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THE VENTRAL NUCLEUS OF THE LATERAL LEMNISCUS OF THE GERBIL (*MERIONES UNGUICULATUS*): ORGANIZATION OF CONNECTIONS WITH THE COCHLEAR NUCLEUS AND THE INFERIOR COLLICULUS

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Abstract

The spatial organization of projections from the ventral cochlear nucleus (VCN) to the ventral nucleus of the lateral lemniscus (VNLL) and from the VNLL to the central nucleus of the inferior colliculus (CNIC) was investigated using neuroanatomical tracing methods in the gerbil. In order to label cells in the VNLL that project to the CNIC, focal injections of biotinylated dextran amine (BDA) were made into different CNIC regions. Retrogradely labeled cells were distributed throughout the dorsal-to-ventral axis of the VNLL in all cases. In contrast, the distribution of labeled cells across the lateral-to-medial dimension of the VNLL was related to the location of the injection site along the dorsolateral to ventromedial (frequency) axis of the CNIC. Cells projecting to dorsolateral (low-frequency) regions of the CNIC were located peripherally in the VNLL, mainly laterally and caudally, whereas those projecting to ventromedial (high-frequency) regions of the CNIC tended to be clustered centrally. Projections to the VNLL were labeled anterogradely following injections of BDA in the VCN. The distribution of terminal fields in the VNLL closely paralleled the topographic arrangement of cells projecting to the CNIC; projections from ventrolateral (low-frequency) areas of the VCN terminated mainly along the lateral and caudal borders of the VNLL, whereas projections from dorsomedial (high-frequency) areas terminated in more central regions. The results demonstrate a topographic organization of the major afferent and efferent connections of the gerbil VNLL.

Keywords

auditory pathways; biotinylated dextran amine; neuroanatomy

INTRODUCTION

The ventral nucleus of the lateral lemniscus (VNLL) projects almost exclusively to the inferior colliculus (IC) and represents a major source of its ascending inhibitory input (Whitley and Henkel, 1984; Roberts and Ribak, 1987; Saint Marie and Baker, 1990; Winer et al., 1995; González-Hernández et al., 1996; Saint Marie et al., 1997; Vater et al., 1997; Zhang et al., 1998; Riquelme et al., 2001). The majority of input to the VNLL arises from the contralateral ventral cochlear nucleus (VCN; Friauf and Ostwald, 1988; Smith et al., 1991, 1993, 2005; Huffman and Covey, 1995; Adams, 1997; Schofield and Cant, 1997; Vater et al., 1997), with additional projections originating in the superior olivary complex and the ipsilateral VCN (Warr, 1966, 1969, 1972; Glendenning et al., 1981; Henkel and

Spangler, 1983; Spangler et al., 1985; Sommer et al., 1993; Huffman and Covey, 1995; Warr and Beck, 1996; Smith et al., 1998). Several studies have indicated that the VNLL is involved in analyzing temporal aspects of auditory signals (Covey and Casseday, 1991; Covey, 1993; Batra and Fitzpatrick, 1999; Wu, 1999; Nayagam et al., 2005; Batra, 2006; Zhang and Kelly, 2006b). It has been hypothesized that neurons in the VNLL may play a role in temporal pattern recognition and in the processing and interpretation of complex sounds (Covey and Casseday, 1986, 1991; Oertel and Wickesberg, 2002).

The central auditory pathways are characterized by their systematic projection patterns (topography), which form the basis for tonotopy. Several studies, however, have suggested that in some species the VNLL might not conform to the principle of either a topographic or tonotopic organization. In bats, certain regions of the VNLL or INLL do demonstrate a tonotopic order (Covey and Casseday, 1991; Metzner and Radtke-Schuller, 1987), but in both cats and rats a frequency-related organization is much less obvious. In the cat, electrophysiological experiments initially suggested a dorsal-to-ventral tonotopic arrangement, with low best frequencies represented dorsally and high best frequencies ventrally (Aitkin et al., 1970), but subsequent studies failed to confirm this finding (Guinan et al., 1972; Adams, 1997). Evidence from connectional studies in cats indicated either a lack of topography (Adams, 1979; Brunso-Bechtold et al., 1981; Glendenning and Hutson, 1998) or a complex interdigitation of cell clusters projecting to different frequency regions of the IC (Malmierca et al., 1998). Similarly, in the rat, some studies failed to find a tonotopic organization in the VNLL (Saint Marie et al., 1999b; Zhang and Kelly, 2006a; Nayagam et al., 2006), while others provided evidence for either a patchy, horizontally organized frequency representation (Friauf, 1992) or a concentric, helically structured topography (Merchán and Berbel, 1996).

Our aim in the present study was to investigate the topography of the main connections of the gerbil VNLL. We used the bidirectionally transported neuronal tracer, biotinylated dextran amine, to analyze both the distribution of projections from the cochlear nucleus to the VNLL and also the organization of neurons projecting from the VNLL to the central nucleus of the IC (CNIC). Our results indicate that both of these projections are topographically arranged and share a similar concentrically based organization in the VNLL.

MATERIALS AND METHODS

The brains of 37 female gerbils (*Meriones unguiculatus*) were used in the present study. Gerbils were obtained from Charles River Laboratories when they were approximately eight weeks old and were housed in Duke University animal facilities until use (average age at use was approximately 11 weeks). All animal procedures were in compliance with NIH guidelines and were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. The experimental material generated from each animal was used in multiple studies.

Experimental procedures

Retrograde tracing experiments—The 24 gerbils whose brains were evaluated in this part of the study were the subjects of a previous report (Cant and Benson, 2006b). Before all surgical procedures, the animals were anesthetized with Nembutal (50 - 70 mg/kg, i.p.). When withdrawal reflexes were no longer present, an incision was made in the skin over the occipital bone and a small hole was opened in the skull. Iontophoretic injections of a 10% solution of BDA (molecular weight 10,000, D-1956; Molecular Probes, Eugene, OR) in saline were made through glass micropipettes (tip diameter approximately 15 μ m) lowered into the IC. Following the injection, the skin was sutured and the animals were placed on a heating pad for the recovery period. After an appropriate survival time (5 - 11 days), the

gerbils were given an overdose of Nembutal (>100 mg/kg, i.p.). When breathing had just ceased, they were perfused through the left ventricle with a rinse of 0.1M phosphate buffer (pH 7.4), followed by buffered 4% paraformaldehyde. On the following day, the brain was removed from the skull and stored in a buffered solution of 30% sucrose overnight. Frozen sections, 40 μ m thick, were cut on a sliding microtome. Twenty of the brains were cut in the horizontal plane and four in the transverse plane. In all but two of the cases, one series of alternate sections was processed for BDA detection using standard avidin-biotin-peroxidase histochemistry (ABC Elite Kit, Vector Laboratories, Burlingame, CA) with metal enhancement (Adams, 1981), and the other set of sections was processed for cytochrome oxidase (CO) activity (Wong-Riley, 1979; Cant and Benson, 2005). In the remaining two cases (both cut in the transverse plane), all sections were reacted for BDA.

Anterograde tracing experiments—The gerbils used in this part of the study were also the subjects of another study (Cant and Benson, 2008). Before surgery, gerbils (n = 12) were anaesthetized with Nembutal as described above. In 11 of the gerbils, an incision was made in the scalp and a small hole was opened in the skull overlying the right cerebellum. Glass micropipettes (20 – 30 μ m inside tip diameter), containing a 10% solution of BDA in saline, were lowered through the cerebellum into the right cochlear nucleus. In one gerbil, a different surgical approach was used; a small hole was made in the left temporal bone and the pipette was advanced into the left anteroventral cochlear nucleus through the parafloccular lobe of the cerebellum (Frisina et al., 1982). Multiple injections of BDA (usually three) were made iontophoretically (5 μ A positive current pulses, 7 seconds on / 7 seconds off for 10-12 minutes) at slightly different depths in the cochlear nucleus. Following surgery, the skin was sutured and the gerbils placed on a heating pad for the recovery period. After a 7 – 10 day survival period, animals received an overdose of Nembutal (>100 mg/kg, i.p.), and were perfused through the left ventricle, as described above. The brains were removed the next day and kept in 30% sucrose in phosphate buffer at 4°C for 1 – 4 days. Frozen sections, 40 μ m thick, were cut in the horizontal plane. Alternate sections were processed for BDA detection or for CO activity, as outlined above.

Nissl stain—One non-operated gerbil was perfused as described above, the brain was embedded in Parlodion and horizontal sections, 18 μ m thick, were cut on a sliding microtome. Every 4th section was stained with cresyl violet.

Wisteria floribunda agglutinin histochemistry—The procedures used for lectin histochemistry have been described in detail in a previous report from our laboratory (Cant and Benson, 2006a). We used material generated in that study to examine the characteristics of *Wisteria floribunda* lectin labeling in the gerbil VNLL. Briefly, the procedure for lectin histochemistry was as follows: frozen sections, 40 μ m thick, were incubated in 0.6% hydrogen peroxide for 30 min, rinsed, and placed in a solution of phosphate buffered saline containing 2% bovine serum albumin and 0.5-1.0 μ g/ml of biotinylated *Wisteria floribunda* agglutinin (WFA; Sigma L-1766) for 15-20 h at 4 °C. Sections were then rinsed and processed further using a buffered Vector Elite ABC solution (Vector Laboratories), followed by 0.05% diaminobenzidine with heavy metal intensification.

Data collection and analysis

Sections were analyzed using a Zeiss AxioSkop 2 compound light microscope. Digital micrographs of the sections were obtained using a Zeiss AxioCam HRC camera controlled by Zeiss AxioVision software.

Localization and counts of labeled neurons in VNLL—In the 24 cases in which BDA was injected into the IC, digital images of every BDA-reacted section through the

ipsilateral VNLL were imported into Adobe Photoshop CS (Adobe Systems, San Jose, CA) and adjusted for brightness and contrast. Retrogradely labeled neurons were almost always very darkly stained and were thus readily distinguishable against the much paler tissue background (see Cant and Benson, 2006b, their Figure 3). BDA-labeled cell bodies were counted in each section in cases cut in the horizontal plane. Only neurons that were unequivocally labeled were included in the counts. Labeled neurons were plotted on the images while the sections were viewed through a 20x plan-neofluar objective (N.A. 0.50) and 10x eyepieces (final magnification = 200x).

In one case, all BDA-reacted horizontal sections through the entire VNLL were aligned and a three-dimensional (3-D) reconstruction of the distribution of labeled neurons was made using Neurolucida software (Microbrightfield, Inc.; Colchester, VT).

Anterograde labeling of terminal fields in VNLL—High-resolution digital images of BDA-labeled terminal fields in the VNLL were acquired in 10 of the cases in which BDA was injected into the cochlear nucleus. The remaining 2 cases were excluded from the analysis as there was insufficient label in the VNLL. For illustrative purposes, labeled fibers and terminals were traced from digital images in 3 representative cases using Adobe Photoshop CS software running on a Macintosh G5 computer.

RESULTS

Anatomical characteristics of the gerbil VNLL

The VNLL is a curved, elongated nucleus that extends from the rostral end of the superior olivary complex to the ventral pole of the DNLL (Fig. 1A-C). On average there were 54 horizontal sections through the nucleus, corresponding to a dorsal-to-ventral length of approximately 2.2 mm in perfused and processed tissue.

The neurons which form the VNLL are embedded among the fibers of the lateral lemniscus, which form its lateral, medial and rostral borders and are also interspersed among its constituent cells. The appearance of the VNLL along its dorsoventral axis is not uniform; the cellular packing density, orientation of cell bodies, and arrangement of lemniscal fibers all show marked variations along this axis (Figs. 1, 2). For the purpose of describing and analyzing the results of our tracing studies, we have subdivided the VNLL into three zones: dorsal, middle, and ventral (Fig. 1A-C). Our nomenclature is based on the cytoarchitectonic subdivisions originally proposed by Adams (1979) for the cat. As noted by Adams, the boundaries between these three regions are indistinct.

In sections processed for CO activity, the ventral zone of the VNLL is densely and fairly uniformly stained (Fig. 1A-C). In sections processed for WFA histochemistry, most of the neurons in the ventral zone appear to be round and are only lightly stained (Fig. 1D). These cells presumably correspond to the spherical or globular cells which have been described in this region of the VNLL in other species (e.g., Kudo et al., 1990; Vater and Feng, 1990; Adams, 1997; Schofield and Cant, 1997). A few darkly stained multipolar cells are interspersed among the dense accumulation of round cells (Fig. 1D, arrows). Dorsal to this highly cellular zone, the middle zone of the VNLL is characterized by the presence of prominent fascicles of lemniscal fibers (Figs. 1A-C,E; 2B,E), which run in an approximately ventral to dorsal direction. The middle zone lacks the profusion of lightly stained round cells characteristic of the ventral zone. Vertical strands of cells, darkly stained with WFA (Fig. 1E, arrows; Fig. 2E), are interspersed among the fibers, forming an irregular columnar arrangement. Most of the cells have round or oval cell bodies, and the dendrites that are visible are oriented in many different directions. Cells with vertically elongated cell bodies are common, especially in rostral regions. Not all cells in the middle region are densely

stained with WFA; in the horizontal plane, unstained or lightly stained areas are visible in many sections (Fig. 2E, white arrow).

The columnar arrangement characteristic of the middle zone of the VNLL continues into the dorsal zone (Figs. 1A-C,F; 2A,D). At this level, some of the fibers in the medial limb of the lateral lemniscus turn and course obliquely before entering the DNLL, becoming intertwined with those of the lateral limb (Fig. 1A, clear arrow). The fibers surround vertically oriented islands of cells (Fig. 1F, arrowheads), which are heavily stained with WFA and, although more densely packed, appear similar to the multipolar cells in the middle zone. Some of these cells are horizontally elongated and have dendrites oriented mediolaterally, orthogonal to the lemniscal fibers.

The boundary between the dorsal VNLL and the ventral DNLL is readily apparent in transverse sections (black arrow, Fig. 1A). The caudoventral border of the VNLL, where it merges with periolivary nuclei of the rostral superior olivary complex, is less distinct. In the horizontal plane, the caudoventral VNLL can be seen to adjoin a group of rostral periolivary cells, intercalated between the VNLL and the rostral border of the medial superior olive (Fig. 2C,F). In the gerbil, as in the guinea pig (Schofield and Cant, 1997), the VNLL can be distinguished from this adjacent periolivary region by the presence of more loosely packed cells and the horizontally elongated shape of some cell bodies in the latter.

Projections from the VNLL to the IC

In an earlier study, we analyzed the distribution of retrogradely labeled cells in brainstem auditory nuclei following focal injections of BDA into different regions of the gerbil IC (Cant and Benson, 2006b). In the VNLL, retrogradely labeled cells were found almost exclusively ipsilaterally and were present in all of the cases, including those in which the injection site lay outside the CNIC. This is consistent with the widespread distribution of projections from the VNLL to the IC reported in the cat (Whitley and Henkel, 1984). In several cases a few isolated labeled cells were also present in the contralateral VNLL, as has been previously reported for the gerbil (Nordeen et al., 1983). The contralateral cells were scattered and are not considered further here.

Dorsoventral distribution of labeled cells in the VNLL—In order to determine whether regions of the CNIC in which different frequencies are represented might be differentially connected to the VNLL along its dorsoventral axis, we analyzed the patterns of retrograde labeling in the ipsilateral VNLL in 20 of the horizontally sectioned cases used in our earlier study. The cases selected for analysis included 8 cases assigned to the low- (L), 7 to the middle- (M) and 5 to the high-frequency (H) range (Cant and Benson, 2006b, their Table 1). The locations of the injection sites in the CNIC for these cases are shown in Figure 3. Details of the methods we used to identify the center of the injection site and to assign it to a putative frequency range are given in our previous report (Cant and Benson, 2006b).

Retrogradely labeled cells were found along the entire dorsoventral length of the VNLL in all of the cases. A comparison of the proportion of labeled cells in the dorsal, middle and ventral zones of the VNLL in different cases is presented in Table 1. The proportion of labeled cells in the middle zone of the VNLL was quite consistent between cases (range 32.1 - 46.5%; Table 1). Proportions in the dorsal (11.3 - 47.4%) and the ventral (13.6 - 47.4%) zones, however, showed considerable variation. The relative distribution of cells in the three zones of the VNLL did not show any consistent relationship to the frequency range of the injection site. Cases with relatively low percentages of cells dorsally (e.g. 462, 568, 596, 545) were present in both the low- and high-frequency groups. Cases with relatively low percentages of cells ventrally (e.g. 475, 443, 460, 449, 454) were present in all three frequency groups.

Topographic organization of retrogradely labeled cells in the VNLL—The distribution of labeled cells was related to the location of the injection site along the frequency axis of the IC (Figs. 4 - 7). In the dorsal and middle zones of the VNLL, cells projecting to dorsal (low-frequency) regions of the CNIC (Fig. 4, B1-B6 and C1-C6, cases 427 and 419) were located primarily around the periphery of the VNLL, particularly near its lateral and caudal borders (e.g., Fig. 4, B5,B6 and C2,C4,C5, black arrows). A few labeled cells were present along the medial border (e.g. Fig. 4, B3,B4, arrowheads). In the ventral zone of the VNLL, labeled cells in some of the low-frequency cases were segregated into bands or patches (Fig. 4, B8 and C8; see also Fig. 6,E-H). When the injection was in the ventral (high-frequency) region of the CNIC, labeled cells were distributed differently in the VNLL (Fig. 4, D1-D8, case 450). Most of the labeled cells occupied the central region of the nucleus (Fig. 4, D3,D6, clear arrows), with only a few cells positioned along the lateral and caudal borders. Cells projecting to low- versus high-frequency regions of the CNIC were to a large degree segregated at all levels along the dorsoventral axis of the VNLL, occupying complementary positions across its mediolateral and rostrocaudal dimensions. The topographical organization was usually clearest in the middle region of the VNLL (Fig. 4, B3-B6, C3-C6, D3-D6). There tended to be more scatter and a less well-defined pattern of distribution in the dorsal VNLL in almost all of the cases.

In our previous study, the middle-frequency group of cases was further subdivided into low-to-middle (LM), middle (M), and middle-to-high (MH) ranges (Cant and Benson, 2006b). All three of these ranges were represented in the present analysis (LM, cases 420, 469; M, cases 412, 417, 460; MH, cases 443, 444; injection sites are shown in Fig. 3). The four cases shown in Figure 5 illustrate that as the putative frequency at the injection site increases from LM — M — MH — H, the position of labeled cells moves progressively away from the periphery of the VNLL towards the central region. There appears to be a concentric component to the organization that is most evident in the middle zone of the VNLL (Fig. 5, A4-D4, A5-D5, arrows).

A direct comparison of the location of cells projecting to different parts of the CNIC is presented in Figure 6. Segregation of cells projecting to low- versus high-frequency regions of the CNIC was most obvious in the middle and ventral zones of the VNLL. In the middle zone of the VNLL (Fig. 6A-D, I-L, Q-T), most of the labeled cells in the high-frequency case (Fig. 6, red dots) were located medial, or rostromedial, to the more peripherally located cells labeled in the low-frequency case (black dots). In the ventral VNLL, the labeled cluster of cells in high-frequency cases often extended towards the lateral border of the VNLL, forming a band that stretched across the mediolateral dimension of the nucleus (Fig. 6M-P). This band was positioned between the bands or patches of labeled cells in low-frequency cases. In the most ventral aspect of the VNLL, labeled cells in high-frequency cases were generally located caudal to those in low-frequency cases (Fig. 6H,P,X; see also Fig. 4, compare B8,C8 to D8).

In the transverse plane, spatial differences in the distribution of cells that project to low- versus high-frequency regions of the CNIC were less easy to visualize, especially in caudal sections (Fig. 7). Differences were more evident in rostral sections through the nucleus (Fig. 7, compare sections C-F with I-L). In the most rostral regions (sections E,F and K,L), cells projecting to the low-frequency CNIC were located mainly along the lateral margin of the VNLL, with a few also positioned near its medial border (Fig. 7K,L arrows). Labeled cells in the high-frequency case occupied a more central position (Fig. 7E,F, arrows), appearing to fill in the area left unlabeled in case 363 (Fig. 7K,L, arrowheads).

A 3-D reconstruction of the VNLL was made in one case with a BDA injection in the ventrolateral, rostral CNIC (case 420, for injection site see Fig. 3). In our earlier study, this

case was assigned to the LM-frequency range (Cant and Benson, 2006b). In individual sections from this case, labeled cells were located mainly around the periphery of the nucleus (Fig. 5, case 420), as in other cases with injection sites in this frequency range. When the distribution of labeled neurons through the entire nucleus is reconstructed, the complex spatial arrangement of the labeling becomes apparent; the labeled arcs and patches of cells that are seen in individual horizontal sections form curving sequences of cells which wind along the caudoventral-to-rostr dorsodorsal axis of the nucleus (Fig. 8). In some places, groups of labeled cells form roughly circular patterns. The spatial distribution of labeled cells in this reconstruction appears very similar to that observed in a 3-D reconstruction of retrogradely labeled fibrodendritic laminae in the rat VNLL (Merchán and Berbel, 1996, their Fig. 13).

Projections from the cochlear nucleus to the VNLL

Anterograde tracing studies with BDA—Axons projecting to the contralateral VNLL were labeled by the anterograde transport of BDA from the cochlear nucleus. Densely labeled terminal fields were present along the entire dorsoventral axis of the contralateral VNLL in all of the 10 cases analyzed. Most of the labeled axons were thin, and gave rise to *en passant* boutons that were generally quite closely spaced. Terminal boutons, sometimes arranged in clusters, were also present. In two cases in which the injection site included the PVCN, calyceal endings were seen in the ventral region of the VNLL only. Although some labeled fibers and boutons were also present ipsilaterally, the density was much lower than on the contralateral side.

The location of labeled axons and terminal fields in the VNLL varied systematically according to the location of the BDA injection site in the cochlear nucleus (injection sites are shown in Fig. 9). The three cases shown in Figure 10 illustrate labeling patterns that were typical of all of the cases. In case 566, the injection site incorporated ventrolateral (low-frequency) regions of the DCN (Fig. 9A). As projections from the DCN to the VNLL are sparse, or absent, in almost all species studied (reviewed in Cant and Benson, 2003), we assume that most of the labeled axons and terminals in the VNLL in this case arose from multipolar cells in the ventral PVCN that became labeled through their collateral projections to the DCN (Fig. 9A; Adams, 1983; Doucet and Ryugo, 1997). Terminal label in this case was located primarily along the lateral and caudal borders of the VNLL (Fig. 10A-C).

When the injection was centered in the middle (mid-frequency) region of the PVCN (Fig. 9B, case 544), the majority of the labeled field occupied a more central position in the VNLL (Fig. 10D-F). The terminal field appeared to closely adjoin, and in some places partially overlap, that in case 566 but extended further medially. This was particularly evident in the middle and ventral regions of the VNLL where the heaviest accumulation of labeled fibers and terminals in case 544 was positioned rostromedially to that in case 566 (Fig. 10, compare B-C with E-F). The terminal field was located still more medially following injections in dorsal (high-frequency) regions of the VCN (Figs. 9C, 10G-I, case 564). The distribution was particularly clear in the middle VNLL, where labeled fibers and terminals were clustered together in a single region in the rostromedial part of the nucleus (Fig. 10H), medial to the main accumulation of labeled fibers in case 544. In the ventral zone of the VNLL the labeled elements had a banded appearance (Fig. 10I). There were almost no labeled terminals along the peripheral margins of the VNLL after injections in high-frequency regions of the cochlear nucleus.

DISCUSSION

The main conclusion of this study is that the principal connections of the gerbil VNLL are organized topographically. Cells that project to the dorsal CNIC (low-frequency) are located

on or near the periphery of the VNLL, surrounding a more centrally located cluster of cells that project to the ventral CNIC (high-frequency). The position of labeled cells shifts progressively from the periphery to the core of the nucleus as the location of the injection site moves from low- to mid- to high-frequency CNIC regions. Projections from the VCN to the VNLL demonstrate a similar topographic arrangement with projections from ventral (low-frequency) regions terminating mainly along the lateral border of the VNLL, and those from dorsal (high-frequency) regions terminating in the central part of the nucleus. The anatomical demonstration that both the terminations from the cochlear nucleus and also the neurons that project to the inferior colliculus are topographically organized predicts, although it does not directly demonstrate, that the nucleus is tonotopically organized.

Cytoarchitecture and organization of the gerbil VNLL: comparison to other species

The anatomical and cytoarchitectonic organization of the VNLL has been studied in several species, including the cat (Glendenning et al., 1981; Malmierca et al., 1998), rat (Merchán and Berbel, 1996), guinea pig (Schofield and Cant, 1997), mole (Kudo et al., 1990), opossum (Willard and Martin, 1983), and several species of echolocating bats (big brown bat: Covey and Casseday, 1986; Huffman and Covey, 1995; Vater et al. 1997; horseshoe bat: Schweizer, 1981; Vater and Feng, 1990; mustache bat: Zook and Casseday, 1982a, 1982b; Ross et al. 1988). In the VNLL of echolocating bats, different cell types show a high degree of regional segregation; this has led to a division of this area into several subnuclei, or cell groups, that can be distinguished not only on cytoarchitectural grounds, but also by differences in response patterns, tonotopic organization, transmitter neurochemistry, and the relative proportions of ascending input from different sources (Covey and Casseday, 1986, 1991; Metzner and Radtke-Schuller, 1987; Frisina et al., 1989; Vater and Feng, 1990; Huffman and Covey, 1995; Winer et al., 1995; Vater et al., 1997). A clearly differentiated intermediate nucleus of the lateral lemniscus (INLL) is present dorsally in the bat (e.g. Schweizer, 1981; Zook and Casseday, 1982a), mouse (Willard and Ryugo, 1983), and mole (Kudo et al., 1990). In contrast, in the rat and opossum the VNLL appears more uniform along its length (Willard and Martin, 1983; Merchán and Berbel, 1996; Merchán et al., 1997). In the gerbil, as in the cat, different cell types are partially segregated in different regions of the VNLL, but precise borders between areas are hard to define (Adams, 1979). In our material this was especially true for the transition between the dorsal zone (presumably equivalent to the INLL of other species) and the middle zone, which both contain high proportions of multipolar cells and have a similar, although not identical, columnar appearance to their structure. We therefore followed the strategy adopted by several previous investigators and, for the purpose of describing the topography of its connections, treated the VNLL as a single nucleus with dorsal, middle and ventral zones (Adams, 1979, 1997; Whitley and Henkel, 1984; Merchán and Berbel, 1996; Malmierca et al., 1998).

In our WFA-labeled material, at least two different cell types could be distinguished. The ventral VNLL is characterized by a high density of lightly labeled round cells that appear similar to the globular or spherical cells described in the cat (Adams, 1979; Glendenning et al., 1981), mouse (Willard and Ryugo, 1983), guinea pig (Schofield and Cant, 1997), mole (Kudo et al., 1990), rat (Zhao and Wu, 2001), echolocating bats (Schweizer, 1981; Zook and Casseday, 1982a; Covey and Casseday, 1986; Vater and Feng, 1990) and human (Adams, 1997). In the gerbil, a few heavily labeled multipolar cells are interspersed among the globular cells of the ventral zone, as has also been observed in some other species (Adams, 1979; Schofield and Cant, 1997). In contrast, the middle and dorsal regions of the VNLL consist predominately of densely labeled multipolar cells, including some which are horizontally elongated and some which are vertically elongated. More than one subtype of multipolar cell may be present in the middle region of the gerbil VNLL, as not all of the

cells are labeled by WFA. Subtypes of multipolar cells have been distinguished in the rat VNLL on the basis of differences in morphology and firing patterns (Zhao and Wu, 2001). In the gerbil, the position of the unlabeled area, in the central core of the VNLL, appears to correspond quite well to the location of cells that project to ventromedial (high-frequency) regions of the CNIC.

Our observations are in agreement with those of Budinger and colleagues (2000) who described the cytoarchitecture of the gerbil VNLL using Nissl, myelin and parvalbumin staining. They also identified a combination of globular, multipolar, and vertically elongated cells in ventral regions of the VNLL and horizontally elongated multipolar cells in more dorsal regions.

Several different cell types (multipolar, octopus and bushy cells) in the contralateral VCN project to the VNLL (Friauf and Ostwald, 1988; Smith et al., 1993, 2005; Huffman and Covey, 1995; Adams, 1997; Schofield and Cant, 1997; Vater et al., 1997). Although the distribution of terminations from these cell types may differ, our data do not allow us to differentiate among their respective contributions to the VNLL. Our injection sites in the VCN were relatively large and thus encompassed areas containing more than one cell type, and the anterograde tracing method we used does not distinguish between multipolar and bushy cell axons. When the injection site included the caudal PVCN, we did observe that calyceal endings, presumably arising from thick octopus cell axons (Adams, 1997), appeared to be confined to the ventral VNLL, consistent with previous reports in the cat, guinea pig and horseshoe bat (Vater and Feng, 1990; Adams, 1997; Schofield and Cant, 1997).

Organization of cells projecting to the CNIC

The clearest evidence for a tonotopic organization in the VNLL or INLL comes from studies in the bat. Orderly tonotopic arrangements have been found in the INLL of the horseshoe bat (Metzner and Radtke-Schuller, 1987), in the ventral VNLL of the mustache bat (Ross et al., 1988), and in the columnar area of the VNLL of the big brown bat (Covey and Casseday, 1991). In contrast, in both the cat and the rat, consistent evidence for a topographic organization related to tonotopy has so far not been obtained. While some tracing studies in the cat provided evidence for a complex topographic organization of cells projecting to the IC (Roth et al., 1978; Whitley and Henkel, 1984; Merchán et al., 1997; Malmierca et al., 1998), others indicated an absence of any orderly arrangement (Adams, 1979; Glendenning and Hutson, 1998). Recordings of best frequencies from single neurons in the cat VNLL likewise produced conflicting evidence of a tonotopic sequence (Aitkin et al., 1970; Guinan et al., 1972), and recent electrophysiological studies in the rat VNLL failed to reveal a tonotopic progression (Nayagam et al., 2006; Zhang and Kelly, 2006a).

Our results demonstrating a topographic organization of VNLL neurons that project to the CNIC in the gerbil are remarkably similar to those reported in retrograde tracing studies in the rat (Merchán and Berbel, 1996). In those experiments, restricted injections of BDA or biocytin in the rat CNIC gave rise to a concentric pattern of labeling in the middle region of the VNLL, with a lateral to medial frequency progression. On the basis of 3-D reconstructions, the authors proposed a helically based topography, consisting of elongated fibrodendritic laminae that twist along the dorsoventral axis of the VNLL. Consistent with our findings in the gerbil, the peripheral laminae in the rat project to dorsolateral CNIC laminae and central ones to ventromedial laminae (Merchán and Berbel, 1996). A helical organization of the VNLL was first proposed by Willard and Martin (1983), based on observations of the spiraling arrangement of interlacing dendritic arbors in the core of the opossum VNLL. Concentrically based patterns of organization in the VNLL or INLL have also been described in other studies. In the mouse INLL, the dendrites of peripheral neurons

are arranged in a “series of concentric sheaths” (Willard and Ryugo, 1983), and in the big brown bat both the INLL and the multipolar region of the VNLL display a roughly concentric tonotopicity, with middle frequencies surrounding a more centrally located high-frequency region (Covey and Casseday, 1991). The DNLL of the rat also demonstrates a concentric topographic organization (Merchán et al., 1994; Kelly et al., 1998; Saint Marie et al., 1999a); high frequencies are found in a peripheral ring and lower frequencies occupy progressively more central positions. As is the case for the VNLL, the topography of the gerbil DNLL strongly resembles that described in the rat (unpublished observations) but differs from that reported in the cat (Bajo et al., 1999).

Our results suggest that, at least in the gerbil, the entire frequency range of the CNIC is represented at every horizontal level through the VNLL. The close proximity in the VNLL of cells projecting to different frequency regions in the CNIC might serve to maximize neuronal interactions across different frequencies and facilitate the processing of complex sounds (Malmierca et al., 1998; Oertel and Wickesberg, 2002). This form of organization may provide an explanation for the widespread, diffuse distribution of terminations from the VNLL that has been observed in the CNIC in anterograde tracing studies (Kudo, 1981; Whitley and Henkel, 1984; Kudo and Nakamura, 1988). Unless the injection site were extremely small, one would expect the tracer to label a group of cells in the VNLL that collectively project to the entire frequency range of the CNIC; the spread of label across the CNIC would consequently be large. In addition, the VNLL projects to areas of the IC outside the CNIC (Whitley and Henkel, 1984; Cant and Benson, 2006b). It is also understandable why electrophysiological studies in species other than bats have so far failed to uncover a convincing tonotopic organization (Aitkin et al., 1970; Guinan et al., 1972; Adams, 1997; Nayagam et al., 2006; Zhang and Kelly, 2006a), although many neurons in the VNLL do have a clearly defined characteristic frequency (Adams, 1997; Portfors and Wenstrup, 2001; Nayagam et al., 2006; Zhang and Kelly, 2006a). Our results indicate that electrode penetrations along a horizontal, lateral-to-medial trajectory would be more likely to show a consistent gradient of best frequencies than along the more usual dorsal-to-ventral trajectory. It is also possible, however, that a tonotopic map might not match the topographic sequence seen in our studies. In the big brown bat, neurons in the columnar area show a highly precise topography related to the tonotopic organization of both the AVCN and the inferior colliculus but have extremely broad tuning curves (Covey and Casseday 1986, 1991; Vater et al., 1997). This might reflect the convergence and integration of excitatory and inhibitory inputs from a variety of sources (Vater et al., 1997; Wu, 1999; Irfan et al., 2005). The majority of VNLL neurons are themselves inhibitory (Winer et al., 1995; Saint Marie et al., 1997; Zhang et al., 1998; Riquelme et al., 2001) and, as noted by Oertel and Wickesberg (2002), even those that are sharply tuned may not share the characteristic frequency of the CNIC neurons to which they project.

Dorsoventral differences

Although we observed a segregation of cells projecting to different CNIC regions throughout the VNLL, the distribution pattern was often less obvious in the dorsal zone. A difference between the distribution of labeled cells in the dorsal zone compared to the middle zone has also been observed in tracing studies in the cat (Malmierca et al., 1998), and rat (Merchán and Berbel, 1996). Variations in the pattern of labeling may be related to the different transmitter content of the dorsal zone; in both the rat and cat, neurons immunoreactive for glycine or GABA predominate in ventral regions of the VNLL, whereas immunonegative (presumably excitatory) neurons predominate dorsally (Saint Marie et al., 1997; Riquelme et al., 2001). In addition, Merchán and Berbel (1996) observed that the helical coils of fibrodendritic laminae in the rat VNLL appear to be wider and more obliquely oriented in the dorsal zone, compared to the tighter coiling pattern of the middle

zone. This might contribute to differences in labeling patterns between the two regions. In the bat, some regions of the lemniscal nuclei similarly manifest a more clearly defined organization than others; in the big brown bat, the tonotopic arrangement of the INLL lacks the regularity of that in the columnar area (Covey and Casseday, 1991), and in the horseshoe bat a unidirectional tonotopy is found only in the INLL (Metzner and Radtke-Schuller, 1987).

Organization of projections from the cochlear nucleus

The distribution of the terminal fields of axons projecting from different regions of the VCN to the VNLL was very similar to that of cells projecting from the VNLL to the CNIC. Topographically organized projections from the cochlear nucleus to the VNLL have not previously been reported in non-echolocating mammals, but have been described in bats. In the big brown bat, topographically organized projections from defined frequency regions of the AVCN terminate in a single sheet, extending through the entire rostrocaudal extent of the columnar area (Covey and Casseday, 1986). Projections from the AVCN to the INLL showed a distinct tonotopic gradient in horseshoe bats, with low frequencies represented laterally and higher frequencies progressively more medially (Vater and Feng, 1990), mirroring the tonotopic organization of INLL neurons (Metzner and Radtke-Schuller, 1987). In the cat, however, the tonotopicity of projections to the VNLL from the cochlear nucleus has been questioned (Glendenning and Hutson, 1998). This conclusion was based partly on observations of the distribution of degenerating fibers in the VNLL after ablations of large areas of the cochlear nucleus (Glendenning et al., 1981). As the cat VNLL appears to have a complex organization (Malmierca et al., 1998), it would seem improbable that large lesions would yield reliable indications of topographic connections. Indeed, Warr (1982) provided preliminary evidence of a mediolateral frequency axis in the cat VNLL, based on degeneration arising from more localized lesions of specific regions of the VCN.

In both our anterograde and retrograde experiments, the concentrically based organization characteristic of the middle VNLL appeared to transition into a more banded or clustered arrangement in the ventral zone of the VNLL. Repetitive horizontal bands or 'cross bridges' of labeled neuronal structures in the VNLL have been noted in other species after injections of tracers in either the IC or cochlear nucleus (Roth et al., 1978; Covey and Casseday, 1986; Friauf and Ostwald, 1988; Kandler and Friauf, 1993; Merchán and Berbel, 1996; Kelly et al., 1998), and also with *c-fos* labeling in response to pure tones (Friauf, 1992). Banding of terminations from the superior olivary complex may be even more pronounced than those from the cochlear nucleus (Glendenning et al., 1981; Spangler et al., 1985). The banded configuration observed in these studies might be related to the horizontal bands of alternating areas of high and low cell packing density that have been described in the ventral VNLL of the guinea pig (Schofield and Cant, 1997). In the rat, a 'staircase' pattern of retrograde label from the IC was associated with low- or mid-frequency labeling, but was absent after injections in high-frequency regions (Kelly et al., 1998). This might be explained by the clustering of labeled neurons in the central core of the VNLL after high-frequency injections, observed in both the gerbil and the rat (Merchán and Berbel, 1996), which would tend to result in a more uniform distribution of labeled cells in transverse sections. Our reconstruction of the distribution of BDA-labeled neurons (Figure 8), and the similar reconstructions presented by Merchán and colleagues (Merchán and Berbel, 1996; Merchán et al., 1997), suggest that the bands and patches of label seen in sectioned material form part of a complex 3-D structure, encompassing the entire length of the VNLL.

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LIST OF ABBREVIATIONS

Aq	cerebral aqueduct
AVCN	anteroventral cochlear nucleus
BIC	brachium of the inferior colliculus
BDA	biotinylated dextran amine
CNIC	central nucleus of the inferior colliculus
cnr	cochlear nerve root
CO	cytochrome oxidase
DCN	dorsal cochlear nucleus
DNLL	dorsal nucleus of the lateral lemniscus
GR	granule cell layer
H	high-frequency
IC	inferior colliculus
INLL	intermediate nucleus of the lateral lemniscus
L	low-frequency
LL	lateral lemniscus
LM	low-to-middle frequency
M	middle-frequency
mcp	middle cerebellar peduncle
MH	middle-to-high frequency
MSO	medial superior olive
PAG	periaqueductal gray
PO	periolivary area
PVCN	posteroventral cochlear nucleus
scp	superior cerebellar peduncle
V	fifth nerve root
VCN	ventral cochlear nucleus
VNLL	ventral nucleus of the lateral lemniscus
WFA	<i>Wisteria floribunda</i> agglutinin

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Figure 1.

A-C: Digital images of transverse sections through the VNLL reacted for CO activity. Cellular areas with high CO activity stain darkly while fibers remain pale. Caudal to rostral progression is from A to C, and the sections are 240 μ m apart. Horizontal lines demarcate the approximate boundaries of the dorsal (d), middle (m), and ventral (v) zones of the VNLL, as defined in the text. Black arrow in A points to the ventral border of the DNLL; clear arrow points to fibers coursing obliquely through dVNLL. Scale bar in C (applies to A-C) = 0.5 mm. D-F: Digital images of one section through the caudal VNLL processed for WFA histochemistry. The section is from the same case as those in panels A-C and is the section adjacent to the one shown in panel A. The dorsoventral levels in the 3 panels

correspond to the ventral (D), middle (E) and dorsal (F) zones of the VNLL. Arrows in D point to heavily labeled multipolar cells. The density of labeled cells is higher in the middle zone (arrows in E). In the dorsal zone, almost all cells are heavily labeled with WFA and appear as islands (arrowheads in F) surrounded by lemniscal fibers. Scale bar in F (applies to D-F) = 0.1 mm. In this figure, as well as in Figure 2, high-resolution color images of the sections were converted to grayscale images in Adobe Photoshop CS. Minor adjustments to the contrast and brightness of the images (also performed in Photoshop) were applied uniformly and equally across each series of sections. In panels A-C, the fifth nerve was torn during processing and has been erased from the images. Tissue debris outside the boundaries of the sections was also erased (applies also to Figs. 2 and 4-6). For abbreviations, see list.

Figure 2.

Differences in cellular organization in the dorsal, middle and ventral zones of the VNLL. A-C: Digital images of Nissl-stained horizontal sections (18 μm thick) through the 3 zones of the VNLL. D-F: Sections (40 μm thick) at approximately equivalent horizontal levels to those in A-C, processed for WFA histochemistry. Arrow in C points to the border between periolivary regions (PO) and the adjacent ventral VNLL. White arrow in E indicates a relatively unlabeled area in the middle zone of the VNLL. Sections in A and B are 648 μm apart, sections in B and C are 792 μm apart. Minor changes to the contrast and brightness of the images were applied equally to each series of sections in Adobe Photoshop. Scale bar = 0.2 mm.

Figure 3.

Location of injection sites in the CNIC (modified from Fig. 2 in Cant and Benson, 2006b). The outlines of the horizontal series of sections shown are from an atlas of the gerbil IC (Cant and Benson, 2005). The most dorsal section (H2120, approximately 2.12 mm from the ventral boundary of the IC) is at the upper left and the most ventral section (H120, approximately 120 μ m from the ventral boundary) is at the lower right. The center of the injection site for each case is shown as a circle plotted on the appropriate section (case numbers are inside each circle). Cases 454 and 455, in which multiple injections were made, each had two distinct injection sites in the CNIC (labeled 1 and 2). Cases plotted on each section are those with injection sites assigned to that section and also those with sites assigned to the section 80 μ m dorsal to it. On section H120, the 2nd injection site for case 455 was on the section 80 μ m more ventral (H40). The CNIC, as defined by Cant and Benson (2006b), is indicated by grey shading. The rostral border of the IC is indicated by a thin line (e.g. arrows on H1240, H600). M, medial; R, rostral.

Figure 4.

Distribution of BDA-labeled cells in horizontal sections through the VNLL in three cases with injections in the CNIC. Cases 427 (B1-B8) and 419 (C1-C8) had injections in the low-frequency region of the CNIC (L) and case 450 (D1-D8) in the high-frequency region (H; see Fig. 3 for locations of injection sites). In each column, the sections are arranged in a dorsal to ventral progression from top to bottom. Sections are 240 μ m apart and are at comparable horizontal levels in each row, so that direct comparisons between different cases can be made. For orientation, the adjacent CO-reacted sections are shown for case 427 (A1-A8). The sections in the first two rows (A1-D2) are in the dorsal zone of the VNLL, sections in the third row (A3-D3) are at the border between the dorsal and middle zones, the fourth and fifth rows (A4-D5) are in the middle zone, the sixth row (A6-D6) is at the border between the middle and ventral zones, and the bottom two rows (A7-D8) are in the ventral zone. Each BDA-labeled cell is indicated by a single black dot. Arrows and arrowheads point to labeled cells and are referenced where appropriate in the text. Rostral is toward the left of the figure, lateral toward the top. To enable the symbols marking the positions of labeled cells to be seen more clearly, the opacity of the BDA-labeled images was reduced uniformly across all sections shown for each case, using the opacity command in Adobe Photoshop (applies also to Figure 5). The edge of the sections was accentuated with a thin line. Scale bar = 0.2 mm.

Figure 5.

Distribution of BDA-labeled cells in the VNLL after injections in the CNIC. The arrangement of this figure is the same as for Figure 4. LM, low-to-middle frequency; M, middle frequency; MH, middle-to-high frequency. Scale bar = 0.2 mm.

Figure 6.

Comparison of the location of BDA-labeled cell bodies in middle and ventral regions of the VNLL after injections in low- (L) versus high-frequency (H) regions of the CNIC. Labeled cells from two cases are shown plotted on the series of CO-reacted sections from case 462. A-D, I-L, Q-T: Sections from the middle zone of the VNLL. E-H, M-P, U-X: Sections from the ventral zone. The most dorsal section is at the top of each column and the sections are 160 μ m apart. Left column (A-H): BDA injection in the low-frequency CNIC (case 462). In each panel, labeled cells (black dots) from two BDA-reacted sections 80 μ m apart are shown re-plotted on the adjacent CO section from the same case. Center column (I-P): Injection in the high-frequency CNIC (case 545). Labeled cells (red dots) from two BDA-reacted

sections are shown re-plotted on the same CO sections as in the left panels. BDA-reacted sections from case 545 were first aligned with their own adjacent CO sections and the positions of labeled cells re-plotted. This CO section was then aligned with the comparable CO section from case 462 and the positions of labeled cells were transferred onto it. The opacity of the images of the CO-reacted sections was reduced uniformly for all sections shown, as described for Figure 4. Right column (Q-X): Labeled cells from both cases have been overlaid on the same section. C, caudal; L, lateral; M, medial; R, rostral. Scale bar = 0.2 mm.

Figure 7.

Distribution of labeled cells in the VNLL in transverse sections from two cases with injections in different regions of the CNIC. A-F: A series of sections from a case with an injection in the high-frequency region of the CNIC (551 H). G-L: A series of sections at comparable levels through the VNLL from a case with an injection in the low-to-middle frequency region (363 LM). In both panels the most caudal section is on the left (A, G) and the most rostral is on the right (F, L); sections are 160 μm apart. Labeled cells from two BDA sections 80 μm apart have been collapsed onto a single section (each black dot = one cell). Labeled cells are located mainly along the borders of the nucleus in case 363 but occupy a more central position in case 551 (arrows); very few labeled cells are located in the central region of the nucleus in case 363 (arrowheads). D, dorsal; L, lateral. Scale bar = 0.5 mm.

Figure 8.

3-D computer reconstruction of labeled cells in the VNLL. The stereo pair shown is from a case (420) with an injection in the LM-frequency region of the CNIC (also illustrated in Fig. 5). All labeled cells in 27 alternate horizontal sections through the VNLL were included in the reconstruction. The left image has been rotated 7° from the right image on the vertical axis. The reconstruction is viewed from a position caudal and medial to the nucleus. Each black dot represents one labeled cell body. D, dorsal; L, lateral. Arrow points in the rostral direction.

Figure 9.

Injection sites in the cochlear nucleus for the 3 cases illustrated in Figure 10. One BDA-reacted horizontal section through the approximate center of the injection site is shown for each case, and an additional more ventral section through the VCN is shown for case 566.

A: Injection site for case 566 in the ventrolateral DCN (low-frequency region, L). In this case, heavily labeled cells were present in the ventral PVCN (small black dots in lower section). B: Injection site for case 544 in the middle PVCN (mid-frequency region, M), C: Injection site in the dorsal AVCN for case 564 (high-frequency region, H). Black shading in the outline drawings of each section depicts the area of the densest BDA accumulation.

Figure 10.

A-I: Distribution of BDA-labeled axons and terminals in dorsal, middle, and ventral regions of the VNLL in 3 cases with BDA injections in different regions of the cochlear nucleus (injection sites are shown in Fig. 9). Labeled fibers and terminals were traced from high-resolution digital images of the sections using Adobe Photoshop CS software. Case numbers are on the right of each row. J-L: CO-reacted sections adjacent to the BDA-reacted sections shown for case 544 and at comparable horizontal levels to the sections shown for the other two cases. Scale bar = 0.2 mm.

Table 1

Percentage of retrogradely labeled neurons in different regions of the VNLL after BDA injections in the CNIC.

Case # ^I	Dorsal VNLL	Middle VNLL	Ventral VNLL
Low-frequency			
419	20.5	43.5	36.0
427	25.8	42.0	32.2
462	19.4	38.8	41.8
475	43.2	34.3	22.5
510	16.9	46.5	36.6
528	22.8	43.7	33.5
568	11.3	41.3	47.4
596	14.9	44.7	40.4
Range	11.3-43.2	34.3-46.5	22.5-47.4
Mid-frequency			
412	26.7	43.2	30.1
417	44.9	32.5	22.6
420	32.9	40.5	26.6
443	47.4	39.0	13.6
444	30.6	39.4	30.0
460	43.1	36.6	20.3
469	25.8	41.5	32.7
Range	25.8-47.4	32.5-43.2	13.6-32.7
High-frequency			
449	45.2	36.3	18.5
450	25.4	40.5	34.1
454	47.2	32.5	20.3
455	43.0	32.1	24.9
545	19.5	38.0	42.5
Range	19.5-47.2	32.1-40.5	18.5-42.5

^I Cases were classified as low-, mid- or high-frequency according to the location of retrogradely labeled cells in the contralateral cochlear nucleus (see Cant and Benson, 2006b for details).