Intrinsic and Synaptic Properties of Neurons in an Avian Thalamic Nucleus During Song Learning

MINMIN LUO AND DAVID J. PERKEL
Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Luo, Minmin, and David J. Perkel. Intrinsic and synaptic properties of neurons in an avian thalamic nucleus during song learning. J Neurophysiol 88: 1903–1914, 2002; 10.1152/jn.01043.2001. The anterior forebrain pathway (AFP) of the avian song system is a circuit essential for song learning but not for song production. This pathway consists of a loop serially connecting area X in the basal ganglia, the medial portion of the dorsolateral nucleus of thalamus (DLM), and the pallial lateral magnocellular nucleus of the anterior neostriatum (IMAN). The majority of DLM neurons in adult male zebra finches closely resemble mammalian thalamocortical neurons in both their intrinsic properties and the strong GABAergic inhibitory input they receive from the basal ganglia. These observations support the hypothesis that the AFP and the mammalian basal ganglia-thalamocortical pathway use similar information-processing mechanisms during sensorimotor learning. Our goal was to determine whether the cellular properties of DLM neurons are already established in juvenile birds in the sensorimotor phase of song learning when the AFP is essential. Current- and voltage-clamp recording in DLM of juvenile male zebra finches revealed that, as in adults, most area X neurons have intrinsic properties largely similar to those of their respective adult counterparts. Immunostaining for glutamic acid decarboxylase (GAD) in juvenile zebra finches revealed that, as in adults, most area X somata are large and strongly GAD+ and that their terminals in DLM form dense GAD+ baskets around somata. GAD immunoreactivity in DLM was depleted by lesions of area X, indicating that a strong GABAergic projection from area X to DLM is already established in juveniles. Some of the DLM neurons exhibited large, spontaneous GABAergic synaptic events. Stimulation of the afferent pathway evoked an inhibitory postsynaptic potential or current that was blocked by the GABA_\text{A} receptor antagonist bicuculline methiodide. The decay of the GABA_\text{A} receptor-mediated currents was slower in juvenile neurons than in adults. In addition, the reversal potential for these currents in juveniles was significantly more depolarized both than that in adults and than the Cl\textsuperscript{−} equilibrium potential; yet the reversal potential was still well below the firing threshold and thus inhibitory in the slice preparation. Our findings suggest that the signal-processing role of DLM during sensorimotor learning is generally similar to that in adulthood but that quantitative changes in synaptic transmission accompany the development of stereotyped song.

INTRODUCTION

Vocal learning in oscine songbirds is increasingly used as a model system for studying the mechanisms of sensorimotor learning. The male zebra finch is one of the best-studied songbirds, both in its singing behavior and the underlying neural mechanisms. A male zebra finch acquires its song by learning from another male, usually his father. Song learning occurs through two phases (Fig. 1A) (Arnold 1975; Immelmann 1969). During the initial sensory phase, which starts ~20 days post hatching (DPH) and lasts ~30 days for male zebra finches, he listens to a tutor singing and forms a memory or “template” of the tutor song. After 30 DPH, the sensorimotor phase begins. The bird starts practicing to sing and, using auditory feedback, gradually learns to match his own song to the template tutor song. By 60 DPH, his song has many adult-like features and by 90 DPH, the song becomes highly stereotyped or crystallized.

More than a dozen interconnected nuclei comprise a circuit known as the song system, which underlies song learning and production. The song system is often simplified into two main neural pathways with different functions (reviewed in Brenowitz et al. 1997). The motor pathway is essential for song production, as lesions in this pathway disrupt singing. It includes nucleus HVc (as used in the proper name), nucleus robustus archistriatalis (RA), and brain stem motor and premotor nuclei controlling the syrinx and respiration (Fig. 1B) (Nottebohm et al. 1976; Vicario 1991).

The anterior forebrain pathway (AFP) provides an indirect connection between HVc and RA. It consists of a topographically organized loop connecting area X, the medial portion of the dorsolateral nucleus of the thalamus (DLM) and the lateral portion of the magnocellular nucleus of the anterior neostriatum (IMAN) (Bottjer et al. 1989; Luo et al. 2001; Nottebohm et al. 1976). The AFP in adults has many anatomical and physiological similarities to the mammalian cortico-basal ganglia-thalamocortical loop. Many neurons in area X have intrinsic properties resembling those of mammalian basal ganglia neurons (Farries and Perkel 2002). The area X neurons projecting to DLM provide a strong GABAergic input to DLM (Luo and Perkel 1999a,b). In addition, the majority of DLM neurons have intrinsic properties strikingly similar to those of mammalian thalamocortical neurons (Luo and Perkel 1999a). While lesions of this pathway dramatically interrupt song learning in juveniles, they do not alter song production in adult birds (Bottjer et al. 1984; Scharff and Nottebohm 1991; Sohrabji et al. 1990), indicating that the AFP plays a critical role in song learning. It could do so by providing feedback signals...
to the motor pathway to guide vocal learning in juveniles (Brainard and Doupe 2000). However, our finding of an inhibitory connection between area X and DLM in adults indicates that this loop does not simply serve an excitatory relay function. Whatever the precise function of the AFP, knowing that this loop does not simply serve an excitatory relay function between area X and DLM in adults indicates that processing by this synaptic connection undergoes developmental changes during the sensorimotor phase of song learning.

**Methods**

Juvenile (29–60 DPH) or adult (>110 DPH) male zebra finches (*Taeniopygia guttata*) were obtained from our breeding colony at the University of Pennsylvania or from a local breeder. All procedures used here were in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were consistent with the policy of the American Physiological Society.

**Slice preparation**

Slice preparation was similar to that described previously (Luo and Perkel 1999a). Briefly, halothane or isoflurane was used to anesthetize a bird deeply. The bird was then decapitated and the brain was rapidly removed. The brain was blocked by first cutting nearly parasagittally ~4 mm from the midline with the anterior end of the blade rotated ~20° laterally and then by cutting midsagittally to separate the hemispheres. The separated hemispheres were glued to the stage of a vibrating microtome with the lateral surface down, and 300-μm-thick slices were then prepared. To improve slice viability, NaCl was replaced with sucrose in the artificial cerebrospinal fluid (ACSF) for slice preparation (Aghajanian and Rasmussen 1989). The ACSF for slicing contained (in mM) 238 sucrose; 1.3 KCl, 1.3 MgCl₂, 2.5 CaCl₂, 1.0 NaH₂PO₄, 26.2 NaHCO₃, and 11 glucose. Slices were incubated for 30 min. in 30°C oxygenated solution that had half of the NaCl replaced by sucrose and for ≥30 additional min at 22–25°C in normal recording solution, which was identical to the slicing solution except that it contained 119 mM NaCl and no sucrose and had an osmolarity of 304 mosM. To improve the quality of cell filling, in some experiments the osmolarity of the ACSF was increased to 314 by increasing the sucrose concentration to 248 mM for slice preparation or by increasing the NaCl to 124 mM for recording (see results).

**Electrophysiological recording**

Slices were submerged and superfused with ACSF at 1–2 ml/min at 22–25°C. Neurons in DLM were recorded using the “blind” whole cell method (Blanton et al. 1989) in current- or voltage-clamp mode. All electrodes were pulled with a P-97 micropipette puller (Sutter Instruments, Novato, CA). The intracellular solution in the recording pipette contained (in mM) 120 K-glucosate, 10 HEPES, 10 EGTA, 8 NaCl, 2 MgATP, and 0.3 Na₃GTP (pH = 7.2–7.4). Electrodes typically had a resistance of 5–12 MΩ for current-clamp recording and 3–6 MΩ for voltage-clamp recording. Whole cell recordings were routinely obtained and usually remained stable for ≥45 min and sometimes up to 3 h. To evoke synaptic activity, afferents were activated with a single pulse (100 μs; 2–100 V or 0.1–10 mA) with a bipolar stainless-steel stimulating electrode (FHC, Brunswick, ME; 2–5 MΩ) placed within the axonal pathway anterior to DLM, 400–800 μm away from the anterior edge of the nucleus.

Current-clamp signals were amplified using an Axoclamp 2 amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 2–5 kHz, and digitized at twice the filter cutoff frequency. Voltage-clamp signals were amplified using an Axopatch 1D amplifier (Axon Instruments), low-pass filtered at 6–10 kHz, and digitized at twice the filter cutoff frequency. Input resistance and series resistance were monitored throughout the recording by delivering 10-pA hyperpolarizing signals filtered at 6–20 kHz.
current pulses or −5-mV voltage pulses and measuring the cellular responses. Only the cells with stable membrane potential, input resistance and series resistance were analyzed. Except for those illustrating firing patterns, current or voltage records are presented here as averages of three to five consecutive traces.

Cell filling

A subset of neurons was filled with neurobiotin (0.5%, Vector Laboratories, Burlingame, CA), which was included in the whole cell patch recording electrodes. Slices were fixed with 4% formaldehyde, cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB), and sectioned (60 μm) with a freezing microtome. Filled cells were visualized using Cy3-conjugated streptavidin, and data were collected with confocal microscopy. Camera lucida drawing was used in some cells to illustrate their morphology.

Lesions and GAD immunostaining

Surgery and histology were similar to those described previously (Luo and Perkel 1999b). Briefly, animals were anesthetized with pentobarbital sodium (40 mg/kg) and mounted in a stereotaxic apparatus. The skull was opened, and a small craniotomy was performed over the desired targets. Ibotenic acid (0.1–0.5 μL, 10 μg/μL) was unilaterally pressure injected into area X with a glass micropipette (tip: 30–50 μm) glued to a Hamilton syringe. After the injection, the pipette was withdrawn, and the wound was closed with surgical adhesive (Nexaband, Closure Medical, Raleigh, NC). After a survival time of 3–4 days, the animal was killed with pentobarbital (250 mg/kg) and was perfused transcardially with saline followed by 4% formaldehyde in 0.1 M PB. Each brain was then postfixed in 4% formaldehyde for 2 h and cryoprotected in 30% sucrose in 0.1 M PB for 48 h at 4°C. Parasagittal sections were prepared (30 μm thickness) using a freezing microtome.

For GAD immunostaining, sections were incubated in 1:1,500 anti-GAD antibody (AB108, Chemicon, Temecula, CA) and 10% normal goat serum (NGS) in 0.1 M PB for 48 h at 4°C. After rinsing in PB, sections were incubated with goat anti-rabbit biotinylated secondary antibody (1:200) and 10% NGS in 0.1 M PB overnight at 4°C. After rinsing in PB, sections were incubated with 1:1000 Cy3-conjugated streptavidin. After rinsing in PB, sections were mounted with Vectashield (Vector Laboratories), coverslipped, and sealed with nail polish. All data were collected with confocal microscopy (Leica TCS).

Materials

Except as noted, chemicals were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Research Biochemicals (Natick, MA) supplied the N-methyl D-aspartate (NMDA) receptor antagonist (±)-2-amino-5-phosphonovaleric acid (APV) and the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). All drugs were added to the superfusion medium by dilution of a stock solution. Stock solutions of CNQX were prepared in DMSO; all other stock solutions were made in water.

Results

Current-clamp data described here are from 19 juvenile neurons whose resting membrane potential was more negative than −50 mV and showed some overshooting action potentials. In addition, 57 cells recorded under voltage-clamp conditions met our criteria of a stable holding current and a series resistance <30 MΩ. Of these 57 neurons, 12 neurons were from adult male zebra finches and the rest were from juvenile male zebra finches of 29–60 DPH. To facilitate examination of the developmental pathway, some data from our previous studies of current-clamp recording of adult DLM neurons were incorporated. GAD immunostaining was performed on four juvenile males (40–48 DPH), two of which had ibotenic acid injected into area X in one hemisphere (1 right and 1 left). We first report on the intrinsic properties of the DLM neurons, then the GAD immunoreactivity patterns, and finally the synaptic responses to stimulation of the DLM afferent pathway.
Intrinsic properties

Similar to their adult counterparts, juvenile DLM neurons could be classified by their intrinsic properties into two major types, closely resembling the two types found in the adult (Luo and Perkel 1999a). Type I neurons resemble mammalian thalamocortical neurons in having rebound burst firing (Llinás and Jahnsen 1982), and type II neurons resemble mammalian thalamic interneurons (Pape and McCormick 1995; Turner et al. 1997; Williams et al. 1996). In addition, we used voltage-clamp techniques to study the conductances underlying some intrinsic and synaptic properties.

**TYPE I NEURONS.** Every type I neuron (n = 18) exhibited either a low-threshold Ca spike (Fig. 2A1) or current (BI), which none of the type II neurons (n = 0/6) exhibited. On hyperpolarization, type I neurons exhibited a depolarizing sag ~200 ms after the onset of hyperpolarization (Fig. 2A1, ↑), which was very small or nonexistent in type II neurons. Current-voltage relations revealed fast outward rectification which none of the type II neurons (Fig. 2B1) exhibited. On depolarization, type I neurons exhibited a depolarizing sag which was much smaller than that produced by the same amount of hyperpolarizing current (Fig. 2A, 1 and 2). When recorded in voltage-clamp mode, inward currents were usually activated during large hyperpolarizing pulses with an activating time constant ranging from 300 to 1,100 ms (Fig. 2B, ↑). Following the end of hyperpolarizing pulses from holding potentials near the resting potential (approximately −55 mV), a low-threshold inward current (probably a t-type calcium current) was usually seen (Fig. 2B1). In many cases, rapidly decaying transient outward currents were activated at the beginning of depolarizing pulses, suggesting the existence of $I_A$ (Fig. 2B, 1 and 2).

The time-dependent inward current activated during hyperpolarization was likely to be $I_h$, as it was completely (98 ± 2%) blocked by Cs$^+$ (3–10 mM; n = 9; Fig. 3A). The amplitude of the current increased significantly when the cell was hyperpolarized beyond −75 mV and reached a maximum when the potential was stepped to −110 mV (Fig. 3B). The activation time constant, measured by fitting a single exponential to the current, decreased gradually from 1,100 to ~300 ms when the cell was hyperpolarized from −60 to −105 mV (Fig. 3B). The dependencies of current amplitude and time constant on membrane potential were similar to those observed in mammalian thalamic relay neurons (McCormick and Pape 1990).

In all juvenile type I neurons, depolarizing current pulses elicited action potentials with an intermittent train pattern exhibiting subthreshold oscillations at 40–100 Hz between trains (Fig. 4Aa). Stronger current pulses elicited more action potentials within each train and shorter intervals between trains. When the current was strong enough (usually >150 pA), cells fired repetitively and regularly with a firing frequency as high as 150 Hz. When the cell was steadily hyperpolarized, depolarizing current pulses instead elicited bursts with two or three action potentials on top of a Ca$^{2+}$ spike (Fig. 4B). During prolonged hyperpolarization, spontaneous bursts were also seen in some cells (Fig. 4C). This spontaneous firing could recur at 0.5–4 Hz (Fig. 4D), which is within the delta frequency range (Curro Dossi et al. 1992; McCormick and Pape 1990). We measured a variety of parameters and found that juvenile and adult type I neurons do not show statistically significant differences (Table 1). Further analysis of these parameters within the juvenile age group indicated no correlation of any parameter with age, except for threshold current. This parameter was positively correlated with age (range: 44–53 DPH; n = 13; P < 0.002; r$^2$ = 0.6).

Eighteen juvenile type I cells were well filled with neurobiotin during recording, and their morphology appeared qualitatively to fall into two different classes (Fig. 5). The first class was characterized by a medium-sized (12–16 μm) round or polygonal soma and four to six primary dendrites that could reach 200–300 μm in distance (n = 6, Fig. 5A1). Although varicosities were observed on the dendritic processes (Fig. 5A2), they were much less prevalent than those observed in adults. Axonal processes extending outside of DLM in the anterior direction were observed in two of these neurons. The second class (Fig. 5B) was characterized by a larger soma (15–25 μm) and three to eight dendrites, which were usually shorter than 100 μm and located toward one side of the soma (n = 11). The morphology of this class is similar to that of retrogradely labeled neurons after tracer injection of IMAN (unpublished observations). Highly varicose dendrites were also seen in six of these neurons, five of which were recorded with lower osmolarity ACSF (304 mosM). Five of six neurons recorded with
higher osmolarity ACSF (314 mosM) did not have these highly varicose processes yet had other features of this class, suggesting that the varicosities observed in the adult neurons (Luo and Perkel 1999a) could be, in part, the result of slice preparation. Three of the filled neurons were observed to have axonal processes leaving DLM, further suggesting that many of the neurons in this class may be projection neurons that innervate IMAN. One neuron (not shown) had a morphology that was very different from all the others in that its soma was small (~10 μm) and its dendrites were few and short (~30 μm).

**TYPE II NEURONS.** The basic features of type II neurons are already established by 35 DPH. They have much larger input resistance (usually >1 GΩ; range: 655–1,885 MΩ) than type I cells and lack low-threshold Ca2+ spikes (Table 1). Six type II neurons were recorded in current-clamp mode. The firing patterns in two of these were different from those of adult neurons (see following text). Voltage-clamp recording revealed additional details regarding this difference. Resting membrane potential and input resistance were not significantly different in juveniles and adults, and no significant correlation was observed between either of these parameters and age within the juvenile category (range: 34–55 DPH; n = 13; P > 0.49).

On hyperpolarization, rapid inward rectification was observed in most of the juvenile type II neurons (n = 4/6 for current-clamp recording and 8/11 for voltage-clamp recording). Thus when recorded in current-clamp mode, depolarizing current was more effective at deflecting the membrane potential than hyperpolarizing current of the same intensity (Fig. 6A, 1 and 2). In voltage-clamp mode, currents activated by hyperpolarizing voltage pulses were larger than those activated by depolarizing pulses (Fig. 6B).

Most type II neurons also exhibited time-dependent hyperpolarization-activated inward currents (n = 10/11 for juveniles, and 4/4 for adults). These currents had an activation time constant of ~400 ms and thus likely represent the I_{h}. Compared to that found in type I neurons, this current had a much smaller amplitude (5–10 pA at ~95 mV vs. 40 pA at ~95 mV for type I neurons). Possibly because of this small amplitude, we did not observe the depolarizing sags during hyperpolarizing current injections in current-clamp mode.

**TABLE 1.** Intrinsic properties of DLM neurons

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Juvenile Type I (n = 13)</th>
<th>Adult Type I (n = 13)</th>
<th>Juvenile Type II (n = 6)</th>
<th>Adult Type II (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-56 ± 4 (n = 38)</td>
<td>-55 ± 4 (n = 46)</td>
<td>-59 ± 8 (n = 17)</td>
<td>-58 ± 3 (n = 10)</td>
</tr>
<tr>
<td>Membrane input resistance (MΩ)</td>
<td>525 ± 180** (n = 39)</td>
<td>482 ± 222 (n = 46)</td>
<td>1205 ± 340 (n = 16)</td>
<td>1232 ± 362 (n = 11)</td>
</tr>
<tr>
<td>Membrane time constant (ms)</td>
<td>21.8 ± 8.1**</td>
<td>23.2 ± 11.6</td>
<td>48.3 ± 21.6</td>
<td>44.6 ± 10.8</td>
</tr>
<tr>
<td>Threshold current to elicit first action</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>potential at rest (pA)</td>
<td>86 ± 55*</td>
<td>91 ± 61</td>
<td>22 ± 10</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>Spike duration at half amplitude (ms)</td>
<td>0.69 ± 0.2**</td>
<td>0.73 ± 0.22</td>
<td>1.47 ± 0.39</td>
<td>1.70 ± 0.50</td>
</tr>
<tr>
<td>Amplitude of fast AHP (mV)</td>
<td>21.3 ± 6.9</td>
<td>18.5 ± 6.7</td>
<td>18.0 ± 5.4</td>
<td>15.8 ± 4.2</td>
</tr>
<tr>
<td>Time to reach the peak of fast AHP (ms)</td>
<td>1.4 ± 0.5**</td>
<td>1.6 ± 0.9</td>
<td>10.7 ± 8.5</td>
<td>8.6 ± 7.4</td>
</tr>
</tbody>
</table>

Data are means ± SD. Age range for juveniles 44–53 DPH. AHP, after hyperpolarization; DLM, medial portion of the dorsolateral nucleus of thalamus; DPH, days posthatching. * Significant difference (unpaired t-test; P < 0.05) for comparison between juvenile type I and type II neurons. ** Significant difference (unpaired t-test; P < 0.01) for comparison between juvenile type I and type II neurons.
Most type II neurons had firing properties similar to adult type II neurons ($n = 4/6$). Like their adult counterparts, these neurons had regular firing patterns in response to depolarizing current injection. Stronger currents caused increased firing frequency ($\leq 50$ Hz) and decreased spike amplitude during firing (Fig. 7A, 1–3). These cells usually exhibited adaptation of the firing rate, especially when injected with large currents (Fig. 7A4).

Two juvenile type II neurons exhibited slightly different firing patterns not seen in adult neurons. They fired bursts comprising two and five action potentials at $\sim 50$ Hz at the beginning of depolarizing pulses and quickly adapted thereafter (Fig. 7B). Although stronger currents increased the overall firing frequency, the frequency of initial bursting was usually $\sim 50$ Hz, well below the frequency observed for the stereotypical bursts of action potentials on top of the $Ca^{2+}$ spike observed in type I neurons (100–150 Hz). This cell had a biphasic afterhyperpolarization after action potentials (Fig. 7B1).

**GAD immunostaining**

To begin to address the synaptic input to DLM, we carried out GAD immunostaining in juvenile animals ($n = 4$). This revealed that by 40 days the juveniles had an adult-like GAD immunoreactivity pattern in the song system. Large, intensely GAD+ somata were found sparsely distributed in area X (Fig. 8A). Strong GAD immunoreactivity was observed throughout DLM (Fig. 8B1). At high power, dense GAD+ terminals were observed in DLM but no GAD+ somata were found (Fig. 8B2). In many cases, these GAD+ terminals formed baskets

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**FIG. 5.** Morphology of type I neurons. A1: camera lucida drawing of a 49-DPH neuron. This class of neuron was characterized by a medium-sized soma and long dendrites. A2: confocal microscopic image of the dendritic processes within the box in 1. B: a 43-DPH type I neuron showing the second type of morphology seen with a large soma and short processes that are often restricted to one side of the soma. Scale bars: A1 = 25 µm, A2 = 10 µm, B = 25 µm.

Most type II neurons had firing properties similar to adult type II neurons ($n = 4/6$). Like their adult counterparts, these neurons had regular firing patterns in response to depolarizing current

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**FIG. 6.** Membrane properties of type II DLM neurons from juvenile birds. A1: the membrane potential deflections of a 48-DPH type II DLM neuron are shown in response to hyperpolarizing and depolarizing current pulses (shown below). A2: the current-voltage relation for this cell. The dashed line is determined by the resting potential and the membrane potential change with a 10-pA depolarizing pulse. All the points were on or above the line, suggesting rapid inward rectification. B1: the membrane currents activated by clamping a 37-DPH cell to various potentials between $-40$ and $-100$ mV from the resting membrane potential of $-60$ mV. B2: the current-voltage plot for the same cell. The dashed line is determined by the rest condition and the currents immediately following a step to $-55$ mV. The inward rectification was also revealed by voltage-clamp recording in that most of the points were below the dashed line. Time-dependent inward currents like $I_h$ were activated in some cells, but the amplitude was much smaller ($< 5$ pA at $-90$ mV) than that observed in type I neurons. Open circles and filled circles represent current at the beginning and end of pulse, respectively.
neurons, especially GABAergic ones, than in adult cells. In most cases, higher-intensity stimulation (>1 mA or >70 V) and substantial repositioning of the stimulating electrodes was required. A number of recordings were lost during this process.

Stimulating the afferent pathway ~500 μm anterior to DLM often evoked both inhibitory and excitatory postsynaptic responses. All of the excitatory postsynaptic potentials or currents (EPSPs or EPSCs) were blocked by the AMPA-type glutamate receptor blocker CNQX (10 μM). Although some of the excitatory responses appeared to be monosynaptic, most were polysynaptic, as the latency could be >100 ms.

In the presence of CNQX, GABAergic postsynaptic responses were evoked in 12 juvenile and 4 adult type I neurons. This population represents all cells in which a response was seen prior to addition of CNQX. In all juvenile neurons tested (n = 9), the GABA_A receptor antagonist bicuculline methiodide (BMI) blocked the inhibitory PSP or PSC (IPSP or IPSC, n = 1/1 for IPSP and n = 8/8 for IPSC) in a reversible manner (Fig. 9A), indicating that these responses were mediated by GABA_A receptors. At resting membrane potentials of −51 and −54 mV, the two IPSPs recorded in juvenile cells had peak amplitudes of −3.2 and −2.5 mV, respectively.

To test for developmental changes of IPSC waveform, we examined the latency, rise time and decay time constant of the IPSC in juveniles and adults. The IPSC decay time constant for juveniles (15.2 ± 6.3 ms; range: 7.7–25.0 ms), was significantly longer than for adults (9.3 ± 1.8 ms; range: 7.4–10.9 ms; P < 0.05). Within the juvenile group (age range: 34–55 DPH), there was no significant correlation of decay time constant with age (P > 0.17; n = 12). There was no significant difference in either the latency or rise time between juveniles (n = 10) and adults (n = 5, Table 2), or significant linear correlation of these parameters with age within the juvenile group (P > 0.1; n = 12).

Evidence of functional GABAergic input to DLM neurons also came from spontaneous IPSCs. While the vast majority of the type I neurons exhibited spontaneous PSCs with frequencies ranging from 1 to 100 Hz, many of these PSCs had fast decay time constants (3–9 ms) and amplitudes <40 pA at −60 mV. Application of CNQX blocked most of these small PSCs, suggesting they were glutamatergic EPSCs (Fig. 9B). In five cells, however, some of the spontaneous PSCs were resistant to the application of CNQX. These PSCs had longer decay time constants (8–12 ms) and a wide range of amplitudes (40–500 pA). In the one cell tested, these PSCs were completely blocked by BMI and this blockade was reversible (Fig. 9C). The wide range of spontaneous IPSC amplitudes observed suggests the possibility of a spontaneously active GABAergic cell population remaining within the slice and projecting to DLM neurons.

The reversal potential of the evoked IPSCs was measured by stimulating the afferent pathway in the presence of CNQX while clamping the cell at different membrane potentials (Fig. 10A1) and then calculating the reversal potential by using linear regression (Fig. 10A2). In juvenile cells, the IPSC had a reversal potential of −55.4 ± 3.4 (mean ± SE, n = 7). This value is significantly different from −70.5 mV, the equilibrium potential for Cl− calculated using the Nernst equation using the concentrations of Cl− in the pipette and extracellular solutions (paired t-test; P < 0.01). It is also significantly less negative.

Synaptic physiology

To test the functionality of inputs to DLM neurons, we examined several aspects of synaptic physiology. In general, it was much more difficult to elicit synaptic responses in juvenile

FIG. 7. Firing pattern of type II DLM neurons from juvenile birds. A, 1–3: a type II neuron from a 47-DPH bird exhibited a regular firing pattern in response to depolarizing pulses of 30, 50, and 70 pA. A4: the instantaneous firing rates shown in A, 1–3. Adaptation of firing rates was observed for most of the type II neurons. B, 1–3: another type II neuron from a 38-DPH bird exhibiting initial bursting behavior followed by large adaptation of firing rate in response to depolarizing pulses of 30, 50, and 70 pA. B4: instantaneous firing rates shown in B, 1–3.

the size of DLM somata. In addition, ibotenic acid lesions of part of ipsilateral area X depleted the GAD immunoreactivity in part of DLM (n = 2). The lesioned area in area X (Fig. 8C1) corresponded largely to the GAD-depleted area in DLM (Fig. 8C2). For example, lesion of posterior area X substantially reduced GAD immunoreactivity in posterior and slightly dorsal DLM only, consistent with the topographic organization of the X → DLM projection in adults (Luo et al. 2001). These data suggest that by 40 DPH, the area X → DLM projection is already GABAergic and at least a coarse topography is already established.

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than the reversal potential of IPSPs and IPSCs in adult cells ($-65.9 \pm 1.5$, $n = 8$; $P < 0.05$; Fig. 10B). Within the juvenile group, the reversal potential showed a weak but statistically significant positive correlation with age (range 34–55 DPH; $P < 0.035$; $r^2 = 0.049$). Based on the reversal potential and the amplitude of IPSC, the peak conductance of the IPSCs was $12.8 \pm 4.1$ (SE) nS.

In each of three cells examined, the amplitude of the GABAergic PSC exhibited all-or-none properties with regard to the stimulation intensity. In the presence of CNQX, stimulation below threshold intensity elicited no response, but slightly stronger stimulation elicited a full-amplitude IPSC, whose amplitude was not increased even by much stronger stimulation (Fig. 11).

Stimulation of the afferent pathway elicited PSCs in juvenile type II neurons that were mostly blocked by the application of CNQX ($n = 4/4$) when the cell was held at its resting membrane potential (Fig. 12A). The latency of the EPSC was $4.4 \pm 2.3$ ms, longer than that of the IPSC of type I neurons ($2.4 \pm 1.0$ ms; $P < 0.05$). Spontaneous EPSCs were observed in many of the type II neurons ($n = 9/11$), and all spontaneous EPSCs were completely blocked by CNQX ($n = 2/2$; Fig. 12B).

**FIG. 8.** GAD immunoreactivity pattern in area X and DLM in juvenile male zebra finches. A1: low-power ($\times 5$) image of GAD immunoreactivity in area X (- - -). ↑, pallial lateral magnocellular nucleus of the anterior neostriatum (IMAN); A2: high-power ($\times 40$) image of GAD immunoreactivity in area X showing sparsely distributed, large and strongly GAD+ somata. B1: low-power view of GAD immunoreactivity in DLM; B2: high-power view of GAD immunoreactivity in DLM showing that only terminals were GAD+. C1: a lesion in the posterior and dorsal portion of area X was made by injection of ibotenic acid in the hemisphere contralateral to that shown in B. C2: the area within the posterior and dorsal portion of DLM ipsilateral to the lesioned area X was depleted of GAD immunoreactivity. The ibotenic acid was injected at 44 DPH, and the animal was perfused at 48 DPH. In A–C, anterior is to the right and dorsal is up. Scale bar for A1: 200 $\mu$m; A2: 25 $\mu$m; B1: 100 $\mu$m; B2: 25 $\mu$m; C1: 100 $\mu$m; and C2: 100 $\mu$m.
DISCUSSION

The major results of this study are that the intrinsic properties of DLM neurons are largely established by post-hatch day 44, near the beginning of the sensorimotor phase of song learning; the area X \( \rightarrow \) DLM projection is GABAergic and functional during this phase, and in many features resembles that in adults; two physiological properties of this synaptic connection in juveniles, the IPSC decay time and reversal potential, differ from those in the adult. These results suggest that the processing mechanism of the area X \( \rightarrow \) DLM projection is basically similar during the sensorimotor learning phase and adulthood but that quantitative changes in synaptic function occur during development that could affect neural information processing.

Intrinsic properties

By the onset of sensorimotor learning, juvenile DLM neurons have already established their adult-like phenotypes. They fall into two categories, each of which has features similar to those of its adult counterpart. Type I neurons have low-threshold \( \text{Ca}^{2+} \) spikes and time-dependent inward rectification activated by hyperpolarization. The current underlying the inward rectification closely resembles the \( I_h \) observed in mammals. In addition, more detailed studies of the current amplitude and activation time constant as a function of membrane potential revealed strong similarities between the \( I_h \) of DLM type I neurons and that of mammalian thalamic relay neurons (McCormick and Pape 1990), further strengthening the similarities between avian thalamic neurons and those in mammals (Strohmann et al. 1994).

The morphological properties observed in juvenile DLM neurons suggests the possibility of at least two subtypes of the type I class. It is possible that the morphological variation among type I neurons corresponds to cells with different physiological properties. Heterogeneity was observed in the firing properties of both juvenile and adult type I neurons, such as the delayed firing in some but not all type I neurons. Quantitative morphometry coupled with a more detailed characterization of intrinsic properties should help test for subtypes of type I neurons. In adults, however, we reported one main type of morphology (Luo and Perkel 1999a). Instead of reflecting a developmental change in morphology, this difference is more likely the result of the larger number of cells filled (18 vs. 6) and improvements in slice preparation and cell filling techniques (e.g., higher osmolarity of ACSF) in the present study.

This study revealed some additional detailed intrinsic properties of type II neurons. Similar to the adult type II neurons, they have large input resistances and lack a low-threshold Ca spike (Luo and Perkel 1999a). With voltage-clamp recording, we found that these neurons have rapid inward rectification and a small degree of time-dependent inward rectification. In addition to the cells with regular firing patterns (\( n = 4/6 \)), two of these neurons showed bursts of action potentials at \( \sim 50 \text{ Hz} \) at the beginning of current pulses followed by strong adaptation. This firing pattern is not observed in adult type II neurons, suggesting some developmental change in their firing properties. We had great difficulty in filling the type II neurons, which

**TABLE 2. IPSC properties for juvenile and adult type I DLM neurons**

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Juvenile (( n = 10 ))</th>
<th>Adult (( n = 5 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency (ms)</td>
<td>2.4 ± 1.0</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>Rise time (ms)</td>
<td>1.3 ± 0.7</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td>Decay time constant (ms)(^a)</td>
<td>15.4 ± 5.9</td>
<td>8.8 ± 1.7</td>
</tr>
</tbody>
</table>

Data are means ± SD. Rise time was measured from 10–90% of peak amplitude. The IPSC decay was fitted with a single exponential function. IPSC, inhibitory postsynaptic current; DLM, medial portion of the dorsolateral nucleus of thalamus. \(^a\)Significant difference (unpaired t-test; \( P < 0.05 \)).
might be explained by their small somata, an inference based on their large input resistance. The single type II neuron that we filled had a soma 10 μm in diameter and had short and twisted dendrites. More type II neurons need to be filled to examine whether these are characteristic features.

**Synaptic input**

Similar to their adult counterparts, juvenile type I neurons receive both glutamatergic and GABAergic input in response to stimulation anterior to DLM, while type II neurons appear to receive only glutamatergic input. Evoked and spontaneous glutamatergic synaptic activity occurs in both types of neurons.
Evoked glutamatergic EPSCs with long latencies are likely polysynaptic; apparently monosynaptic glutamatergic EPSCs are also observed, however. These could come from orthodromic or antidromic activation of other DLM neurons making collateral excitatory synapses within DLM or through orthodromic activation of neurons outside of DLM, although it is unclear where the somata of such neurons would lie. One possibility is nucleus RA, which makes a weak anatomical projection to DLM (Vicario 1993; Wild 1993). Another possibility is an as-yet undescribed glutamatergic projection from the forebrain to DLM. It seems unlikely that such a projection could come from area X because all area X somata retrogradely labeled after tracer injections in DLM were GAD positive (Luo and Perkel 1999b).

Evidence for a strong GABAergic projection from area X to DLM in juveniles comes from three observations. First, immunostaining for GAD shows that DLM has dense GAD+ terminals but no GAD+ somata. Strong GAD immunoreactivity is observed in large area X neurons, the soma size of which is comparable to that of the projection neurons (Luo and Perkel 1999b). Lesions of area X substantially reduce GAD immunoreactivity in the corresponding portion of DLM. The indistinguishable GAD immunoreactivity pattern and lesion effect in adult and juvenile animals indicates that by 40 days, the GABAergic area X projection to DLM is well established.

Second, type I neurons have spontaneous synaptic activity that is resistant to the glutamatergic AMPA receptor blocker CNQX but is completely blocked by the GABA_\text{A}_\text{A receptor blocker BMI. The spontaneous GABAergic synaptic activity suggests that GABAergic synapses are functional in juvenile animals. Last and most important, stimulating the afferent pathway elicits large all-or-none IPSCs in type I neurons that are resistant to CNQX and completely blocked by BMI. These data indicate that DLM receives strong GABAergic input from outside of the nucleus. Because lesions of area X deplete the GAD immunoreactivity in DLM and DLM itself does not contain GAD+ somata, this GABAergic input most likely arises from area X. The all-or-none nature of the synaptic input in juveniles suggests that, as in adults, there is a low degree of convergence of area X axons onto DLM neurons. In fact, our voltage-clamp data from juveniles complement our current-clamp data from adults (Luo and Perkel 1999a), in which nonlinear summation could conceivably have masked convergent inputs.

While both juvenile and adult DLM type I neurons receive strong GABAergic input that is mediated by GABA_\text{A} receptors, developmental changes do occur in this pathway. As in some mammalian systems, the GABA_\text{A receptor-mediated synaptic currents in juvenile DLM neurons have a significantly longer decay time constant than those in adult neurons (Tia et al. 1996; Vicini 1999). Slower decay of the GABAergic input would result in longer and thus more effective inhibitory action on the postsynaptic cell, which could more reliably generate postinhibitory rebound in thalamic neurons with a low threshold Ca^{2+} spike. This effect is confounded, however, by the more depolarized reversal potential in juveniles (see following text). In any case, a longer-duration synaptic current would enhance the efficacy of the synaptic response.

We observed that the reversal potential of the GABA_\text{A} receptor-mediated currents in juvenile DLM cells is more depolarized than that in adults. This connection could thus have a different effect on the computations performed during sensorimotor learning. In many mammalian systems, the GABA_\text{A} receptor-mediated PSC in neonatal animals is much more depolarizing than that in adults (LoTurco et al. 1995) and is also implicated in a neurotrophic effect (Ben-Ari et al. 1997; Lauder et al. 1998). The changed reversal potential is due to a higher concentration of intracellular Cl\textsuperscript{-} in the young cells (Owens et al. 1996; Rohrbough and Spitzer 1996). If the reversal potential is above the membrane potential threshold for spike generation, it could be excitatory rather than inhibitory to the postsynaptic cell. The reversal potential of GABA_\text{A} receptor-mediated currents in juvenile DLM neurons (−55 mV) is significantly more depolarized than in adults (−65 mV) and also more depolarized than the E_{Cl} determined from internal and external chloride concentrations (−70 mV). Cells were recorded using the whole cell technique and thus the intracellular [Cl\textsuperscript{-}] was likely affected by the [Cl\textsuperscript{-}] in the recording pipette due to dialysis. This suggests that the intracellular [Cl\textsuperscript{-}] in juveniles is higher than that in the adult and higher than that in the pipette, possibly because of active transport through a Cl\textsuperscript{-} transporter in the postsynaptic cell (Rohrbough and Spitzer 1996). At a reversal potential of −55 mV, the GABAergic current evoked in this pathway is still well below the firing threshold of about −40 mV and is thus inhibitory. More accurate methods, such as perforated cell-attached recording (Horn and Marty 1988) or sharp electrode recording, are needed to determine more accurately the intracellular [Cl\textsuperscript{-}] and IPSP reversal potential in the juvenile and cells.

Whether area X induces bursting in juvenile DLM neurons depends on the membrane potential of DLM neurons. If their resting membrane potential is hyperpolarized in vivo, firing of the GABAergic afferent pathway will generate depolarizing currents in the DLM neurons and possibly activate Ca^{2+} spikes, which in turn will generate bursts of action potentials and thus become excitatory. Overall, whereas the GABAergic input in juveniles has a reversal potential below the firing threshold and is inhibitory to the postsynaptic cells in the slice preparation, accurate measurement of intracellular [Cl\textsuperscript{-}] and the resting membrane potential in vivo will be needed to correctly understand the action of the GABAergic input from area X to DLM during sensorimotor learning (Owens et al. 1996; Verheugen et al. 1999).

**Summary**

The data from this study directly demonstrate that in juveniles in the midst of the sensorimotor learning phase, the area X → DLM projection in the male zebra finch is both anatomically and physiologically GABAergic. In addition, the intrinsic properties of the DLM neurons are largely the same as those of the adult neurons. Our data thus suggest that the basic processing mechanism of the area X → DLM projection has been established by the start of the sensorimotor phase of song learning. More detailed characterization of the intrinsic properties of DLM neurons, such as the I_{spike}-like current, has strengthened the similarity between avian and mammalian thalamic neurons (McCormick and Pape 1990) and added to the parallels between the AFP and the mammalian basal ganglia-thalamocortical pathway at a cellular level. The computations performed by the AFP during song learning may have...
many similarities with those made by the mammalian basal ganglia-thalamocortical pathway in sensorimotor learning.

Some properties of the GABA_A receptor-mediated synaptic currents in juvenile DLM neurons are different from those in adult neurons. The change in reversal potential may be especially important, as it could indicate a switch in the area X → DLM pathway from excitation during song learning to inhibition after the song is learned. Our measurement of the reversal potential in this study is, however, limited by our invasive method of recording. To understand better the function performed by this pathway in vivo in juveniles, less invasive methods are necessary to accurately measure the reversal potential of GABA_A receptor-mediated currents and the resting membrane potential in vivo.

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Present address: M. Luo, Dept. Neurobiology, Duke University Medical Center, Durham, NC 27710.

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