Intrinsic and Thalamic Excitatory Inputs Onto Songbird LMAN Neurons Differ in Their Pharmacological and Temporal Properties

CHARLOTTE A. BOETTIGER AND ALLISON J. DOUPE
Keck Center for Integrative Neuroscience and Neuroscience Graduate Program, Departments of Psychiatry and Physiology, University of California, San Francisco, California 94143-0444

Boettiger, Charlotte A. and Allison J. Doupe. Intrinsic and thalamic excitatory inputs onto songbird LMAN neurons differ in their pharmacological and temporal properties. J. Neurophysiol. 79: 2615–2628, 1998. In passerine songbirds, the lateral portion of the magnocellular nucleus of the anterior neostriatum (LMAN) plays a vital role in song learning, possibly by encoding sensory information and providing sensory feedback to the vocal motor system. Consistent with this, LMAN neurons are auditory, and, as learning progresses, they evolve from a broadly tuned initial state to a state of strong preference for the bird’s own song and acute sensitivity to the temporal order of this song. Moreover, normal synaptic activity in LMAN is required during sensory learning for accurate tutor song copying to occur (Basham et al. 1996). To explore cellular and synaptic properties of LMAN that may contribute to this crucial stage of song acquisition, we developed an acute slice preparation of LMAN from zebra finches in the early stages of sensory learning (18–25 days posthatch). We used this preparation to examine intrinsic neuronal properties of LMAN neurons at this stage and to identify two independent excitatory inputs to these neurons and compare each input’s pharmacology and short-term synaptic plasticity. LMAN neurons had immature passive membrane properties, well-developed spiking behavior, and received excitatory input from two sources: afferents from the medial portion of the dorsolateral thalamus (DLM), and recurrent axon collaterals from LMAN itself (“intrinsic” input). These two inputs differed in both their pharmacology and temporal properties. Both inputs were glutamatergic, but LMAN responses to intrinsic inputs exhibited a larger N-methyl-d-aspartate component than responses to DLM inputs. Both inputs elicited temporal summation in response to pairs of stimuli delivered at short intervals, but DL-2-amino-5-phosphonovalerate (APV) significantly reduced the temporal summation only of the responses to intrinsic inputs. Moreover, responses to DLM inputs showed consistent paired-pulse depression, whereas the responses to intrinsic inputs did not. The differences between these two inputs suggest that intrinsic circuitry plays an important role in transforming DLM input patterns into the appropriate LMAN output patterns, as has been suggested for mammalian thalamocortical networks. Moreover, in LMAN, such interactions may contribute to the profound temporal and spectral selectivity that these neurons will acquire during learning.

INTRODUCTION

The lateral portion of the magnocellular nucleus of the anterior neostriatum (LMAN) contributes critically to song learning: electrolytic or pharmacological disruptions of LMAN in juvenile birds during sensory learning or early sensorimotor learning prevent normal song development, whereas similar disruptions in adult birds do not affect normal song production (Fig. 1) (Basham et al. 1996; Bottjer et al. 1984; Scharff and Nottebohm 1991; Sohrabji et al. 1990). LMAN belongs to a set of nuclei unique to songbirds termed the song system (Fig. 2A), and is the output nucleus of a song system circuit known as the anterior forebrain pathway. The anterior forebrain pathway also includes Area X and the medial portion of the dorsolateral thalamus (DLM) (Bottjer et al. 1989; Okuhata and Saito 1987; Sohrabji et al. 1990). In contrast to the anterior forebrain pathway, the motor pathway of the song system must remain intact throughout life for normal song production; this circuit includes nucleus HVc (the abbreviation used here as the proper name), the robust nucleus of the archistriatum (RA), and the tracheosyringeal portion of the hypoglossal nucleus (nXIIts; Fig. 2A) (Nottebohm et al. 1976). The motor pathway is the target of the anterior forebrain pathway via LMAN projections to RA, consistent with a possible role of the anterior forebrain loop in guiding development and learning in the motor circuit.

Neurons selectively tuned to the bird’s own song are found in LMAN of adult male zebra finches: they respond more strongly to the bird’s own song than to similar conspecific songs or the bird’s own song played either in reverse or with the syllables out of order (Doupe 1997; Doupe and Konishi 1991). Thus adult LMAN is extremely sensitive to the complex spectral and temporal properties of song. This selectivity is not innate but emerges during vocal learning (Doupe 1997; Solis and Doupe 1997). The selective auditory properties of LMAN coupled with its projection to the motor pathway suggest that LMAN could provide the auditory feedback that is essential during sensorimotor learning (Konishi 1965; Price 1979).

The cellular and synaptic bases of LMAN auditory selectivity are unknown. This selectivity likely reflects processing both within LMAN circuitry, and in upstream nuclei, which also show song selectivity (Doupe 1997; Doupe and Konishi 1991; Lewicki and Arthur 1996; Margoliash 1983, 1986; Margoliash and Fortune 1992; Solis and Doupe 1997; Volman 1996). Although LMAN circuitry has been little studied, the nucleus is known to express the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Grisham and Arnold 1994) and several neuromodulators (Aamodt et al. 1992; Bottjer 1993; Bottjer et al. 1997; Carrillo and Doupe 1995; Sakaguchi and Saito 1991; Saha et al. 1996), to send a glutamatergic projection to RA by day 12–15 posthatch (Johnson and Bottjer 1994; Mooney 1992; Mooney and Rao 1994; Nordeen et al. 1992), and to express very high levels of N-methyl-d-aspartate (NMDA) receptors (NMDARs).
early in development (Aamodt et al. 1992). One functional site of these NMDARs recently was demonstrated to be the DLM afferent synapses onto LMAN projection neurons (Livingston and Mooney 1997). From ~25 to 35 days to adulthood, as development and song learning proceed, the level of NMDARs in LMAN declines, LMAN neurons show dendritic regression and the LMAN projection to RA partially retracts (Fig. 1) (Aamodt et al. 1992; Herrmann and Arnold 1991; Nixdorf-Bergweiler et al. 1995b). Early in song system development and sensory learning, zebra finch LMAN shows little sexual dimorphism in morphology, neuronal number, or in its connections with other song nuclei (Bottjer et al. 1985; Nordeen et al. 1987, 1992). A marked divergence between the song systems of males and females begins at around the onset of sensorimotor learning (Fig. 1), unless females are treated with the masculinizing hormone estradiol (Gurney 1981; Konishi and Akutagawa 1988; Nordeen et al. 1987, 1992). Less is known about the LMAN of adult females, but it has been shown to express less tyrosine hydroxylase, somatostatin, and calcitonin gene-related peptide than male LMAN (Bottjer 1993; Bottjer et al. 1997).

To begin to understand the circuitry of LMAN at the crucial early stage of learning, we developed an in vitro slice preparation of the zebra finch anterior forebrain (Fig. 2, B and C) from birds in the sensory phase of learning but not yet singing and not yet obviously sexually dimorphic (18–25 days; Fig. 1). The juvenile brain slice preparation affords the opportunity to determine the pharmacology, modulation, and plasticity of synaptic transmission in LMAN at the time when sensory learning is occurring. Intracellular recordings from LMAN projection neurons in these brain slices revealed glutamatergic inputs from both DLM afferents and LMAN axon collaterals (‘‘intrinsic’’ input). Responses to intrinsic inputs were found to have an apparently greater NMDAR-mediated component than responses to DLM inputs. Moreover, the temporal properties of responses to the two pathways differed: both exhibited temporal summation in response to paired-pulse (P-P) stimulation, but only responses to DLM inputs demonstrated consistent paired-pulse depression (PPD). These data thus begin to resolve LMAN circuitry as well as point to possible mechanisms and sites of the temporal information processing and experience-dependent plasticity characteristic of this nucleus. Some preliminary results of this study have been presented previously in abstract form (Boettiger and Doupe 1996).

METHODS

Slice preparation

All experiments were conducted with 18- to 25-day-old zebra finches reared in our breeding colony (mean age: 21.5 days). Because of the uncertainty of noninvasive sex determination at this age, slices were prepared from both males and females (males: n = 16/50 animals; n = 36/113 cells from females). Data from males and females were considered together as there was no statistical difference in their intrinsic properties, pharmacological sensitivity, or paired pulse ratios (Student’s unpaired t-test, two-tailed, P > 0.05 for all comparisons). The lack of sexual dimorphism in cellular and synaptic properties is not surprising given that many anatomic studies have found no difference between male and female LMAN at this stage (Bottjer et al. 1985; Mooney and Rao 1994; Nordeen et al. 1987, 1992), and that the animals used here were well within the window for masculinization of the female song system with estradiol (Konishi and Akutagawa 1988). Furthermore, other physiological studies in zebra finches aged ~25 days also have reported no differences between males and females (Mooney 1992; Mooney and Rao 1994).

Techniques are similar to those described by Mooney and Konishi (1991). Birds were anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL) and rapidly decapitated. The skull was removed, and the brain was blocked in situ and rapidly placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). The blocking angle was varied initially to determine the angle that produced slices with maximally complete DLM afferents and adequate LMAN outflow fibers. The best angle resulted from angling the blocking blade ~30–45° lateral to the sagittal plane in both the dorsal-ventral and rostral-caudal dimensions. The cut, lateral surface of the brain was glued to a plastic stage with cyanoacrylate glue (Krazy-glue), and 400 µm slices were cut on a VibroSlice (Stoelting, Wood Dale, IL). Initial experiments used coronal slices prepared otherwise identically (n = 38 cells). The slices were transferred to a submersion-type incubation chamber and maintained at room temperature until time of use. After a recovery period of ~1 h, individual slices were submerged in the recording chamber and superfused with ACSF at a flow rate of ~2 ml/min. Experiments were done at room temperature. The normal bathing solution contained the following (in mM): 134 NaCl, 3 KCl, 1.3 NaH2PO4, 1.3 MgSO4, 2.4 CaCl2, 25.7 NaHCO3, and 12 dextrose. The solution was saturated continuously with a gas mixture of 95% O2-5% CO2, which maintained a pH of 7.4. Chemicals were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), except where noted.

Electrophysiological techniques

Intracellular electrodes were pulled (Sutter Instrument, Novato, CA) from 1.2 mm OD borosilicate capillary tubing (A-M Systems, Everett, WA). Electrodes tips were filled with 2% Biocytin in 1.2 M potassium acetate (pH 7.2), and the remainder of the electrode was filled with 3 M potassium acetate (80–285 MΩ). The slice...
was transilluminated and LMAN was visualized with a dissecting microscope (Nikon). Penetrations were made “blind” within the visible borders of the nucleus (see Fig. 2, B and C). A penetration was maintained when a cell met the following criteria: resting membrane potential ($V_m \leq -50$ mV), input resistance ($R_i \geq 20$ MΩ), threshold current to elicit an action potential $< 1$ nA, and action potentials overshot 0 mV. One bipolar stainless steel stimulating electrode (FHIC, Brunswick, ME) was placed in the loose bundle of axons exiting LMAN dorsocaudally and another was placed in the bundles of fibers in the ventral forebrain, which include DLM axons entering LMAN ventrally (Fig. 2, B and C). In most experiments, the DLM pathway stimulating electrode was placed in the ventral-most forebrain, always several hundred micrometers below Area X, to avoid antidromic stimulation of LMAN axonal collaterals projecting to Area X. In some cases, however, it was possible to place the electrode even farther from Area X, in the thalamic fiber bundle just as it exited DLM. A few cells also are included from early experiments, when the DLM pathway stimulating electrode was placed between LMAN and Area X ($n = 3/20$ cells used for pharmacology). Input pathways were activated using monopolar current pulses (100 μsec duration), and neuronal recordings were made with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) in “bridge” mode and filtered at 3 kHz. Data was digitized at 10 kHz and analyzed off-line using the DataWave Experimenter’s Workbench hardware and software package (DataWave Technologies, Longmont, CO).

**Histological methods**

Cells were filled with Biocytin by injection of low-intensity current during the course of both intrinsic (see description of current injection protocol) and synaptic property experiments. During synaptic property experiments, low-intensity current ($-100$ pA for most experiments) was injected periodically throughout the course of the experiment to monitor the cell’s input resistance. After the end of experiments, slices were fixed by immersion in 4% paraformaldehyde at 4°C. Subsequently, slices were rinsed three times with 0.025 M phosphate buffer (PB), and incubated for 1 h in Cy3-conjugated streptavidin (1.5 μg/ml; Jackson ImmunoResearch Laboratories, West Grove, PA) in 0.025 M PB with 0.3% Triton X-100. After the reaction, slices again were rinsed three times in PB, then mounted in 90% glycerol/10% 0.025 M PB, coverslipped and sealed.

**Confocal microscopy**

Fluorescence was viewed with an MRC-600 laser scanning confocal imaging system (Bio-Rad, Hercules, CA) equipped with a filter optimized for imaging Cy3 fluorescence. The soma diameter of each cell was measured from a confocal image using the interactive scale bar in Bio-Rad’s COMOS software. Because of shrinkage caused by fixation, the soma diameters given here underestimate the true diameter. Each cell’s primary dendrite number also was counted from confocal images.

**Synaptic properties**

To examine monosynaptic responses, we selected for pharmacological and temporal analysis those excitatory postsynaptic potentials (EPSPs) that showed a fixed latency with increasing current intensity and a stable amplitude in response to stimulation at 1 Hz. The current intensity was set for each stimulating electrode to minimize response failures as well as polysynaptic EPSPs and inhibitory postsynaptic potentials (IPSPs).
Data analysis

Each cell’s $R_i$ and membrane time constant ($\tau$) were estimated from responses to 510–590 ms, −100 pA current injections. Responses to this magnitude of current fell within the linear response range of the neurons. Single exponential curves were fit to the charging phase of single voltage traces using the Origin software package’s nonlinear curve fit tool with $\chi^2$ error minimization (Microcal Software, Northampton, MA). Current-voltage relationships were determined by injecting subthreshold current steps at a range of intensities. The range varied for different cells, but in all cases was between −1 nA and +1 nA. For each cell, the series of intensities was repeated five times and means ± SD were calculated (example shown in Fig. 3). To examine firing properties, suprathreshold current pulses also were delivered over a range of intensities (±1 nA). For each current level, mean firing rate was calculated by dividing the total number of spikes by the duration of the pulse. Plots of mean rate versus current intensity were fit with a straight line.

In the synaptic property experiments, responses were monitored on-line, but for analysis purposes EPSP initial slopes and peak amplitudes were measured off-line using the DataWave Common Processing Analysis software package (DataWave Technologies). Slope and peak amplitude values were measured for each individual EPSP. Responses during each drug condition were compared with the mean value from a 10-min predrug baseline period. In the P-P experiments, we made slope and peak amplitude measurements for the responses to both pulses and separately calculated (EPSP 2)/(EPSP 1) in terms of peak amplitude and slope for each cell. These are referred to collectively as P-P ratios and individually as peak ratios and slope ratios. P-P ratios were calculated for 10 consecutive trials at each of three interstimulus intervals (ISI; 50, 100, and 200 ms), and from these the mean P-P ratio was computed for each interval. To examine the second pulse responses without the contribution of residual voltage from the first pulse response found in many of the traces, a second “subtracted” ratio was calculated for each cell. To generate the subtracted ratio, first, the same 10 consecutive traces for each ISI were averaged and the slope and peak amplitude of the first EPSP were measured. Then, in lieu of a single pulse average trace, the first pulse of the 200 ms ISI average traces was subtracted from the 50 and 100 ms ISI average traces. The purpose of this was to subtract the residual voltage from the first EPSP from the second pulse of the 50 and 100 ms ISI traces, to determine the slope and peak amplitude of the second EPSP without the contribution of temporal summation. Finally, the slope and peak amplitude of the subtracted second pulses were measured, and the ratio of these values to those from the first EPSP of the 50 and 100 ms ISI traces are termed the subtracted P-P ratios.

Pre- and postdrug data were compared, for each input type, using paired two-sample $t$-tests (two-tailed). For both pharmacology and paired-pulse experiments, DLM and intrinsic pathway data (collected from the same cells) were compared using paired two-sample $t$-tests (two-tailed). To determine whether P-P ratios were significantly different from 1, one-sample $t$-tests with a hypothesized mean of 1.0 were used. Error values given are SEs, with exceptions as noted.

RESULTS

Intrinsic properties

Intrinsic property data were collected from 113 neurons from 50 animals. Cells in this sample had an average resting potential of −65.2 ± 7.5 (SD) mV; $n = 113$), an average input resistance of 130.1 ± 52.9 MΩ ($n = 112$), and an average time constant of 40.6 ± 17.5 ms ($n = 112$). One hundred-six of these neurons were classified according to their firing behavior in response to suprathreshold current steps. Intrinsic property data for each class are given in Table 1. All classes displayed characteristics of immature mammalian neurons; that is, less hyperpolarized resting potentials than those of adult neurons, high-input resistances, long membrane time constants, and linear current-voltage relationships over a broad range of applied current intensities.

Linearity of subthreshold current-voltage relationships

Intracellular injections of subthreshold current pulses revealed the markedly linear current-voltage relationships of juvenile LMAN neurons. A series of hyperpolarizing and depolarizing pulses was repeated five times for each cell, and the mean voltage change for each current intensity was measured. Responses of a cell illustrating typical linearity are shown in Fig. 3. To determine $R_i$ and $\tau$, curves were fit to the membrane responses to −100 pA injections (see METHODS; Table 1). At the offset of hyperpolarizing current injections, most cells exhibited rebound depolarization, often reaching suprathreshold levels (81% of cells, $n = 81$). This property was present with differing frequency across firing types (Table 1).

Responses to suprathreshold current injections

In response to prolonged suprathreshold intracellular current injections, cells showed one of three types of firing behavior. The most common (73%, $n = 77$) was the adapting type I (ADI; Fig. 4 A), which exhibited monotonically increasing interspike intervals over the duration of the current injection. The next most common firing type (16%, $n = 17$) was the adapting type II (ADII; Fig. 4 B).

FIG. 3. Linearity of current-voltage relationships. A: superimposed responses to subthreshold current injections made at resting $V_m$. Top: voltage records; bottom: applied current. B: $V-I$ plot of cell shown in A. Each point represents mean maximal voltage change ±SD for 5 repetitions. This cell had values near the mean for intrinsic properties (see text) and demonstrated typical phenomena, such as rebound spiking (shown) and postburst afterhyperpolarization (AHP; see A). Rebound action potentials are clipped. Resting membrane potential was −65 mV.
TABLE 1. Properties of three classes of LMAN neurons (class determination based on intrinsic firing properties)

<table>
<thead>
<tr>
<th></th>
<th>ADI</th>
<th>ADII</th>
<th>NM</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>n</em></td>
<td>77</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>−63.9 ± 6.8 (−76.8−−50.7)</td>
<td>−68.5 ± 7.6 (−77.9−−51.3)</td>
<td>−70.8 ± 8 (−80.6−−50.6)</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>133.3 ± 49.3 (56.2−252.2)</td>
<td>120.6 ± 62.9 (21.2−269.4)</td>
<td>134.4 ± 68.1 (51.8−267.4)</td>
</tr>
<tr>
<td>Time constant, ms</td>
<td>40.0 ± 16.0 (8.4−86.2)</td>
<td>36.8 ± 19.5 (6.4−81.6)</td>
<td>44.1 ± 19.2 (13.7−71)</td>
</tr>
<tr>
<td>Minimum current to elicit an action potential, pA</td>
<td>96 ± 6 (20−250)</td>
<td>179 ± 27 (40−400)</td>
<td>126 ± 23 (40−300)</td>
</tr>
<tr>
<td>Percent of cells with post-burst AHP</td>
<td>94</td>
<td>67</td>
<td>82</td>
</tr>
<tr>
<td><em>n</em></td>
<td>70</td>
<td>15</td>
<td>11</td>
</tr>
<tr>
<td>Percent of cells with rebound depolarization</td>
<td>97</td>
<td>57</td>
<td>56</td>
</tr>
<tr>
<td><em>n</em></td>
<td>58</td>
<td>14</td>
<td>9</td>
</tr>
</tbody>
</table>

For each value, the mean ± SD is given, and the range of each value is given in parentheses. The three cell types differed significantly in their resting potentials (_P_ < 0.003) and in the minimum current required to elicit an action potential (_P_ < 0.00007), but not in their input resistance (_P_ > 0.67) or time constant (_P_ > 0.53) (by one-way analysis of variances). LMAN, lateral portion of the magnocellular nucleus of the anterior neostriatum; ADI, adapting type I; ADII, adapting type II; NM, nonmonotonic; AHP, after hyperpolarization.

ADII cells generated a single spike or a short-duration adapting burst of spikes. The spike burst of ADII cells often was followed by a long, slow depolarization like that shown in Fig. 4B. The third most common firing type (10%, _n_ = 11) was the nonmonotonic cell (NM; Fig. 4C). NM cells generated firing characterized by postburst afterdepolarization (ADP, Fig. 4C), nonmonotonically modulated interspike intervals, and minimally decreasing spike amplitude. Most cells (89%, _n_ = 96) exhibited after-hyperpolarization at the offset of suprathreshold current injections, as shown in the examples in Fig. 4. A cell’s firing type was not correlated with the age or sex of the bird nor with the _R_ of the cell. Although the firing types had significantly different resting potentials (Table 1), the range of resting potentials was similar, and changing the resting potential with DC injections did not change the cell’s firing type (data not shown). The three types also significantly differed in the minimum current intensity necessary to elicit an action potential, with the ADII type showing the highest thresholds (Table 1). All firing types appear to be projection neurons, as antidromic spikes could be elicited by LMAN efferent stimulation in cells of each firing type.

To assess how a cell’s firing profile affected that cell’s responses to suprathreshold current injections of increasing intensity, we also examined the mean firing rate versus current intensity relationships of the three major firing types. The ADI and NM cells had rate/intensity relations with slopes that were significantly steeper than those of the transiently responding ADII cells (Fig. 5, A and B; ADII vs. ADI: _t_ = 4.937, _P_ < 0.03; ADII vs. NM: _t_ = 16.938, _P_ < 0.0004; unpaired _t_-tests).

**Morphology of cells appears uniform across classes**

Of the cells recorded, 24 cells were filled successfully with Biocytin and visualized by confocal microscopy (see Methods for details). Of these, 20 were ADI type, 2 were ADII type, and 2 were NM type; 19 were from males and 5 were from females. All of the cells had similar size, morphology, and spininess; an example is shown in Fig. 6. The average diameter was 25.1 ± 3.1 μm, and the cells had an...
average of 6.8 ± 0.3 primary dendrites distributed evenly around the cell soma. Two morphological classes of neurons, large spiny and small aspiny, have been described from Golgi studies of LMAN (Nixdorf-Bergweiler et al. 1995b). The cells filled here corresponded to the large spiny class and likely represent the projection neurons for several reasons. First, the reported soma size of LMAN projection neurons is consistent with the large spiny class (Korsia and Bottjer 1989). Second, the morphology of cells filled here was similar to that of adult male LMAN neurons retrogradely labeled from RA (Boettiger, unpublished data). Third, antidromic spikes could be elicited in many of the recorded cells when stimulating the LMAN outflow tract, as would be expected for projection neurons.

**Synaptic properties**

Pharmacology data were analyzed from 20 of the cells included in the intrinsic property data above (12 from males, 8 from females; n = 16 animals). To examine monosynaptic excitatory responses that were stable over time, we selected synaptic responses that met the following criteria: they were stable during a predrug baseline period of ≥10 min, their initial peak was not truncated by a disynaptic IPSP, and they recovered by ≥50% after washout of synaptic antagonists. Mean recovery for responses to intrinsic inputs was 87.3 ± 3.7%; mean recovery for responses to DLM inputs was 87.4 ± 3.9%. Data for both pathways are included from 11 cells (6 from males, 5 from females), data for the intrinsic path only are included from 5 cells (3 from males, 2 from females), and data for the DLM path only are included from 4 cells (3 from males, 1 from a female). All statistical comparisons between pathways are made using only those cells with data from both pathways.

**DLM afferent and LMAN intrinsic synapses are glutamatergic**

To identify excitatory inputs to LMAN neurons, we stimulated both the DLM afferent fiber bundles entering the nucleus ventrally and the fiber bundle exiting LMAN dorsocaudally (Fig. 2B). In this way, we demonstrated that LMAN cells receive excitatory inputs from both DLM afferents and intrinsic synapses. In the majority of cells from which we recorded, disynaptic IPSPs could be elicited by slightly higher intensity stimulation of either input (data not shown), providing evidence that both feedforward and feedback inhibition are present in LMAN by this early stage in development. The current intensity for each stimulation electrode was set to the minimum value that generated no response failures, which produced responses to DLM inputs that were generally of equal or greater magnitude than those of the responses to intrinsic inputs, in both peak amplitude and initial slope (amplitude: 3.5 ± 0.5 vs. 2.5 ± 0.4 mV; slope: 1.1 ± 0.2 vs. 0.7 ± 0.2 mV/ms), although neither difference reached statistical significance (P > 0.1 and P > 0.09 respectively). Both responses were nearly eliminated (mean residual response for both pathways was 0.4 mV) by bath application of 100 μM APV plus 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Research Biochemicals, Natick, MA). Intrinsic response peak amplitudes were reduced by an average of 85.8 ± 2.2%, and their slopes were reduced by an average of 89.8 ± 2.7%. Similarly, DLM response peak amplitudes were reduced by an average of 89.1 ± 2.4%, and their slopes were reduced by an average of 83.4 ± 3.3%. All the reductions were significant at the P < 0.0003 level.

**FIG. 6.** Example of a filled juvenile LMAN neuron, demonstrating typical morphology. This cell was filled with Biocytin, reacted with Cy3-streptavidin, and visualized with confocal microscopy. Inset: enlarged view of a few dendrites with white arrowheads pointing to some of the dendritic spines.
Figure 7, A–C, shows an example of a cell with typical antagonist sensitivity. This virtual elimination of both responses by APV and CNQX establishes that both pathways are glutamatergic.

**Responses to intrinsic inputs have a relatively larger NMDAR-mediated component**

To assess the contribution of NMDARs to both types of LMAN responses, we measured the effect of 100 μM APV alone on the responses to both pathways. We first evaluated the contribution of NMDAR’s to the monosynaptic component of each response by measuring APV’s effect on the initial peak of the EPSP. APV reduced the amplitude of the intrinsic EPSPs by an average of 24.9 ± 3.6% (P < 0.0002, t15 = 4.9). APV also significantly reduced the DLM EPSPs, but relatively less so (13.1 ± 3.5%; P < 0.003, t14 = 3.7). This difference was not attributable to differing times to peak for the two types of EPSP (intrinsic: 5.5 ± 0.6 ms; DLM: 4.9 ± 0.5 ms; P > 0.3). As NMDAR-mediated responses have relatively slow kinetics, we also measured the effect of APV at a later time after EPSP onset (28 ms), closer to the peak of the NMDAR-mediated potential. This time point is likely to include additional polysynaptic components, despite the care taken to minimize polysynaptic activation. At this time point, the responses of both pathways were again significantly reduced but still differed from one another: the responses to intrinsic inputs were reduced by an average of 46.2 ± 4.4%, whereas the responses to DLM inputs were reduced by an average of 26.7 ± 3.1% (intrinsic: P < 0.0001, t11 = 6.7; DLM: P < 0.0001, t10 = 8.0). An example of the effect of APV on both input pathways in a single cell is shown in Fig. 7, A–C.

Statistical comparisons between the APV sensitivity of both pathways, measured and compared in the same cells, confirmed that the responses to intrinsic inputs were more sensitive to APV than the responses to DLM inputs, both at the initial peak and at 28 ms after EPSP onset (Fig. 7, D and E). This result is further strengthened by that fact that responses to intrinsic inputs tended to have slightly smaller EPSP peak amplitudes than did responses to DLM inputs. Because NMDARs are less blocked by Mg2+ at more depolarized membrane potentials, for a given NMDAR to non-NMDAR synaptic ratio, the NMDAR contribution increases with increasing EPSP amplitude. Thus if these two inputs had similar NMDAR synaptic ratios, the NMDA component should have been more evident in the DLM EPSPs because they are larger. Our results therefore suggest that NMDAR-mediated currents account for a larger proportion of the responses to intrinsic inputs than of the responses to DLM inputs.

**Effects of P-P stimulation**

Because LMAN neurons in vivo are sensitive to the temporal patterning of auditory stimuli, we investigated the cellular responses to temporally patterned stimulation of either input. We delivered pairs of pulses to each pathway, in alternation, at 50, 100, and 200 ms intervals (interleaved at 20 s intervals) and calculated the resulting P-P ratio of both the EPSP peaks and slopes for each interval. Multi-interval P-P data were collected from 26 of the cells included in the intrinsic property study (16 from males, 10 from females; n = 14 animals). This experiment revealed differences in the temporal dynamics of responses to these two inputs, with potential implications for the role of each pathway in processing temporally patterned stimulation.

**INTRINSIC PATHWAY.** Responses to paired stimulation of the intrinsic pathway generated P-P peak ratios significantly >1.0 for all three intervals. Example traces are shown in Fig. 8A, and Fig. 8C shows a plot of EPSP 1 versus EPSP 2 for each interval for the same cell. Both figures demonstrate the effect of the first pulse on responses to subsequent pulses; group data are shown in Fig. 9A. In contrast to the peak ratios, the slope ratios for the intrinsic pathway were not significantly different from 1.0 for any of the intervals,
as shown in Fig. 9B. Although, on average, the P-P slope ratios showed no significant modulation from 1.0, a minority of cells showed paired-pulse facilitation (PPF) or PPD (Table 2). The slope ratios had a weak negative correlation with first EPSP peak amplitude, significantly so at the 50 and 200 ms ISIs ($R^2 = 0.22, P < 0.02; R^2 = 0.15, P > 0.05; R^2 = 0.18, P < 0.04$; for 50, 100, and 200 ms ISIs, respectively). The fact that the intrinsic P-P slope ratios were not significantly different from 1.0, while the P-P peak ratios were, suggested that the modulation of P-P peak ratios $>1.0$ was due to temporal summation of EPSPs and not PPF.

To distinguish temporal summation from PPF, we assumed that there were no voltage-activated conductances and digitally subtracted the 200 ms ISI average trace from both the 50 and 100 ms average traces. This subtraction procedure removed the portion of the second EPSP that was attributable to residual response to the first pulse (see Methods). We then measured the peak amplitudes and slopes of the second EPSP from these subtracted traces and divided these values by the first EPSP values to determine the subtracted P-P ratios. Examples of subtracted traces together with the 200 ms ISI average traces that were subtracted from them are shown in Fig. 9C. As predicted by the slope ratios, the positive modulation of P-P peak ratios of intrinsic responses was eliminated by the subtraction (Fig. 9C). As further evidence in favor of temporal summation,

![Graph](image_url)
the subtraction did also not reveal a significant facilitation of the slope, as shown in Fig. 9D.

**THALAMIC PATHWAY.** Responses to paired thalamic afferent stimulation were significantly different from intrinsic pathway responses in a number of ways. DLM-elicited EPSPs also displayed P-P peak ratios significantly >1.0 at the 50- and 100 ms ISIs but not at 200 ms. Example traces for each interval are shown in Fig. 8B, along with an EPSP 1 versus EPSP 2 plot from the same neuron (Fig. 8D); group data are shown in Fig. 9A. The P-P slope ratios, however, showed strong PPD, largest at the 50 ms ISI and decreasing with longer intervals (Fig. 9B). Statistically significant PPD of responses to DLM inputs occurred in 20 of 25 cells (Table 2). PPD of the responses to DLM inputs was not simply caused by a loss of driving force due to residual voltage, as the P-P slope ratio was not correlated with the $V_m$ at the second pulse ($R^2 = 0.004, R^2 = 0.0004, R^2 = 0.018$ for the 50, 100, and 200 ms intervals, respectively). The DLM P-P slope ratios were also uncorrelated with the peak amplitude of the first pulse ($R^2 = 0.0011, R^2 = 0.03305, R^2 = 0.0072$ for the 50, 100, and 200 ms ISIs, respectively). The discrepancy between the peak and slope ratios suggested that temporal summation masked an underlying PPD of the responses to DLM inputs. Consistent with this, subtracted DLM P-P peak ratios revealed significant depression (example trace in Fig. 8F; group data in Fig. 9C). Furthermore, subtraction did not reverse the depression of the slope ratios, as shown in Fig. 9D.

Thus the P-P responses of juvenile LMAN neurons to these two excitatory inputs are distinct. Indeed, statistical comparison of the two pathways showed greater P-P ratios of the responses to intrinsic inputs at all three intervals measured in terms of both peaks and slopes (Fig. 9). Summation of responses to intrinsic inputs was larger and longer lasting than that of responses to DLM inputs, despite the smaller peak amplitude of intrinsic EPSPs (2.2 vs. 3.5 mV, $P < 0.002$).

To examine the contribution of NMDARs to the temporal dynamics of LMAN membrane responses to synaptic stimulation, we also performed P-P experiments in the presence of APV. The results showed that blockade of NMDARs had differential effects on the temporal properties of the two types of responses. Although APV significantly reduced temporal summation of the responses to intrinsic inputs, it had no significant effect on the DLM P-P ratios. Figure 10, A and B, shows group data for the effect of APV on temporal summation, and Fig. 10, C and D, shows the effect of APV on the temporal summation of both types of responses in a single cell. This experiment also provided further evidence that the DLM PPD was not simply due to loss of driving force: despite the fact that APV reduced the membrane voltage at the time of the second pulse, it did not change the P-P slope ratios. APV also had no significant effect on the intrinsic P-P slope ratios (data not shown).

**DISCUSSION**

In this study, the intrinsic properties of LMAN neurons in the early sensory phase of learning were determined, and two sources of excitatory input to these cells were established. The neurons had immature passive membrane properties and were grouped into three classes based on their firing behavior. Inputs from DLM afferents and intrinsic synapses, although both glutamatergic, differed in a number of ways. The responses to intrinsic inputs possessed a relatively greater NMDAR-mediated component than the responses to thalamic inputs. Moreover, although both responses were characterized by temporal summation in response to paired stimuli, the temporal summation of responses to intrinsic inputs was greater, and it was significantly reduced by APV. Finally, the two responses were distinguished further by the occurrence of consistent PPD in responses to DLM inputs, whereas the intrinsic inputs showed no consistent short-term synaptic plasticity.

**Intrinsic properties**

The LMAN neurons recorded here from birds aged 18–25 days had passive intrinsic properties that appeared immature compared with LMAN neurons of both adults and of birds aged 27–51 days (Livingston and Mooney 1997). Instead, their properties were more similar to those described in immature neurons in mammalian neocortex: their resting potentials were high, their $R'_i$’s were large and their $\tau$’s were long (Burgard and Habilitz 1993; McCormick and Prince 1987). In addition, most LMAN neurons in our study generated a continuously adapting spike train in response to suprathresh-
old current injections; we have called these adapting type I or ADI cells. ADI firing is similar to the spiking behavior described for neocortical regular spiking (or RS) neurons (McCormick et al. 1985). A smaller number of LMAN neurons, termed adapting type II or ADII cells, generated single spikes or a very short initial burst of spikes, often followed by a slow depolarization, in response to the same types of inputs. Such spiking behavior is similar to that described for nonpyramidal low-threshold spike (LTS) neurons found in rat frontal cortex (Kawaguchi 1995). Calcium-mediated LTSs have been described in neurons from another zebra finch song nucleus, HVc (Kubota and Saito 1991). A minority of LMAN cells had novel spiking behavior; we have called these the nonmonotonic or NM type. NM cells generated bursts that were like the bursts of cortical chattering (CH) cells in that interspike intervals changed nonmonotonically, spike amplitudes decreased minimally within a burst, and bursts were followed by ADPs (Gray and McCormick 1996). The interburst intervals of NM cells were longer, however, than those described for CH cells and were more similar to those of cortical intrinsic bursting cells (McCormick et al. 1985). ADII cells were distinguished from ADI and NM cells not only by their firing behavior but also by their inability to markedly increase their firing rate in response to increasing current intensity. A recent report described only two firing types in LMAN slices from young finches, a NM type, which predominated, and an adapting mode that appears intermediate between ADI and ADII (Livingston and Mooney 1997). A possible explanation for this difference in firing types and their incidence is the age difference in the animals used: while we investigated birds between 18 and 25 days of age, that study examined intrinsic properties in birds >27 days.

Despite their functional differences, the three firing classes were not obviously morphologically distinct nor did firing class, at this stage of development, correlate with age or sex. Because each cell’s firing behavior did not depend on its passive membrane properties, one possibility is that the three classes may be generated by differing ion channel and calcium buffering profiles. Alternatively, these “types” may represent different firing regimes of a single neuron type, controlled by modulatory neurotransmitters such as dopamine or acetylcholine, as has been described in other systems (McCormick and Nowak 1996; Turrigiano and Marder 1993). The presence of cholinergic and catecholaminergic fibers in LMAN (Bottjer 1993; Sakaguchi and Saito 1991; Soha et al. 1996) lends support to this hypothesis.

The firing behaviors of LMAN neurons recorded here in vitro were consistent with LMAN responses in vivo, including burst firing and response adaptation (Doupe 1997; Hessler and Doupe 1997; Solis and Doupe 1997). Moreover, burst firing may be critical to the temporal sensitivity of these neurons, as has been suggested from in vivo intracellular studies of similarly song-selective neurons in HVc (Lewicki 1996).

Differing pharmacology of intrinsic and thalamic synapses

Our data confirm the recent finding that DLM inputs onto the LMAN projection neurons are excitatory and glutamatergic (Livingston and Mooney 1997). Our data also show that LMAN neurons receive significant excitatory and glutamatergic input from recurrent collaterals as well. Moreover, we found that the responses to recurrent or “intrinsic” inputs had a relatively larger NMDAR-mediated component than the responses to DLM inputs. This difference between inputs was even more marked at a late time point after response onset, which may reflect not only a greater NMDAR-mediated component of monosynaptic responses to intrinsic inputs but also greater activation of excitatory polysynaptic circuitry by the intrinsic connections. One interpretation of the functional difference between the pharmacology of these two inputs is that there is a larger proportion of NMDARs to non-NMDARs at intrinsic synapses relative to DLM synapses. This explanation assumes, however, that each input makes synapses onto LMAN neurons at similar electrotonic locations and thus that the local level of depolarization at each synapse type is reflected with equal accuracy at the cell soma. The locations of DLM afferent and LMAN intrinsic synapses onto LMAN neurons are presently unknown. A second possible explanation for the difference in NMDAR-mediated component between the two inputs is that undetectable disynaptic IPSPs act to attenuate the NMDA component of responses to DLM inputs but not of the responses to intrinsic inputs. This possibility was not explored here by using GABA_A antagonists, as the intrinsic pathway was prone to a high degree of polysynaptic excitation even in the presence of normal inhibition. Whether or not there is an absolute difference in the pharmacology of intrinsic and DLM synapses, there remains a functional difference in how much NMDARs contribute to responses to the two inputs.

Functional pharmacological differences analogous to those in LMAN are evident in the thalamic and intrinsic connections in mammalian neocortical areas (Gil and Amitai 1995; Pirot et al. 1995). This similarity suggests that intrinsic LMAN circuitry could serve to modify and amplify inputs from the thalamus, as has been proposed for cortical circuitry (Daw et al. 1993; Nelson and Sur 1992).

Differing temporal dynamics of responses to intrinsic and thalamic stimulation

Little is known about the cellular mechanisms that may underlie the ability of LMAN neurons to develop sensitivity to complex temporal patterns. A recent theoretical investigation of temporal processing demonstrated that time-dependent properties of neurons, such as short-term synaptic plasticity, can generate temporal sensitivity in neuronal networks (Buonomano and Merzenich 1995). We therefore also examined responses of LMAN neurons to pairs of stimuli separated by different time intervals and found that the responses of LMAN neurons to DLM and intrinsic inputs differed in their temporal properties.

Responses to paired-pulse stimulation of either input showed temporal summation but, despite the smaller peak amplitude of intrinsic EPSPs, the summation of the intrinsic responses was larger and longer lasting than that of the responses to DLM inputs. There are several possible explanations for this difference. First, individual intrinsically evoked-EPSPs may decay more slowly than DLM-evoked EPSPs, consistent with a proportionally greater NMDAR-mediated component in the intrinsic responses. The long τ’s
of these neurons likely contribute to the long duration of both responses, as seen in immature rat neocortical neurons (Burgard and Hablitz 1993), and may exaggerate a difference in decay times. Second, there may be greater activation of excitatory polysynaptic circuitry by intrinsic inputs. DLM inputs may generate less polysynaptic excitation if they induce more polysynaptic inhibition or if they project to a more circumscribed set of excitatory LMAN neurons relative to intrinsic projections. Such a restriction of the DLM projection could reflect either the in vivo situation or could be an artifact of our slice preparation. Finally, the presence of significant PPD only in DLM-evoked EPSPs also could account for the differences in temporal summation between the two pathways. The fact that temporal summation of responses to intrinsic inputs, and not of responses to DLM inputs, is APV-sensitive lends further support to our conclusion that responses to intrinsic inputs are more NMDAR dependent. This result, together with the basic difference in temporal summation, also raises the further possibility that the two excitatory synapse types on LMAN neurons express NMDARs with differing subunit compositions, with the intrinsic inputs activating NMDARs with slower kinetics or less susceptibility to Mg$^{2+}$ block (Monyer et al. 1992).

Most DLM-evoked responses showed considerable PPD. Among significantly depressed EPSPs, the average reduction neuron dendritic spines are being eliminated (Fig. 1) (Nix and Doupe 1995). As NMDAR levels decline, three other phenomena are also occurring in LMAN: projection neuron dendritic spines are being eliminated (Fig. 1) (Nixdorf-Bergweiler et al. 1995b), the DLM afferents are regressing (Johnson and Bottjer 1992), and single-unit auditory responses are becoming selective (Doupe 1997; Solís and Doupe 1997). The coincidence of these phenomena suggests that NMDAR-dependent long-term plasticity phenomena, such as those studied in mammalian systems (Bear and Malenka 1994; Crair and Malenka 1995; Dudek and Friedlander 1996; Kirkwood et al. 1995; Linden 1994; Scanzi et al. 1996), may play a role in selective synapse retention and/or elimination in LMAN. Such synaptic modifications could contribute to the experience-dependent development of selectivity in LMAN (see Doupe 1998 for review). Because birds are undergoing sensory learning during this time, Basham and colleagues tested the functional importance of NMDARs in song learning (Basham et al. 1996). These experiments demonstrated that intact NMDAR function in LMAN during exposure to tutor song is required for normal tutor song learning to occur. That study did not determine, however, whether adult LMAN selectivity was abnormal in APV-treated birds nor whether neuronal morphology was immature. Thus the connection between selectivity, morphology and NMDAR-dependent processes remains to be tested directly. Moreover, our data suggest that, rather than simply subserving plasticity within LMAN, an essential function of LMAN NMDARs involves basic neuronal processing, such as amplifying DLM input or prolonging LMAN responses. For example, if activation of LMAN intrinsic circuitry is needed for repetitive DLM-driven responses to reach threshold, blockade of NMDARs in LMAN may simply eliminate much of the output of the nucleus rather than selectively blocking plasticity; such a situation has been described previously in mammalian visual cortex (Fox et al. 1989; Miller et al. 1989). Basic processing functions of NMDARs could play an important role in shaping the level or timing of LMAN output. In other pattern-generating systems such as the lamprey spinal cord and the
electric organ pacemaker nucleus of electric fishes, activation of NMDARs can switch the firing state of output neurons (Grillner 1995; Kawasaki and Heiligenberg 1990). Thus NMDAR activation in LMAN is predicted to have consequences not only within LMAN but also in its target motor nucleus RA.

**Hypothesized function of LMAN circuitry during song learning**

The data from juvenile birds presented here begin to suggest how LMAN circuitry may be functioning during the sensory acquisition phase of song learning. First, the DLM synapses, carrying information from HVC via Area X, will activate fast, large, somewhat quickly decaying responses that depress rapidly with repeated high-frequency stimulation. With adequate stimulation, perhaps through synchronous DLM firing or by coactivation of modulatory inputs, the DLM afferents will trigger activation of LMAN intrinsic excitatory circuitry, although the time-dependent properties of the DLM synapses should make this activation strongly dependent on the recent temporal context. Activation of the longer lasting intrinsic circuitry then could generate prolonged firing in the LMAN output neurons. An unresolved but intriguing issue is how LMAN may modulate its input from DLM via the ‘recurrent’ loop through Area X (Nixdorf-Bergweiler et al. 1995a; Yates and Nottebohm 1995).

The long time course of responses within the LMAN circuitry of young birds could contribute to both the lack of selectivity and the tendency toward more tonic responses typical of juvenile LMAN (Doupe 1997). This long response time course also may allow coincident activation and strengthening of temporally distinct inputs, not only within LMAN but also within its target nuclei RA and X. Examining how the synaptic and temporal properties of LMAN neurons change as song learning proceeds should shed light on basic cellular mechanisms of learning.

We thank D. Buonomano, G. Carrillo, M. Crair, S. du Lac, S. Gomperts, R. Malenka, D. Perkel, M. Silver, and A. Tam for valuable technical help and advice and D. Buonomano, N. Hessler, R. Malenka, C. Ramsey, and M. Solis for critical comments on the manuscript.

This work was supported by the Klingenstein Fund, the Lucille P. Markey Charitable Trust, the McKnight Foundation, the John Merck Fund, the Searle Scholars Program, the Sloan Foundation, National Institute of Mental Health Grant (NIMH) MH-55987 (to A. J. Doupe), and by a University of California San Francisco Graduate Opportunity Fellowship and a predoctoral National Research Service Award from the NIMH (to C. A. Boettiger).

Address for reprint requests: C. A. Boettiger, Physiology Dept., Box 0444, UCSF, 513 Parnassus Ave., San Francisco, CA 94143-0444.

Received 27 October 1997; accepted in final form 9 January 1998.

**REFERENCES**


NELSON, S. B. AND SOR, N. M. Different classes of glutamate receptors and GABA mediate distinct modulations of a neuronal oscillator, the medullary pacemaker of a gymniform electric fish. J. Neurosci. 10: 3299±3304, 1990.


