innervate RA (Mooney 1992). Stimulus intensity was adjusted to generate an evoked EPSC with consistent amplitude that was monosynaptic, i.e., the interval between the stimulus artifact and the onset of the synaptic current was <5 ms, and the rising and falling phases of the synaptic current appeared smooth and monotonic. In LMAN, this approach resulted in EPSC peak amplitudes that did not vary across age or treatment. In RA, EPSC amplitudes were lower in slices from younger animals because lower stimulus intensities were required to avoid recruiting polysynaptic responses. We did not test for statistical significance of any differences in the peak amplitudes of evoked EPSCs because they varied depending on the slice preparation, the stimulating electrodes, and the stimulus intensity.

Data acquisition and analysis

Data acquisition and analysis for intracellular recordings were performed with a National Instruments (Austin, TX) data acquisition board (AT-MIO-16E2), controlled by custom Labview software written by F. Livingston and R. Neummann. Five to 10 individual events were collected from a single neuron and digitally filtered (low-pass, 2 kHz) with an 8-pole Bessel filter and then averaged to obtain a representative cellular EPSC. The EPSCs shown in the figures are the averages of these cellular EPSCs for all the cells in each treatment group. The peak amplitude of the currents, the 10–90% rise time of the currents from baseline to peak amplitude, the time from the peak amplitude to $1/e$ of the amplitude, and the relative charge (measured by integrating currents with normalized peak amplitudes) were calculated using automated Labview software written by F. Livingston and S. White. Statistical analyses were conducted using JMP IN software (SAS Institute, Cary, NC). Nonparametric statistical tests were used because the data were not assumed to be normally distributed. Mann Whitney U tests were used to assess the significance between experimental and age-matched controls, as well as between two different developmental stages. In all cases, the minimum significance level was set at $P < 0.05$ using two-tailed comparisons. Only significant differences are reported in the text, unless otherwise noted. Averages are reported as means ± SE.

Song analysis

Song was analyzed by placing an individual male zebra finch in a sound-proof recording chamber (Industrial Acoustics Corporation, Bronx, NY) with a female finch. Digital audio recordings were collected using a National Instruments data acquisition board (AT-MIO-16E2) and Labview software written by R. Balu and F. Livingston. After 24 h, recordings were visually scanned for the presence of song. If no song was detected, then the animal remained in the chamber for another 24 h until a minimum of 30 bouts of song were collected. In one case, no song was detected until after the female finch was removed on the fourth day. Thirty examples of song were selected and imported into Avisoft Software (SASLab Pro 3.4, Raimond Specht, Berlin) for further analysis and generation of sonograms (frequency intensity vs. time). From these 30 renditions, one of us selected two representative 7-s sweeps for presentation to each of six observers.

Observers, blind to the experimental condition of the animal, were instructed to assign a song score ranging from 1 (very abnormal) to 5 (normal, adultlike) by listening to audio playback of the song and by visually inspecting the oscillograms (voltage vs. time) and sonograms. Observers were instructed to base their song quality judgments on the

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stereotypy of syllables and motifs. A song syllable was defined as a continuous marking on the song spectrogram. Mature zebra finch song is composed of one or more motifs that each contain the same stereotyped sequence of song syllables. One bout of song is typically composed of several introductory notes, followed by one or more motifs. Observers ranked the song quality of one juvenile male (*PHD 57*) in addition to the four castrates (>*PHD 200*) as well as one intact adult that had been housed in the same cage with the castrated animals from when they were *PHD 35*.

Morphology

To obtain filled neurons that were of sufficient quality for morphological analysis, sharp microelectrode intracellular recordings were made in LMAN and RA from brain slices (prepared as described above) that were held in an interface-type chamber (30°C, Medical Systems, Greenvale, NY). Electrodes were made from borosilicate glass pulled to a final resistance of 100–150 MΩ when filled with 3 M K-acetate (Mallinkrodt) and 4–8% neurobiotin. After establishing stable intracellular recordings, depolarizing currents (+0.5–1.5 nA, 1 s in duration at 2-s intervals) were applied for 20–40 min. The slice was fixed in 4% paraformaldehyde in 25 mM sodium phosphate buffer for at least 24 h at 4°C, resectioned on a freezing microtome or embedded in a gelatin-albumin mixture and cut on a vibratome at 75 μm, and visualized with avidin-HRP (diluted 1:100; Vector Laboratories, Burlingame, CA) and 3′3′-diaminobenzidine tetrahydrochloride (Sigma); the reaction was intensified with 1% $CoCl_2$ (Sigma) and 1% $NiSO_4(NH_4)_2SO_3$ (Sigma). Camera lucida drawings were made with the aid of a drawing tube attached to a Zeiss Axioskop, using a ×63 oil-immersion objective (nA 1.3).

Total dendritic length was measured from the camera lucida drawings of filled cells using a PlanWheel (Scalex, Carlsbad, CA). To determine the spine frequency, the total number of dendritic spines was counted for each individual neuron and then divided by the total dendritic length. To measure the extent of an individual cell's processes, a Sholl-like analysis (Sholl 1956) was performed from the drawings. Briefly, a series of evenly spaced concentric circles (20-μm increments) was placed over the cell drawing, centered around the soma, and the number of dendrites that intersected each of these circles was plotted as a function of radius from the cell body. For areal measurements of individual RA or LMAN cell bodies, drawings were scanned into a computer, and the borders of the soma were traced using Scion Image software (Scion Corporation, Frederick, MD) to convert pixel values into μm^2. No corrections were made for tissue shrinkage. Mann-Whitney *U* tests were used to compare the anatomic data from control and T-treated fledglings. For the Sholl analysis, two-way ANOVAs were used to test for any effects of radius or experimental group on the number of intersections.

RESULTS

NMDA-EPSCS in LMAN and RA change during development

To track developmental changes in NMDA receptors, we isolated NMDA-EPSCs at DLM-LMAN and LMAN-RA synapses in male zebra finches by applying NBQX (to block AMPA-EPSCs) and by holding the membrane potential 20 mV positive of the reversal potential (to relieve the voltage-dependent magnesium blockade). Whole cell recordings revealed that NMDA-EPSC decay times became markedly faster in LMAN and RA between fledgling, juvenile, and adult time points (Fig. 2). These faster currents were reflected by a decrease in the time it took the current to decay to 1/*e* of the peak amplitude (*e*-fold decay time), as well as by a decrease in the relative charge that was transferred by the current (Fig. 2*A*), which was measured as the area underneath the normalized current trace (see METHODS). Because these measurements generally covaried, only the *e*-fold decay times are reported here (but one exception is noted). The intrinsic and synaptic properties for LMAN and RA neurons throughout development are shown in Table 1. Below, significance values are reported in the text only when they do not appear in the figure legends.

Within LMAN, the *e*-fold decay times of NMDA-EPSCs decreased by ~50% over development, (94 ± 7 vs. 48 ± 4 ms,

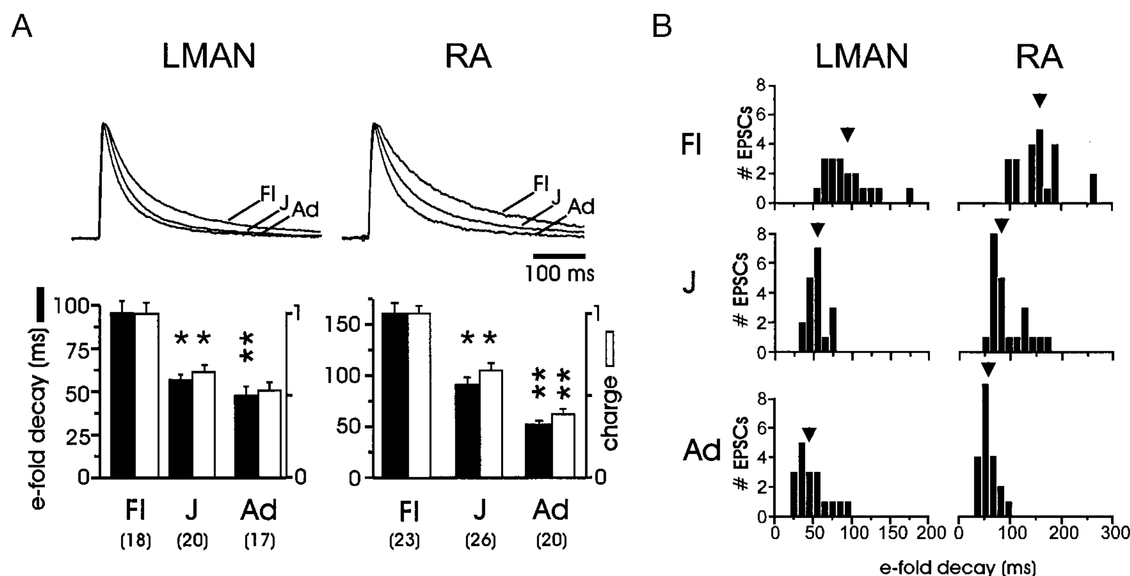

FIG. 2. NMDA-EPSCs in LMAN and RA become faster over the course of song development. *A*, *top*: average NMDA-EPSCs recorded in LMAN and RA at fledgling (Fl), juvenile (J), and adult (Ad) time points. *Bottom*: bar graphs show the *e*-fold decay times (■, left ordinate) and relative charge transfer (□, right ordinate) for the currents (mean ± SE) at these 3 times; the number of cells recorded in each case is shown in parentheses. * Significantly different from fledglings, $P < .0001$ (LMAN and RA). ** Significantly different from juveniles, $P < 0.03$ (LMAN), $P < .0001$ (RA). *B*: histograms show the distribution of NMDA-EPSC *e*-fold decay times at LMAN and RA synapses over development. Arrowheads mark the mean values.

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TABLE 1. *Intrinsic properties of LMAN and RA neurons: NMDA-EPSC experiments*

Group	Fledgling	Juvenile	Adult	Fledgling Control Implant	Fledgling Testosterone	Fledgling DHT	Juvenile DHT	Adult DHT
				LMAN				
Age (PHD)	25.7 (21–32)	45.3 (41–49)	128.3 (107–166)	24.2 (22–26)	25.4 (20–29)	25.1 (23–28)	44.9 (44–46)	115 (108–132)
n: cells, animals	18, 8	20, 10	23, 11	29, 12	25, 10	18, 8	19, 4	15, 6
Peak amplitude*, pA	106 ± 15	124 ± 20	121 ± 20	83 ± 10	106 ± 11	110 ± 17	114 ± 14	129 ± 22
10–90% rise time, ms	6.5 ± 0.5	5.6 ± 0.4	3.9 ± 0.2† (J)	6.9 ± 0.5	6.2 ± 0.4	5.3 ± 0.2‡ (Ctr)	4.6 ± 0.2§ (J)	4.9 ± 0.2
Input resistance, MΩ	104 ± 10	114 ± 10	100 ± 11	112 ± 11	86 ± 14§ (Ctr)	114 ± 12	92 ± 15	82 ± 9
e-fold decay, ms	94 ± 7	56 ± 3† (Fl)	48 ± 4§ (J)	104 ± 8	68 ± 4† (Ctr)	60 ± 3† (Ctr)	48 ± 2§ (J)	44 ± 4
				RA				
Age (PHD)	24.8 (21–30)	43.0 (38–49)	135.0 (90–387)	24.8 (21–28)	24.8 (22–28)	24.1 (22–29)	45.0 (44–46)	117.5 (108–132)
n: cells, animals	23, 11	26, 10	20, 11	31, 7	24, 11	23, 9	17, 4	15, 6
Peak amplitude*, pA	41 ± 5	93 ± 13	100 ± 12	53 ± 3	54 ± 8	52 ± 5	72 ± 9	65 ± 6
10–90% rise time, ms	9.5 ± 0.5	7.0 ± 0.4† (Fl)	5.8 ± 0.4§ (J)	10.6 ± 0.8	9.2 ± 0.7	9.4 ± 1.1§ (Ctr)	6.8 ± 0.5	6.9 ± 0.6
Input resistance, MΩ	238 ± 18	150 ± 11† (Fl)	138 ± 18	264 ± 20	219 ± 13	261 ± 28	221 ± 16‡ (J)	133 ± 9
e-fold decay, ms	159 ± 10	91 ± 7† (Fl)	58 ± 3† (J)	159 ± 10	117 ± 7‡ (Ctr)	124 ± 9§ (Ctr)	70 ± 6§ (J)	64 ± 4

Values are means ± SE. Numbers in parentheses are ranges. LMAN, lateral part of the magnocellular nucleus of the archistriatum; RA, robust nucleus of the archistriatum; NMDA, *N*-methyl-D-aspartate; EPSC, excitatory postsynaptic current; DHT, 5α-dihydrotestosterone; PHD, posthatch day; J, juvenile; Ctr, control; Fl, fledgling. * See METHODS for statistics. † $P < 0.0005$. ‡ $P < 0.005$. § $P < 0.05$. Letters in parentheses indicate comparison group.

mean ± SE; Fig. 2.) Almost all (~90%) of this shift took place between fledgling and juvenile life. However, a small decrease in *e*-fold decay time was revealed between juveniles and adults (56 ± 3 vs. 48 ± 4 ms). This small change in NMDA-EPSCs was not apparent in the relative charge transfer (Fig. 2*A*), nor seen in an earlier study that used double exponential fits to describe the EPSCs (Livingston and Mooney 1997). Distributions of *e*-fold decay times demonstrated that the range of values narrowed over development (Fig. 2*B*). The decrease in NMDA-EPSC *e*-fold decay times that occurred between fledgling and juvenile ages was not accompanied by changes in EPSC rise times or in the input resistances of LMAN neurons (Table 1). These data indicate that the changes in *e*-fold decay times are not part of a more general alteration of neuronal electrotonic properties (see DISCUSSION). In summary, these results show that developmental changes in NMDA-EPSCs within LMAN occur predominantly during early posthatch development (Livingston and Mooney 1997), before sensorimotor learning is complete.

In RA, NMDA-EPSCs also became faster over development, as the mean *e*-fold decay times declined by almost two-thirds between fledgling and adult life (159 ± 10 vs. 58 ± 3 ms; Fig. 2). These developmental changes were not only greater in magnitude than those seen in LMAN, but were also more protracted: in RA, close to one-third of the total change in *e*-fold decay times occurred between juvenile and adult life (i.e., 91 ± 6 vs. 58 ± 3 ms). Similar to the results obtained in LMAN, the distribution of *e*-fold decay times in RA narrowed markedly over development (Fig. 2*B*). In contrast to observations made in LMAN, the rise times of NMDA-EPSCs decreased at each developmental time point, and RA neuronal input resistance decreased between fledgling and juvenile life (Table 1). In summary, NMDA-EPSCs become faster in both RA and LMAN between fledgling and adult life, but the magnitude and timing of these changes differs between the two nuclei. Additionally, other cellular changes occur within RA over development.

Androgens hasten NMDA-EPSC maturation in fledglings and juveniles

In many types of songbirds, exogenous steroids produce dramatic effects on both song behavior and the morphology of song control nuclei (for review see Bottjer and Johnson 1997). In zebra finches, androgen treatment of juvenile males at early time points (<*PHD 40*) perturbs song development (Korsia and Bottjer 1991). One hypothesis is that androgens affect synaptic transmission in LMAN and RA, making NMDA-EPSCs faster, which would be reflected by decreases in the *e*-fold decay times. If such effects play a role in song learning, then they should occur during the same developmental periods when androgens affect song behavior. As a first test of these ideas, we implanted young birds (~*PHD 15*) with either testosterone-containing or blank pellets and examined NMDA-EPSCs in LMAN and RA neurons 10 days later (~*PHD 25*).

This chronic testosterone (T)-treatment induced premature changes in NMDA-EPSCs within both LMAN and RA (Fig. 3). In LMAN, T-treated fledgling birds had NMDA-EPSCs that were faster than those of age-matched control-implanted birds, as reflected by a 35% decrease in the mean *e*-fold decay time (68 ± 4 vs. 104 ± 8 ms; Fig. 3*A*). The NMDA-EPSC decay times in T-treated fledglings were equivalent to those in normal juvenile birds (68 ± 4 vs. 56 ± 3 ms, $P = 0.09$). In RA, NMDA-EPSCs from T-treated fledglings were also faster than those of control-implanted fledglings, with a 26% decrease in *e*-fold decay times (117 ± 10 vs. 159 ± 8 ms; Fig. 3*A*), but were not as fast as those of juvenile birds. In both LMAN and RA, T-treatment narrowed the distribution of *e*-fold decay times, similar to the tightened distribution that emerges during normal maturation (Fig. 3*B*). In LMAN, T-treatment produced a slight decrease in neuronal input resistance relative to control-implanted fledglings, but this decrease was not observed with DHT treatment (Table 1, see below regarding DHT treatment). In RA, T-treatment made NMDA-EPSCs faster relative to control fledglings, without the concomitant decrease in input resistance observed between fledglings and juveniles. It should be noted that in subsequent experiments, age-matched controls

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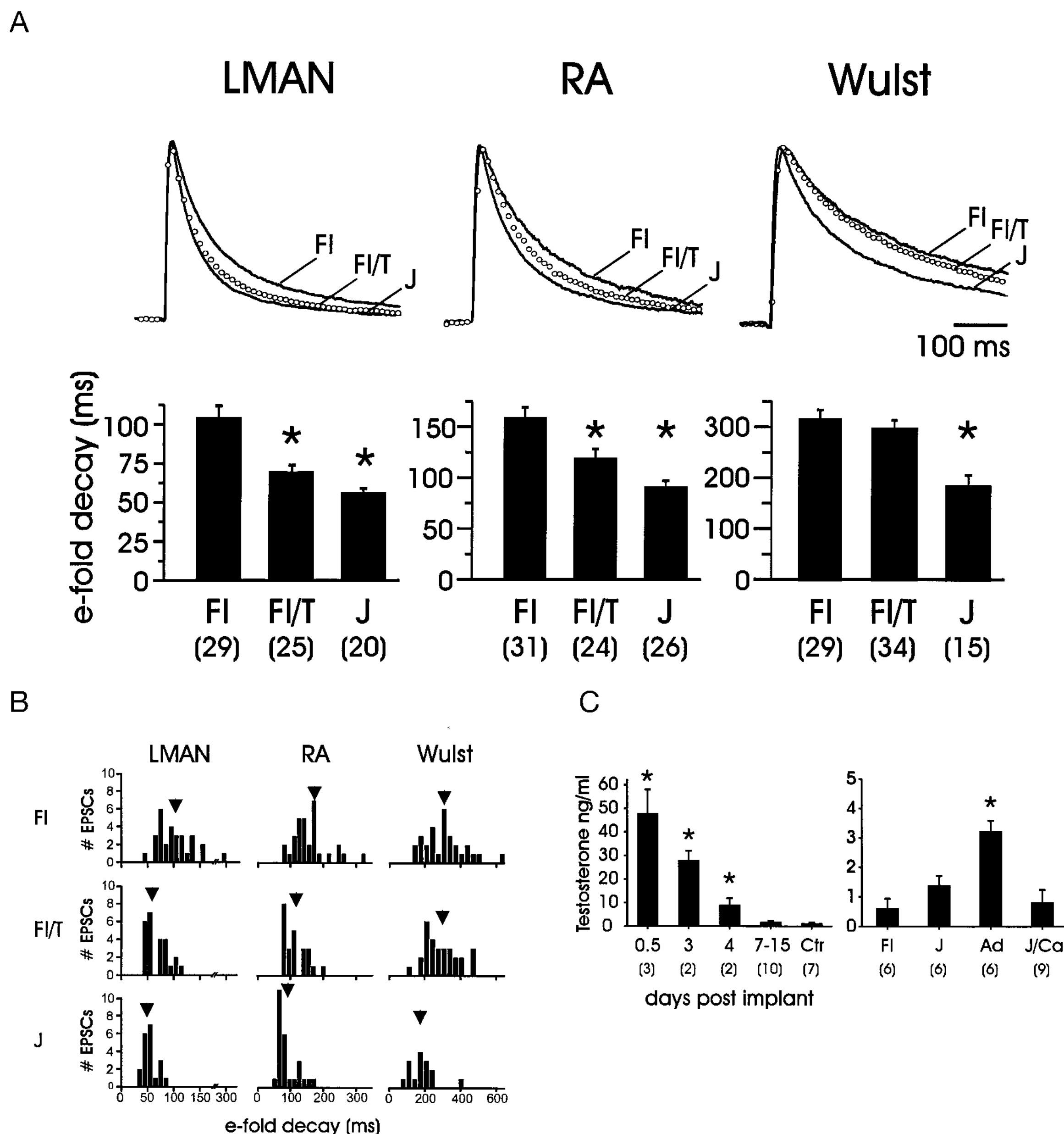

FIG. 3. Testosterone accelerates the maturation of NMDA-EPSCs in the song nuclei LMAN and RA, but not in the Wulst (a brain area not involved in singing), during the 1st month of posthatch life. *A*, *top*: average NMDA-EPSCs recorded in LMAN, RA, and the Wulst from fledglings [Fl; approximately *posthatch day 25* (∼*PHD 25*)], testosterone-treated fledglings (Fl/T; ∼*PHD 25*), and juveniles (J; ∼*PHD 45*). For the FL and FL/T groups, pellets were implanted in young birds (∼*PHD 15*), and recordings were made 10 days later. Control birds used for LMAN and RA recordings contained blank pellets, but those used for the Wulst did not. EPSCs from testosterone-treated groups are depicted with open circles for clarity. *Bottom*: histograms show the *e*-fold decay times (mean± SE) for these currents; * significantly faster than fledgling controls [Fl/T vs. Fl: $P < 0.0001$ (LMAN); $P < 0.002$ (RA). J vs. Fl: $P < 0.0001$ (LMAN and RA); $P < 0.0002$ (Wulst)]; the number of cells recorded in each group is shown in parentheses. *B*: distributions of *e*-fold decay times measured at LMAN, RA, and Wulst synapses in testosterone-treated and control birds. Means are marked by arrowheads. *C*, *left*: plasma testosterone levels in *PHD 25* male zebra finches determined by radioimmunoassay 0.5–15 days following pellet implantation. * Significantly higher T levels relative to control-implanted fledglings (Ctr). *Right*: plasma testosterone levels in normal fledgling, juvenile, and adult male zebra finches and in juvenile castrates (J/Ca). * Significantly higher T levels relative to fledglings

were not implanted with blank pellets, because there was no observed difference in any of our measures between control-implanted and unimplanted fledglings in LMAN and RA (Table 1).

Androgen receptors within LMAN and RA neurons provide a direct mechanism to mediate the effects seen here. However, it is possible that androgens affect NMDA-EPSC development in many types of telencephalic neurons, even those lacking androgen receptors, via an indirect mechanism (see DISCUSSION and Fig. 7). To test whether these androgen effects could occur in neurons devoid of androgen receptors, we recorded NMDA-EPSCs in the Wulst, an avian telencephalic visual area that lacks androgen receptors and is not implicated in song development (Balthazart et al. 1992). As in LMAN and RA,

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NMDA-EPSCs within the Wulst became faster between fledgling and juvenile time points (312 ± 19 ms, $n = 23$ vs. 185 ± 27 ms, $n = 15$; Fig. 3, *A* and *B*). In contrast to LMAN and RA, however, in the Wulst, T-treatment of young birds (implanted ~*PHD 15,* recorded 10 days later, ~*PHD 25*) did not modulate NMDA-EPSCs (mean *e*-fold decay time in T-treated fledglings: 306 ± 27 ms; $n = 34$ cells, $P = 0.90$ relative to control). Thus although NMDA-EPSCs become faster in both song and nonsong areas over development, androgens do not globally influence this transition throughout the avian telencephalon.

Steroid hormones can exert short-term effects on neuronal excitability via nongenomic pathways, as well as long-term effects that require changes in gene expression. Although we implanted testosterone (~*PHD 15*) and then recorded NMDA-EPSCs 10 days later, it is possible that androgens exerted their actions within a much shorter time frame. Therefore we implanted fledgling birds (~*PHD 24.5*) with testosterone 12 h (rather than 10 days) before recording, and then compared the EPSC *e*-fold decay times to those of untreated age-matched controls. Unlike the effects of longer androgen exposure, the 12-h treatment did not alter EPSC *e*-fold decay times in either LMAN or RA (LMAN: 12 h = 99 ± 12 ms, $n = 10$, control = 104 ± 8 ms, $n = 29$, $P = 0.58$; RA: 12 h = 157 ± 15 ms, $n = 14$, control = 159 ± 10, $n = 31$, $P = 0.94$), even though a radioimmunoassay confirmed that the implants significantly elevated serum androgen levels before recording (Fig. 3*C*).

Because endogenous neuronal aromatases can convert testosterone to 17β-estradiol (Schlinger 1997), we used the non-aromatizable androgen DHT to confirm that the effects of testosterone were androgen-specific. As with T-treatment, young birds were implanted with DHT (~*PHD 15*), and recorded from 10 days later (~*PHD 25*). Indeed, this chronic DHT-treatment produced faster EPSCs in both LMAN and RA as reflected in a 42 and 22% decline, respectively, in the *e*-fold decay times from control values to those of DHT-treated fledglings (LMAN: 104 ± 8 vs. 60 ± 3 ms, RA: 159 ± 10 vs. 124 ± 9 ms; Table 1). In LMAN, the EPSC *e*-fold decay times in DHT-treated fledglings were indistinguishable from normal juveniles (60 ± 3 vs. 56 ± 3 ms, $P = 0.26$) but in RA, EPSC *e*-fold decay times from DHT-implanted fledglings were still slower than those of juveniles (124 ± 9 vs. 91 ± 6 ms, $P < 0.005$). In both LMAN and RA, DHT-treatment produced a slight decrease in EPSC rise times, an effect not seen with T-treatment (Table 1). In LMAN, DHT did not alter the input resistance, although testosterone did (Table 1). Except for these small differences, DHT caused similar effects to those of testosterone, which suggests that androgens, rather than estrogens, are responsible for the faster NMDA-EPSCs seen here.

Song development is disrupted by exogenous testosterone administered as late as *PHD 40* (Korsia and Bottjer 1991). To test whether changes in NMDA-EPSCs within LMAN and RA are also sensitive to such later treatment, recordings were made from juvenile male finches (~*PHD 45*) that had received DHT implants 10 days earlier (~*PHD 35*). DHT was chosen to ensure that any observed effects were androgenic and not due to estrogens. In parallel with the effects seen at earlier ages, this later treatment yielded NMDA-EPSCs that decayed more quickly than those of age-matched controls (Fig. 4). In LMAN, the *e*-fold decay times of NMDA-EPSCs in DHT-treated juveniles were slightly (14%) faster than in normal juveniles (48 ± 2 vs. 56 ± 3 ms; Fig. 4) and were equivalent to adult

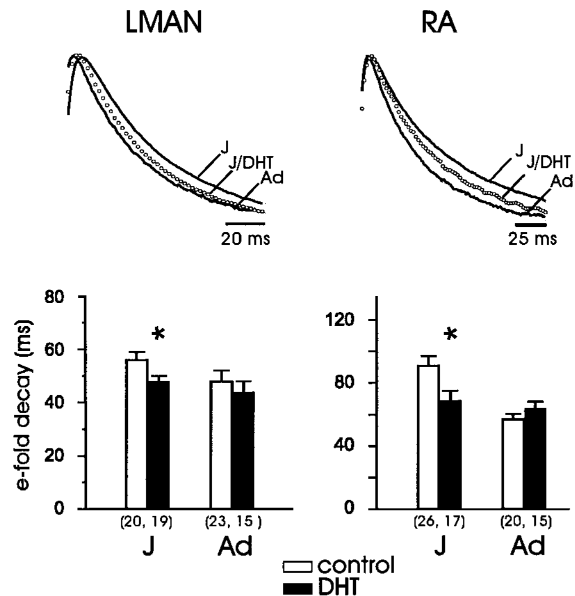

FIG. 4. NMDA-EPSCs are modulated by androgen treatment in juveniles but not in adults. 5α-Dihydrotestosterone (DHT) pellets were implanted 10 days before recordings made at ~*PHD 45* for DHT-treated juveniles (J/DHT) and >*PHD 100* for DHT-treated adults. *Top*: average traces from LMAN (*left*) or RA (*right*) of NMDA-EPSCs from juvenile (J), and adults (Ad); J/DHT EPSCs are shown with open circles. *Bottom*: histograms show the average *e*-fold decay times (mean ± SE) for each condition shown above, plus adults treated with DHT; the number of cells in each case is shown in parentheses. Solid bars represent animals treated with DHT; * significantly faster than juvenile controls ($P < 0.05$). Androgen treatment did not alter decay times in adults (LMAN, $P = 0.82$; RA, $P = 0.15$).

values (48 ± 4 ms; $P = 0.22$ relative to juvenile-DHT birds). In RA, DHT-treated juveniles had NMDA-EPSCs that were also faster than those of normal juveniles (70 ± 6 vs. 91 ± 6 ms; Fig. 4) and equivalent to those of adults (64 ± 4 ms, $P = 0.10$; Fig. 4); this treatment additionally resulted in higher input resistances of RA neurons relative to controls (Table 1). In summary, NMDA-EPSCs in both LMAN and RA are sensitive to androgen treatment during the developmental period when these currents normally become faster, when endogenous androgen levels fluctuate (Prove 1983), and when exogenous androgen disrupts song learning (Korsia and Bottjer 1991).

Endogenous androgens could provide an intrinsic signal regulating the maturation of NMDA-EPSCs in LMAN and RA, because testosterone levels increase over the course of song development in male zebra finches (Adkins-Regan et al. 1990; Prove 1983). To confirm these hormonal changes, we measured plasma testosterone levels from a subset of the animals from which electrophysiological recordings were made (Fig. 3*C*). Adult testosterone levels (3.2 ± 0.4 ng/ml) were higher than those of juveniles (1.4 ± 0.3 ng/ml). Although mean values for fledglings (0.6 ± 0.3 ng/ml) were half of the juvenile measure, these two groups were not significantly different ($P = 0.95$).

Adult NMDA-EPSCs are not altered by androgens

To test whether exogenous androgens affect NMDA-EPSCs throughout life or, instead, only during a restricted developmental period, adult birds (>*PHD 90*) were implanted with DHT pellets and then recorded from 10 days later. In both

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LMAN and RA, this adult DHT-treatment did not affect NMDA-EPSC *e*-fold decay times (see Fig. 4 and Table 1). This lack of effect demonstrates that NMDA-EPSCS are sensitive to exogenous androgens only during juvenile life, and that the transition to adultlike NMDA-EPSCs is the final step both for development and for our experimentally induced androgen-mediated effects. That this limit exists experimentally suggests that the androgen sensitivity of the NMDA-EPSCs seen in young animals is not merely due to pharmacological actions of the steroid. Thus in the song system, androgen treatment can modulate NMDA-EPSCs during a fledgling and juvenile sensitive period that overlaps with periods when song learning can be disrupted by exogenous androgens (Fig. 4 and Table 1).

NMDA-EPSCs development in castrates

In addition to being sensitive to exogenous androgens, normal development of NMDA-EPSCs within LMAN and RA might be regulated by endogenous androgens, i.e., androgen dependent. If so, in the absence of androgens, NMDA-EPSCs should remain in the fledgling state. To test whether developmental changes in NMDA-EPSCs were androgen dependent, zebra finch chicks were castrated ~*PHD 14* and chronically treated with the androgen receptor antagonist flutamide (implanted every 10 days until recordings were made). Previous work has shown that similar treatment produces aberrant song in a majority of adults (Bottjer and Hewer 1992). We were not able to assess any deleterious effects of early castration and flutamide treatment on song behavior in juveniles because, at that age, song is highly variable and not well-developed (Immelmann 1969). Nevertheless, we initially tested the androgen dependency of NMDA-EPSC *e*-fold decay times in slices made from juvenile animals, rather than adults, because this is an age when the currents are androgen sensitive. We found no differences in *e*-fold decay times in either LMAN or RA between intact and castrated juveniles (LMAN: juvenile = 56 ± 3 ms, n = 20, castrate = 57 ± 5 ms, n = 16, P = 0.81; RA: juvenile = 91 ± 6 ms, n = 26, castrate = 94 ± 5 ms, n = 41, P = 0.35). To test whether the effects of castration emerged at later time points, we also compared *e*-fold decay times between castrated adults and age-matched controls. Once again, however, no difference was observed between intact and castrated animals (LMAN: adult = 48 ± 4 ms, n = 23, adult castrate = 54 ± 3 ms, n = 20, P = 0.09; RA: adult 58 ± 3 ms, n = 20, adult castrate = 70 ± 8 ms, n = 16, P = 0.20).

A potential confound is that, although it is relatively easy to augment androgen levels, it is difficult to abolish them in zebra finches (Adkins-Regan et al. 1990; Marler et al. 1988). Indeed, a radioimmunoassay revealed that our juvenile and adult castrates had serum testosterone levels similar to age-matched controls (see Fig. 3*C,* adult castrate data not shown). Thus despite the absence of observable gonadal tissue, significant levels of androgens were present in the blood of castrates, presumably arising from extra-gonadal sources (Adkins-Regan et al. 1990).

Despite the lack of effect of castration on plasma testosterone levels, flutamide levels may have been sufficient to block receptor-mediated actions of extragonadal androgens. To determine whether our protocol of flutamide treatment had prevented androgen signaling, we analyzed several androgen-sensitive features of songbirds. We measured syringeal mass (Luine et al. 1980), frequency of singing (Arnold 1975), and song quality (Bottjer and Hewer 1992) in four surviving adults from the castrate group. Syringeal weights were normalized for body weight to derive a syringeal-somatic index (SSI; syrinx weight/body weight × 100). SSIs were lower in these four successful castrates compared with normal adults (0.11 ± 0.01, n = 4, vs. 0.20 ± 0.01, n = 10, $P < 0.01$), and also compared with intact fledglings (0.13 ± 0.01, n = 13, $P < 0.02$ as compared with adult castrates). These SSI data indicate that flutamide treatment achieved at least a partial peripheral block of androgen action in each of the four adult castrates.

In contrast to the somatic effects, behavioral measures did not reflect consistent effects among flutamide-treated castrates. To obtain 30 renditions of song for the behavioral analyses, three of the four castrates required more than two recording sessions. The fourth castrate (*Blue 7*) required just 2 days, as did the intact adult and the juvenile. Song quality (1–5) of the four castrates and the two intact males was judged by six observers blind to the experimental group. By these evaluations, the song quality of *Blue 7* as well as that of another castrate was deemed normal (above 4), whereas those of two castrates and of a control juvenile were ranked as disrupted relative to the song of an untreated adult. These behavioral data from castrates, although from a small sample size, are similar to those of Bottjer and Hewer (1992), which indicate that castration and flutamide treatment are ineffective in altering song quality in roughly one quarter of the cases. Given the presence of circulating androgens in our castrates (i.e., those that were used to determine NMDA-EPSC *e*-fold decay times; Fig. 3), one possibility for the lack of effect of castration on NMDA-EPSCs is that the flutamide treatment was not consistent in blocking central androgen receptors from these residual androgens (see DISCUSSION).

Androgen treatment alters AMPA-EPSCs in LMAN, but not in RA

Androgenic effects at LMAN and RA synapses could be specific to NMDA-EPSCs, or might involve more general actions on synaptic transmission at these sites. To address the specificity of androgenic actions, we examined another postsynaptic component of glutamatergic transmission, the AMPA-EPSC. Previous studies have demonstrated that synaptic transmission at DLM terminals onto LMAN neurons occurs via both NMDA and AMPA receptors (Livingston and Mooney 1997), whereas EPSPs at the LMAN terminals onto RA neurons are mediated largely by NMDA receptors (Mooney 1992). Similar to the previous experiments on NMDA-EPSCs, we measured the *e*-fold decay times of AMPA receptor–mediated EPSCs in fledgling controls and fledglings that had been implanted with DHT 10 days earlier (~*PHD 15*). Additionally, we tested whether the relationship between the AMPA-EPSC and the total glutamate-EPSC was altered by DHT. To do this, we first measured the peak amplitude of the glutamate-EPSC at 20 mV positive of the reversal potential, and then that of the pharmacologically isolated AMPA-EPSC (in 100 μM D,L-APV, see METHODS for details). We calculated the ratio of these two EPSC amplitudes (i.e., Glu_{peak}:$AMPA_{peak}$), and compared these values in control and DHT-implanted fledglings.

In LMAN, androgens affected the *e*-fold decay times of AMPA-EPSCs, reducing the mean by 30% (7.6 ± 0.6 ms vs. 5.4 ± 0.7 ms; Fig. 5, Table 2). There was also a difference in

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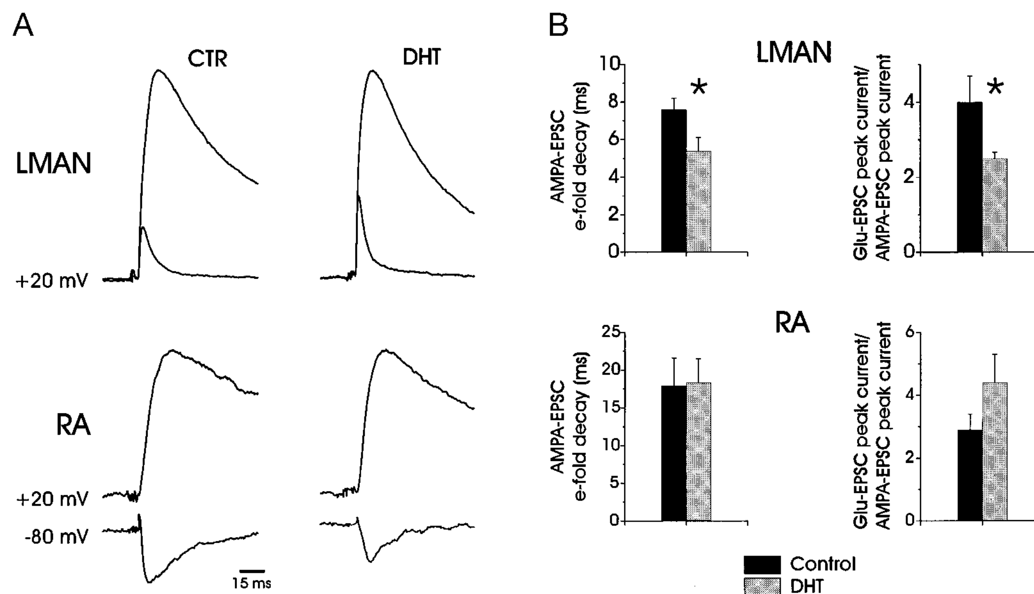

FIG. 5. Androgens decrease both the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprianate (AMPA)–EPSC *e*-fold decay times and the ratio of the peak glutamate-EPSC to the peak AMPA-EPSC (Glu_{peak}: $AMPA_{peak}$) in LMAN, but not in RA. Young birds (~*PHD 15*) were implanted with DHT pellets and recorded from 10 days later (~*PHD 25*). *A*, *top*: average glutamate-EPSCs and AMPA-EPSCs recorded in LMAN from controls (CTR) and DHT-implanted fledglings are superimposed; to illustrate the effect of DHT on Glu_{peak}: $AMPA_{peak,}$ the peak amplitudes of glutamate currents were normalized across the control and DHT-treated groups, while preserving the within-group Glu_{peak}: $AMPA_{peak}$. Glutamate currents were recorded 20 mV positive of reversal potential (+20 mV) in picrotoxin (50 μM), and AMPA currents were isolated by adding 100 μM D,L-2-amino-5-phosphonovaleric acid (D,L-APV). *Bottom*: the corresponding synaptic currents from RA are shown. However, because AMPA currents were too small to measure accurately at +20 mV, RA cells were clamped at 80 mV negative of the reversal potential (−80 mV) to produce larger currents. *B*: bar graphs show the average *e*-fold decay times (mean ± SE) and average Glu_{peak}: $AMPA_{peak}$ (mean ± SE) in LMAN (*top*) and RA (*bottom*). Data from DHT-treated animals are shown in gray, and control data are in black; * significantly different from control ($P < 0.05$).

the Glu_{peak}:$AMPA_{peak}$ between control and DHT-implanted fledglings (4.0 ± 0.7 vs. 2.5 ± 0.17; Table 2 and Fig. 5). In contrast, no differences were seen in either the AMPA-EPSC rise times or the input resistances (confirming our earlier findings, see Tables 1 and 2).

At the LMAN-RA synapse, following the application of D,L-APV, only a very small current (~4 pA) remained at +20 mV, consistent with previous observations at this synapse (Mooney 1992; Stark and Perkel 1999), which showed that nearly all of the synaptic response was mediated by NMDA receptors. Therefore we clamped the membrane potential to 80 mV negative of the reversal potential to increase the driving force and augment AMPA-EPSCs in RA. The peak amplitudes for these AMPA-EPSCs were then measured at this new holding potential, and the ratio of the peak amplitudes for glutamate (at +20 mV) and AMPA (at −80 mV) receptor–mediated synaptic currents was calculated. In RA, neither the *e*-fold decay times of AMPA-EPSCs nor the Glu_{peak}:$AMPA_{peak}$ from birds implanted with DHT differed from those of controls (Table 2 and Fig. 5). These results reveal that, in LMAN, androgens act more generally to alter both AMPA receptor and NMDA receptor–mediated synaptic transmission, whereas at the LMAN-RA synapse, androgen effects are specific to the NMDA receptor–mediated component of the synaptic current.

TABLE 2. *Intrinsic properties of LMAN and RA neurons: AMPA-EPSC experiments*

Group	Fledgling	Fledgling DHT
LMAN		
Age (PHD)	25.6 (22–28)	25.6 (21–29)
n: cells, animals	13, 10	15, 11
Peak amplitude, pA	39.0 ± 5.7	49.3 ± 7.1
10–90% rise time, ms	1.5 ± 0.1	1.8 ± 0.4
Input resistance, MΩ	114 ± 19	96 ± 11
e-fold decay, ms	7.6 ± 0.6	5.4 ± 0.7*
$Glutamate_{peak}$: $AMPA_{peak}$	4.0 ± 0.7	2.5 ± 0.2*
RA		
Age (PHD)	26.3 (23–28)	25.7 (23–29)
n: cells, animals	13, 10	16, 11
Peak amplitude (−80 mV), pA	14.4 ± 2.9	14.6 ± 3.0
10–90% rise time, ms	5.1 ± 1.0	5.9 ± 0.9
Input resistance, MΩ	204 ± 22	225 ± 26
e-fold decay, ms	17.9 ± 3.7	18.3 ± 3.2
$Glutamate_{peak}$: $AMPA_{peak}$	2.9 ± 0.5	4.4 ± 0.9

Values are means ± SE with age ranges in parentheses. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprianate; for other abbreviations, see Table 1. * $P < 0.05$.

Testosterone treatment alters neuronal morphology in LMAN, but not in RA

The sexual dimorphism of the song system (Nottebohm and Arnold 1976) is a compelling example of how sex steroids affect the structure of song nuclei, including neuronal morphology (DeVoogd and Nottebohm 1981; DeVoogd et al. 1985; Gurney 1981). The effects of augmenting early testosterone on LMAN and RA volume, neuron number, and soma size has been examined in adult male zebra finches (Schlinger and Arnold 1991), but no morphological analysis has been

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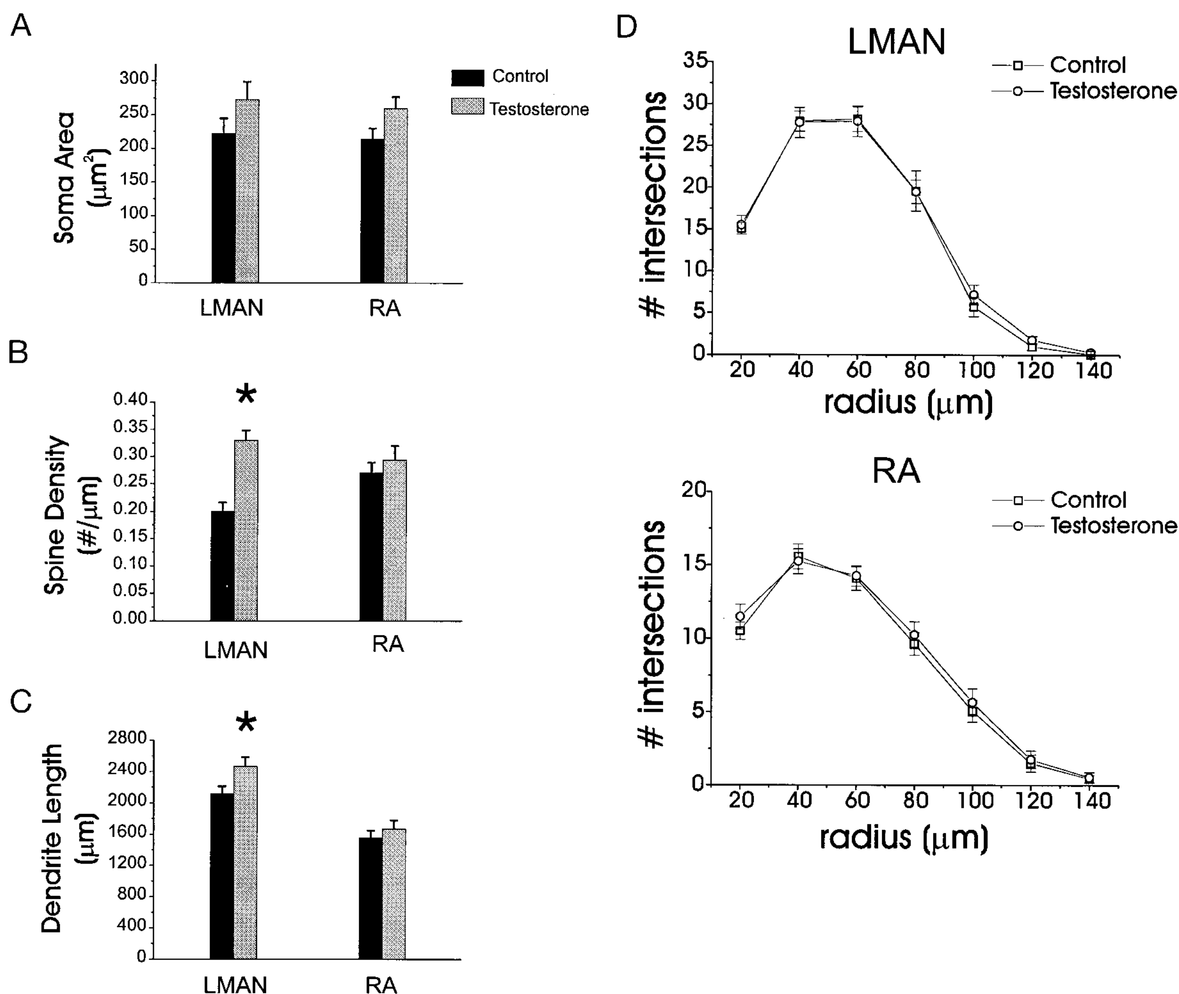

FIG. 6. Dendritic length and spine frequency change in LMAN, but not in RA, as a function of testosterone-treatment in fledgling male zebra finches. Young birds (∼*PHD 15*) were implanted with testosterone pellets and recorded from 10 days later (∼*PHD 25*). *A*: soma size was measured in controls (CTR) and T-treated fledglings (T) in LMAN (CTR: $n = 15$; T: $n = 18$) and RA (CTR: $n = 15$; T: $n = 18$) and was not affected by testosterone. *B*: spine frequencies were increased by testosterone in LMAN (CTR: $n = 15$; T: $n = 18$), but not in RA (CTR: $n = 15$; T: $n = 17$). *C*: dendritic length was also increased by testosterone in LMAN (CTR: $n = 15$; T: $n = 18$), but not RA (CTR: $n = 14$; T: $n = 17$). *D*: dendritic branching complexity was assayed using a Sholl analysis, which plots the number of dendritic branches (intersections) as a function of radial distance, in 20-μm intervals, from the soma. Testosterone did not alter branching complexity (2-way ANOVA) in either nucleus. * Significantly different from control, $P < 0.05$.

made at the earlier time points studied here. Because changes in dendritic or somatic morphology could exert electrotonic effects on synaptic currents, we assessed neuronal soma size, total dendritic length, dendritic branching complexity via a Sholl analysis (Sholl 1956), and spine density of LMAN and RA neurons in both control and T-treated fledglings (young birds implanted with testosterone ∼*PHD 15,* and recorded from 10 days later). Within LMAN, T-treatment had no effect on neuronal soma size or on dendritic complexity (Fig. 6*A* and *D*), although there was a slight effect on total dendritic length (T-treated = 2,464 ± 122 μm, control = 2,118 ± 92 μm; Fig. 6*C*). Within RA, androgen treatment had no effect on soma size, total dendritic length, or dendritic complexity (Fig. 6). Finally, measurements of dendritic spine densities revealed a striking contrast in the androgen sensitivity of LMAN and RA neurons. In LMAN, T-treatment resulted in a 64% increase in spine density relative to control animals (control = 0.20 ± 0.016, T-treated = 0.33 ± 0.08 spines per μm; Fig. 6*B*) but exerted no effect on spine density in RA. As with the contrasting effects between LMAN and RA on AMPA-EPSCs, these effects of androgens on spine density indicate a qualitative difference in the androgen sensitivity of LMAN neurons relative to those in RA.

DISCUSSION

Here we show that developmental changes in NMDA-EPSCs within the song system can be potently modulated by androgens during fledgling and juvenile periods, and we provide the first demonstration that sex steroids can alter synaptic transmission in the song system. Treatment of young zebra finches with testosterone caused NMDA-EPSCs in LMAN and RA to become faster. The nearly identical effect of treatment with DHT, a nonaromatizable androgen, indicates that androgens rather than estrogens mediate this effect. NMDA-EPSCs in LMAN and RA remain sensitive to exogenous androgens

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from fledgling through juvenile life, in parallel with behavioral studies that have shown that song development in zebra finches is disrupted by testosterone implants as late as *PHD 40* (Korsia and Bottjer 1991). In LMAN and RA, testosterone treatment of juveniles yielded NMDA-EPSC *e*-fold decay times that were as fast as those in adult animals. In contrast, exogenous androgens did not alter NMDA-EPSCs in adult animals, suggesting that there is a sensitive period for these steroid effects.

In addition to showing that androgens can modulate NMDA-EPSCs, our data confirm that NMDA-EPSCs become faster within LMAN during normal song development (Livingston and Mooney 1997), and reveal a similar developmental trend in RA. These observations add to those of others that document developmental changes in the physiological properties of LMAN neurons (Boettiger and Doupe 1998; Bottjer et al. 1998; Livingston and Mooney 1997). Further, In LMAN, NMDA-EPSCs become fast early in posthatch development, primarily during sensory acquisition and early sensorimotor learning. In RA, changes in NMDA-EPSC *e*-fold decay times are more protracted, extending further into the period of sensorimotor learning. Although developmental changes in NMDA-EPSCs also occurred in the Wulst, there this process was not altered by T-treatment. Thus androgens do not globally influence NMDA-EPSCs throughout the zebra finch telencephalon.

Whether the developmental changes in NMDA-EPSCs in LMAN and RA are androgen dependent remains unknown, because the castration did not lower endogenous androgens, and the efficacy of the flutamide treatment is not clear. If flutamide was completely effective in blocking androgen signaling, then this would suggest that NMDA-EPSC development, although sensitive to exogenous androgens, is not regulated by endogenous androgens, or in the absence of androgen signals, alternate mechanisms compensate for NMDA receptor development. Alternatively, endogenous androgens could be crucial for normal NMDA-EPSC development, but the early castration and flutamide treatment may not have blocked androgen signaling in all birds (Adkins-Regan et al. 1990; Bottjer and Hewer 1992). Although the low syringeal weight of each of the four adult castrates is consistent with a peripheral blockade or reduction of androgens (Luine et al. 1980), behavioral measures of androgen sensitivity such as song frequency (Arnold 1975) and song quality were variable between birds, similar to what was found in a previous study (Bottjer and Hewer 1992). These differing effects may indicate that peripheral and central androgen signaling have different androgen and/or flutamide sensitivities, and that central androgen receptors were activated in a subset of the castrates. If so, then the *e*-fold decays obtained following our castration protocol were gathered from a heterogeneous population of animals, some with continual blockade of androgen receptors, and others with persistent androgen signaling. Given the natural variability of *e*-fold decay times seen here, a contaminant population (potentially 25%, as suggested by behavioral data) within one experimental group would preclude our ability to detect differences in decay times between groups. In any case, the present results show that androgen treatment can functionally regulate NMDA-EPSCs, and provide one mechanism by which endogenous androgen signaling could alter synaptic development within the song system.

Androgen effects on NMDA-EPSCs are not well described in any system, even though steroids have been broadly implicated in the modulation of neuronal excitability. In electric fish, androgens and estrogens reciprocally modulate voltage-dependent sodium channels that underlie sexually dimorphic electric organ discharges (Dunlap et al. 1997; Ferrari et al. 1995), and in *Xenopus*, estrogens increase quantal content at laryngeal neuromuscular junctions (Tobias and Kelley 1995). In the mammalian CNS, estrogens can modulate synaptic transmission through a variety of mechanisms (Joels 1997), including the augmentation of NMDA receptor currents in the CA1 region of the rat hippocampus during estrus (Woolley et al. 1997). The present results suggest that in the zebra finch song system, androgens can regulate the development of NMDA-EPSCs by making them decay faster.

Although changes in NMDA-EPSC time course occur over development at a number of central synapses, the androgen sensitivity of these changes in LMAN and RA affords a previously unidentified means for regulating this process in the song system. In other vertebrate systems, stimulus-driven electrical activity plays a major regulatory role: in the visual system, dark-rearing of the young animal or TTX treatment of its cortex prevents the normal maturation of NMDA-EPSCs in cortical neurons (Carmignoto and Vicini 1992). In LMAN and RA, androgens could also affect NMDA-EPSC development through activity-dependent pathways, either by directly altering neuronal excitability in these areas or by inducing behavioral changes that then alter activity (such as increased singing with a concomitant increase in auditory stimulation, or increased attention to their own song or that of a tutor). In contrast, androgens could alter NMDA-EPSCs via activity-independent pathways that involve direct activation of steroid receptors in LMAN and RA neurons (Balthazart et al. 1992). These various possibilities for the actions of androgens are schematized in Fig. 7. The present findings do not allow us to discriminate between activity-dependent and -independent mechanisms. However, the NMDA-EPSC *e*-fold decay times were not altered within 12 h after steroid implantation, even though this treatment successfully elevated serum testosterone levels. This lack of effect argues against a fast action of androgens on NMDA receptors, although acute effects (approximately minutes) of another sex steroid, estradiol, have been seen on NMDA receptor–mediated potentials in rat hippocampal neurons (Foy et al. 1999). Further, the persistence of fast NMDA-EPSCs in steroid-implanted birds even days after their testosterone levels had returned to normal values (Fig. 3*C*) suggests that testosterone produces a lasting change in the functional properties of LMAN and RA neurons. Finally, androgens did not affect NMDA-EPSCs within the Wulst, an area devoid of androgen receptors (Balthazart et al. 1992), which indicates that local androgen receptors may be required to mediate the effects of androgens seen here. Together, these results are consistent with the idea that androgens achieve their effects through slow and long-lasting processes, such as altered gene expression.

In LMAN and RA, a likely mechanism for the change in NMDA-EPSC *e*-fold decay times is an altered pattern of NMDA receptor subunit expression. A major developmental transition in the mammalian brain involves an increase in the NR2A subunit relative to NR2B (reviewed in Flint et al. 1997; Scheetz and Constantine-Paton 1994). For example, in rat superior colliculus, the slower NMDA-EPSCs recorded at de-

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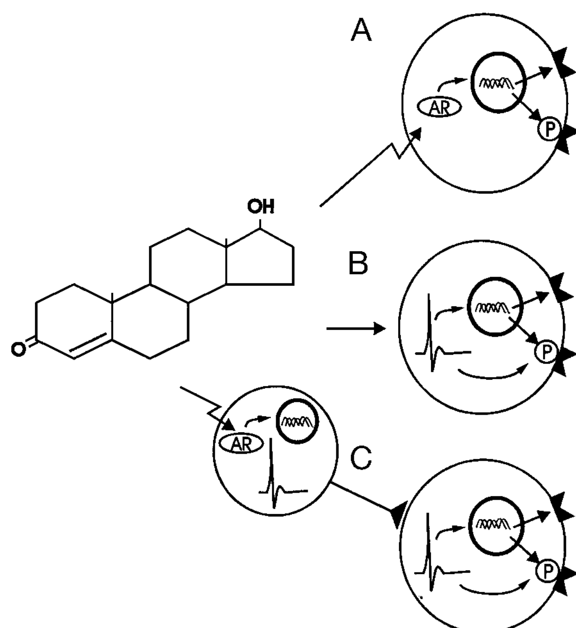

FIG. 7. Schematic indicating some of the mechanisms by which androgens could affect synaptic currents mediated by NMDA receptors (black polyhedrons) on LMAN and RA neurons. In each case, alterations of the currents could be produced by transcriptional changes of either NMDA receptor subunits or of another molecule that then makes posttranslational modifications to the NMDA receptor [e.g., via phosphorylation (P)]. These steroid effects could be activity independent as shown in *A,* where direct binding of androgens to their receptors (AR) within LMAN and RA neurons alters gene expression. NMDA-EPSCs could also be altered via changes in activity due to *B* androgen effects on membrane excitability of LMAN and RA neurons that then result in either genomic or nongenomic alterations to the NMDA receptor, or *C* androgenic effects on activity of neurons presynaptic to LMAN and RA which, in turn, would act through a mechanism as in *B*. Action potential signifies increased activity.

veloping synapses can be accounted for by the single-channel properties of the NMDA receptor (Hestrin 1992), and the subsequent development of faster NMDA-EPSCs is accompanied by elevated levels of NR2A subunit protein and transcript expression (Shi et al. 1997). As visual experience regulates changes in NMDA receptor subunit expression in the rat visual cortex (Quinlan et al. 1999), androgen-induced fluctuations in activity could trigger a subunit switch in song system nuclei. We have not yet examined the subunit composition of NMDA receptors on LMAN or RA neurons, but NR2B-containing receptors decline in LMAN over song development (Basham et al. 1999), and our results are consistent with the idea that androgens promote a relative increase in the expression of the NR2A subunit over NR2B. In addition to changes in subunit composition, posttranslational modifications of the NMDA receptor by phosphatases/kinases have been reported and could contribute to the changes in decay times seen here (Lieberman and Mody 1994; Tong et al. 1995).

Androgen-induced effects on NMDA-EPSCs are likely due to changes in the receptors themselves, and not to alterations of other cellular properties of song system neurons. Although changes in membrane resistance can have pronounced effects on EPSC decay times (Spruston et al. 1993), no changes in input resistances occurred within LMAN either over development or with DHT treatment. Changes in RA input resistances that do occur with development were not reproduced by androgen treatment (Table 1). Thus in RA, androgen treatment does not reproduce all of the developmental changes that occur in these neurons, but instead has more selective effects on NMDA-EPSCs. In addition, it should be noted that the morphological complexity of the neurons studied here could introduce space-clamp problems, which would interfere with the measurement of input resistance from distal dendritic regions. Altered dendritic filtering, which can arise from the remodeling of dendritic arbors, also can affect decay times of synaptic currents (Spruston et al. 1994). However, our Sholl analyses show that LMAN and RA dendritic structure does not change with androgen treatment (Fig. 6). Although in RA there is no change in dendritic length, there was a slight increase in LMAN. It is doubtful that this small (16%) increase in length could produce a significant increase in dendritic filtering, because dendritic lengthening would be expected to increase decay times, rather than decrease them as seen here. Finally, androgens did not increase the cell body sizes in either LMAN or RA. These observations lend further support to the idea that androgens alter NMDA-EPSCs by regulating the NMDA receptor, and not by changing other properties of the postsynaptic membrane.

One intriguing difference between LMAN and RA is that androgens caused a large increase in spine frequency in LMAN, but not in RA. Another difference is that androgens caused AMPA-EPSCs to become faster, and decreased the Glu_{peak}:$AMPA_{peak}$ evoked at the DLM-LMAN synapse, without altering these features at the LMAN-RA synapse. Thus although androgens had comparable effects on NMDA-EPSCs in LMAN and RA, androgens affected neuronal morphology and other aspects of glutamatergic synaptic transmission only in LMAN, even though neurons in both nuclei contain androgen receptors (Balthazart et al. 1992). The selective action of androgens within the two song nuclei studied here, coupled with the lack of any effect of androgens on NMDA receptor–mediated EPSCs in the Wulst, illustrate that androgens can have specific actions on different glutamatergic synapses in the telencephalon of juvenile male zebra finches. Future work could reveal whether there are different androgen sensitivities of AMPA receptors within RA itself, between the LMAN-RA synapse studied here, and the HVc-RA synapse, which is primarily AMPA receptor mediated (Mooney 1992; Stark and Perkel 1999).

The effect of testosterone on LMAN spine density is notable because raising zebra finches in isolation from adult tutors, which affects song learning, also affects spine density in LMAN, and suggests that changes in this structural feature may be one hallmark of synaptic change important for song learning. Further, during normal development, DLM efferent projections to LMAN initially increase, then later retract (Johnson and Bottjer 1992), which suggests that new synaptic connections are being shaped at this time. Assuming that the new spines seen here reflect functional synapses, one possibility is that new synapses induced by testosterone express a new physiological phenotype that is distinct from preexisting synapses. Alternatively, androgens could induce physiological changes at both new and preexisting synapses.

In the song system, as in other systems modified by experience, a major question is how sensitive periods are regulated. Several central synapses that display experience-dependent remodeling initially exhibit slow NMDA-EPSCs that become faster as synaptic reorganization draws to a close (Carmignoto

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and Vicini 1992; Hestrin 1992; Shi et al. 1997). Because NMDA receptor activation at these same synapses is critical for their modification (Bear et al. 1990; Schnupp et al. 1995), these slower NMDA-EPSCs could permit sufficient postsynaptic Ca^{2+} entry to enable experience-dependent plasticity (Bliss and Collingridge 1993). In both LMAN and RA, the slower synaptic currents of immature animals could allow synaptic modification important to song learning. In LMAN, where tutor song-evoked NMDA receptor activity is crucial to sensory acquisition (Basham et al. 1996b), slower NMDA-EPSCs could sustain auditory experience-dependent changes that then instruct vocal motor learning. The more prolonged expression of slow NMDA-EPSCs in RA could then afford a means by which early auditory experience might continue to guide vocal motor learning, until pubertal increases in androgens curtail plasticity and result in song crystallization (Marler et al. 1988).

Here we have linked androgens, which could act to synchronize song learning and sexual maturation, with NMDA receptors, which are known to be important for sensory acquisition. While we have studied the effects of exogenous androgens, behavioral studies in other songbirds suggest that endogenous androgens play an important role in controlling various stages of song development. In swamp and song sparrows, high levels of androgens are needed for the transition from plastic to crystallized song (Marler et al. 1988). A more recent finding is that juvenile white-crowned sparrows display an extended capacity for sensory acquisition when reared in acoustical isolation, but only when isolation is combined with a photoperiod regimen that delays the onset of adult testosterone levels (Whaling et al. 1998). Perhaps this delay in the rise to adult testosterone levels allows adult birds to copy song because NMDA-EPSC maturation in the song system of these birds is also delayed. An important goal will be to determine whether the functional changes in NMDA-EPSCs induced by androgens seen here do indeed limit sensitive periods for song learning. Further, it will be interesting to explore other neuronal properties that are modulated by androgens that could influence song learning.

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