Published in final edited form as:

J Comp Neurol. 2010 April 15; 518(8): 1199–1219. doi:10.1002/cne.22269.

Three subdivisions of the auditory midbrain in chicks (*Gallus gallus*) identified by their afferent and commissural projections

Yuan Wang^{1,2} and Harvey J. Karten¹

¹ Department of Neurosciences, University of California at San Diego, La Jolla, CA 92093-0608, USA

² Department of Otolaryngology and Virginia Merrill Bloedel Hearing Research Center, University of Washington, Seattle, WA 98195, USA

Abstract

The auditory midbrain is a site of convergence of multiple auditory channels from the brainstem. In birds, two separate ascending channels have been identified, through which time and intensity information is sent to nucleus mesencephalicus lateralis, pars dorsalis (MLd), the homologue of the central nucleus of mammalian inferior colliculus. Using *in vivo* anterograde and retrograde tracing techniques, the current study provides two lines of anatomical evidence supporting the presence of a third ascending channel to the chick MLd. First, three non-overlapping zones of MLd receive inputs from three distinct cell groups in the caudodorsal brainstem. The projections from nucleus angularis (NA) and nucleus laminaris (NL) are predominately contralateral and may correspond to the time and intensity channels. A rostromedial portion of MLd receives bilateral projections mainly from the *Regio Intermedius*, an interposed region of cells lying at a caudal level between NL and NA, as well as scattered neurons embedded in 8th nerve tract, and probably a very ventral region of NA. Second, the bilateral zones of MLd on two sides of the brain are reciprocally connected and do not interact with other zones of MLd via commissural connections. In contrast, the NL-recipient zone projects contralaterally upon the NA-recipient zone. The structural separation of the third pathway from the NA and NL projections suggests a third information-processing channel, in parallel with the time and intensity channels. Neurons in the third channel appear to process very low frequency information including infrasound, probably utilizing different mechanisms than that underlying higher frequency processing.

Keywords

cochlear nuclei; MLd; inferior colliculus; parallel organization; infrasound

Introduction

In birds, ascending axons of the inner ear mainly terminate in two major auditory cell groups, the nucleus magnocellularis (NM) and the nucleus angularis (NA), as well as a number of vestibular nuclei in the brainstem (Holmes, 1903; Boord and Rasmussen, 1963; Parks and Rubel, 1978). In addition, fibers from the inner ear also terminate in less well circumscribed areas adjacent to, and between these nuclei (Boord and Rasmussen, 1963; Boord and Karten, 1974; Parks and Rubel, 1978; Kaiser and Manley, 1996). These nuclei

Correspondence to: Yuan Wang, Ph.D., Department of Otolaryngology, Mail Stop 357923, University of Washington, Seattle, WA 98195, Phone: (206) 616-4113; Fax: (206) 221-5685, wangyuan@u.washington.edu.

Dedication: This work is dedicated to Masakazu (Mark) Konishi. Inspiring scientist, generous teacher, and dear friend. He continues to provide guidance and share his unique understanding of neuroethology with another generation of students.

contribute in differing manner and degree to ascending projections upon the nucleus mesencephalicus lateralis, pars dorsalis (MLd), the homologue of the central nucleus of the mammalian inferior colliculus. Convergence of these ascending projections within MLd, as well as their interactions with the commissural projection from the contralateral MLd, is critical for the computation of sound localization (for review see Knudsen, 1987; Klump, 2000) and the encoding of a wide range of complex sounds (Woolley and Casseday, 2004, 2005; Woolley et al., 2006).

The organization of the afferent and commissural projections to the avian MLd has mainly been studied in barn owls, a highly specialized species with well-developed sound localization. In barn owls, MLd receives intensity and time information through two separate ascending channels. The intensity channel arises from the contralateral NA, and the time channel from the contralateral nucleus laminaris (NL) which receives bilateral inputs from NM (Sullivan and Konishi, 1984; Takahashi et al., 1984; Konishi et al., 1985; Takahashi and Konishi, 1986). Both channels contain monosynaptic and polysynaptic pathways, the latter via cell groups embedded in the lemniscus lateralis and the superior olive (Takahashi and Konishi, 1988a, 1988b). Within the MLd, NA and NL terminate in two non-overlapping zones that are biochemically distinct from each other (Takahashi et al., 1987; Takahashi and Konishi, 1988a; Adolphs, 1993; Wagner et al., 2003). This differential distribution is associated with the organization of the commissural connection between two MLds. The NL-recipient zone of MLd projects in a restricted manner upon the lateral portion of the NA-recipient zone on the other side of the brain (Takahashi et al., 1989). This projection integrates time and intensity information and contributes to the computation of the spatial location of auditory stimuli (Takahashi et al., 1989).

Data from two more commonly studied avian species, chickens and pigeons, show similarities in the general pattern of two separate pathways from NA and NL upon MLd (Leibler, 1975; Conlee and Parks, 1986; Wild, 1995). However, notable discrepancies across species in the organization of MLd and its afferent and commissural projections have been reported. One of the most distinct variations is a bilateral projection upon MLd in chickens from the regions adjacent to, as well as partially embedded within NM, NL and NA (Conlee and Parks, 1986). The separation of this bilateral projection from those arising from the contralateral NA and NL has not been adequately clarified. In addition, the NL-recipient zone of MLd in pigeons is reported to be reciprocally connected (Leibler, 1975), in contrast to the commissural projection from the NL-recipient zone upon the contralateral NA-recipient zone in barn owls. Whether these discrepancies reflect significant functional and evolutionary divergence remains unknown.

The present study reconciles diverse studies in chicks, a worthy goal in view of its great utility in developmental and auditory studies and availability of the chick genome. The organization of the afferent projections of MLd was examined by placing anterograde and retrograde tracers into either NA, NL, or various locations of MLd. These studies provide additional details of the organization of the chick MLd with an emphasis on the clarification of the characteristics of the third channel. This knowledge is prerequisite to understanding the relationship between time, intensity, and frequency processing at the midbrain level, as well as at later stages in the thalamus and telencephalon along the ascending pathway (Karten, 1967, 1968). Polysynaptic projections from NA and NL upon MLd via the cell groups embedded in the lemniscus lateralis and the superior olive will be the topic of a future report.

Materials and Methods

Experiments were performed on White Leghorn chick hatchlings of less than five days age (*Gallus gallus*). The general organization of the auditory pathway is considered stabilized after hatching (See review, Rubel and Parks, 1988). All experimental procedures were approved by the University of California, San Diego Animal Care Committee and conformed to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals.

In vivo CTB and PHA-L track tracing

Chicks were anesthetized with a mixture of 40 mg/kg ketamine and 12mg/kg xylazine and placed in a stereotaxic head holder. The skull was exposed and a hole was made above the injection target area estimated from stereotaxic coordinates (Kuenzel and Masson, 1988). A solution of 1% cholera toxin B-subunit (CTB; List Laboratories, Campbell, CA) was injected through a glass micropipette using a pressure device (PicoSpritzer II; General Valve, Fairfield, NJ). In a separate group of subjects, *Phaseolus vulgaris* leucoagglutinin (PHA-L; Vector Laboratories, Burlingame, CA) was iontophoretically deposited by means of glass micropipettes with the tip diameter of 2-10 μm and filled with a solution of 2.5% PHA-L. The parameters of iontophoretic injection were a constant positive current of 2-10 μA for 10 minutes to deposit a small amount of tracer. The site of each successful injection, as well as the tracer used in each case, is listed in the Table 1. Following CTB or PHA-L injections, the micropipette was retracted, the wound was closed and the animal was allowed to recover. After a survival time of 3 days, animals were transcardially perfused with 0.9% saline followed by chilled 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, 7.2-7.4 pH). The brains were removed from the skull, postfixed overnight in the same paraformaldehyde solution, and then transferred to 30% sucrose in PB until they sank. Each brain was frozen and cut coronally at 30 μm on a freezing sliding microtome, and each section was collected in phosphate-buffered saline (PBS; 0.1 M, 7.2-7.4 pH).

Standard immunohistochemistry procedures were applied to reveal the distribution of CTB and PHA-L. Briefly, sections were incubated with primary antibodies solution diluted in PBS (anti-CTB, 1:12,000; anti-PHA-L, 1:1000) with 0.3% Triton X-100 overnight at 4°C, followed by biotinylated IgG antibodies (1:200; Vector Laboratories) for one hour at room temperature. Polyclonal anti-CTB made in goat, purchased from List Laboratories (Lot No. 703), was raised against unconjugated, purified cholera toxin B subunit isolated from *Vibrio cholerae*. Anti-PHA-L, purchased from Vector Laboratories (Catalog No. AS-2300), was produced by hyperimmunizing rabbits with pure lectins. Following conventional purification steps, specific antibody was isolated by affinity chromatography on lectin-agarose columns. Sections were then incubated in avidin-biotin-peroxidase complex solution (ABC Elite kit; Vector Laboratories) diluted 1:100 in PBS with 0.3% Triton X-100 for an hour at room temperature. Sections were incubated for 3-5 minutes in 0.025% 3-3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) with 0.01% hydrogen peroxide in PB. Sections were mounted on gelatin-coated slides and were either stained with 0.05% osmium tetroxide for 30 seconds or counterstained with Giemsa (Iiguez et al., 1985). Sections were then dehydrated, cleared, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA). To further test the specificity of the primary antibodies, sections were prepared from an animal that did not receive an injection with either CTB or PHA-L, and probed immunohistochemically with these antibodies. No specific staining was detected in any brain regions, as compared with control sections that were probed without the primary antibodies.

Imaging and cell counting

All CTB or PHA-L labeled neurons and terminals were reconstructed with a camera lucida on a Zeiss WL microscope (Carl Zeiss, Inc., Thornwood, NY). The drawings were then digitized and edited in Canvas (Deneba Systems, Miami, FL). Digital images of selected sections were captured with a Nikon D100 digital camera mounted on a Nikon photomicroscope (Nikon Corp., Tokyo, Japan) or a Phase One FX+ digital studio camera system (Phase one A/S Denmark, Frederiksberg, Denmark). Image contrast adjustments and photomontages were performed using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Labeled neurons were counted and the somatic area was measured in Image J software (version 1.38X; National Institutes of Health). Data are shown as mean $\pm$ 1 SD in the text. Labeled neurons without an obvious nuclear profile were excluded from counting and measuring. Abercrombie's formula was used to correct cell profile counting in each group (see Tab. 2), except for that in NM where only two labeled neurons were found.

$$N = \frac{T}{T+D} \times n$$

N, number of cells; T, section thickness; D, mean of diameters of 12 nuclei; n, number of counted nuclei.

Results

Figures 1-2 illustrate the location and general organization of the major nuclei examined in the current study in Nissl-stained sections. In chicks, NL is located ventrolaterally and rostrally to NM with its neurons arranged in a single layer (Fig. 1 A), except in its caudolateral region where multilayers of neurons are present (Fig. 1 B). NA is a heterogeneous cell group located laterally and caudally to NL (Fig. 1 C; Soares et al., 2002). MLd is situated in the caudal midbrain below the tectal ventricle, and surrounded by the nucleus intercollicularis and the stratum griseum periventriculare (Fig. 1 D; Karten, 1967; Puelles et al., 1994).

In addition, scattered neurons and/or small neuronal clusters are identified in the surrounding region lying between NM, NL, and NA at the caudal level. These neurons are less prominent in Nissl-stained sections compared to NM/NL/NA due to their loose packing and light staining. Three such neuronal clusters that contained retrogradely labeled neurons following injections into MLd are illustrated in Figure 2. The first cluster is located lateral to NM at the level caudal to NL (Fig. 2 A-B). In some sections, the distribution of these neurons appears to extend medially along the ventral edge of NM. The second cluster is a group of scattered neurons distributed along the outer edge of the ventral neuropil of NL (Fig. 2 C-D). Although less frequently, some neurons are also found along the outer edge of the dorsal neuropil of NL. The third cluster extends from the gap between the NL and the ventral NA and sweeps ventrally and medially (Fig. 2 E-F). Many neurons in this region display elongated cell body oriented parallel to the long axis of the cluster. Complete separation of these three neuronal clusters from each other and from surrounding regions in Nissl-stained sections is impractical as these clusters appear continuous with each other and located immediately adjacent to NM, NL, and NA. As described below, these three neuronal clusters are the most prominent groups beyond the boundaries of NM, NL, and NA that contain retrogradely labeled neurons following injections into MLd. To facilitate further

description, we designate them collectively as the *Regio Intermedius* (RI) based on their location.

Injections of CTB or PHA-L into NL

For injections into NL, the micropipette was inserted vertically through the overlying cerebellum into NL. To avoid the micropipette passing through or the tracer diffusing into the adjacent NA, the injections specifically avoided a small portion of the caudolateral NL that is immediately ventral and medial to NA (Fig. 3). Other than that, different injections in NL covered the whole nucleus throughout its rostrocaudal and mediolateral extents. In addition, every injection into NL involved a portion of NM (Fig. 3).

Following injections into NL and NM, the distribution pattern of anterograde labeling within MLd fall into two groups. In the first group, anterogradely labeled terminals within MLd were found mostly, if not exclusively, contralaterally (Fig. 4 A). These terminals were highly concentrated in a small central, though relatively ventral, region along the medial border of the nucleus at the intermediate level (Fig. 5 B). Remaining areas were totally lacking labeling (Fig. 5 C), except for a very low density of scattered terminals in the rostral MLd. Labeled terminals in MLd often formed clusters with axonal bouton-like swellings (Fig. 5 D). Cases in the first group tended to have a relatively restricted injection in the NL/NM region and did not involve the most caudal portion of NL or neuronal clusters in RI. Cases 469 and 472 in Figure 3 represent such examples.

The second group exhibited anterogradely labeled terminals bilaterally in the rostromedial portion of MLd, in addition to the small central region of the contralateral MLd (Fig. 4 B). Labeled terminals in these two regions were discontinuous from each other, a pattern especially evident in the cases with heavy staining. The density of labeled terminals in the rostromedial MLd was consistently higher on the side contralateral to the injection than the other side. Injection sites of the second group either extended ventrally across the fiber bundle deep into the ventral vestibular nuclei and/or encroached the most caudal region of NL and the surrounding area. Cases 468 and 471 in Figure 3 represent such examples.

Injections of CTB or PHA-L into NA

For injections into NA, the micropipette went tangentially from the cerebellum across the midline and into the contralateral NA. Different injections in NA covered the whole nucleus except for its most caudodorsal portion (Fig. 3). Most injections into NA did not involve NL, except in one case where the tracer diffused into a very small portion of the caudal NM and NL (case 481 in Figure 3). This case also involved neuronal clusters in RI.

Injections into NA produced a similar distribution of anterograde labeling within MLd in all cases (Fig. 6; except for case 480). On the side contralateral to the injection, labeled terminals were found extensively and widely within MLd. The terminal density varied in relationship to the injection site within NA. Following an injection concentrated in the ventral portion of NA, labeled terminals were substantially denser in the dorsal and lateral portion than in the caudal and caudoventral portion of MLd (Fig. 6 B and 7 A-B), while injections involving both the dorsal and ventral NA produced a relatively uniform distribution of labeling in MLd (Fig. 6 A). However, a small central region along the medial border of the nucleus consistently contained the sparsest terminals (Fig. 7 B-C), whose location approximately corresponds to where dense NL terminals were found. The density of labeled terminals in the rostromedial portion of the nucleus was also consistently lower than the adjacent ventral portion (Fig. 6 and 7 D, 7 F).

On the side ipsilateral to the injection, anterograde labeling was predominantly concentrated in the rostromedial portion of MLd (Fig. 6 and 7 E), with occasional terminals scattered in

other portion of the nucleus. As a result, a boundary between the rostromedial and other portions of MLd could be easily drawn, as the terminal density in the rostromedial MLd was substantially lower on the side contralateral to the injection, and significantly higher on the side ipsilateral to the injection, than the adjacent ventral region (Fig. 7 D-F).

The injection in case 480 involved only a very small portion of the dorsal NA and produced sparse anterogradely labeling in MLd. Although labeled terminals were found in different regions of the nucleus, specific patterns of distribution can not be determined.

Injections of CTB into MLd

Retrograde labeling in the caudodorsal brainstem—Regardless of the injection sites within MLd (Fig. 8), retrogradely labeled neurons were found in both NA and NL, although the percentage of labeled neurons in each nucleus varied across cases. This may be due to a substantial mixture of afferent fibers from NA and NL within MLd, or alternately, may reflect the difficulty of obtaining injections limited to sufficiently restricted regions of MLd. On the other hand, these retrograde tracing studies confirmed that the projections from NA and NL upon the majority of the MLd (except for its rostromedial portion) are mostly, if not exclusively, contralaterally. Statistical analyses among all cases with injections into MLd (excluding case 418 with an injection in the rostromedial portion) demonstrated that the percentage of labeled neurons on the ipsilateral side is $2.9 \pm 2.3\%$ ($n=10$; ranging from 0.3% to 6.6%) in NA and $2.8 \pm 4.2\%$ ($n=10$; ranging from 0 to 10.7%) in NL. In addition, distributions of labeled neurons within the contralateral NA support the topography of the projection from NA upon MLd. Injections into the caudal or ventral portion of MLd retrogradely labeled neurons mainly in the dorsal NA, while the majority of neurons were found in the ventral NA following injections into the dorsal MLd (Fig. 9). No labeled neurons were found in NM.

An injection into the rostromedial portion of MLd (case 418) clarified the nature of the bilateral inputs to this region (Fig. 10 and 11). Retrogradely labeled neurons in the caudodorsal brainstem were found bilaterally and symmetrically in caudal NL, ventral NA, and neuronal clusters in RI. Labeled neurons in the caudal NL were mostly located in the multilayered portion and predominantly on the side contralateral to the injection (Fig. 10 C-D, 11 D-F). The average soma area was $196 \pm 37 \mu\text{m}^2$ ($n=29$). Labeled neurons in NA were located along the ventromedial edge of NA at the caudal level (Fig. 10 D, 11 E) and concentrated in the very ventral region of the nucleus throughout the intermediate and rostral levels (Fig. 10 E, 11 H-I). The majority of these neurons were small with the average soma area of $114 \pm 31 \mu\text{m}^2$ ($n=86$). All three neuronal clusters of RI that we identified in Nissl-stained sections contained a substantial number of labeled neurons (Fig. 11 A-F). A few labeled neurons were also found in surrounding areas beyond these clusters (Fig. 11 G). Some of these neurons were located clearly beyond the boundaries that delimit the major body of NM, NL, and NA, while others were distributed along the edges of these nuclei that we were not able to unambiguously determine whether they belong to one of these three nuclei. The soma area of these neurons and labeled neurons in RI varied largely and averaged $134 \pm 57 \mu\text{m}^2$ ($n=176$). In addition, a number of labeled neurons were identified in the region ventral to the NM/NL/NA/RI complex (Fig. 11 J). They were embedded among large cell bodies of vestibular neurons and often located along the tract of the 8th nerve (Fig. 11 K-L). They had very small cell bodies with a soma area of $80 \pm 21 \mu\text{m}^2$ ($n=43$) and did not form clusters as in RI.

To determine whether these labeled neurons do provide bilateral projections to the rostromedial MLd or some of them were labeled due to tracer diffusing into regions of MLd that are mainly innervated by the contralateral projections from NL and NA, we counted the number of labeled neurons in each cell group on each side. Since injections into other

regions of MLd produced averagely 3% neurons on the ipsilateral side of NL and NA, if a certain cell group in case 418 is labeled due to tracer diffusion into these regions, we expect to see a small percentage (~ 3%) of labeled neurons on the ipsilateral side. In general, the density of labeled neurons was substantially lower on the ipsilateral side than on the contralateral side. Specifically, the percentage of labeled neurons on the ipsilateral side among the total on two sides is 2.9% in NL, 7.3% in NA, 21.6% in RI, and 49.2% in the region ventral to the NM/NL/NA/RI complex and within the 8th nerve tract (Tab. 2). This analysis indicates that RI and scattered neurons along the 8th nerve tract, and probably the ventral NA, do send bilateral inputs to the rostromedial MLd. Labeled neurons in NL have a low percentage of distribution on the ipsilateral side, which does not support a bilateral projection from this region to the midbrain.

In addition, we detected two labeled neurons that appeared to be located within the caudoventral NM on the contralateral side (Fig. 11 C). Both neurons were located close to the periphery, which may reflect the fact that NM, NL, and NA are not sharply and absolutely delineated from each other and from the surrounding area, at least in chick hatchlings, and may not indicate that NM projects to the midbrain.

Commissural connections of MLd—The topography of commissural connections of MLd was mapped following unilateral injections in various locations of MLd. To facilitate the description of this topography in relationship to the organization of the ascending projections from the NA and NL, we name the small central region along the medial border of MLd as the *NL-recipient zone*, as it is innervated predominately by the contralateral NL. The rostromedial portion of MLd receives bilateral inputs from the caudodorsal brainstem, and is designated as the *bilateral zone*. The remaining region of MLd in receipt of the contralateral NA is designated as the *NA-recipient zone*.

The NA-recipient zone is the largest subdivision of MLd. Our injections into this subdivision usually only involved a portion of it and could be organized into three groups, the ones concentrated in the caudal, ventral, or dorsal portion. Injections in all three groups displayed a similar distribution pattern of retrograde and anterograde labels in the contralateral MLd. Retrogradely labeled neurons were widely distributed, with the predominant population in the NL-recipient zone (Fig. 12 and 14 A). Anterogradely labeled neuropil was distributed mainly along the dorsal, lateral, and ventral borders of the nucleus rather than within the major body in receipt of NA.

In contrast, the NL-recipient zone occupies a small volume of the nucleus; our injection centered in the NL-recipient zone involved a substantial portion of the adjacent NA-recipient zone. In the contralateral MLd, anterogradely labeled terminals were found extensively in the NA-recipient zone (Fig. 13 and 14 B). These terminals were not uniformly distributed with the highest density in the dorsal portion. Notably, neurons in the contralateral NL-recipient zone were densely labeled. However, this region was lacking anterogradely labeled terminals (Fig. 14 C), demonstrating that the two NL-recipient zones were not reciprocally connected. These neurons were labeled probably due to the tracer spreading into the adjacent NA-recipient zone. Retrogradely labeled neurons were also found in the bilateral zone of MLd.

Following an injection into the bilateral zone of MLd (case 418; Fig. 15), the NL- and NA-recipient zones on the contralateral side were totally lacking any retrograde or anterograde labeling. Labeled neurons and neuropil densely overlapped in the contralateral bilateral zone (Fig. 14 D). This pattern indicates that labeled neurons in the bilateral zone following injections in the NA- or NL-recipient zone described above were due to tracer spreading

directly into the bilateral zone or inadvertently labeled by fibers of passage. In addition, a prominent bundle of fibers were labeled along the rostrolateral margin of the nucleus.

These results demonstrate a heavy commissural projection from the NL-recipient zone upon the contralateral NA-recipient zone and a highly restricted reciprocal connection between the bilateral zones of two sides. Neurons in the NA-recipient zone of MLd may also send their axons contralaterally which are distributed mainly along the borders of the MLd.

Discussion

The current study has identified three subdivisions of MLd in chicks based on the organization of their afferent and commissural projections. The main results are summarized and schematically illustrated in Figure 16. These results, as well as a comparison with the data from other avian species and mammals, may contribute to a better understanding of the organization and function of the auditory midbrain.

Three ascending auditory pathways to the midbrain

One of the most common features of auditory information processing in birds is that acoustic information is conveyed to the midbrain in at least two separate ascending pathways from NA and NL upon two non-overlapping subdivisions of the contralateral MLd (barn owl-Takahashi and Konishi, 1988a; chicken-Conlee and Parks, 1986; pigeon-Leibler, 1975; Wild, 1995). Both NA and NL contain a comparable set of tonotopic representations with a wide frequency range (Boord and Rasmussen, 1963; Hotta, 1971; Rubel and Parks, 1975; Warchol and Dallos, 1990; Köppl, 2001). The current study further demonstrated that the separation of these two pathways has developed in hatchling chicks, consistent with the view that the general organization of the auditory system is stabilized in chicks after hatching (See review, Rubel and Parks, 1988). In addition, we described the general topography of the projection from NA upon MLd, approximately consistent with the previous observation in pigeons (Leibler, 1975). Although the tonotopic organization of MLd has not been mapped in chicks, this topography is consistent with the tonotopic organization of MLd in zebra finches (Woolley and Casseday, 2004), with the dorsal NA (high frequency) projecting to the ventral MLd (high frequency), and the ventral NA (low frequency) to the dorsal MLd (low frequency). The NA-recipient zone of MLd may be further divided into several smaller subzones. Discharging pattern of the neurons in the NA-recipient zone of MLd varies in relationship to their location, with mainly 'simple' neurons in the ventral portion and a high proportion of 'complex' neurons in the dorsal portion (Scheich et al., 1977). In addition, different portions of the NA-recipient zone of MLd terminate restrictively within several cytoarchitecturally distinct subdivisions of the auditory thalamus (Wang and Karten, unpublished observations). The small volume of the NL-recipient zone in chicks and our relatively large injection sites in NL do not allow us to map the topography of the projection from NL upon MLd. In barn owls, this projection is organized in a topographical matter (Takahashi and Konishi, 1988a) and units in the NL-recipient zone of MLd (also called ICCcore in barn owls) are arranged in a frequency-dependent order (Knudsen and Konishi, 1978; Wagner et al., 1987, 2002).

More importantly, we clarified the presence of a third separate ascending pathway from the caudodorsal brainstem upon the rostromedial MLd. This pathway was first differentiated by Conlee and Parks (1986) due to its distinct bilateral property. The current study further identified the major neuronal sources of this projection in the regions adjacent to NM, NL, and NA at their caudal level which is named RI in the current study, as well as from scattered neurons along the 8th nerve tract, and probably a very ventral portion of NA. Although our data do not support a bilateral projection from the caudal NL upon MLd, they do not exclude the possibility that the caudal NL sends a contralateral projection to the

rostromedial MLd. At the midbrain level, the target of the bilateral projections constitutes a unique and cytoarchitecturally distinct portion of the auditory midbrain. They possess unique reciprocal commissural connections (the current study) and maintain intensive 2-deoxyglucose staining and display frequency selectivity with an oscillating discharge pattern after binaural cochlear removal (Heil and Scheich, 1986; Schwarz et al., 1993), features which are not common to neurons in other portions of MLd. In addition, the rostromedial portion of MLd in zebra finches shows a distinctive cytoarchitecture, although it is less pronounced in chicks (Karten, unpublished observations). These unique features characterizing the neurons and their innervation in this pathway may provide the anatomic basis for a third, functionally unique channel. In barn owls, the first two channels code time and intensity information, respectively (see review, Carr and Code, 2000). Bilateral properties of the third channel suggest that these neurons may involve in interaural processing. In addition, Theurich et al. (1984) reported that the rostromedial MLd contains neurons sensitive to acoustic stimuli between 2-10 Hz, suggesting the involvement of this channel in processing acoustic information at very low frequencies. Warchol and Dallos (1989) reported that some neurons in NA are sensitive to low frequency and infrasound between 10-50 Hz. While the authors did not specify the location of these neurons, they did mention that non-auditory neurons were found “immediately ventral and medial” to low-frequency units. The authors assumed these non-auditory neurons were vestibular neurons. If this assumption is valid, the low-frequency neurons should distribute along the ventral and medial margins of NA, where we found neurons with bilateral projections upon MLd.

A pathway comparable to this third channel in chicks has not been identified in barn owls or pigeons. Neither retrograde nor anterograde tracing studies in barn owls showed evidence of a bilateral projection from NM/NL/NA and/or their adjacent region to MLd (Takahashi and Konishi, 1988a). In pigeons, a very small number of labeled neurons were found in the ipsilateral NL and NA following large injections into MLd (Leibler, 1975). Following more restricted injections into the rostromedial portion of MLd, labeled neurons were detected surrounding the caudal NL and between the caudal NL and NA (Leibler, 1975; Wild, 1995). However, these neurons were reported only on the contralateral side. These interspecies variations raise interesting questions regarding species-specific animal behaviors in relationship to the organization of the auditory midbrain.

Two different views on nomenclature

Due to immediately adjacent and partially overlapped location of the bilateral projecting neurons in the caudorostral brainstem with remaining neurons in NM, NL, and NA, there are two different views on how to name the bilateral input to the avian auditory midbrain to facilitate further research in the system.

One view is that the bilateral projecting neurons in the caudodorsal brainstem should be treated as the very low frequency regions of NL and NA. First, the distribution of RI, one of the major origins of the bilateral projection to MLd, overlaps with the termination and nerve tract of a fiber bundle of the 8th nerve arising from the inner ear. The exact origin of this fiber bundle was reported as the lagenar in pigeons (Boord and Rasmussen, 1963; Boord and Karten, 1974) and more recently described as the low frequency region of the cochlea in chicks (Kaiser and Manley, 1996). Second, although NA contains several physiologically and anatomically distinct cell types (Häusler et al., 1999; Soares et al., 2001; Köppl and Carr, 2003), only one tonotopic representation was recorded in NA (Konishi, 1965; Boord and Rasmussen, 1963; Hotta, 1971; Warchol and Dallos, 1990; Köppl, 2001). In barn owls, the same cell types are distributed along the tonotopic axis (Soares and Carr, 2001). An obvious disadvantage of grouping these neurons as parts of NA and NL is increased ambiguity in the definition and description of NM, NL, and NA, since a large portion of the

bilateral projecting neurons are located clearly outside of the traditional boundaries of these three nuclei.

An alternative view is that these bilateral projecting neurons should be considered as distinct cell groups in addition to NM, NL, and NA. The major rationale under this view is the unique connectional features of these bilaterally projecting neurons. Primary cochlear nuclei NM and NA and secondary cochlear nucleus NL have been extensively studied both physiologically and anatomically. Two well-established definitions are that NL neurons do not receive inputs from the periphery (Parks and Rubel, 1978) and that NM neurons do not project upon the midbrain (Boord, 1968; Leibler, 1975; Conlee and Parks, 1986; Takahashi and Konishi, 1988a). Direct inputs from the inner ear and outputs to the midbrain distinguish the neurons in RI from NM and NL neurons. In addition, the bilateral property of the projection from neurons in RI and the ventral NA to MLd clearly indicates their distinct roles in processing auditory signals compared to the neurons in NL and the major body of the NA. Furthermore, scattered neurons embedded in the 8th nerve tract clearly do not belong to NM, NL, or NA. Since the bilaterally projecting neurons are innervated in a similar manner, it is reasonable to treat them as a whole structure with distinct functions from NM, NL, and NA.

Commissural connections of MLd

Equally importantly, the current study demonstrated that the three subdivisions of MLd, identified on the basis of their differential afferent projections, also play distinct roles in the commissural connections of MLd, which further supports the notion that these three subdivisions are functionally distinct. Two major components of the commissural connections of MLd have been identified: a heavy projection from the NL-recipient zone upon the contralateral NA-recipient zone and a highly restricted reciprocal connection between the RI-recipient zones of two sides. The commissural projection from the NL-recipient zone to the NA-recipient zone has been previously identified in barn owls (Takahashi et al., 1989). However, this projection innervates the whole range of the NA-recipient zone in chicks but is restricted to the lateral portion of the owl NA-recipient zone. As this projection integrates time and intensity information and has been considered to contribute to the computation of the spatial location of auditory stimuli in barn owls, it would be interesting to study whether this is the case in chicks and, if so, why this projection expands in a species with less sensitivity to sound localization than barn owls. Comparison of commissural connections of MLd between chicks and pigeons is somewhat confusing. Studies in pigeons reported that the rostromedial MLds on two sides of the brain are reciprocally connected (Leibler, 1975; Wild, 1995), consistent with our observation in chicks. However, both authors reported that the rostromedial portion of the pigeon MLd is the major target of the contralateral NL. Reciprocal connections between the NL-recipient zones of MLd are not found in chicks or barn owls. Functional significance of this second integration of binaural time information at the midbrain level in pigeons after the first convergence of acoustic information from the two ears within NL remains unknown.

Comparison with mammals

The central nucleus of the inferior colliculus (ICC) in mammals is the major recipient of the ascending projections from the auditory brainstem including the cochlear nuclei, the superior olive complex, and the lateral lemniscus nuclei (Oliver and Huerta, 1992), comparable to the information convergence in the avian MLd. A recent tracing study in gerbils divided ICC into two subdivisions, one receiving inputs from both the cochlear nuclei and the superior olive, and the other from only the cochlear nuclei (Cant and Benson, 2006). The avian NL is thought to be homologous or analogous to the medial superior olivary nucleus of mammals (Cajal 1909-11, Burger and Rubel, 2008), although the extent

of this postulated homology remains controversial (Grothe et al., 2004). Neurons in NA display a similar range of response types to sound stimuli as in the mammalian cochlear nuclei (Köppl and Carr, 2003). Thus, two ICC subdivisions identified in gerbils may be generally compared to the NL- and NA-recipient zones of the avian MLd, respectively. Birds and mammals may differ in the organization of the commissural connections of the auditory midbrain. In mammals, this connection is reciprocal and topographically organized throughout the nucleus (Saldaña and Merchán, 1992; Malmierca et al., 1995, 2005), in contrast to the region-region specificity in birds.

Conclusion

Multiple, parallel auditory brainstem pathways converge in the avian MLd and the mammalian ICC. As a consequence, the auditory midbrain has been suggested to be a major site of neural integration and involved in a number of auditory tasks, including sound localization and coding of complex acoustic signals such as birdsong and vocalization. One of the requirements necessary for performing these auditory tasks is the capability of organizing and integrating various properties of acoustic signals (time, intensity, and frequency) in a highly specific pattern. The heterogeneous organization of the auditory midbrain, with differential afferents to several distinct subdivisions and specialized interactions among these subdivisions, may provide the anatomical basis for this capability. Further studies on the detailed organization of the neuronal network in each subdivision will be essential to understand cellular substrates underlying the function of the auditory midbrain.

Acknowledgments

Grant sponsors: National Institute of Neurological Disorders and Stroke [NS 24560-15]; National Institute of Mental Health [MH 60975-07]; National Institute on Deafness and Other Communication Disorders [DC-03829, DC-04661, and DC-00018]

The authors are grateful to Dr. Catherine Carr (University of Maryland) and Dr. Edwin W Rubel (University of Washington) for criticisms of the manuscript.

Literature Cited

- Adolphs R. Acetylcholinesterase staining differentiates functionally distinct auditory pathways in the barn owl. *J Comp Neurol.* 1993; 329:365–377. [PubMed: 7681456]
- Boord RL. Ascending projections of the primary cochlear nuclei and nucleus laminaris in the pigeon. *J Comp Neurol.* 1968; 133:523–541. [PubMed: 4185132]
- Boord RL, Rasmussen GL. Projection of the cochlear and lagenar nerves on the cochlear nuclei of the pigeon. *J Comp Neurol.* 1963; 120:463–475. [PubMed: 14013817]
- Boord RL, Karten HJ. The distribution of primary lagenar fibers within the vestibular nuclear complex of the pigeon. *Brain Behav Evol.* 1974; 10:228–235. [PubMed: 4141918]
- Burger, RM.; Rubel, EW. Encoding of interaural timing for binaural hearing. In: Dallos, P.; Oertel, D., editors. *The senses: a comprehensive reference*. San Diego: Academic Press; 2008. p. 613-630.
- Cant NB, Benson CG. Organization of the inferior colliculus of the gerbil (*Meriones unguiculatus*): differences in distribution of projections from the cochlear nuclei and the superior olivary complex. *J Comp Neurol.* 2006; 495:511–528. [PubMed: 16498677]
- Carr, CE.; Code, RA. The central auditory system of reptiles and birds. In: Dooling, RJ.; Fay, RR.; Popper, AN., editors. *Comparative hearing: birds and reptiles*. New York: Springer-Verlag; 2000. p. 197-248.
- Grothe, B.; Carr, CE.; Casseday, JH.; Fritzsche, B.; Köppl, C. The evolution of central pathways and their neural processing patterns. In: Manley, GA.; Popper, AN.; Fay, RR., editors. *Evolution of the vertebrate auditory system*. New York: Springer-Verlag; 2004. p. 289-359.

- Code RA. Efferent neurons to the macular lagena in the embryonic chick. *Hear Res.* 1995; 82:26–30. [PubMed: 7744710]
- Conlee JW, Parks TN. Origin of ascending auditory projections to the nucleus mesencephalicus lateralis pars dorsalis in the chicken. *Brain Res.* 1986; 367:96–113. [PubMed: 3697720]
- Heil P, Scheich H. Effects of unilateral and bilateral cochlear removal on 2-deoxyglucose patterns in the chick auditory system. *J Comp Neurol.* 1986; 252:279–301. [PubMed: 3793978]
- Holmes G. On the comparative anatomy of the nervus acusticus. *Trans Roy Irish Acad Section B.* 1903; 32:101–144.
- Hotta T. Unit responses from the nucleus angularis in the pigeon's medulla. *Comp Biochem Physiol A.* 1971; 40:415–424. [PubMed: 4400939]
- Iiguez C, Gayoso MJ, Carreres J. A versatile and simple method for staining nervous tissue using Giemsa dye. *J Neurosci Methods.* 1985; 13:77–86. [PubMed: 3887046]
- Kaiser A, Manley GA. Brainstem connections of the macula lagenae in the chicken. *J Comp Neurol.* 1996; 374:108–117. [PubMed: 8891950]
- Karten HJ. The organization of the ascending auditory pathway in the pigeon (*Columba Livia*). 1. Diencephalic projections of the inferior colliculus (nucleus mesencephali lateralis, pars dorsalis). *Brain Res.* 1967; 6:409–427. [PubMed: 6076249]
- Karten HJ. The ascending auditory pathway in the pigeon (*Columba Livia*). II. Telencephalic projections of the nucleus ovoidalis thalami. *Brain Res.* 1968; 11:134–153. [PubMed: 5749228]
- Klump, GM. Sound localization in birds. In: Dooling, RJ.; Fay, RR.; Popper, AN., editors. *Comparative hearing: birds and reptiles.* New York: Springer-Verlag; 2000. p. 249–307.
- Knudsen EI. Neural derivation of sound source location in the barn owl. An example of a computational map. *Ann N Y Acad Sci.* 1987; 510:33–38. [PubMed: 3324875]
- Knudsen EI, Konishi M. Space and frequency are represented separately in auditory midbrain of the owl. *J Neurophysiol.* 1978; 41:870–884. [PubMed: 681991]
- Konishi M, Sullivan WE, Takahashi T. The owl's cochlear nuclei process different sound localization cues. *J Acoust Soc Am.* 1985; 78:360–364. [PubMed: 4031243]
- Köpl C. Auditory nerve terminals in the cochlear nucleus magnocellularis: differences between low and high frequencies. *J Comp Neurol.* 1994; 339:438–446. [PubMed: 8132870]
- Köpl C. Tonotopic projections of the auditory nerve to the cochlear nucleus angularis in the barn owl. *J Assoc Res Otolaryngol.* 2001; 2:41–53. [PubMed: 11545149]
- Köpl C, Carr CE. Computational diversity in the cochlear nucleus angularis of the barn owl. *J Neurophysiol.* 2003; 89:2313–2329. [PubMed: 12612008]
- Kuenzel, WJ.; Masson, M. A stereotaxic atlas of the brain of the chick (*Gallus domesticus*). Johns Hopkins University Press; 1988.
- Leibler, LM. Ph D Thesis. Massachusetts Institute of Technology; Cambridge, MA: 1975. Monaural and binaural pathways in the ascending auditory system of the pigeon.
- Malmierca MS, Rees A, Le Beau FE, Bjaalie JG. Laminar organization of frequency-defined local axons within and between the inferior colliculi of the guinea pig. *J Comp Neurol.* 1995; 357:124–144. [PubMed: 7673462]
- Malmierca MS, Hernandez O, Rees A. Intercollicular commissural projections modulate neuronal responses in the inferior colliculus. *Eur J Neurosci.* 2005; 21:2701–2710. [PubMed: 15926918]
- Oliver, DL.; Huerta, MF. Inferior and superior colliculi. In: Webster, DB.; Popper, AN.; Fay, RR., editors. *The mammalian auditory pathway: neuroanatomy.* New York: Springer-Verlag; 1992. p. 168–221.
- Parks TN, Rubel EW. Organization and development of the brain stem auditory nuclei of the chicken: primary afferent projections. *J Comp Neurol.* 1978; 180:439–448. [PubMed: 659669]
- Puelles L, Robles C, Martinez-de-Ia-Torre M, Martinez S. New subdivision schema for the avian torus semicircularis: neurochemical maps in the chick. *J Comp Neurol.* 1994; 340:98–125. [PubMed: 8176005]
- Rubel EW, Parks TN. Organization and development of brain stem auditory nuclei of the chicken: tonotopic organization of n. magnocellularis and n. laminaris. *J Comp Neurol.* 1975; 164:411–433. [PubMed: 1206127]

- Rubel, EW.; Parks, TN. Organization and development of the avian brain-stem auditory system. In: Edelman, GM.; Gall, WE.; Cowans, WM., editors. Auditory function: neurobiological bases of hearing. New York: John Wiley; 1988. p. 3-92.
- Saldana E, Merchán MA. Intrinsic and commissural connections of the rat inferior colliculus. *J Comp Neurol.* 1992; 319:417–437. [PubMed: 1376335]
- Scheich H, Langner G, Koch R. Coding of narrow-band and wide-band vocalizations in the auditory midbrain nucleus (MLD) of the guinea fowl (*numida meleagris*). *J Comp Physiol.* 1977; 117:245–265.
- Schwarz DW, Dezso A, Neufeld PR. Frequency selectivity of central auditory neurons without inner ear. *Acta Otolaryngol.* 1993; 113:266–270. [PubMed: 8517126]
- Sullivan WE, Konishi M. Segregation of stimulus phase and intensity coding in the cochlear nucleus of the barn owl. *J Neurosci.* 1984; 4:1787–1799. [PubMed: 6737041]
- Takahashi T, Konishi M. Selectivity for interaural time difference in the owl's midbrain. *J Neurosci.* 1986; 6:3413–3422. [PubMed: 3794779]
- Takahashi T, Konishi M. The projections of the cochlear nuclei and nucleus laminaris to the inferior colliculus of the barn owl. *J Comp Neurol.* 1988a; 274:190–211. [PubMed: 2463286]
- Takahashi T, Konishi M. Projections of nucleus angularis and nucleus laminaris to the laterallemniscal nuclear complex of the barn owl. *J Comp Neurol.* 1988b; 274:212–238. [PubMed: 2463287]
- Takahashi T, Moiseff A, Konishi M. Time and intensity cues are processed independently in the auditory system of the owl. *J Neurosci.* 1984; 4:1781–1786. [PubMed: 6737040]
- Takahashi T, Carr CE, Brecha N, Konishi M. Calcium binding protein-like immunoreactivity labels the terminal field of nucleus laminaris of the barn owl. *J Neurosci.* 1987; 7:1843–1856. [PubMed: 2439666]
- Takahashi T, Wagner H, Konishi M. Role of commissural projections in the representation of bilateral space in the barn owl's inferior colliculus. *J Comp Neurol.* 1989; 281:545–554. [PubMed: 2708580]
- Theurich M, Langner G, Scheich H. Infrasound responses in the midbrain of the guinea fowl. *Neurosci Lett.* 1984; 49:81–86. [PubMed: 6493602]
- Wagner H, Takahashi T, Konishi M. Representation of interaural time difference in the central nucleus of the barn owl's inferior colliculus. *J Neurosci.* 1987; 7:3105–3116. [PubMed: 3668618]
- Wagner H, Mazer JA, von Campenhausen M. Response properties of neurons in the core of the central nucleus of the inferior colliculus of the barn owl. *Eur J Neurosci.* 2002; 15:1343–1352. [PubMed: 11994128]
- Wagner H, Güntürkün O, Nieder B. Anatomical markers for the subdivisions of the barn owl's inferior-collicular complex and adjacent peri- and subventricular structures. *J Comp Neurol.* 2003; 465:145–159. [PubMed: 12926022]
- Warchol ME, Dallos P. Neural coding in the chick cochlear nucleus. *J Comp Physiol A.* 1990; 166:721–734. [PubMed: 2341992]
- Wild JM. Convergence of somatosensory and auditory projections in the avian torus semicircularis, including the central auditory nucleus. *J Comp Neurol.* 1995; 358:465–486. [PubMed: 7593743]
- Woolley SM, Casseday JH. Response properties of single neurons in the zebra finch auditory midbrain: response patterns, frequency coding, intensity coding, and spike latencies. *J Neurophysiol.* 2004; 91:136–151. [PubMed: 14523072]
- Woolley SM, Casseday JH. Processing of modulated sounds in the zebra finch auditory midbrain: responses to noise, frequency sweeps, and sinusoidal amplitude modulations. *J Neurophysiol.* 2005; 94:1143–1157. [PubMed: 15817647]
- Woolley SM, Gill PR, Theunissen FE. Stimulus-dependent auditory tuning results in synchronous population coding of vocalizations in the songbird midbrain. *J Neurosci.* 2006; 26:2499–2512. [PubMed: 16510728]

Figure 1.

The chick NL at intermediate (A) and caudal (B) levels, NA (C), and MLd (D) in Nissl-stained sections in the coronal plane. Dashed lines in C and D indicate the outlines of NA and MLd, respectively. Abbreviations: NL, nucleus laminaris; NA, nucleus angularis; NM, nucleus magnocellularis; MLd, nucleus mesencephalicus lateralis pars dorsalis; TeO, optic tectum; SGPv, stratum griseum periventriculare; ICo, nucleus intercollicularis. Scale bar = 200 μ m in A (applies to A-C), 500 μ m in D.

Figure 2.

Three neuronal clusters, named as RI in the current study, in Nissl-stained sections in the coronal plane. **A, C, and E:** low-power images illustrating approximate location of the clusters (dashed lines) and their relationship to NM/NL/NA. Loose packing, light staining, and their location adjacent to NM/NL/NA of these clusters do not allow us to draw their exact boundaries. Images from A to E represent sections at the levels from caudal to rostral, although they were all caudal to the level in Fig. 1B. Arrow in A points to a neuronal cluster with more prominent Nissl staining than that in RI. No retrogradely labeled neurons in this cluster were found following injections into MLd. **B, D, and F:** high-power images of the boxed regions in A, C, and E, respectively. Arrowheads indicate neuronal cell bodies in each cluster. Note many neurons in F are elongated and oriented parallel to the long axis of the cluster. Abbreviations see Figure 1. Scale bar = 400 μ m in E (applies to A, C, and E), 100 μ m in F (applies to B, D, and F).

Figure 3.

Injection sites in NA (cases 466, 480, 481, and 484) and NL (cases 468, 469, 471, and 472). Drawings from A to L represent sections at the levels from caudal to rostral. Dashed circles indicate injection sites that are defined as the blackened region in a section under bright-field observation. As an example, case 469 at the level of H was drawn from the section illustrated in Figure 5A. Injection sites of cases 461, 477, and 483 largely overlap with that of cases 469, 468, and 481, respectively, and are not included in the drawing for the clarity of the figure. Abbreviations see Figure 1.

Figure 4.

Line drawings of two different patterns of anterograde labeling in MLd following injections into NL/NM. **A:** Anterograde terminals were found contralaterally in a small central region along the medial border of MLd. The injection site is illustrated in Figure 3 (case 469). **B:** Anterograde terminals were detected bilaterally in the rostromedial portion of MLd, in addition to the small central region. The injection site is illustrated in Figure 3 (case 471). Black dots represent anterograde terminals. Numbers below each drawing indicate the approximate anterior-posterior stereotaxic levels. Abbreviations see Figure 1.

Figure 5. Bright-field photomicrographs of anterograde labeling in MLd following an injection of CTB into NL (case 469). **A:** The injection site in NL. **B:** The restricted distribution of the anterograde labeling within the contralateral MLd. **C:** A closer view of the box in B illustrating the sharp edge formed by labeled terminals from adjacent unstained area. **D:** A high-magnification photo showing terminal clusters. A was taken from a Giemsa-counterstained section and B-D from non-counterstained sections. Abbreviations: VeM, nucleus vestibularis medialis; VT, ventriculus tecti mesencephali. Other abbreviations see Figure 1. Scale bar = 500 μ m in A and B; 50 μ m in C; 30 μ m in D.

Figure 6.

Line drawings of anterograde labeling in MLd following injections into NA. **A:** Labeled terminals were distributed heterogeneously throughout the contralateral MLd and within the rostromedial portion of the ipsilateral MLd. Within the contralateral MLd, a small central region along the medial border of the nucleus contained the lowest density of labeling. The injection site is illustrated in Figure 3 (case 484). **B:** Anterogradely labeled terminals in the contralateral MLd were sparser in the caudal and caudoventral portion than in the dorsal and lateral portion of the nucleus following an injection in the ventral NA. The injection site is illustrated in Figure 3 (case 481). Black dots represent anterograde terminals. Short lines in A indicate PHA-L labeled fiber fragments. Numbers below each drawing indicate the approximate anterior-posterior stereotaxic levels. Abbreviations see Figure 1.

Figure 7.

Photomicrographs of anterograde labeling in MLd following an injection of CTB into NA (case 481). **A:** The injection site in NA. Dashed lines indicate the boundary of NA **B:** Widely distributed anterograde labeling in the contralateral MLd. **C:** Abrupt changes in the density of labeled terminals (arrows) between the small central region and the surrounding area. **D:** Bright-field image of the rostromedial portion of the contralateral MLd. **E-F:** Dark-field images of the rostromedial portion of the ipsilateral (E) and contralateral (F) MLd. Dashed lines in D-F indicate the boundaries of MLd. Note changes in the density of labeled terminals between the rostromedial portion and the adjacent ventral region (thick dotted lines). A was taken from a Giemsa-counterstained section and B-F from non-counterstained sections. D and F were taken from the same section. Abbreviations see Figure 1. Scale bar = 500 μ m in A and B; 100 μ m in C; 500 μ m in D (applies to D-F).

Figure 8.

Injection sites in MLd. Drawings from A to F represent sections at the levels from caudal to rostral. Case 319 is an injection involved the major portion of the NL-recipient zone and is illustrated in grey to distinguish from other cases.

Figure 9.

Line drawings and photomicrographs of the distribution of retrogradely labeled neurons in the contralateral NA following injections into the caudal (A-D, case 419), ventral (E-H and M, case 322), or dorsal (I-L and N, case 333) portions of MLd. The majority of retrogradely labeled neurons were situated in the dorsal NA following injections into the caudal or ventral portion of MLd, and in the ventral NA following an injection into the dorsal portion of MLd. Black dots in A-L represent retrogradely labeled neurons. Dashed lines in M-N indicate the boundaries of NA. Numbers below each lane of drawings indicate the approximate anterior-posterior stereotaxic levels. Scale bar = 200 μ m in N (applies to M-N).

Figure 10.

Retrogradely labeled neurons in the caudodorsal brainstem following a CTB injection into the rostromedial portion of MLd (case 418). **A:** The injection site in the rostromedial portion of MLd. **B-E:** Series of sections through the contralateral NM, NA, and NL. **F:** A section through the ipsilateral NM, NA, and NL. Note retrogradely labeled neurons were not located in the major body of NL or NA. Approximate anterior-posterior stereotaxic levels are indicated at the lower left corner of each photo. Images were taken from Giemsa-counterstained sections. Abbreviations see Figure 1. Scale bar = 1 mm in A; 500 μ m in F (applies to B-F).

Figure 11.

High-magnification photomicrographs of labeled neurons following the injection into the rostromedial portion of MLd (case 418). **A-B:** Labeled neurons in the neuronal cluster depicted in Figure 2A on the ipsilateral side. **C:** One labeled neuron (arrowhead) that appeared to be located within the caudoventral region of the contralateral NM. **D:** Labeled neurons in the neuronal cluster depicted in Figure 2C on the ipsilateral side. **E-F:** Labeled neurons in the neuronal cluster depicted in Figure 2E on the contralateral (E) and ipsilateral (F) side. **G:** Two scattered neurons (arrowheads) labeled in the surrounding region. **H-I:** Labeled neurons in the ventral region of NA on the ipsilateral (H) and contralateral (I) side. **J-L:** Labeled neurons in the region ventral to NA on the contralateral (J-K) and ipsilateral (L) side. Arrowheads in K and L point out labeled neurons. K is a closer view of the box in J. Empty arrowheads in L indicate the course of one axonal collateral of a labeled neuron. All images were taken from Giemsa-counterstained sections. Up is dorsal. Right is medial and lateral for images taken from the ipsilateral and contralateral side, respectively. Abbreviations: RI, regio intermedius; VeL, nucleus vestibularis lateralis; nVIII, 8th nerve. Other abbreviations see Figure 1. Scale bar = 100 μ m in A (apply to A-B, D-I, and L), 50 μ m in C and K, and 200 μ m in J.

Figure 12.

Low-magnification dark-field images and line drawings of anterograde and retrograde labeling in the contralateral MLd following injections of CTB into the NA-recipient zone of MLd. **A-D:** Images of labeling following an injection into the caudal portion of MLd (case 419). Numbers below each image indicate the approximate anterior-posterior stereotaxic levels. **E-H:** line drawings of A-D, respectively. Solid black circles and lines indicate retrogradely labeled neurons and anterogradely labeled neuropil, respectively. **I-L:** line drawings of labeled neurons and terminals following an injection into the ventral portion of MLd (case 322). The approximate anterior-posterior levels of I-L are comparable to those in A-D. **M-P:** line drawings of labeled neurons and terminals following an injection into the dorsal portion of MLd (case 333). The approximate anterior-posterior levels of M-P are comparable to those in A-D. All three cases produced a similar distribution of labeled neurons and terminals in the contralateral MLd. A central region along the medial border of the nucleus, corresponding to the NL-recipient zone, contained a high density of labeled neurons. Anterogradely labeled terminals were mainly distributed along the periphery of the nucleus. Injection sites are illustrated in Figure 8. Scale bar = 500 μ m.

Figure 13.

Low-magnification dark-field images (**A-D**) and line drawings (**E-H**) of anterograde and retrograde labeling in the contralateral MLd following an injection of CTB into the NL-recipient zone of MLd (case 319). The injection also involved the adjacent region of the NA-recipient zone, as illustrated in Figure 8. E-H are line drawings of A-D, respectively. Numbers below each image indicate the approximate anterior-posterior stereotaxic levels. Solid black circles and lines indicate retrogradely labeled neurons and anterogradely labeled neuropil, respectively. In the contralateral MLd, anterogradely labeled terminals were found extensively in the NA-recipient zone, especially in its dorsal portion. The NL-recipient zone contained densely labeled neurons but was lacking anterogradely labeled terminals. Scale bar = 500 μ m.

Figure 14.

High-magnification bright-field images of the anterograde and retrograde labeling in MLd following injections of CTB in the contralateral MLd. **A:** Retrogradely labeled neurons in the NL-recipient zone following the injection into the caudal portion of the NA-recipient zone (case 419). **B:** Anterogradely labeled terminals in the NA-recipient zone following the injection into the NL-recipient zone (case 319). **C:** Retrogradely labeled neurons in the NL-recipient zone in the case 319. **D:** Retrogradely labeled neurons and anterogradely labeled terminals in the bilateral zone of MLd following the injection into the same zone on the other side of the brain (case 418). In addition to darkly labeled cell bodies, a high density of small boutons and terminals formed a dark granular appearance of staining in this region, in contrast to the unstained, pale background in C. Scale bar = 50 μ m.

Figure 15.

Low-magnification dark-field images (**A-D**) and line drawings (**E-H**) of anterograde and retrograde labeling in the contralateral MLd following the injection into the bilateral zone of MLd (case 418). The retrograde and anterograde labeling was restricted to the bilateral zone of the contralateral MLd. Solid black circles and lines indicate retrogradely labeled neurons and anterogradely labeled neuropil, respectively. Numbers below each photo indicate the approximate anterior-posterior stereotaxic levels. The injection site is illustrated in Figure 8. Scale bar = 500 μ m.

Figure 16.

Schematic drawing of the afferent and commissural organization of MLD. The dorsal auditory brainstem sends three separate pathways (green, red, and blue) upon three distinct subdivisions of MLD. Filled circles and lines indicate neurons and connections in each pathway, respectively. Solid and dashed lines indicate connections issued from the right and left sides of the brainstem, respectively. For the purpose of clarity, the red and blue pathways in A are not illustrated; the organization of these connections is the same as demonstrated in B. The green pathways are illustrated in A as the majority of the neurons in the green channel at the brainstem level are located in the caudal portion. The NA-recipient zone of MLD, shown as black cells, receives information from both red and blue channels. However, whether individual neurons in the NA-recipient zone receive direct bilateral inputs requires further investigation. The ipsilateral projections from NL and NA upon MLD, the projection from NA upon the NL-recipient zone of MLD, and the connections between the NA-recipient zones of two sides, are very sparse, and not included in the drawing. Abbreviations: NL, nucleus laminaris; NA, nucleus angularis; NM, nucleus magnocellularis; RI, regio intermedius; MLD, nucleus mesencephalicus lateralis pars dorsalis.

Table 1

The injection sites in NA, NL, and MLd, as well as the tracer used in each case.

Case	Injection site	Tracer
461	NL, NM	CTB
468	NL, NM	CTB
469	NL, NM	CTB
471	NL, NM	PHA-L
472	NL, NM	PHA-L
477	NL, NM	PHA-L
466	NA	CTB
480	NA	CTB
481	NA	CTB
483	NA	PHA-L
484	NA	PHA-L
419	caudal MLd	CTB
327	caudal MLd	CTB
339	caudal MLd	CTB
322	ventral MLd	CTB
323	ventral MLd	CTB
324	ventral MLd	CTB
333	dorsal MLd	CTB
338	dorsal MLd	CTB
340	dorsal MLd	CTB
319	central MLd	CTB
418	rostromedial MLd	CTB

Table 2

Numbers of labeled neurons in NM, NL, NA, RI, and the region ventral to the NM/NM/NA/RI complex following the injection into the rostromedial MLd (case 418). Numbers of labeled neurons on each side (contralateral and ipsilateral) are listed separately. The percentages of labeled neurons on the ipsilateral side among all labeled neurons are calculated. Neurons whose location can not be unambiguously determined are grouped into the fourth category.

	number of labeled neurons		ipsi / (contra + ipsi) (%)
	contral	ipsi	
caudoventral NM	2	0	0
caudolateral NL	100	3	2.9
ventromedial and ventral NA	101	8	7.3
RI and scattered neurons in surrounding regions	174	48	21.6
Region ventral to the NM/NL/NA/RI complex	30	29	49.2