Published in final edited form as:

J Comp Neurol. 2006 March 10; 495(2): 185–201. doi:10.1002/cne.20862.

Interaural timing difference circuits in the auditory brainstem of the emu (*Dromaius novaehollandiae*)

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Abstract

In the auditory system, precise encoding of temporal information is critical for sound localization, a task with direct behavioral relevance. Interaural timing differences are computed using axonal delay lines and cellular coincidence detectors in nucleus laminaris (NL). We present morphological and physiological data on the timing circuits in the emu, *Dromaius novaehollandiae*, and compare these results with those from the barn owl (*Tyto alba*) and the domestic chick (*Gallus gallus*). Emu NL was composed of a compact monolayer of bitufted neurons whose two thick primary dendrites were oriented dorsoventrally. They showed a gradient in dendritic length along the presumed tonotopic axis. The NL and nucleus magnocellularis (NM) neurons were strongly immunoreactive for parvalbumin, a calcium-binding protein. Antibodies against synaptic vesicle protein 2 and glutamic acid decarboxylase revealed that excitatory synapses terminated heavily on the dendritic tufts, while inhibitory terminals were distributed more uniformly. Physiological recordings from brainstem slices demonstrated contralateral delay lines from NM to NL. During whole-cell patch-clamp recordings, NM and NL neurons fired single spikes and were doubly-rectifying. NL and NM neurons had input resistances of $30.0 \pm 19.9 \text{ M}\Omega$ and $49.0 \pm 25.6 \text{ M}\Omega$, respectively, and membrane time constants of $12.8 \pm 3.8 \text{ ms}$ and $3.9 \pm 0.2 \text{ ms}$. These results provide further support for the Jeffress model for sound localization in birds. The emu timing circuits showed the ancestral (plesiomorphic) pattern in their anatomy and physiology, while differences in dendritic structure compared to chick and owl may indicate specialization for encoding ITDs at low best frequencies.

Indexing terms

avian; nucleus laminaris; nucleus magnocellularis; dendrite; coincidence detection; sound localization

INTRODUCTION

Emus (*Dromaius novaehollandiae*) are members of the ratite family, as are the ostrich, rhea, cassowary and kiwi. Ratites and Tinamiformes make up the Paleognathae, a group of flightless birds that show a number of ancestral and/or neotenic features. Thus the structure of their central auditory system would be expected to be relatively unspecialized, and suitable for comparisons with the better-understood chicken and barn owl. The inner ear of the emu is also more plesiomorphic than any bird so far investigated (Fischer et al., 1994; Manley et al., 1997). Furthermore, emus make booming vocalizations that may be heard over long distances, and analysis of their brainstem auditory system may therefore be

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expected to shed light on low best frequency auditory processing and localization (Marchant and Higgins, 1990; Halkin and Evans, 1999).

In the auditory system, encoding of temporal information is critical for sound localization. Timing cues are preserved in the phase locked firing of the auditory nerve, which enters the brain and innervates the two cochlear nuclei (Boord and Rasmussen, 1963; Takahashi et al., 1984; Warchol and Dallos, 1990). The cochlear nucleus magnocellularis processes phase information, and projects bilaterally to the nucleus laminaris (Parks and Rubel, 1975; Parks and Rubel, 1978; Sullivan and Konishi, 1984; Young and Rubel, 1986; Carr and Konishi, 1990). Sensitivity to interaural time differences first appears in the nucleus laminaris (Sullivan and Konishi, 1986; Carr and Konishi, 1990; Overholt et al., 1992; Funabiki et al., 1998).

The projection from the cochlear nucleus magnocellularis to the nucleus laminaris forms a circuit that conforms to the model proposed by Jeffress to explain sound localization by detection of interaural time differences (Jeffress, 1948). In the Jeffress model, axonal delay lines project to neurons that require simultaneous arrival of spikes from the two sides to elicit a maximal discharge. In the owl and chicken brainstem, the magnocellular projections to the nucleus laminaris function as delay lines (Carr and Konishi, 1990), while the neurons of the nucleus laminaris act as coincidence detectors (Sullivan and Konishi, 1986; Funabiki et al., 1998). Magnocellular inputs form maps of delay with axonal paths that may be unequal for the two sides (Carr and Konishi, 1990). NL neurons respond maximally when the axonal delays are opposed by equivalent interaural phase differences.

This paper describes the cell types and connections of the circuit responsible for the detection of interaural phase differences in the brainstem of the emu. Examination of this circuit by analyzing evoked field potentials and intracellular whole-cell recordings in the brainstem slice preparation has shown that the emu circuits conform to the Jeffress model. The monolayer organization of the emu's brainstem displayed a plesiomorphic pattern similar to other birds (Rubel and Parks, 1988; Carr and Code, 2000). However, the cellular morphology of the emu NL neurons, bitufted neurons with thick primary dendrites which receive distal excitatory input, were distinct from those found in chick and barn owl NL, suggesting specialization in dendritic structure for the computation of ITD at low best frequencies.

MATERIALS AND METHODS

Immunohistochemistry and anatomy

Emu embryos (embryonic day (E) 36–50 of a ~50 day incubation) were processed for immunohistochemistry. After cooling the egg, the embryos were deeply anesthetized with an overdose of pentobarbital IM. The emus were then perfused transcardially with saline, followed by 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB, pH 7.4). The brains were postfixed in the perfusion solution for 1–2 days, and cryoprotected in 30% sucrose in PB at 4°C. Brains were embedded in 7.5% gelatin/ 30% sucrose/ 0.1 M PB and placed in 30% sucrose/ 4% PF/ 0.1 M PB overnight. The brains were sectioned on a freezing microtome at 30 µm thickness and the sections collected in PB. Most brains were cut in the coronal plane, while three were cut at a 45° angle to the main axis of the hindbrain, and thus roughly parallel to the isofrequency plane on the one side, and orthogonal on the other side.

The primary antisera, their sources, and the antiserum dilutions were as follows: The anti-glutamic acid decarboxylase (GAD) was provided by Dr. J. White from a source (1440-4) developed at the National Institutes of Health by Drs. Irwin J. Kopin, Wolfgang Oertel, Donald E. Schmechel, and Marcel Tappaz (Oertel et al., 1981). It was used at a

concentration of 1:1,000. 1440-4 is a polyclonal antibody raised in sheep against GAD partially purified from rat brain synaptosomes. After polyacrylamide gel electrophoresis, the one band containing GAD activity was injected into a sheep, producing a polyvalent GAD-antiserum. A GAD-inhibitor-antibody complex was produced, and injected into a non-immunized sheep that had been previously bled to obtain a preimmune serum. The resultant sheep anti-GAD (1440) antibody detected one antigen in rat brain homogenate supernatant (1440, Oertel et al., 1981), although later reports show that although the 1440 antibody preferentially recognizes GAD65, it also binds to GAD67 (Kaufman et al., 1991). Western blot analyses of zebra finch brain revealed a doublet at 61 and 59 kD in the cerebellum and forebrain (Fig. 5, Spiro et al., 1999). In another bird species, the barn owl, serial dilution controls showed optimal staining at a concentration of 1:1,000, and no specific staining with the preimmune serum (Carr et al., 1989).

The anti-synaptic vesicle protein 2 (SV2) was provided by Dr. K. Buckley and the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City (1:1,000). SV2 is a transmembrane glycoprotein whose distribution and localization have been well characterized. Immunolabeling of the SV2 antigen was achieved using a mouse-derived monoclonal antibody (Buckley and Kelly, 1985) that is known to react with each of the three SV2 isoforms, but not with other synaptic or neural proteins (Janz and Sudhof, 1999). Interspecific sequence analyses demonstrate that SV2 is highly conserved among vertebrates, with strong homology among isoforms (Bajjalieh et al., 1993; Janz and Sudhof, 1999) and across species (Bajjalieh et al., 1992; Bindra et al., 1993). The monoclonal antibody was prepared by immunizing mice with synaptic vesicles purified from the elasmobranch *Discopyge ommata* (Buckley and Kelly, 1985). Western blot analysis of the anti-SV2 monoclonal antibody against a zebra finch brain tissue homogenate yielded a broad band over a range of protein sizes (66–200 kDa) distinct from control tissue labeling (Nealen, 2005). This is in agreement with previously published reports that show difficulty in electrophoretically resolving glycosylated proteins (Schivell et al., 1996).

The anti-Parvalbumin (PV; product No. P3171; clone PA-235) was a mouse monoclonal antibody obtained from Sigma (1:1,000). The Sigma product information sheet states that purified carp muscle parvalbumin was used as the immunogen, and that the anti-Parvalbumin was derived from a hybridoma and originally characterized by Celio (Celio et al., 1988). This anti-PV labeled a single spot on a 2-dimensional gel-electrophoretic immunoblot of rat brain tissue at a molecular weight of 12 kD and pI of 4.9, and was distinct from other calcium-binding proteins. The Sigma product information further states that the antibody reacted specifically with chicken tissue, and that western blots yield a 12 kD band. Specificity of this anti-PV label has also been previously demonstrated in chicken brainstem sections in which staining was abolished by preadsorption of the primary antiserum with the immunizing protein (Fujii and Lucaj, 1993). The cellular localization of the anti-PV label in our emu material (uniformly distributed throughout the cell body and processes), as well as the intense label we found in Purkinje neurons in the cerebellum, is consistent with the staining described in chicken, finch and several mammals using this antibody (Celio et al., 1988; Fujii and Lucaj, 1993; Lohmann and Friauf, 1996; Reiner et al., 2004). Anti-PV label similar to that we describe in the emu cochlear and laminaris nuclei has been found in the brainstem auditory nuclei in the rat (Lohmann and Friauf, 1996).

Standard immunohistochemical procedures were followed using the avidin-biotin-peroxidase complex (ABC) method with reagents from Vectastain elite kits (Vector laboratories, Burlingame, CA). For immunohistochemistry, sections were pre-incubated for 1 hour in 0.01 M phosphate buffered saline pH = 7.6 with 4% normal serum and 0.4% Triton-X, then incubated overnight in antisera. Floating sections were incubated for 10 minutes in 3% H₂O₂ in PBS:Butanol (1:1), washed, incubated for 1 hour in biotinylated

rabbit anti-sheep secondary antisera (GAD) or biotinylated horse anti-mouse (PV, SV2), both diluted at 1:1,000. Sections were washed (PBS, $3 \times 15\text{min}$), incubated in ABC for 1 hour, then washed for 20 minutes in Tris-HCl buffer followed by 20 minutes in acetate-imidazole buffer. Sections were treated with diaminobenzidine tetrahydrochloride (DAB, 0.48 mg/ml) and 0.03% H_2O_2 with nickel sulfate intensification (26.3 mg/ml) in acetate imidazole buffer. Sections were mounted onto subbed slides, dehydrated, cleared, and cover slipped with Permount. Some sections were additionally counterstained with neutral red.

The results revealed that SV2 and PV could be visualized within the nucleus laminaris. To determine the relationship between the presynaptic terminals and the dendritic tufts in the nucleus laminaris, immunohistochemical double labeling was carried out. The primary antisera used were the same as in the single-label studies. The tissue was incubated with 1% normal goat serum, then incubated in 1:1,000 anti-PV for 12 hours at 4°C , washed well, then incubated in tetramethylrhodamine (TRITC) conjugated secondary antiserum at 1:250 dilution. The tissue was then washed, and incubated in 1:1,000 anti-SV2 as before. Sections were washed and incubated in Cy5 conjugated secondary antiserum at 1:250 dilution. The secondary antibodies were all raised in goat and obtained from Jackson ImmunoResearch (West Grove, PA). The sections were washed, mounted and coverslipped with an Antifade kit (Molecular Probes, Eugene, OR). They were examined by using a Bio-Rad 1024 krypton-argon laser scanning, confocal imaging system coupled to a Nikon Diaphot 300 inverted epifluorescence microscope. Digital photographs of immunohistochemistry sections were imported into Adobe Photoshop 7.0 and slightly adjusted for brightness and contrast.

Using the computer-assisted tracing program, Neurolucida (Microbrightfield, Williston, VT), 3-dimensional reconstructions of the nucleus laminaris and nucleus magnocellularis were created from transverse Nissl stained sections (see Fig. 3). Cell bodies of NL and NM were marked and nuclear outlines were drawn around cell body clusters in each section.

Dorsoventral dendritic length measurements were made from parvalbumin stained sections that had been cut orthogonal to presumed isofrequency bands. A 3-dimensional plot was constructed with the mediolateral position and rostrocaudal position as the x- and y-axes, respectively. Each dendritic length data point at a given position was represented as a color-coded pixel with red pixels for short dendrites and blue for long (Figure 6A). The iso-length axis was then calculated as the best multi-dimensional linear fit (red line in Figure 6A). The line orthogonal to the multi-dimensional linear fit, and positioned to traverse the shortest and longest lengths in the plot, was defined as the “dendritic length gradient” (black line in Figure 6A); the panel in Figure 6B is the cross-section of the data plot in Figure 6A along the dendritic length gradient, averaged across a 10-data-point window on either side of the line. While the slope that we report in the Results was derived from sections orthogonal to the iso-frequency bands, we performed a similar analysis on sections parallel to the isofrequency (‘iso-length’) axis and determined similar gradient slopes (data not shown).

To map a putative best frequency to dendritic length, we used data on the frequency representation in the emu basilar papilla, which showed a log mapping of frequency with position (Köppl and Manley, 1997). Although physiological recordings in chick showed a linear mapping of best frequency across NL (Rubel and Parks, 1975), lacking any direct evidence from the emu NL, we assumed a frequency representation in emu NL identical to that in the papilla (i.e., exponential).

Total dendritic length and other morphological measurements (e.g., cell body size) were made from NL neurons that had been passively filled with biocytin during whole cell physiological recordings. Brainstem slices containing filled neurons were processed according to standard procedures. After completion of the recording, slices were fixed in 4%

paraformaldehyde in 0.1 M phosphate buffer overnight, and processed with Vectastain ABC (avidin-biotinylated HRP complex) Elite Kit (Vector Labs) with DAB visualization. Completely filled neurons were drawn using the Neurolucida system. We used subjective criteria for determining the outline of the cell body: an ovoid outline was drawn whose dorsal and ventral limits (where the dendrites began) were determined by an inflection in the curvature at the transition from cell body to dendrite. For most neurons, this was unambiguous and reliably determined by different observers. In some cases, cell bodies tapered gradually into the thick primary dendrites; in these cases, a criterion of width narrowing to $\sim 1/3$ was used to determine where the cell body ended and the dendrite began. Each cell body was drawn in Neurolucida as a contour, and we report as length and width the Neurolucida calculation of the 'Ferret' maximum and minimum measurements, respectively, which provides an unbiased long axis length and short axis width of irregular contour shapes. Total dendritic length was calculated as the sum of all dendritic segment lengths, and did not include the cell body. Linear dorsoventral dendritic length of filled cells was measured using the 'Quick measure' straight line tool in Neurolucida, from the dorsal-most dendritic tip to the ventral-most dendritic tip, as projected in the X-Y plane.

Physiological slice preparation

Emu embryos aged E35–45 were rapidly decapitated and an approximately 4 mm segment of the caudal skull containing the brainstem removed with a razor blade and quickly submerged in oxygenated (95 % O₂-5 % CO₂) artificial cerebral spinal fluid (ACSF in mM: 130 NaCl, 26 NaH₂CO₃, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, and 10 dextrose). The brainstem segment was dissected out, mounted with cyanoacrylate glue, supported by a gel solution (4% agarose), and transferred to a vibrating tissue slicer (Campden Instruments, Leicester, UK). Transverse or off-transverse slices cut to a 250–350 μ m thickness and containing NL were collected and maintained in a holding chamber at room temperature (22–25°C) in oxygenated ACSF. For recordings, slices were placed in a submersion-type recording chamber continuously perfused (1–2 ml/min) at room temperature for all experiments except where indicated, when they were heated to 29–33°C using an inline heater (Warner Instruments, Hamden, CT).

Electrophysiological recordings

Local field potential recordings were made with low-resistance glass microelectrodes (~ 1 M Ω) filled with ACSF, using an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA) in current clamp mode. For synaptic stimulation of NL afferents, extracellular stimulation was produced with a tungsten metal monopolar or bipolar electrode. Stimulation and recordings were controlled by a PC computer running custom software written with programming software IGOR Pro (Wavemetrics, Lake Oswego, OR) via a National Instruments A/D board (National Instruments, Austin, TX). A few experiments were carried out using pClamp (Axon Instruments) and later analyzed with IGOR. Recordings were made serially across the nucleus while the stimulation electrode remained in place. Events were recorded as averages of 20–100 sweeps. Field potential latencies were measured from averaged records and the electrical stimulation artifact used as the reference time point. To compare across experiments, all latencies were computed relative to the latency measured most medially. For contralateral stimulation, the metal stimulation electrode was placed in the cross tract, at the midline or in the fiber path just ventromedial to NL. For ipsilateral stimulation, the stimulation electrode was placed in the ipsilateral NM, or in the afferent fiber tract dorsal to the NL.

Whole-cell patch-clamp recordings were made from visually identified NL and NM neurons using IR/DIC (infrared/differential interference contrast) video microscopy (Stuart et al., 1993). Initial micropipette resistance was 3–7 M Ω with an internal solution of (mM): 110–

120 potassium gluconate, 20 KCl, 0.1 EGTA, 2 MgCl₂, 2 Na₂ATP, 10 Hepes, 0.3 NaGTP, 0.01 phosphocreatine, 0.1–0.5% biocytin. Electrical recordings were made using an AxoPatch 200B (Axon Instruments) in fast current-clamp mode. Membrane potentials are reported uncorrected for a measured junction potential of 9 mV.

Fertilized emu eggs were obtained from local farms and incubated until approximately one week before hatching. Efforts were made to minimize animal suffering and to reduce the number of animals used. All animal procedures were performed in accordance with guidelines approved by the University of Maryland Institutional Animal Care and Use Committee, the National Institute of Health *Guide for the Care and Use of Laboratory Animals in Research* (NIH Publication No. 80-23) and the 2000 Report of the AVMA Panel on Euthanasia.

Emu interaural distances were measured from skeletal material made available by the Department of Ornithology at the American Museum of Natural History. Calipers were used to measure the distance between the lateral edge of the squamosal bones at the midpoint of the otic recess in 4 emu skulls. These measurements were used to provide an estimate of interaural distance.

RESULTS

Emu NL is a monolayer of bipolar neurons

In this paper we describe for the first time the anatomy and physiology of the auditory brainstem of the emu involved in encoding timing information of sound localization. The cochlear nuclei, magnocellularis and angularis, and the nucleus laminaris were clearly observed in Nissl material, as described for other avian species (Fig. 1A). Auditory brainstem structures express calcium-binding proteins (Takahashi et al., 1987; for review see (Braun, 1990; Celio et al., 1996). We used antibodies against the calcium-binding protein parvalbumin to investigate the cytoarchitecture of the auditory circuits in emu. Anti-parvalbumin immunoreactivity (PV-IR) was observed in NL (Fig. 1B, D) and the cochlear nuclei (not shown). In NL, PV-IR clearly labeled the cell bodies and dendrites (Fig. 1B, D).

Emu NL was a simple two-dimensional sheet of neurons stretching rostrocaudally and mediolaterally, with dendrites oriented dorsoventrally. Through most of NL, the cell bodies formed a compact monolayer. At the caudolateral extreme, NL was still laminar, but composed of a broader layer of cells, with 3 or 4 cell bodies in a slightly staggered formation. Throughout the nucleus, the most striking feature was the bitufted morphology of the NL neurons: typically a single thick primary dendrite extended from either end of an oblong cell body, which then branched into numerous short secondary and, in lateral areas, tertiary and higher-order dendrites with the appearance of “tufts” (Fig. 1D). A tufted appearance was most apparent in the medial and rostral areas. A second feature apparent in the PV-IR sections was a gradual change in the lengths of the dendrites along the rostromedial to caudolateral axis. An analysis of this dendritic length gradient is reported below.

To investigate whether there might be a functional distinction between the primary dendrites and the tufts we used antisera against a presynaptic marker protein, synaptic vesicle protein 2 (SV2; Fig. 1C, E–G). Anti-SV2 immunoreactivity (SV2-IR) labeled axon terminals in both the cochlear nuclei and the nucleus laminaris (Fig. 1C). In NL, SV2-IR was markedly absent from the afferent tracts containing the NM axons as they approached NL, but was dense within the NL dorsal and ventral dendritic neuropil (Fig. 1C, E–G). SV2-IR was intense within the neuropil of the distal tufts, and inspection of sections double labeled for PV-IR and SV2-IR showed a close correspondence (not shown). Although weaker overall in the

cell body layer, clear punctate staining could be seen along the proximal primary dendrites and cell bodies (Fig. 1E). As with parvalbumin, SV2-IR highlighted the gradient in dendritic length (Fig. 1G).

To determine the distribution of GABAergic inputs to NL, and to differentiate between the locations of excitatory and inhibitory synapses within NL, we used antibodies against GAD (glutamic acid decarboxylase), the enzyme that produces GABA and is found in the terminals of inhibitory axons. Anti-GAD immunoreactivity was observed in axon terminals throughout NL (Fig. 2B–C), as well as in NM (Fig. 2A); the principal neurons in these areas were GAD-negative. Unlike the anti-SV2 label, the GAD immunolabeling was observed as prominently in the cell body layer and around proximal dendrites as in the distal dendritic neuropil (Fig. 2B). GAD-positive fibers were observed to penetrate the NL neuropil (Fig. 2C). Although double labeling experiments for GAD and SV2-IR were not carried out, inspection of adjacent sections labeled with these antibodies suggests that most of the somatic and perisomatic SV2 label originated with GABAergic synapses.

We created 3-dimensional reconstructions of NM and NL using Nissl stained transverse sections (Fig. 3). NL was a flat sheet of neurons, arcing dorsally toward the rostral and lateral edges (red nucleus, Fig. 3A–C). NM (blue nucleus, Fig. 3A–C) was positioned both dorsal and caudal relative to NL. A 2-dimensional horizontal projection of both nuclei is shown in Figure 3D. NM and NL have similar 2-dimensional profiles with their long axes running rostromedial to caudolateral. In birds, a tonotopic map of best frequency is oriented along this axis, with high best frequencies located rostromedially and low best frequencies located caudolaterally (Konishi, 1970; Rubel and Parks, 1975; Warchol and Dallos, 1990). In emus, the basilar papilla has been shown to respond to acoustic frequencies from ~50 Hz to ~4.8 kHz, and is organized logarithmically (Köppl and Manley, 1997). Therefore, we propose a hypothetical mapping of sound frequency as shown in Figure 3D. In this model, approximately two-thirds of NL is dedicated to best frequencies below 1 kHz, and nearly one-half to those below 500 Hz. This model map assumes no transformation of the tonotopic map between papilla and NL (i.e., expansion or compression of a subset of frequencies relative to the others).

We investigated the cytoarchitecture of the NL neurons in emu with PV-IR sections (Fig. 1) and with fills of individual NL neurons that had been stained with the intracellular label biocytin during physiological recordings (Fig. 4; see below). During these physiological recordings we attempted to sample the full range of the nucleus, including the most rostromedial and caudolateral, and thus the filled cells should include examples across the full range of NL morphology. These materials showed two clear findings: first, the PV-IR sections suggested, and the biocytin fills confirmed, that the majority of NL neurons in emu were strictly bipolar, with two primary dendrites, one oriented dorsally, the other ventrally, while a smaller number of neurons had 3 or 4 primary dendrites. Among the filled neurons ($n = 45$), the majority had only two primary dendrites (Fig. 5; 30/45, or 66.7%), while a minority had three primary dendrites (13/45; 28.9%), and only a small number had four (2/45; 4.4%). No neurons were observed with more than 4 primary dendrites, although some dendrites branched very close to the cell body. Nearly all dendrites invaded exclusively the dorsal or ventral neuropil; in one case a dendrite was oriented horizontally.

The second major finding was a gradient of dendritic length, with the shortest lengths in the rostral and medial area, and the longest in the caudal and lateral area. The gradient was observed in both the PV-IR and the SV2-IR sections (e.g., Fig. 1G). Throughout most of NL, the cell bodies formed a compact monolayer, and thus the extent of the neuropil was defined by the dorsoventral neuronal dendritic length. When the dorsoventral depth of the neuropil was measured as an indicator of dendritic length, the lengths ranged from 30 μ m to

430 μm , a ~ 14 -fold range. Filled neurons that were reconstructed and measured as a straight line from dorsal to ventral dendritic tip had lengths that varied from 61 μm to 311 μm , a ~ 5 -fold range. The discrepancy between these two measurements may be due to an underestimate of the extremes by an undersampling of NL during physiological experiments, an overestimate of the maximal length in the PV-IR sections because of the staggered organization of the cell body layer in the caudolateral area, or both.

To investigate the distribution of dendritic lengths throughout the nucleus, we sampled the dorsoventral dendritic neuropil extent at regular intervals from the serial sections of PV-IR material. These lengths were then plotted versus position expressed as percent rostrocaudal position on one axis and percent mediolateral position on the other (Fig. 6A). Dendritic length varied smoothly from shortest in the rostral and medial region to longest in the caudal and lateral region. To quantify this pattern, we calculated the best isolength contour with a multivariable linear regression (red line, Fig. 6A). The dendritic length gradient was calculated as a line orthogonal to the isolength contour (black line, Fig. 6A) and was oriented in a rostromedial to caudolateral direction, similar to the gradient described in the chick (Smith and Rubel, 1979; Smith, 1981). We then used the gradient line to relate the average dendritic length with rostrocaudal-mediolateral position (% RC-ML; Fig. 6B). The dendritic lengths increased linearly ($r = 0.97$; $p < 0.0001$) with an average slope of $\sim 2.4 \mu\text{m}/\text{percentile}$. The exception to this pattern was the decrease in length measurements found at the extreme caudolateral pole; in this area, the neuropil depth measure may have underestimated the true dendritic length because caudolateral-most NL neurons were curved instead of being oriented strictly dorsoventrally. The cumulative distribution of dendritic lengths showed a steep slope at the middle lengths, with shallow tails above and below, suggesting that the median lengths were proportionately overrepresented (Fig. 6C); 80% of the nucleus by area was devoted to lengths between 120 μm and 275 μm .

In order to describe the dendritic morphology of NL neurons, we used detailed reconstructions of individual neurons filled with biocytin during physiological recordings (physiological results are reported below). We were able to fully reconstruct 43 of 45 filled NL neurons with the Neurolucida system (Fig. 4). For these neurons we made two dendritic length measurements. 1) Linear dorsoventral length: a straight line length measure from most distal dorsal extent to most distal ventral extent, comparable to the neuropil depth measurements reported above. 2) Total dendritic length: the sum of all reconstructed dendritic segment lengths, and equivalent to the total dendritic length of Smith and Rubel (1979). Total dendritic length and linear dorsoventral length were well correlated (Fig. 7; linear fit, $r = 0.73$, $p < 0.0001$). The data was slightly better fit by a power law regression ($y = 0.53 \times 1.42^x$, $r = 0.83$, $p < 0.0001$), but with little improvement in the residual distribution. Total dendritic lengths varied widely from 165 μm to 2668 μm (a 16-fold range) and averaged $765 \pm 677 \mu\text{m}$.

To determine whether other morphological features varied across the nucleus, we plotted several measures versus total dendritic length ($n = 43$). Cell bodies were generally ovoid or ellipsoid throughout, with an average area of $244.0 \pm 76.2 \mu\text{m}^2$ and an average length and width of $24.4 \pm 6.6 \mu\text{m}$ and $14.2 \pm 2.5 \mu\text{m}$, respectively (Table 1). The average aspect ratio was 1.7 ± 0.4 . Cell body area did not change significantly across NL (Fig. 8A, $r = 0.036$). There was no correlation between cell body length, width, or aspect ratio with total dendritic length (data not shown).

Emu NL neurons had notable thick primary dendrites that extended some distance before branching into a dense “tuft”. We measured the length of each primary dendrite from the cell body to its first branch point. Individual primary dendritic lengths ranged from 1.0 μm to 92.5 μm . Primary dendrites in our sample had an average length of $22.7 \pm 18.6 \mu\text{m}$ and

thickness of $4.2 \pm 1.4 \mu\text{m}$ ($n = 102$ primary dendrites). Primary dendritic length was not correlated with total length (Fig. 8B, $r = 0.014$); this was because many of the primary dendrites of longer NL neurons branched close to the cell body. Average primary dendritic thickness also did not vary with total dendritic length (Fig. 8C, $r = 0.045$).

The number of branch points, or nodes, was correlated with total dendritic length, however, demonstrating an increase in complexity across the nucleus (Fig. 8D, $r = 0.82$, $p < 0.0001$). Total surface area likewise increased with total dendritic length (Fig. 8E, $r = 0.82$, $p < 0.0001$). The number of primary dendrites was not strongly related to position, as the majority of neurons that had only two primary dendrites were distributed broadly across NL; however, neurons with 3 or 4 primary dendrites were proportionately more likely to have longer dendritic lengths (Fig. 8F).

Ventral total dendritic lengths were longer on average than dorsal total dendritic lengths ($452 \pm 507 \mu\text{m}$, ventral, versus $334 \pm 272 \mu\text{m}$, dorsal; paired Student's t-test, $p < 0.05$; Table 1). This reveals a slight overall bias toward longer total lengths in ventral trees, although dorsal and ventral lengths were correlated (Fig. 9A, $r = 0.64$, $p < 0.0001$). While the ventral dendritic trees had slightly more branch points than dorsal, indicating greater complexity, this difference was not significant on average (ventral, 19.0 ± 19.2 , dorsal, 15.8 ± 13.0 ; $p = 0.086$). The numbers of ventral and dorsal nodes were highly correlated (Fig. 9B, $r = 0.80$, $p < 0.0001$).

Emu nucleus laminaris contains contralateral delay lines

The avian nucleus laminaris is characterized by physiological specializations that contribute to the computation of interaural time difference. One such specialization is the formation of delay lines composed of the axons of nucleus magnocellularis neurons as they approach NL (Young and Rubel, 1983a; Young and Rubel, 1986). We performed *in vitro* physiological experiments to determine whether delay lines were present in emus. In brainstem slices cut in the presumed isofrequency plane, we recorded local field potentials (LFP) in NL in response to electrical stimulation of the afferent inputs from NM (Fig. 10A). These experiments were conducted at room temperature ($22\text{--}24^\circ\text{C}$). We made serial recordings of field potential responses across the (oblique) mediolateral extent of the nucleus, while maintaining the position and stimulation strength of the stimulation electrodes. In some slices, we used two stimulation electrodes and recorded at the same NL site with both contralateral and ipsilateral stimulation. When we stimulated fibers from the contralateral NM, a pronounced, consistent shift occurred in the latency of the evoked postsynaptic field potentials across the medial-to-lateral extent of NL (Fig. 10A and B). In the example in Figure 10B, the most medially recorded field potential evoked by contralateral stimulation (closed circles) had a latency of $470 \mu\text{s}$, while the most laterally recorded field potential, at a distance of $800 \mu\text{m}$ from the first recording, had a latency of 1.5 ms , a shift of $1,030 \mu\text{s}$. This shift can be expressed as the slope of the linear fit to the data, which in this example was 1.3 ms/mm ($r = 0.91$, $p < 0.001$). In contrast, ipsilateral stimulation produced a much smaller shift in latency (0.31 ms/mm ; $r = 0.84$, $p < 0.01$).

Contralateral stimulation evoked LFP responses whose latencies showed clear, positive shifts ($n = 7$; Fig. 10C), with an average slope at $22\text{--}24^\circ\text{C}$ of $0.91 \pm 0.55 \text{ ms/mm}$ (range: 0 to 1.67 ms/mm), which corresponds to an average conduction velocity of 1.09 mm/ms . Ipsilateral stimulation produced LFP responses whose latencies were more difficult to quantify, or which could only be recorded over a portion of the mediolateral length. Ipsilateral stimulation could produce positive, negative, or negligible latency shifts, whose average slope was close to zero ($0.09 \pm 0.37 \text{ ms/mm}$, $n = 8$; Fig. 10D). Individual slopes ranged from -0.47 to $+0.53 \text{ ms/mm}$; most had low correlation constants and thus slopes that were not statistically reliable.

To compare ipsilateral and contralateral latency shifts, we plotted the slope of the linear fit in the range of positions where both ipsilateral and contralateral responses were recorded in the same slice ($n = 5$; closed symbols; Fig. 10E). The average slope shift (gray closed symbols, Fig. 10E) for contralateral stimulation ($1.11 \pm 0.88 \mu\text{s/mm}$) was significantly larger than for ipsilateral stimulation ($0.26 \pm 0.37 \mu\text{s/mm}$; paired Student's t , $p < 0.05$; $n = 5$).

In one experiment, we also made latency measurements while varying the temperature (Fig. 10F). The change in latency with temperature was well fit by a log fit with a slope of -0.027 , and the Q_{10} (factor change for every 10°C change in temperature) was 1.84 . We extrapolated that at physiological temperatures (41°C), the conduction velocity would be $\sim 3.6 \text{ m/sec}$, and thus the maximal delay across the extent of NL in our slice preparation (1 mm) would be $\sim 280 \mu\text{s}$. These results suggest the contralateral conduction velocities found in the emu laminaris could implement delays within the physiological range. Measurements from 4 emu skulls yielded interaural distances of $48.62 \pm 1.85 \text{ mm}$, similar to those measured in the barn owl (Haresign and Moiseff, 1988).

Emu nucleus laminaris and magnocellularis display timing circuit physiological specializations

In chick, it has been well established that the neurons of NM and NL express voltage-gated ionic conductances that contribute to their ability to phase lock and encode the timing cues present in sound (Reyes et al., 1994; Reyes et al., 1996; Trussell, 1997; Rathouz and Trussell, 1998; Kuba et al., 2002a; Kuba et al., 2005). To determine whether these conductances were present in the emu, we performed whole cell intracellular recordings to measure the voltage responses to steps of current input (at room temperature, $22\text{--}24^\circ\text{C}$). We recorded from 45 NL neurons sufficiently to fill with the intracellular label biocytin (see morphology section, above). In 11 of these, we recorded the full range voltage response to current injections; in NM, we made similar recordings from 3 neurons. Both NL and NM neurons showed physiological characteristics similar to those observed in chick NL and NM recordings (Reyes et al., 1994; Reyes et al., 1996; Kuba et al., 2002a; Kuba et al., 2005).

In emu NL, we found a single-spiking profile in response to depolarizing current injection, and a prominent 'sag' following hyperpolarizing current injection, and thus NL demonstrated both inward and outward rectification (Fig. 11A). These results suggest the presence of a low-threshold potassium current activated by depolarization, and an I_h -like current activated by hyperpolarization (Reyes et al., 1996; Trussell, 1997; Carr and Soares, 2002; Kuba et al., 2002a; Kuba et al., 2002b; Kuba et al., 2005). In some recordings, a rebound voltage overshoot was seen with repolarization of the membrane potential, which could result in an action potential (Fig. 11A, inset). There was a significant difference in input resistance above and below resting potential ($n = 11$, $p < 0.01$). For depolarizing steps, emu NL neurons had an average input resistance of $30.2 \pm 19.9 \text{ M}\Omega$, while for hyperpolarizing steps, average input resistance was $177.2 \pm 77.6 \text{ M}\Omega$ (Table 2). Membrane time constants were also different above and below resting potential ($p < 0.05$). For small ($25\text{--}75 \text{ pA}$) current steps around resting potential, the average membrane time constant was $12.8 \pm 3.8 \text{ ms}$ above resting potential, and $30.2 \pm 19.9 \text{ ms}$ below resting potential.

Voltage responses from NM neurons showed a similar profile ($n = 3$; Fig. 11B). For steps depolarizing from the resting potential ($-59.8 \pm 3.1 \text{ mV}$), emu NM neurons had an average input resistance of $49.0 \pm 25.6 \text{ M}\Omega$, and a average membrane time constant of $3.9 \pm 0.2 \text{ ms}$ (Table 2). For steps hyperpolarizing from rest, the input resistance was $260.0 \pm 139.8 \text{ M}\Omega$, and membrane time constant was $14.1 \pm 3.9 \text{ ms}$.

DISCUSSION

We have investigated the brainstem circuits underlying the computation of interaural time differences in the emu (*Dromaius novaehollandiae*). While we found numerous similarities between emu NM and NL anatomy and physiology and the better described chicken and owl, there were also several interesting differences, which may be related to the low best frequency specialization of the emu.

Calcium binding proteins in the avian brainstem

The auditory nuclei in the emu brainstem were strongly immunopositive for calcium binding proteins, a consistent feature in the auditory brainstem of both birds and mammals (Celio et al., 1996). Although their precise role in auditory function is uncertain, calcium binding proteins have been proposed to regulate intracellular Ca^{2+} levels, which may be of critical importance to the health of auditory brainstem neurons due to their high firing rates and the presence of calcium-permeable glutamate receptors (Baimbridge et al., 1992). In the emu, the parvalbumin label was a convenient cellular marker, labeling the laminaris cell but not the magnocellular terminals, while antibodies to calretinin stained the neuropil as well (personal observations). Similar calretinin expression is present in the auditory brainstems of chick, owl, and zebra finch (Rogers, 1987; Takahashi et al., 1987; Braun, 1990; Parks et al., 1997; Kubke et al., 1999), but chick brainstem is negative for both calbindin and parvalbumin (Rogers, 1987; Parks et al., 1997).

Implications for the Jeffress model for interaural time difference computation

Our physiological experiments in the emu further support the Jeffress model for the computation of interaural time differences in birds (Jeffress, 1948; Carr and Konishi, 1990; Overholt et al., 1992). The two fundamental elements in the Jeffress model are delay lines and coincidence detectors. Delay lines are formed from the internal axonal delays in the NM afferent inputs which compensate for the relative acoustic delay of incoming signals to each ear (Young and Rubel, 1983a; Young and Rubel, 1986; Overholt et al., 1992).

We have shown that delay lines exist in emu as they do in chick: electrical stimulation of the contralateral tract results in responses with increasing delay along the mediolateral length of NL. In chick, a shift in latency arises from the longer axonal path length observed morphologically by staining the NM afferent fibers: contralateral afferents project to the ventral neuropil by running nearly horizontally along the mediolateral length (Young and Rubel, 1983b; Rubel and Parks, 1988; Overholt et al., 1992).

We also observed that emu NM and NL neurons responded to current injection by firing a single spike, and displayed strong inward rectification, similar to that found in chick NM and NL (Reyes et al., 1996; Kuba et al., 2002a). In chicks, the inward rectification is due to the activation of low-threshold potassium channels (Kuba et al., 2005). Activation of this potassium conductance is responsible for the low input resistances and short membrane time constants that results in the tight phase-locking of the NM action potentials to their auditory nerve inputs, and the narrow integration time window necessary for coincidence detection (Reyes et al., 1994; Reyes et al., 1996; Carr and Soares, 2002; Kuba et al., 2002a; Kuba et al., 2005).

Anatomical organization

The organization of the emu NL closely resembles that of other basal birds, such as the chicken (*Gallus gallus*), and thus conforms to the ancestral, or plesiomorphic pattern (Ariëns Kappers et al., 1936; Boord, 1968; Boord, 1969; Parks and Rubel, 1975; Jhaveri and Morest, 1982; Rubel and Parks, 1988; Kubke et al., 1999; Carr and Code, 2000; Carr et al., 2001).

This pattern is characterized by a compact, horizontal monolayer of neurons, ventral and rostral to nucleus magnocellularis, that extend polarized dendritic fields into the dorsal and ventral neuropils. In the emu, as in chick, this monolayer alignment is only modified at the caudolateral extreme, where the cell bodies are slightly staggered but polarity of the dendrites is maintained. The basal pattern is in contrast to organization of NL found in an auditory specialist, the barn owl (*Tyto alba*). In the barn owl, the medial NL consists of a thick neuropil, expanded in the dorsoventral dimension with neurons distributed throughout (Schwartzkopff and Winter, 1960; Takahashi et al., 1987; Carr and Boudreau, 1993). This hypertrophy in the high BF region is thought to be a derived, or apomorphic, adaptation that contributes to the barn owl's increased sound localization acuity (Schwartzkopff and Winter, 1960; Carr and Konishi, 1988; Carr and Konishi, 1990; Kubke et al., 2002).

Cellular morphology

Emus make low frequency 'booming' vocalizations (Marchant and Higgins, 1990; Halkin and Evans, 1999). Compared to most other birds, their audiograms show greater sensitivity at lower frequencies and they have a greater proportion of low-BF fibers in the auditory nerve: >30% are below 250 Hz for emu, compared to <10% for chick (Manley et al., 1997; Gleich and Manley, 2000; Yates et al., 2000). We asked whether the properties of NL reflected these specializations.

In chick, NL neurons tuned to low BF are associated with longer dendritic trees and fewer primary dendrites, while neurons tuned to high BF are associated with numerous short primary dendrites (Smith and Rubel, 1979; Smith, 1981; Jhaveri and Morest, 1982; Kuba et al., 2005). The high BF pattern is also exhibited in the very short dendrites in the high BF region of barn owl NL; these short dendrites are unpolarized, unlike in the chick (Carr and Boudreau, 1993). Bitufted neurons are found only in the lowest BF region of the barn owl NL (Carr and Boudreau, 1993; Köppl and Carr, 1997).

In the emu, we show that the general morphology of NL neurons more closely resembled that of the low frequency pattern. Throughout emu NL, all neurons had only two to four primary dendrites that were about 4 μm thick and which branched into dense distal tufts. These tufts were the most striking feature of the emu NL neurons, and one that distinguished them from NL neurons in chicken and barn owl. The tufts appeared to receive the majority of the excitatory synaptic input, which suggests that in emu, inputs arriving from each ear are maximally segregated. In chickens, in contrast, the excitatory inputs appear distributed on both proximal and distal dendrites (Parks et al., 1983). As discussed below, segregation of the synaptic inputs may be a specialization for improving coincidence detection, and one that is most effective at lower frequencies.

Dendritic length gradient in emu NL

The primary difference among neurons within emu NL was the smooth and linear increase in dendritic length with position along the rostromedial to caudolateral axis. The dendritic length gradient has been quantitatively analyzed in hatchling chick (Smith and Rubel, 1979; Smith, 1981), where BF was directly mapped (Rubel and Parks, 1975). The gradient in chick was also linear, and extended from the rostromedial to caudolateral pole, orthogonal to the isofrequency band. To make comparisons with published data from chick, we measured total dendritic length from filled neurons in emu NL; we also measured total dendritic length from our own sample of biocytin-filled neurons from embryonic chick slice (E16–19; data not shown). The overall range of total dendritic lengths was comparable in emu and in chick. The shortest, medial emu and chick neurons had similar total dendritic lengths; however, the longest total dendritic lengths in emu (~2670 μm) were longer than those observed in chick, both according to published work and our own observations (<2000 μm , Smith and Rubel

(1979), Smith (1981); ~2070 μm , personal observation). Whether these longer dendritic length neurons represent the presence of a module of lower-BF tuned cells remains to be tested with direct mapping of frequency in the emu. The distribution of dendritic lengths (Fig. 6C) showed a larger area of NL devoted to mid-range lengths, which would argue against a relative expansion, or hypertrophy, of low best frequency representation as for high best frequencies in the owl. However, longer lengths were found in the non-monolayer region, where NL cell density was 2–4 times higher than in the medial, monolayer region. Thus, the total number of longer dendritic length neurons is probably similar to the total number of mid-length neurons, although shorter dendritic length neurons seem to be less well represented. While we propose a hypothetical relationship of best frequency with dendritic length for the emu, our conclusions regarding frequency representation in the emu NL remain speculative until an *in vivo* mapping of BF is completed.

Why does a gradient in dendritic length exist?

As described in this and other papers, a singular feature of NL neurons is the common morphological organization of bipolar neurons with magnocellular synaptic inputs from each ear segregated on the dendrites, and the relationship between dendritic length and tonotopic frequency. Thus, NL provides an opportunity to investigate the relationship between dendritic structure and neural computation. Detailed biophysical models of these bipolar neurons demonstrated that having dendritic compartments, as opposed to having a point-neuron compartment, can improve the coincidence detection necessary for ITD coding (Agmon-Snir et al., 1998; Simon et al., 1999; Grau-Serrat et al., 2003). The computational advantage of segregating the inputs from each ear depends on dendritic nonlinearities in how the synaptic inputs are summed. Synaptic inputs arriving at the same dendritic compartment sum non-linearly because the driving force decreases with depolarization. As a result, a smaller total synaptic conductance is required to fire the postsynaptic cell when inputs arriving simultaneously, or ‘in phase’, are distributed on different dendrites. The greater the separation of inputs from each ear, as with longer dendrites or with more distally located synapses, the less they affect one another, and the more linear their summation. The more inputs from the same ear can influence each other, such as arriving on the same thin (and therefore electrotonically compact) dendrite, the greater the nonlinearity.

How is the dendritic length gradient related to this computation? The nonlinear dendritic summation effect is frequency-dependent: the advantage of nonlinear dendritic summation for a given BF is limited by the presence of jitter in the phase locking of the NM inputs. Jitter in the timing of the input spikes can create erroneous ‘in phase’ signals, which, when amplified by nonlinear dendritic summation, degrade coincidence detection by causing the neuron to spike in response to ‘out of phase’ signals. Making the dendrites shorter, and therefore reducing the nonlinearity, reduces this jitter effect. Thus for a given BF, these models predict an optimal dendritic length and a tonotopic gradient in ‘best length’ across the nucleus: shorter dendrites for higher BF’s, longer dendrites for lower BF’s.

We conclude that emu timing circuits are plesiomorphic in their gross anatomy, but that the morphology of these NL neurons may be apomorphic. This conclusion is supported by the presence of the plesiomorphic pattern in crocodilians (Soares and Carr, personal observation). Crocodilians are a sister group to the birds, and their NL neurons are bipolar with sparse branching, but they lack the distal tufts. Emus NL neurons may differ from that of the hypothetical ancestral NL neuron in that they are further specialized for low BF by segregating their inputs onto distal dendritic tufts, which would improve coincidence detection. However, emus and chickens have overlapping hearing ranges, as well as total dendritic length ranges, so it is unclear why they differ so strikingly in their morphology, especially in the medial, high BF region. One possibility is that improved coincidence detection is achieved by different strategies in emu and chick. In the emu, inputs from

opposite ears are segregated for maximal separation, while in the chick, inputs from the same ear are clustered on thin dendrites for maximal nonlinear interaction. These results suggest that dendritic computation is an important factor in determining the neuronal morphology in auditory interaural timing circuits.

Acknowledgments

The authors thank Jennifer Holdway and Kai Yan for technical assistance, and Jonathan Simon and Christine Köppl for helpful discussions. The Department of Ornithology at the American Museum of Natural History kindly provided access to emu skeletal material.

Grant sponsor: National Institute on Deafness and Other Communication Disorders, grant numbers: R01 000436 and P30DC04664; grant sponsor: National Institute of Neurological Disorders and Stroke, grant number: NRSA NS10991.

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Figure 1. Auditory brainstem morphology and synaptic localization in emu nucleus laminaris. A) Schematic of transverse section of emu brainstem (left) and a micrograph of Nissl stained section (right). B, D) Anti-parvalbumin immunohistochemistry. C, E–G) Anti-SV2 immunohistochemistry. B) Anti-parvalbumin labeled NL neurons. C) Anti-SV2 produced dense staining of boutons in the dendritic neuropil of NL, and fewer labeled boutons in the cell body layer. Note the absence of staining in fiber tracts surrounding NL dorsally and ventrally, the area occupied by afferents projecting from NM to NL. D) Higher magnification of section in B; anti-parvalbumin labels the cell bodies and full dorsoventral extent of the dendritic trees. E) Higher magnification of section in C; punctate anti-SV2

label surrounded the unlabeled cell bodies and densely labeled the dendritic tuft layer of NL. F) Anti-SV2 label in an oblique section parallel to the assumed isofrequency axis showed dense label in the dendritic tufts, and weaker label in the cell body layer. All NL neurons in this section were equal in dorsoventral length. G) Anti-SV2 label in a section cut orthogonal to the assumed isofrequency axis showed increasing dendritic length from medial (left) to lateral. In B and C, medial is to the right. Scale bars: A) 1 mm, B) 500 μm , C) 1 mm, D and E) 50 μm , F) 200 μm , G) 300 μm .

Figure 2. Anti-GAD immunohistochemistry. A) GAD+ terminals surround unlabeled cell bodies in the nucleus magnocellularis. B) Transverse section through the NL shows the monolayer of cell bodies with the dorsal and ventral dendritic arbors outlined by GAD+ terminals. C) NL neurons have large oval somata (*) and thick primary dendrites, outlined by immunopositive GAD+ terminals. Nomarski optics. Scale bars, A: 10, B: 20 and C: 10 μ m.

Figure 3.

Reconstruction of NM and NL. A 3-dimensional representation of NL (red) and NM (blue; located dorsal and caudal to NL) is shown within the outline of the brainstem (transparent gray). A ~1.15 mm section of brainstem is represented. A) Coronal view from caudal pole. B) Oblique view (~45° rotation from A around dorsoventral axis). C) Horizontal view from dorsal pole (90° rotation from A around mediolateral axis). D) Hypothetical tonotopic projection onto horizontal plane projection of the right NM and NL. Tonotopic frequency map assumes direct projection from equivalent frequency mapping data of auditory best frequency with the apical-basal position on the basilar papilla in emus (bottom two scales, from Köppl and Manley, 1997).

Figure 4.

Nucleus laminaris neuronal reconstructions. Sixteen examples of biocytin-labeled neurons as reconstructed with the Neuroludica computer assisted drawing system, arranged from the shortest dendritic length neurons (most rostromedial) to the longest dendritic length neurons (most caudolateral). NL neuronal morphology varied from bipolar dendrites with a few, short secondary branches to large complex dendritic trees with many higher order branches. Axons originated most often from the soma, but could also branch off from one of the primary dendrites. These examples were taken from 13 separate slices. Dorsal is toward the top. Scale bar, 200 μm . a, axon.

Figure 5. Histogram of the number of primary dendrites per NL neuron showed that most neurons have only two primary dendrites. Data from biocytin-labeled neurons ($n = 45$).

Figure 6.

Dendritic length varies in a gradient across NL. A) Dorsoventral dendritic length measurements were made from stained sections cut nearly orthogonal to the presumed isofrequency bands (30 μm thin sections, anti-parvalbumin immunostaining). Each pixel represents one dendritic length measurement taken at a particular mediolateral position in one section. Color bar: 'dendritic length': red, minimum = 30 μm; violet, maximum, 430 μm; also shown, a hypothetic 'best frequency' mapping according to the model in panel D. Red line shows the multi-dimensional linear fit, which estimates the best orientation of the "iso-length" band. Black line shows the orientation of the dendritic length gradient line, which was calculated to be orthogonal to the "iso-length" band and placed to traverse the length extremes. B) Dendritic length increased linearly along the dendritic gradient line in A. Mean length (y-axis) across a 10-pixel wide averaging window centered on gradient line, versus rostromedial (0%) to caudolateral (100%) position along the gradient line (x-axis). C) Cumulative histogram of dendritic lengths in A. D) Model of best frequency relationship to dendritic length. Best frequency range from Köppl and Manley (1997). This relationship converted dendritic lengths into a best frequency color scale used in A.

Figure 7.

Correlation of total dendritic length with linear dorsoventral dendritic length. Total dendritic length and linear length measurements from biocytin-filled NL neurons ($n = 43$). Total dendritic length is the sum of all dendritic branch lengths drawn with reconstruction; linear dorsoventral length is the straight line measure from ventral to dorsal dendritic tip, including cell body, and is directly comparable to the dendritic length data shown in Fig. 6.

Figure 8.

Morphological characteristics of biocytin-filled NL neurons and their variation with total dendritic length. A) Cell body area. B) Length of primary dendrites, from cell body to first branch point. C) Average primary dendritic thickness. D) Total number of nodes, or branch points. E) Total dendritic surface area. F) Number of primary dendrites per cell. In A, B, and C, there were no correlations with dendritic length. In D, complexity, represented by the number of nodes, increases with length, as did total surface area.

Figure 9.

Comparison of ventral and dorsal dendritic trees. A) Ventral and dorsal dendritic lengths were correlated, although ventral lengths were slightly longer. B) Ventral and dorsal dendritic trees had nearly equal numbers of nodes.

Figure 10.

Emu NM afferents form delay lines to contralateral NL. A) Right: Schematic of the brainstem slice preparation showing electrical stimulation of contralateral tract and/or ipsilateral tract during sequential recordings of the local field potential (LFP) at sites along mediolateral extent of NL. Left: Overlay of LFP responses in the same slice to contralateral stimulation from four separate sites: 1, most medial, 4, most lateral. Note the progressive increase in delay of the first downward deflection. *, stimulus artifact. B) LFP response latencies in a single slice for contralateral and ipsilateral stimulation versus mediolateral position of recording site. Note the large shift in contralateral stimulation latencies with mediolateral distance. Solid, dashed lines; linear fits of the contralateral, ipsilateral data. C)

Contralateral latency shifts in 5 separate slices (closed symbols, solid lines). Similar latency shifts were observed for secondary peaks in two slices (dashed lines). D) Ipsilateral latency shifts in 5 separate slices (open symbols, solid lines). Similar latency shifts were observed for secondary peaks in three slices (dashed lines). E) Contralateral latency shifts were greater than ipsilateral (closed symbols, individual slices; large, gray symbols, average; $p < 0.05$, $n = 5$). Slope was derived from the linear fits of the latency versus position plots as in B. Open symbols, similar plots for secondary peaks (dashed lines in C and D). F) Latencies decreased with increasing temperature. Linear fit with a slope of -0.027 ($r = -0.98$). Q_{10} , the factor by which the latencies changed with every 10°C temperature change.

Figure 11.

Membrane properties of emu NL and NM neurons. A) Left: Voltage response (top traces) of a single NL neuron to current injection steps (bottom traces). Half of NL neurons showed a voltage overshoot at the offset of a hyperpolarizing step (asterisk) that sometimes resulted in an action potential (inset). Right: Voltage-current relationship at time points during the peak response (closed squares) or plateau response (open squares). B) Left: Voltage responses for an emu NM neuron. Right: Voltage current relationship. Inset: camera lucida drawing of a biocytin-filled NM neuron. Note the dearth of dendrites (d); axon, a.

TABLE 1**Emu NL Morphological Properties¹**

Total dendritic length	785.8 ± 715.5 μm
Dorsal	333.5 ± 272.1 μm
Ventral	452.1 ± 506.6 μm
Dorsoventral dendritic length	194.0 ± 123.6 μm
Dorsal	98.1 ± 73.0 μm
Ventral	97.6 ± 64.7 μm
# Primary dendrites	2.4 ± 0.6
Primary dendritic length	22.5 ± 14.9 μm
Primary dendritic thickness	4.2 ± 1.4 μm
Total dendritic surface area	3411.7 ± 2334.3 μm ²
# Nodes	34.0 ± 29.7
# Ends	40.5 ± 32.0
Cell body	
Area (2D)	244.0 ± 76.2 μm ²
Length	24.4 ± 6.6 μm
Width	14.2 ± 2.5 μm
Aspect ratio	1.7 ± 0.4

¹Data from biocytin-filled reconstructed neurons, mean ± SD.

TABLE 2

Emu Neuronal Membrane Properties¹

NL	Membrane potential	-57.5 ± 4.4 (mV)	(n = 29)
	Whole cell capacitance	68.5 ± 13.4 (pF)	(n = 27)
	Input resistance		
	Above V_{rest}	67.3 ± 44.8 (M Ω)	(n = 11)
	Below V_{rest}	177.2 ± 77.6 (M Ω)	
	Membrane time constant		
	Above V_{rest}	12.8 ± 3.8 (ms)	(n = 11)
	Below V_{rest}	30.2 ± 19.9 (ms)	
	Membrane potential	-59.8 ± 3.1 (mV)	(n = 3)
	Whole cell capacitance	24.0 ± 14.9 (pF)	(n = 3)
NM	Input resistance		
	Above V_{rest}	49.0 ± 25.6 (M Ω)	(n = 3)
	Below V_{rest}	260.0 ± 139.81 (M Ω)	
	Membrane time constant		
	Above V_{rest}	3.9 ± 0.2 (ms)	(n = 3)
	Below V_{rest}	14.1 ± 3.9 (ms)	

¹ Values are mean $\pm$ SD.