THE JOURNAL OF COMPARATIVE NEUROLOGY 287:470–483 (1989)

GABAergic Neurons in Brainstem Auditory Nuclei of the Chick: Distribution, Morphology, and Connectivity

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ABSTRACT

The second- and third-order auditory nuclei in the brainstem of the chicken, nucleus magnocellularis (NM) and nucleus laminaris (NL), receive afferents that are immunoreactive to gamma-aminobutyric acid (GABA). In order to investigate the source(s) of these GABAergic afferents, we examined the distribution, morphology, and connectivity of GABAergic neurons in and adjacent to NM and NL in chicks from 7 days of incubation to 12 days posthatch. Immunocytochemical techniques revealed the presence of approximately 150 GABA-labeled neurons within the neuropil surrounding NM and NL on each side of the brainstem. Most of these neurons are located between NM and NL and along the lateral border of NM. GABAergic neurons are multipolar; their thick dendritic processes branch extensively and give rise to several thin, secondary processes. Frequently, the GABA-labeled processes arborize within NM or NL. The morphology of these non-NM/NL neurons was investigated further with Golgi impregnation and specific neuronal markers (antisera to microtubule-associated protein). Our observations suggest that a considerable portion of GABAergic input to NM and NL originates from local GABAergic neurons.

In order to determine other possible sources of GABAergic input to NM and NL, we injected tracers unilaterally into NM/NL. A small number (20–30) of neurons were retrogradely labeled in the trapezoid body, almost exclusively ipsilaterally. No labeled cells were found in other regions of the brainstem, except for the contralateral NM. Unilateral injections of horseradish-peroxidase-labeled wheat germ agglutinin into the paraflocculus revealed only minor terminal labeling in the lateral region of NL bilaterally. The number and distribution of GABAergic terminals in NM and NL appeared normal after transection of the crossed dorsal cochlear tract.

Key words: gamma-aminobutyric acid, nucleus magnocellularis, nucleus laminaris, inhibition, immunocytochemistry, Golgi impregnation

The afferent input and projection patterns of the nucleus magnocellularis (NM) and nucleus laminaris (NL), second- and third-order auditory brainstem nuclei of birds, have been studied extensively (for references, see Rubel, '78; Rubel and Parks, '88). Besides their excitatory afferent input, neurons of NM and NL receive GABAergic terminals (Müller, '87; Code et al., '87, '89; Carr et al., '89) and a sparse glycinergic input (Code and Rubel, '89). To date, the sources of these inhibitory inputs have not been identified. GABAergic neurons occur adjacent to and within NM and NL (Müller, '87; Code et al., '89), but the number and distribution of these cells have not been investigated. It is also unclear whether these local GABAergic neurons actually project into the ipsilateral and/or contralateral NM and NL, and whether other, more distant sources also provide GABAergic afferents to NM and NL.

In mammals, the anteroventral cochlear nucleus (homologous to the avian NM) probably receives input from GABAergic neurons in the superior olivary complex, and this input may be bilateral (Adams and Mugnaigni, '87; Adams and Wenthold, '87; Roberts and Ribak, '87; Spangler et al., '87). Also the medial superior olivary nucleus (possible homologue of NL) presumably receives at least some of its GABAergic afferents from within the superior olivary complex (Moore and Moore, '87; Roberts and Ribak, '87).

Accepted April 27, 1989.

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In the present study, we examine the distribution, morphology, and connectivity of GABAergic cells adjacent to NM and NL in chick embryos and hatchlings. We demonstrate that local GABAergic neurons have ramifying dendritic processes which give rise to thin, secondary fibers within the ipsilateral NM and NL. The possibility of other sources of GABAergic afferents to NM and NL was investigated by injection of tracers into NM/NL and into the parafloccculus and by transection of the crossed dorsal cochlear tract with subsequent processing for GABA immunoreactivity. None of these experiments revealed substantial projections to NM/NL. We conclude that local GABAergic neurons provide most of the GABAergic afferents to NM and NL. A preliminary account of this study has been presented in abstract form (von Bartheld et al., '89).

MATERIALS AND METHODS

Approximately 250 embryonic and posthatch chicks were used in the present study. The age of the animals ranged from 7 days of incubation (E7) to 14 days posthatch (P14). Embryos younger than 14 days of incubation were staged according to the Hamburger and Hamilton ('51) series. GABA-immunostained tissue from older animals (P14 to 1 year old) was available for comparison (Code et al., '87, '89). In addition, Golgi-Kopsch-impregnated sections (Stensaas, '67) through the brainstem of approximately 50 additional chicks and chick embryos from prior studies (Smith and Rubel, '79; Smith, '81; Deitch and Rubel, '84), as well as sections processed with an antibody against microtubule-associated proteins (MAP-2, Matus, '88), were studied (M. Seftel and E.W Rubel, unpublished material).

GABA and MAP-2 immunoreactivity

We investigated the brainstems of 95 chicks for GABA immunoreactivity; two different protocols were used for fixation and sectioning. Under sodium pentobarbital anesthesia (50 mg/kg body weight), one group (n = 51) was perfused with 0.6% glutaraldehyde and 1.5% paraformaldehyde; their brains were dissected and placed in 30% sucrose overnight. Frozen sections were cut at 30 or 40 μm; 48 brains were sectioned in the transverse plane and three in the sagittal plane. Alternate sections were collected on gelatin-coated slides or as free-floating sections. After a 1–2-hour wash in 0.1 M phosphate buffer (PB, pH 7.4) and preincubation in 3% normal goat serum, the sections were processed overnight with GABA antiserum (INCSTAR) diluted 1:2,000 or 1:4,000 in PB. Following incubation in a secondary antibody, biotinylated goat-anti-rabbit IgG (diluted 1:200), the sections were incubated in the avidin-biotin-peroxidase complex (Hsu et al., '81) by using a Vectastain kit (Vector Laboratories). Finally, the sections were processed with 0.02% diaminobenzidine (DAB) for 5–10 minutes. Another group of animals (n = 44) was perfused with 1% paraformaldehyde and 1.25% glutaraldehyde. The brains were sectioned on a vibratome at 30 μm, and every third section was processed free floating for GABA immunoreactivity, as described above.

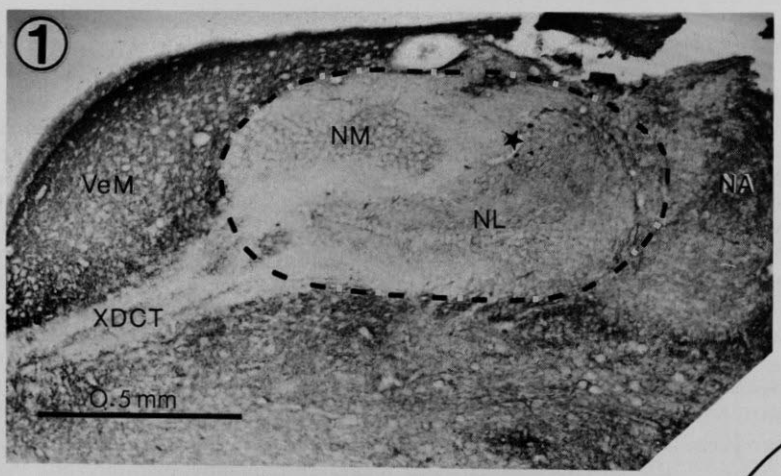
Control sections were incubated without the primary antibody. Other control experiments, which determined the specificity of the GABA antiserum, have been described previously (Code et al., '89). In general, the best immunostaining was obtained with free-floating sections cut on a cryostat. Stained neurons were studied under oil immersion objectives (N.A. 1.3–1.4). A total of 25 neurons and their immunostained processes were documented with the help of a computer-aided neuron reconstruction system (Eutectic Electronics, Inc.); neurons were plotted at a final magnification of ×450. Cell counts were corrected for double counting according to Konigsmark ('70). Since penetration of the antibody (and thus label) in sections processed on the slide appeared to be in the range of 5–10 μm, we corrected for double counting only in sections processed free floating. The criteria for a stained neuron included the intensity of label (clearly above background), the morphology (distinct from peroxidase-stained blood cells; in doubtful cases only cells with a stained process were counted), and the diameter (more than 6 μm). We counted and analyzed only cells stained in the neuropil surrounding the NM and NL nuclei. This area has clear boundaries dorsally, medially, and ventrally because its margins lack GABA-immunostained terminals and fibers (Fig. 1). The NM/NL complex is located medial to nucleus angularis (NA). The NA and its surrounding neuropil were excluded from the area considered for cell counts (Fig. 1). Usually, cells counted were not more than 50 μm from the nearest cell body in NM or NL.

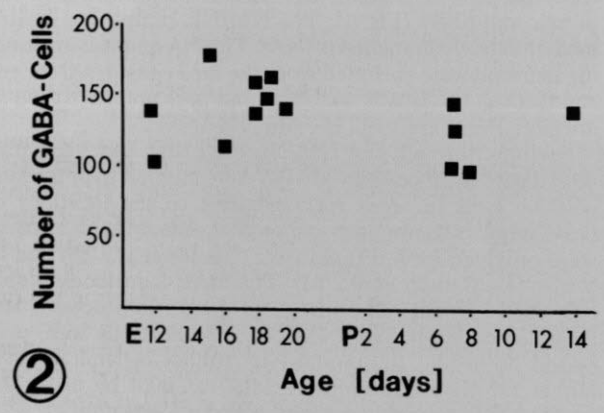
Sections through the NM/NL area that had been processed with antibodies to MAP-2 were studied to determine whether cells in areas corresponding to the locations of GABAergic cells are neuronal or glial cells. MAP-2 antisera were provided by A. Frankfurter (Binder et al., '86) and R. Vallee (De Camilli et al., '84). The MAP-2 antibody is specific for neuronal cell bodies and dendritic processes and does not stain glial cells (Matus, '88). Sections were processed according to standard immunocytochemical techniques (antibody dilutions of 1:250–1:1,000) by using the avidin-biotin-peroxidase complex and a Vectastain kit with DAB as the chromogen.

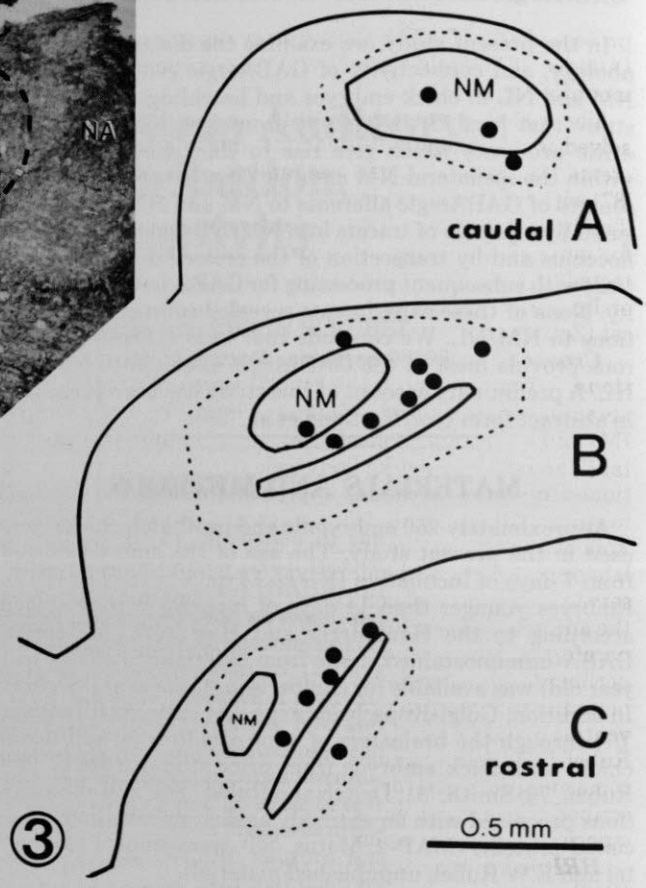
Connectivity

In vitro tracer studies. The NM/NL of 78 chicks and chick embryos was injected with one of the following tracers: fluorogold (FG, n = 37); horseradish peroxidase (HRP, n = 9); dextran-conjugated tetramethylrhodamine (Rh, n = 16); and the carbocyanine dye DiI (n = 18).

The tracers FG (Schmued and Fallon, '86), HRP (Sigma), and Rh (Molecular Probes) were injected into NM/NL in an *in vitro* preparation (Jackson et al., '82). For experiments with HRP injections, the animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body weight) and then perfused intracardially with Tyrode's solution prior to removing the brain. In the FG and Rh tracer experiments, posthatch chicks or chick embryos were anesthetized and then decapitated without perfusion. A chunk of the brainstem (3–6 mm in thickness) was dissected and placed in a chamber containing Tyrode's solution saturated with 95% O_2 and 5% CO_2 (Jackson et al., '82; Hackett et al., '82). A glass micropipette (tip diameter: 20–60 μm) was filled with one of the tracers which was injected into NM/NL with the aid of a Picospritzer (General Valve Corporation). Injections were carried out for a period of 10 minutes to 2 hours. The slice was kept in a fresh oxygenated solution of Tyrode's at 35°C for an additional 5–18 hours and then fixed by immersion in 2% paraformaldehyde and 0.5% glutaraldehyde. The brains were cryoprotected in 30% sucrose and cryosectioned in the transverse plane at 30 μm. Sections containing FG and Rh were coverslipped with Glycergel (DAKO) and observed directly under epifluorescence by using the standard violet exciter filter (Zeiss) for FG and the green exciter filter for Rh. Sections containing HRP were







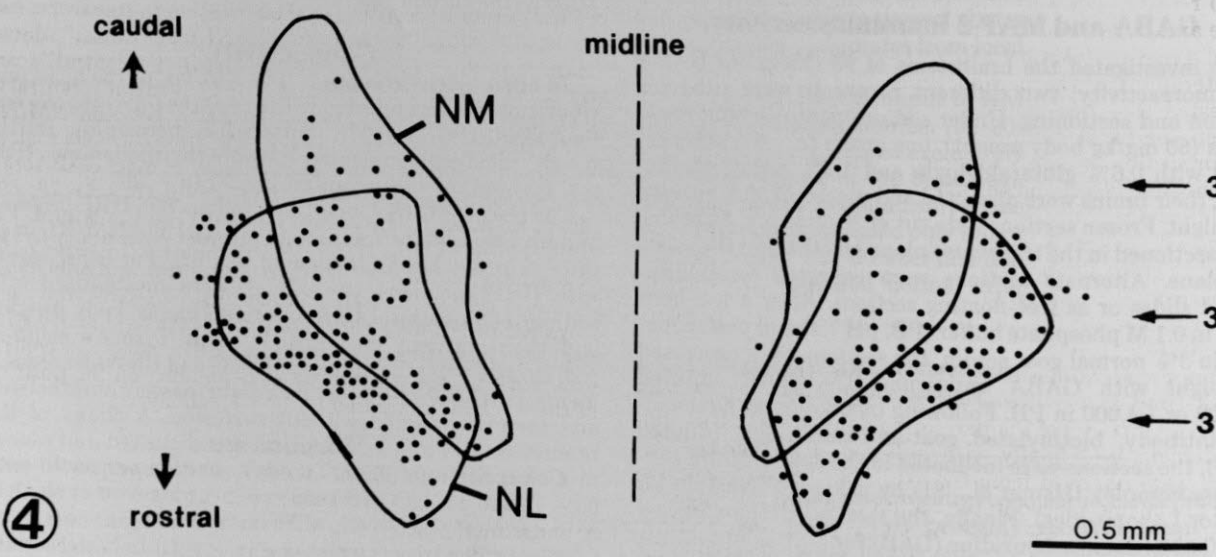


Fig. 1. Transverse section through the auditory brainstem of an E18 embryo processed with an antiserum to GABA. The area of neuropil surrounding the nucleus magnocellularis (NM) and nucleus laminaris (NL) is outlined by dashes. GABAergic neurons were counted in this area, but not in the neuropil surrounding the nucleus angularis (NA). Note GABA-immunoreactive neurons (star) between NM and NL. VeM, medial vestibular nucleus; XDCT, crossed dorsal cochlear tract.

Fig. 2. The number of GABAergic neurons in and adjacent to the nucleus magnocellularis and nucleus laminaris are plotted as a function of age. Counts are from 14 cases, embryonic day (E) 12 to posthatch day (P) 14. Numbers of cells are the average from the two sides of each brain.

Fig. 3. Drawings of representative transverse sections through three levels of the brainstem of an E16 embryo showing the distribution of GABAergic neurons in NM and NL. Lateral is to the right. The dashed line circumscribes the neuropil surrounding NM and NL. Positions of GABAergic neurons are indicated by black circles. The three levels of sectioning (**A–C**) are indicated in Figure 4.

Fig. 4. Plot of the distribution of GABAergic neurons in NM and NL in the horizontal plane (one case shown, E16 embryo). The positions of GABAergic cells of only two-thirds of the sections were plotted; every third section was not immunoprocessed. The dorsoventral extent of NM and NL is collapsed into one plane; only the rostrocaudal and mediolateral distribution of GABAergic neurons is visible in the dorsal view. Most of these neurons are located outside NM or NL (cf. Fig. 3). The levels of transverse sections shown in Figure 3 are indicated.

processed with DAB by using heavy-metal intensification (Adams, '81), dehydrated, and coverslipped with DPX mounting medium.

The carbocyanine tracer DiI (Molecular Probes) was dissolved in 1% dimethylformamide and injected into brainstems that had been perfused and fixed (Godement et al., '87) with 0.6% glutaraldehyde and 1.5% paraformaldehyde. The brainstems were kept in fixative at room temperature for periods of 2 weeks to 6 months and then were cryoprotected, cryosectioned, and observed with or without a coverslip under epifluorescence by using the Rhodamine filter set (Zeiss).

Crossed dorsal cochlear tract (XDCT) transections. Two posthatch chicks (P7) were anesthetized with ketamine hydrochloride (Vetalar, 80 mg/kg body weight, IM) and an intraperitoneal injection of sodium pentobarbital (Nembutal, 20 mg/kg body weight). The XDCT was sectioned by using a caudal approach through the foramen magnum (Benes et al., '77; Deitch and Rubel, '84, '89a,b). Following surgery, the incision was closed with cyanoacrylate adhesive. A third animal, age-matched but unoperated, served as the normal control. After a 1-day survival period, the animals were perfused with 0.1 M PBS followed by 1.0% paraformaldehyde and 1.25% glutaraldehyde. Similar experiments from previous studies show that a 1-day survival period is sufficient to produce terminal degeneration in the ventral neuropil of NL after XDCT transection (Parks and Rubel, '75). Serial sections, 30 μm thick, were cut on a vibratome and processed for GABA immunocytochemistry as described above. Adjacent sections were stained for Nissl substance to verify the lesion.

HRP-WGA injections in vivo. Two posthatch chicks (P12) received pressure injections of the combined anterograde and retrograde tracer horseradish-peroxidase-labeled wheat germ agglutinin (HRP-WGA; Sigma Chemical Co.) unilaterally into the paraflocculus. The paraflocculus was suggested as a possible source of input to NM/NL (H.J. Karten, personal communication[1]). Following anesthesia (see preceding section), an incision was made into the skin overlying the skull, and the skin and muscle were retracted. A hole was made in the lateral aspect of the skull to expose the paraflocculus. A Hamilton syringe was visually positioned to enter the paraflocculus at about a 45° angle from the horizontal plane. Approximately 0.9 μl of a 10% HRP-WGA solution (in 0.1 M PBS) was injected into the paraflocculus (Fig. 10A).

After a survival period of 20–22 hours, the animals were anesthetized and perfused intracardially with 0.1 M PBS (pH 7.4), a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde, and finally with buffered 30% sucrose. The brains were removed from the skull, blocked in the transverse plane, and placed in the buffered sucrose overnight. A sliding microtome was used to cut 50 μm frozen sections through the auditory brainstem, which were collected in 0.1 M PB. Alternate sections were Nissl-stained or processed for tetramethyl benzidine (TMB) histochemistry (Mesulam, '78). The TMB sections were mounted onto chrome-alum-coated slides, counterstained with neutral red, dehydrated, cleared, and coverslipped.

[1]Dr. H. Karten's advice and admirable prediction hereby are acknowledged.

RESULTS

Number and distribution of GABAergic neurons

The number and distribution of GABAergic cells in and adjacent to NM/NL were determined for every or every other section in 14 cases with the best immunocytochemistry; the area under investigation included the neuropil adjacent to NM/NL but not the neuropil of nucleus angularis (NA) (Fig. 1). The neuropil surrounding the NM/NL complex is well demarcated in GABA-stained material due to the lack of GABA-stained terminals and fibers (Fig. 1). GABAergic neurons were detected as early as E7 in the NM/NL complex, but stages prior to E12 were not investigated systematically. Counts revealed 100–200 GABAergic neurons in or adjacent to NM and NL on each side of the brainstem. The number of GABAergic neurons did not change significantly from E12 to P14 (Fig. 2).

Representative drawings of the distribution of GABAergic cells, as seen in transverse sections at three different levels of NM/NL, are shown in Figure 3. In four cases, the position of each stained cell was plotted on a horizontal planar projection of the NM/NL complex (Rubel and Parks, '75). These plots reveal an increasing density of GABAergic neurons from caudomedial to rostrolateral (Fig. 4). Figure 3 shows that GABAergic neurons are concentrated in two areas: between NM and NL and lateral to NM. They are less frequently found in caudal areas around NM or in the rostral pole of the NM/NL complex.

It should be stressed that Figure 4 collapses the dorsoventral dimension. GABAergic neurons are located predominantly not within the borders of NM or NL but in areas surrounding NM and NL. The GABAergic cells were classified into six groups according to their location in transverse sections: 1) "medial" (medial to NM/NL); 2) "dorsal" (dorsal margin of NM); 3) "NM" (within NM); 4) "central" (centrally located between NM and NL); 5) "ventral" (ventral to NL); and 6) "lateral" groups (laterally, between NM/NL and NA) (Fig. 5). Cell counts averaged from these six regions in four embryos aged E15–19 indicate that most GABAergic cells are contained in the central group (N = 61–73; 40–50%), followed by 15–25% (N = 34–41) in the lateral, 10–20% (N = 22–25) in the dorsal, 5–10% (N = 9–12) in the NM group, 5–10% (N = 7–13) in the ventral, and only about 5% (N = 2–7) in the medial group (Fig. 5). GABAergic neurons within NL were very rare (<1%); for the quantitative analysis, such cells were included either with the central group or with the ventral group, depending on the position of the cell body compared to the NL cell layer.

Morphology

Observations from GABA immunocytochemistry. Most of the GABA-stained neurons in the NM/NL area are multipolar, with a diameter varying from 8 to 25 μm. Their dendritic processes give rise to finer secondary fibers (Fig. 6A). The number of arborizations of single GABAergic neurons can be extensive; in one case, we counted 33 arborizations in the plane of the section. Some of the thick processes extend from the cell body for up to 150 μm. The nucleus of GABAergic neurons is large and, in cryosectioned material, usually stained less intensely than the cytoplasm and the proximal processes. In most vibratome sections, on the other hand, the nucleus is heavily immunostained (cf. Code et al., '89). Nuclear staining for GABA may be explained by the suggestion that the GABA molecule can diffuse through nuclear pores (Ottersen and Storm-

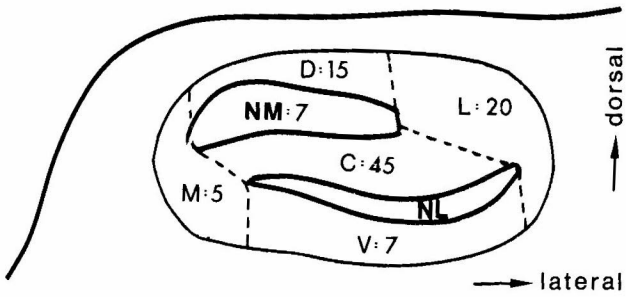


Fig. 5. Schematic drawing of the neuropil region surrounding NM and NL at midlevel showing the relative distribution (percentage) of GABAergic cells within each subdivision (E15–19, n = 4). C, central group; D, dorsal group; M, medial group; L, lateral group; V, ventral group.

Mathisen, '84). This hypothesis, of course, does not explain differential nuclear staining seen with different processing methods.

In about two dozen cases, in which neuronal processes are stained for some distance within the section (thickness: 30 or 40 μm), it is obvious that the thin processes arising from dendrites project into NM (Fig. 6A) or NL; such cases include GABAergic neurons from the medial, dorsal, NM, and central groups (Figs. 6, 7). Stained processes arborize within the dendritic fields of NL or ramify adjacent to the soma of NM cells. Often, the thin secondary processes branch at approximately right angles from the main dendritic process (e.g., neuron at the extreme right in Fig. 7B).

GABAergic neurons of the *ventral group* project into NL and often have another process directed ventrally towards the descending vestibular nucleus and trapezoid fibers. We also observed some GABAergic fibers entering the ventral NL area from outside the ventral neuropil area; possibly, these fibers originate from neurons in the trapezoid body (see below).

The *central group* of GABAergic neurons is located just dorsal to NL, about 20–50 μm from the dorsal margin of the NL cell bodies (cf. Smith and Rubel, '79). Most of the GABA-positive cell bodies are located dorsal to the NL dendrites, but occasionally they are observed among the NL dendrites. Frequently, cells of the central group possess two to four thick dendritic processes, one or two of which will proceed in a medial direction as do the others laterally (Fig. 6B). These processes branch and ramify while entering the dendritic and soma layer of NL. Often the dendritic processes extend more than 100 μm in both medial and lateral directions, and thus cover more than one-third of the width of NL (500–700 μm at E16). Occasionally, these lateral and medial processes originate from one ventral process. The orientation of these processes does not appear to correspond with the isofrequency contours of NL which run orthogonal to the tonotopic axis; the axis of tonotopic organization in NL follows a line from caudolateral (low frequencies) to rostromedial (high frequencies) (cf. Rubel and Parks, '75). On each side of the brain, we counted 15–25 neurons of the cell type with medial and lateral processes. Their number did not change significantly between embryonic and posthatch chicks. Many GABAergic cells of this type have a characteristic dorsal process which is directed towards NM (e.g., neuron in extreme left of Fig. 7A), but could not, in the present material, be demonstrated to terminate in NM. Often, the distal processes of GABAergic neurons are located somewhat caudal to the cell soma. In the present material (30–40 μm-thick sections), cells of the central group seemed only rarely to possess processes that reached both NM and NL, although this might have been a function of limited antibody penetration and/or the thickness of sections.

The *NM group* contains neurons of variable morphology; some have at least two large processes at opposite poles of the soma which branch within NM in a mediolateral direction. Their processes extend over considerable distances (more than 150 μm) within NM (Fig. 6C). Other GABAergic neurons are multipolar with three to five processes radiating in several directions (Fig. 7A). Immunostained neurons in NM are relatively rare; a maximum of 12 cells, in one NM, was counted from one embryo (E12). In older animals (E18 and P7), we counted nine GABAergic neurons within NM on each side of the brain.

Many stained cell bodies of the *lateral group* are located quite distant (more than 100 μm) from NM or NL cell bodies. Their processes form a GABAergic fiber bundle that runs medially towards and into NM, where it disperses among NM neurons. Many GABAergic processes seem to originate from the lateral group; several processes traverse dorsolateral portions of NM, apparently without branching. They may innervate portions of NM, may continue to NL, or have a different destination altogether.

In summary, the morphology of GABA-stained neurons in the NM/NL area shows considerable variability. The shape and size of the somata, as well as the orientation of dendritic processes, are variable. The only cell type that displays a rather consistent morphology is that of the central group with medial and lateral processes. Of those GABAergic neurons in the central group, approximately one-fourth belong to this cell type.

Observations from MAP-2 stains. Sections through the brainstem immunoreacted with an antibody to MAP-2 show labeled neurons in positions corresponding to those revealed by the GABA immunostaining. In the central group, the approximate number of neurons stained with MAP-2 and their morphology correspond to those of the GABA-stained cells. The cell type with medial and lateral processes is easily identified because of its characteristic morphology (cf. Fig. 8B). The number of MAP-2-stained neurons located laterally to NM/NL is considerably larger than the number of GABAergic neurons in the same location. Thus, it appears that only a subpopulation of the (MAP-2 stained) neurons lateral to NM/NL belongs to the GABAergic lateral group, while most or all of the (MAP-2 stained) neurons in the central group are GABA immunoreactive.

Observations from Golgi impregnations. Golgi-impregnated neurons in positions similar to those of the GABAergic cells are encountered rarely. Some, however, are located in the area between NM and NL, corresponding to the central group (Fig. 8C–E). Such neurons have morphological features similar to those described for GABA-stained neurons (Figs. 6, 7). In two cases, the processes of Golgi-impregnated neurons could be traced for considerable distances (Fig. 8C–E). One of these neurons has ventromedially directed processes that branch and terminate in the *ventral* dendritic area of NL, while the ventrolateral processes terminate in the *dorsal* dendritic area of NL (Fig. 8C,D). Since

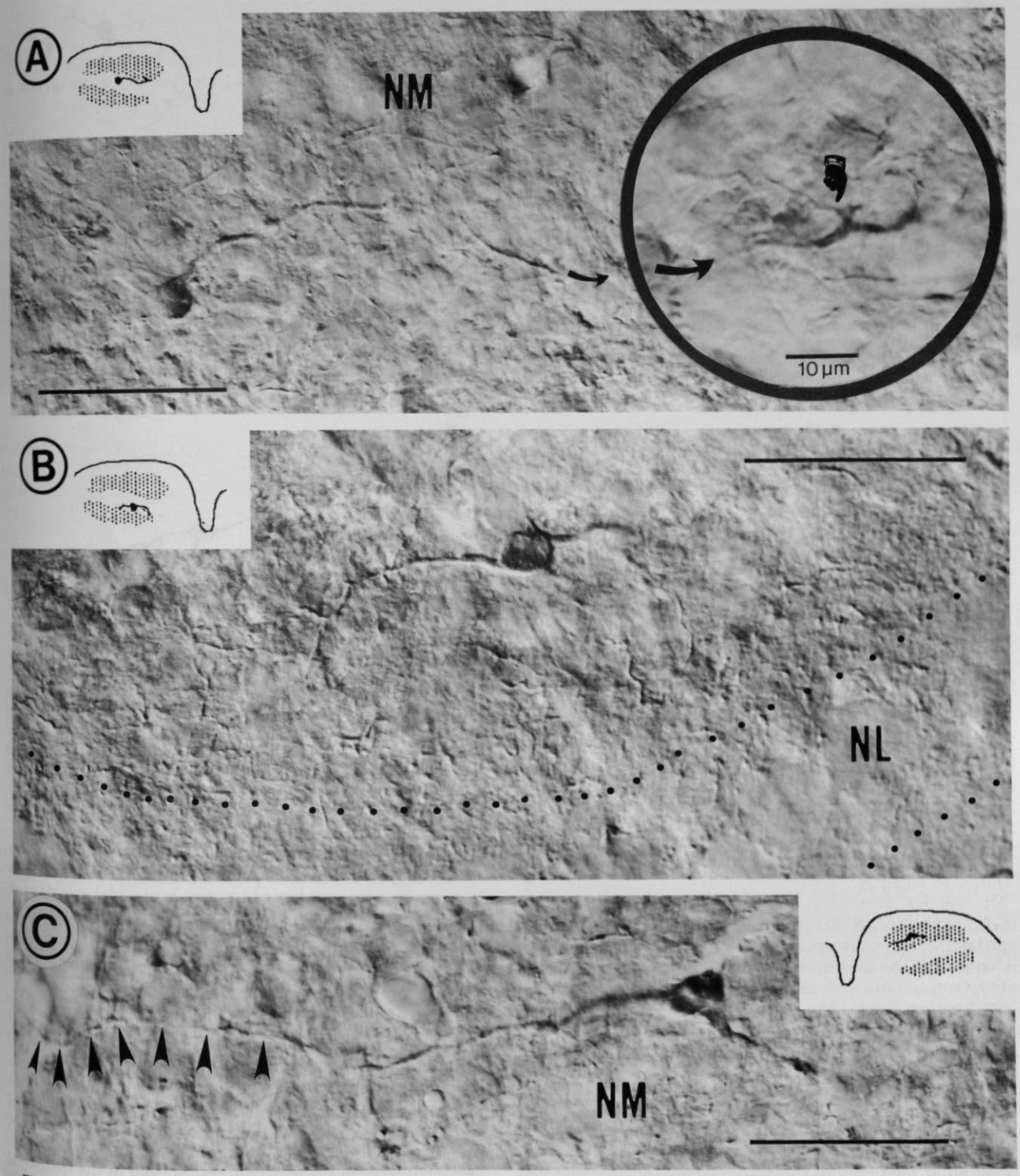


Fig. 6. Photomicrographs of GABA-immunoreactive neurons in the NM/NL area. Cryostat sections (30 µm) were immunoprocessed on the slide. The position of labeled neurons is indicated in the **inserts** and the same cells are among those shown in Figure 7A,B. Scale bars: 50 µm, unless indicated otherwise. **A:** GABAergic neuron immediately ventral to NM with process arborizing in NM. Immunostained process continues medially (arrows), as shown at higher magnification. Note thin, secondary fiber originating from labeled process (pointer) and running dorsally into NM. E16 embryo. **B:** GABAergic neuron with typical morphology, located between NM and NL. Note processes directed medially and laterally, with arborizations in dendritic layer of NL (cf. Fig. 7A). The borders of the cell body layer of NL are indicated by the dotted line. E18 embryo. **C:** GABAergic neuron within NM. Note immunostained process extending more than 150 µm in NM (arrowheads). E12 embryo.

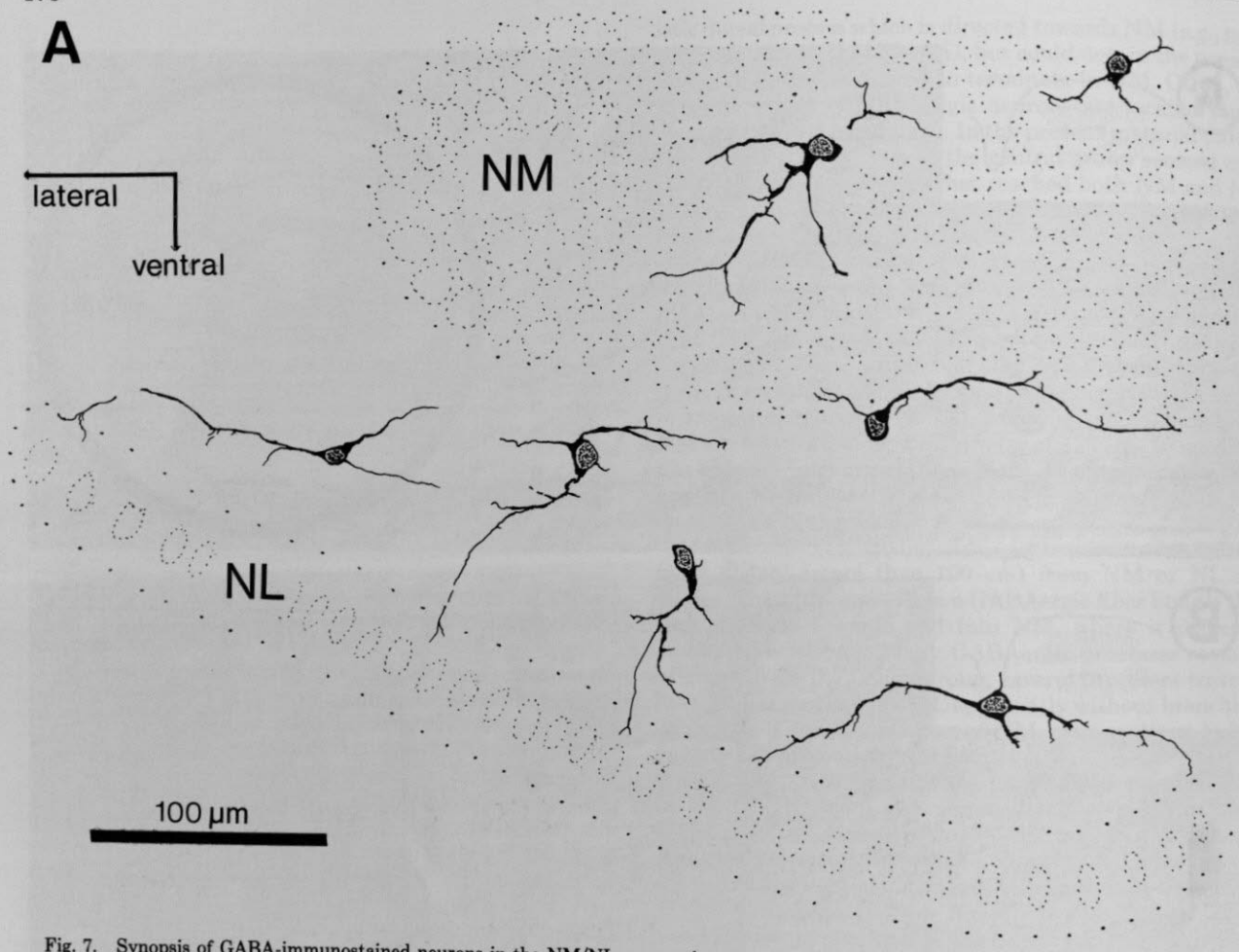


Fig. 7. Synopsis of GABA-immunostained neurons in the NM/NL area. Neurons are from four different animals (E12–18), transverse cryostat sections (30 µm). Neurons were drawn by using a Eutectics neuron reconstruction system with a 100× objective (N.A. 1.32). All neurons are shown at the same magnification and in their original orientation and position relative to NM/NL. The NM and NL nuclei at the midlevel are indicated by stippling; their cell bodies are drawn for illustrative purposes only.

these neurons are impregnated rarely in Golgi-impregnated material, and usually are not sectioned in the optimal plane, it could not be determined whether this is a normal or exceptional feature of neurons located between NM and NL. Another Golgi-impregnated neuron has laterally, medially, and ventrally directed processes that branch extensively in the plane of sectioning (Fig. 8E). This neuron resembles, in position and morphology, the GABAergic neuron shown in the extreme right of Figure 7B. In the Golgi-impregnated cell, we counted 12 thin fibers originating from one main neuronal process over a distance of 50 µm. These Golgi-impregnated neurons are probably GABAergic, because all or nearly all of the neurons between NM and NL (central group) appear to be GABAergic (see above, MAP-2 stains).

Connectivity

In vitro tracers. The best retrograde labeling of cell bodies was obtained with injections of HRP and DiI into NM/NL. For DiI, diffusion times of 6–8 weeks were necessary for retrograde labeling of cell bodies, while good anterograde labeling was obtained after 3–4 weeks. Fluorogold was also transported in a retrograde direction in the in vitro preparation, but its transport was very slow, requiring 12–18 hours in Tyrode's solution.

The ascending projections labeled after injections into NM/NL are essentially similar to those reported by Conlee and Parks ('86) in the posthatch chick. The main targets of the NM/NL include the nucleus mesencephalicus lateralis, pars dorsalis (MLd) contralaterally, and the nucleus of the lateral lemniscus (LL), the ventral nucleus of the lateral lemniscus (VLV), and the superior olive (SO) ipsilaterally. In one embryo (E16), however, we also found some anterograde DiI label in the contralateral SO and in the ipsilateral LL and VLV. These differences may be due to method- or age-dependent parameters.

Following large injections into NM/NL/NA, we found about 20–30 retrogradely labeled neurons in the ipsilateral trapezoid body and the superior olive. In cases with injections restricted to NM/NL (cf. Fig. 9C) but excluding NA, a smaller number of labeled neurons are found scattered throughout the ipsilateral trapezoid fibers (Fig. 9A), with

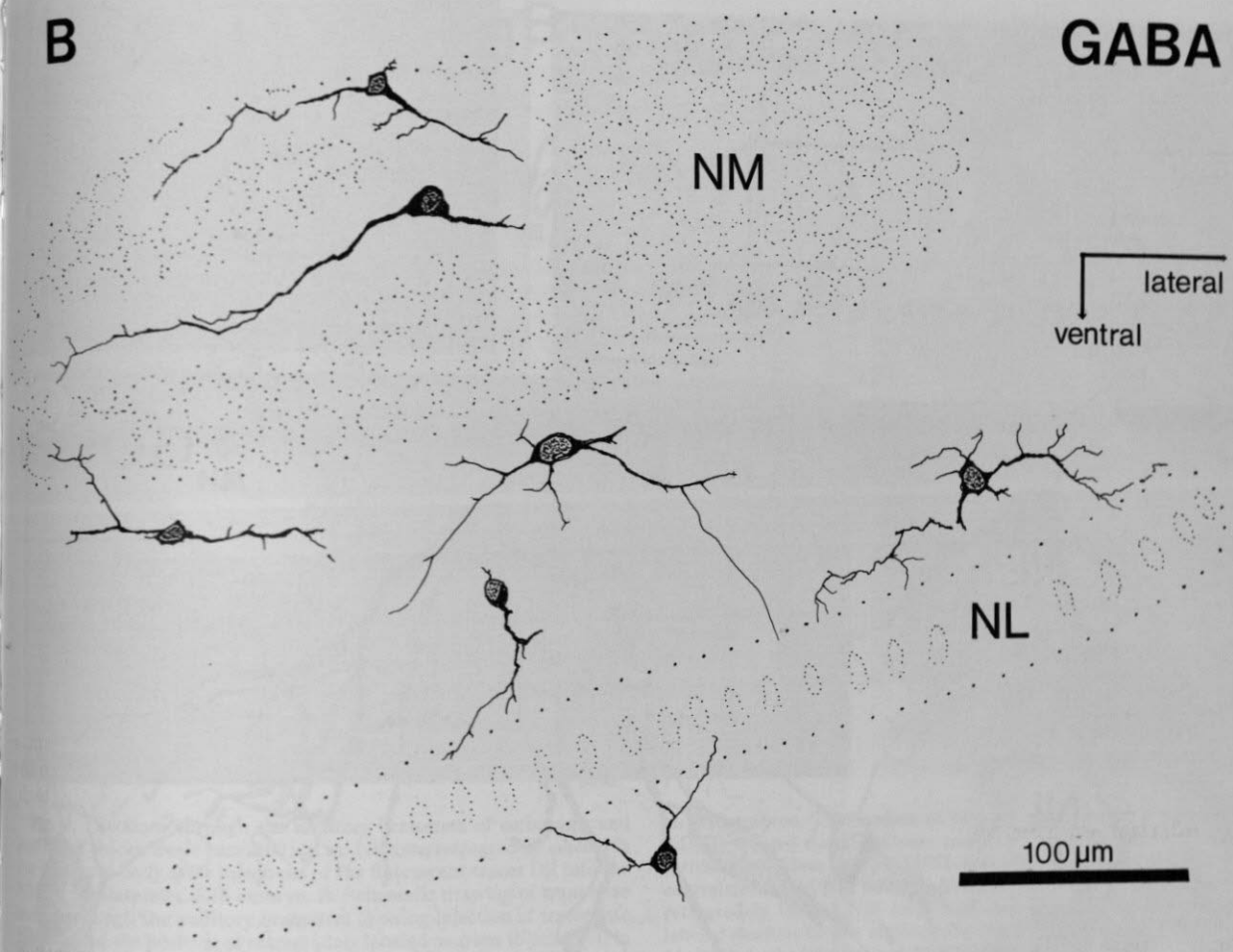


Figure 7 continued

neurons rarely labeled in corresponding areas contralaterally. Injections restricted to NA, on the other hand, resulted in exclusive labeling of neurons in and immediately adjacent to rostral parts of the ipsilateral SO. Since injections into NM/NL usually involved some encroachment into adjacent vestibular nuclei and pathways, it is possible that not all labeled neurons in the trapezoid body project to NM or NL. In addition, retrogradely labeled neurons in this area partly overlap in position with neurons that project to the cochlea (Whitehead and Morest, '81); some of these cells may have been labeled by injection of their efferent processes in the dorsolateral brainstem. Several cells in the trapezoid body and the SO are GABA immunoreactive (data not shown; cf. Müller, '87). Our attempts to double-label neurons in this region with retrograde tracers and the GABA antibody, however, did not render convincing examples of double-labeled neurons. This failure may be due to limitations of the techniques used.

No retrogradely labeled cells were found in the MLd, LL, or in the VLV nuclei. In addition, no cells caudal to NM/NL were labeled, except for cells located immediately adjacent to the injection site. We found no evidence for a projection from cells in NA to NM/NL. In the *contralateral* NM/NL/NA complex, we found no retrogradely labeled neurons located outside the NM nucleus. Since most parts of the cerebellum were removed prior to injections of tracers, the experiments of this series could not determine possible projections from the cerebellum to NM/NL (see below, HRP-WGA injections).

Crossed dorsal cochlear tract (XDCT) transections. The possibility that GABAergic projections to NM/NL run through the XDCT was further examined by transection of the XDCT. In two experiments, the XDCT was completely severed in its dorsoventral dimension. Following subsequent GABA immunocytochemistry, GABAergic terminals were observed in NM on both sides of the brainstem. In neither experiment was there any indication of a decrease in the number of GABAergic terminals relative to normal control animals (Code et al., '89).

HRP-WGA injections. In both experiments in which the paraflocculus was injected, the central area of the injections of HRP-WGA completely filled the paraflocculus while the zone of less intense labeling (the halo) spread into the adjacent cerebellar tissue (Fig. 10A). In neither experi-

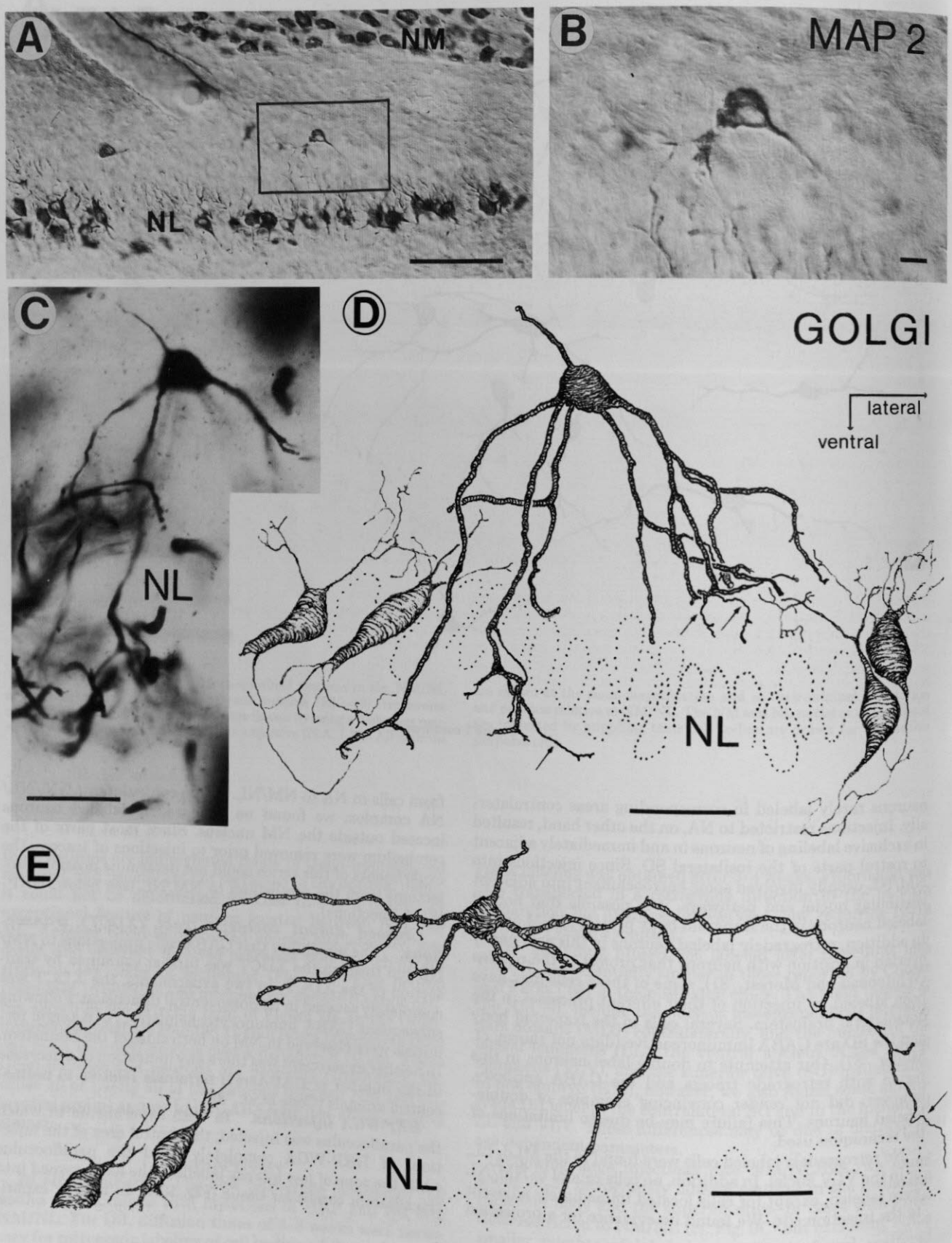


Figure 8

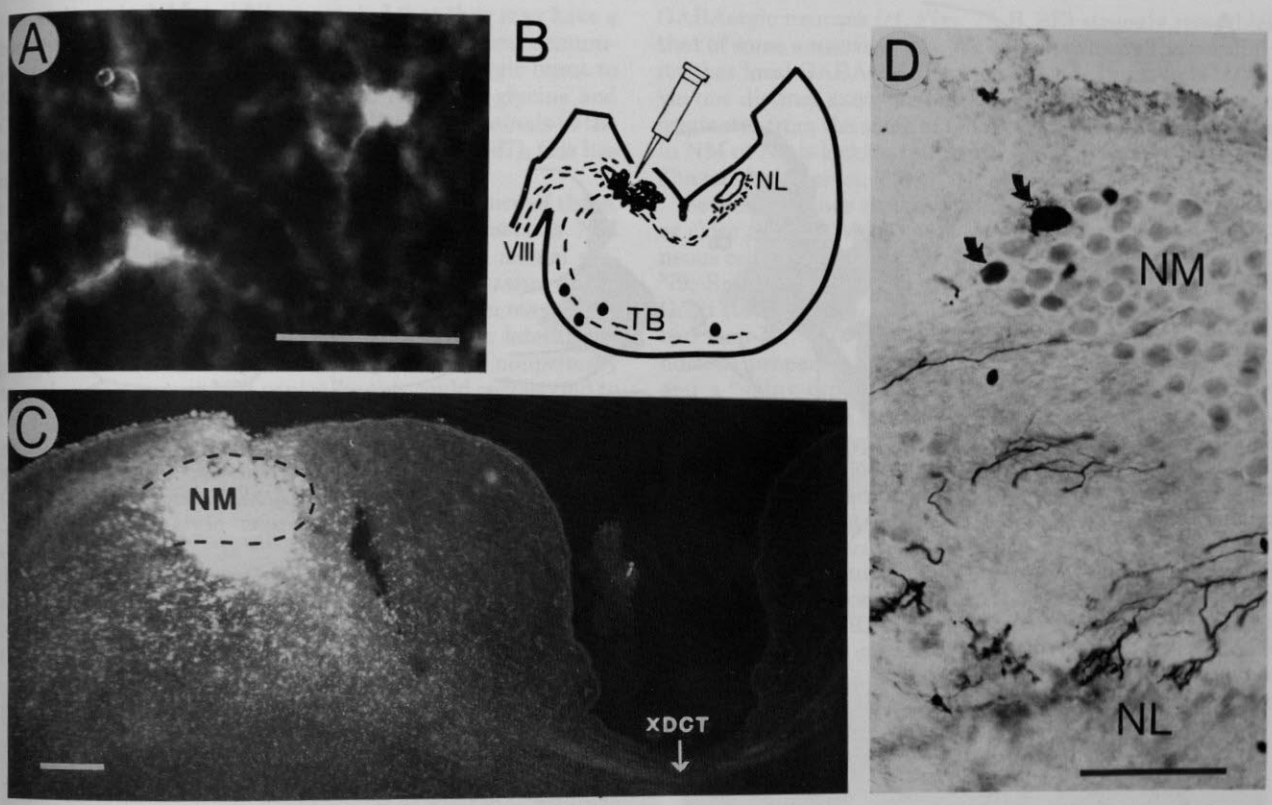


Fig. 9. Sections through the auditory brainstem of embryonic and posthatch chicks. Scale bars: 100 μm. **A:** Neurons retrogradely labeled in the trapezoid body after injections of the fluorescent tracer DiI into the NM/NL ipsilaterally. E18 embryo. **B:** Schematic drawing of transverse section through the auditory brainstem showing injection of tracer into NM/NL and the position of retrogradely labeled neurons (black dots) in the trapezoid body (TB). VIII, eighth nerve; E18 embryo. **C:** Injection site restricted to the NM/NL area following application of fluorogold in an E18 embryo. The borders of NM are indicated by a dashed line. XDCT: crossed dorsal cochlear tract. **D:** Two HRP-labeled NM cells (*arrows*) and fibers in the NM/NL area after injection of HRP into the contralateral NM/NL. Labeled fibers in NL are axon collaterals from retrogradely labeled NM cells. NM cells were the only retrogradely labeled neurons in this region; cells adjacent to NM were not labeled. Medial is to the left. Posthatch (P1) chick.

ment did the injection spread across the midline. The pattern of anterograde and retrograde labeling was the same in both experiments although there were fewer retrogradely labeled cells in the experiment with the more rostral injection. As expected, anterograde labeling was heavy in the medial vestibular nuclei bilaterally. Retrogradely labeled cell bodies were found in the medial, lateral, and dorsal vestibular nuclei bilaterally and in the nucleus paragigantocellularis lateralis ipsilaterally. Injections of HRP-WGA into the paraflocculus resulted in no identifiable anterograde labeling in NM. However, very light, but reliable, anterograde labeling could be seen bilaterally in NL. Most of this label was in the lateral, multicellular region of NL (Fig. 10B), but a very light scattering of label could be observed as well in the central, monocellular region of NL (Fig. 10C). The anterograde labeling in NL was predominantly in the neuropil and not over the cell bodies, whereas the GABAergic terminals are abundant in both areas (Code et al., '89).

DISCUSSION

The excitatory inputs to nucleus magnocellularis (NM) and nucleus laminaris (NL) have been studied extensively (Parks and Rubel, '75, '78; Young and Rubel, '86; Rubel and Parks, '88). Little is known about the source and function of GABAergic terminals in NM and NL. Presumably inhibitory terminals occupy considerable portions of the surface

Fig. 8. Neurons stained with the neuronal marker MAP-2 (A,B) and Golgi-impregnated neurons (C–E) located between NL and NM. **A:** MAP-2-stained section through NM and NL of a posthatch (P12) chick. Monoclonal antibody to MAP-2. Lateral is to the left. Scale bar: 100 μm. **B:** Higher magnification of area outlined in A, showing neuron with typical morphology (cf. Figs. 6B, 7, 8C,D). Scale bar: 10 μm. **C:** Golgi-impregnated neuron located between NM and NL. Note resemblance to MAP-2-stained neuron (B). Posthatch (P10) chick. Scale bar: 50 μm. **D:** Computer-assisted drawing of the same Golgi-impregnated neuron shown in panel C. Note that medial processes appear to terminate in the ventral dendritic area of NL, while lateral processes arborize in the dorsal dendritic area of NL. *Arrows* indicate thin, secondary fibers. Four impregnated NL cells and their processes are also drawn (dendrites are represented by thin lines that do not show their actual thickness); other NL-cell bodies are stippled. Scale bar: 50 μm. **E:** Computer-assisted drawing of Golgi-impregnated neuron with a morphology similar to that shown in Figure 6B. Note fine fibers (*arrows*) arising from thick, dendritic processes. The NL-cell layer is indicated with stippling. Orientation of neuron is the same as in D. Hatchling (P0) chick. Scale bar: 50 μm.

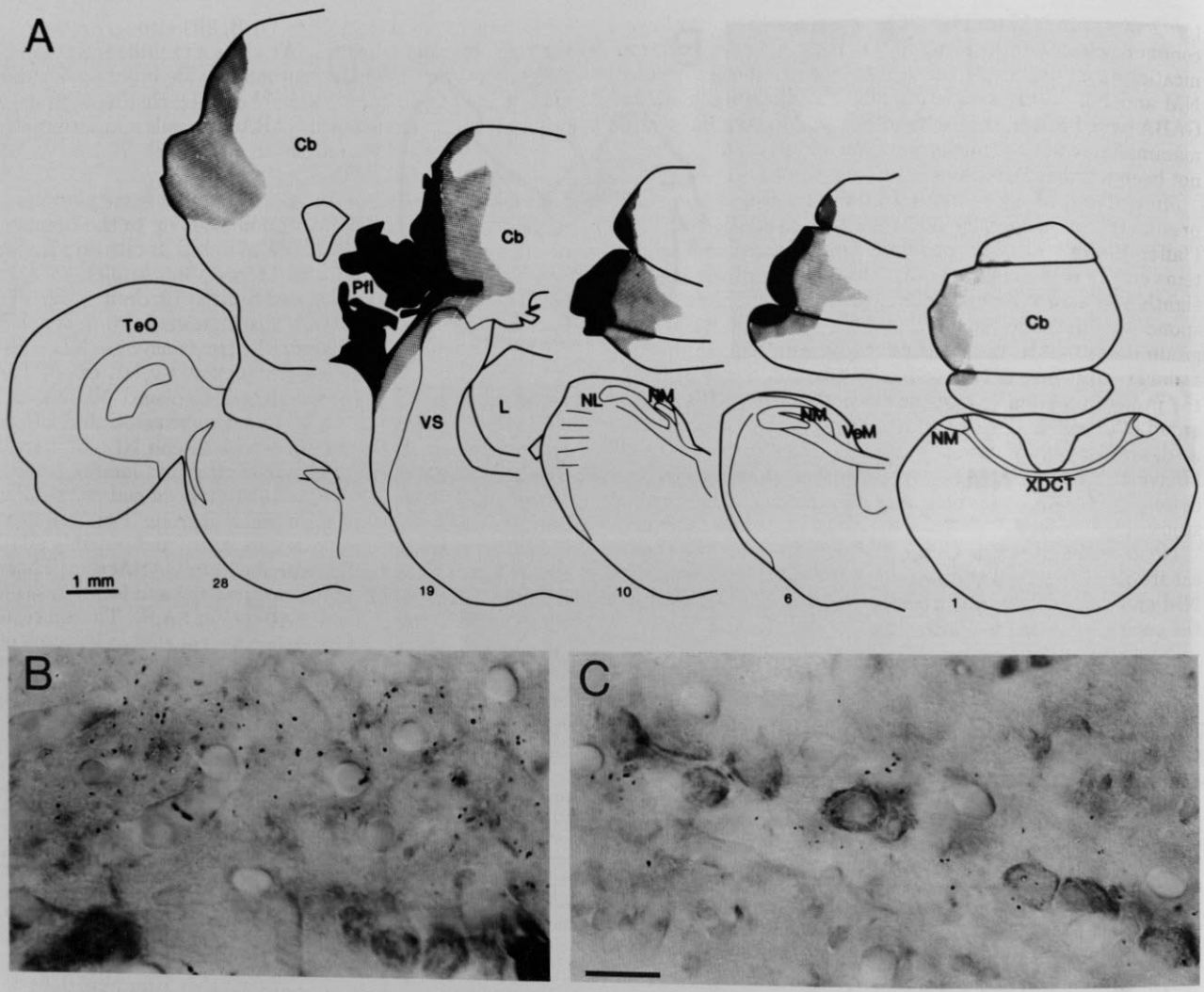


Fig. 10. Unilateral injections of HRP-WGA into the paraflocculus (Pfl) of a posthatch (P12) chick. **A:** Black indicates the central area of the injections; shading indicates the zone of less intense labeling. Numbers to the lower left of each section indicate the section number from caudal (right) to rostral (left). Total volume of 0.9 μl injected over two injection sites. Abbreviations: Cb, cerebellum; L, lingula; NL, nucleus laminaris; NM, nucleus magnocellularis; TeO, optic tectum; VeM, medial vestibular nucleus; VS, superior vestibular nucleus; XDCT, crossed dorsal cochlear tract. **B:** Anterograde labeling in the lateral, multicellular region of the ipsilateral NL after injections of HRP-WGA into the paraflocculus from the experiment shown in panel A. Frozen section, 50 μm thick, TMB chromogen, neutral red counterstain. **C:** Anterograde labeling in the monocellular region of NL from the same tissue section as that in panel B. Scale bar: 20 μm (panel B and C).

of NM and NL neurons (Parks, '81; Parks et al., '83; Carr et al., '89; Code et al., '89). Our data suggest that the GABAergic input originates mainly from local GABAergic neurons that are intrinsic to the NM/NL area. In the following sections, we will discuss the nonprimary afferents to NM and non-NM afferents to NL in relation to the distribution, morphology, and projections of local GABAergic neurons, evaluate the possibility and extent of other sources of GABAergic input to NM/NL, and compare our findings in the chick with those in the mammalian auditory brainstem.

Nonprimary/non-NM afferents to NM/NL

Golgi and electron microscopic studies first documented afferent fiber systems in NM and NL that are not of the primary type, i.e., do not originate in the cochlear ganglion or NM, respectively (Parks, '81; Jhaveri and Morest, '82). More recently, immunocytochemical studies demonstrated that many terminals on NM and NL neurons are GABAergic (Müller, '87; Code et al., '87, '89; Carr et al., '89), and these observations have been confirmed ultrastructurally (Code et al., '89).

Parks ('81) indicates that 13% of the perikaryal surface of NM cells is apposed by presumably inhibitory terminals. Afferents of non-NM origin account for 30% of the terminals on the surface of NL cell bodies and about 10% on their dendritic surface (Parks et al., '83; Deitch and Rubel, '89a). The non-NM afferents may all be GABAergic, since NL neurons appear to receive only two different types of afferents; one presumably is excitatory and arises from NM neurons; the other presumably is inhibitory (Parks et al., '83). The similarity between these types of presumably inhibi-

tory endings in NM and NL suggested that they may have a common source (Parks et al., '83; T. Parks, personal communication). In addition, there is a sparse glycinergic input to NM and NL (Code and Rubel, '89). Although glycine and GABA have been shown to be colocalized in terminals in the mammalian cochlear nucleus (Wenthold et al., '87), this has not been demonstrated in the avian NM or NL.

Jhaveri and Morest ('82) described the presence of three, presumably nonprimary, afferent axonal systems in NM ("afferents of unknown origin"). One of these axonal systems enters NM dorsolaterally, close to the entrance of the eighth nerve into the brainstem. This projection may correspond to the projection from the GABAergic lateral cell group described in the present study. Another nonprimary axonal system enters NM ventrally; this could correspond to the projection from the trapezoid body or from the central group of intrinsic GABAergic neurons. A third type of fiber is described as "extremely fine and delicately beaded" (Jhaveri and Morest, '82). All three fiber types may be processes of GABAergic neurons described in the present study.

None of the previous Golgi, EM, and immunocytochemical studies identified the source of the GABAergic input to NM and NL, although it does not appear to originate from the cochlear ganglion (Parks, '81; Code et al., '88).

GABAergic neurons in the NM/NL area

GABAergic neurons have been described in and around NM and NL in the chick (Müller, '87; Code et al., '89), but their number, distribution, and projections have not been described in detail. The present study demonstrates the presence of approximately 150 intrinsic GABAergic neurons in the NM/NL area. Many of their processes ramify and appear to terminate in the ipsilateral NM and NL.

Our observations from immunocytochemical and Golgi-impregnated material suggest that GABAergic input to NM and NL arises from (presynaptic) dendrites of local GABAergic neurons. GABA-stained neurons in the NM/NL complex typically have several thick dendritic processes that extend for considerable distances from the cell body. Such dendrites give rise to numerous thin fibers which often branch at approximately right angles, similar to axonal processes (Fig. 7A,B). We demonstrate that these thin fibers enter NM and NL and appear to terminate in the vicinity of NM and NL cells. The primary dendritic processes are defined as such because of their initially thick (1–4 μm), then tapering shape and because of the fact that such processes stain with MAP-2, a specific marker for neuronal dendrites and somata (Matus, '88). The thin secondary processes, on the other hand, may be similar to axonal elements: they do not taper, and they resemble processes that have been shown in Golgi-impregnated material to end in tiny swellings on NM cells (Jhaveri and Morest, '82; their Fig. 17). Further identification of the dendritic or axonal nature of these processes will require analysis at the EM level and/or the use of antibodies specific for axonal or presynaptic fibers.

Presynaptic dendrites have been described previously at the ultrastructural level; presumably inhibitory neurons in the superior colliculus (Lund, '69) and thalamic neurons form such contacts (Ralston, '71). More recently, axonlike fibers of dendritic origin were described for GABAergic interneurons in the mammalian brainstem (basilar pontine gray; Thier and Koehler, '87) and for amacrine cells in the retina (Dacey, '88). The morphology of several types of GABAergic neurons (cf. Figs. 7A,B, 8E) strongly resembles that of some amacrine cells. We cannot exclude the possibility that local GABAergic neurons provide input to NM/NL via one distinct axon; evidence, however, that a single axon originates from the soma of GABAergic cells and terminates in NM or NL is lacking (cf. Smith and Rubel, '79; Smith, '80; Jhaveri and Morest, '82).

Several previous studies have noted that some neurons in or close to the NM/NL area do not belong to the homogeneous cell population of either NM or NL (Smith and Rubel, '79; Smith, '80; Jhaveri and Morest, '82; Müller, '87). In Golgi studies of NL (Rubel and Smith, '79; Smith, '80, '81), two non-NL cell types are distinguished from the NL nucleus proper: a "perilaminar" (frequency: 3/100 NL cells) and a "spiny type" neuron (frequency: 1/100 NL cells). The perilaminar cell is interpreted as a displaced NL neuron, while the "spiny type" cell is found to be associated with a group of neurons located between NM and NL (similar to the GABAergic neurons of our central and lateral group). Jhaveri and Morest ('82) refer to one type of neuron (similar to GABAergic neurons of the "central group") as "cell type X." The present study shows that many, and possibly all of the "non NM-, non-NL neurons," are GABAergic. These neurons or ones with similar morphology and location stain with the neuronal marker MAP-2 (Fig. 8A,B). Therefore, it is unlikely that the GABAergic cells are glial cells, some of which possess a GABA-uptake system (Chronwall and Wolff, '80).

Other possible sources of GABAergic afferents to NM/NL

Sparse projections from the trapezoid body/superior olive. Experiments with injections of tracers into NM/NL revealed a few retrogradely labeled cells, mostly ipsilaterally, in the trapezoid body and adjacent to the superior olive (SO). These results confirm unpublished observations by J.W. Conlee (personal communication), who also found "periolivary cells" labeled after injections of HRP into the NM/NL of adult chicken. Recently, Carr et al. ('89) found a sparse projection from the ipsilateral SO to NM in the barn owl. Whether these neurons are GABAergic is not known. The number of labeled projection neurons in the SO, however, appears small compared with the number of GABAergic cells in the NM/NL area (100–200).

No descending projections from higher auditory centers. Injections of tracers into NM/NL failed to reveal retrogradely labeled cells in MLd, LL, or VLV. The lack of such descending projections has also been noted in adult chickens (J.W. Conlee, personal communication) and in the barn owl (Carr et al., '89).

No projections through the crossed dorsal cochlear tract (XDCT). Following transection of the XDCT, the number of GABAergic terminals in NM did not appear different from that in unoperated, control animals. Injections of tracers in NM/NL never revealed retrogradely labeled cells that corresponded to GABAergic neurons in the contralateral NM/NL region. It is unlikely that the absence of contralateral GABAergic projections is due to insensitivity of the methods, because axons crossing the midline from the contralateral NM were labeled in the same material, and NM neurons were retrogradely labeled on the contralateral side, including their axon collaterals to NL on the same side (Fig. 9D).

Sparse projections from the paraflocculus to NL. Unilateral injections of HRP-WGA into the paraflocculus did not produce significant anterograde labeling in NM but did result in appreciable labeling of the lateral, multicellular region of NL bilaterally. In light of the fact that the injections did not cross the midline and that anterograde labeling was seen in the contralateral NL, we believe that the anterograde labeling represents actual transport. The heavy anterograde and/or retrograde labeling seen in the medial, lateral, and dorsal vestibular nuclei after injections of HRP-WGA into the paraflocculus served as a positive control, since cerebellovestibular connections in the chicken have been well documented (Wold, '81).

These data suggest that there is a small projection from the paraflocculus primarily, but not exclusively, to the lateral, multicellular region of NL bilaterally, and possibly a weaker projection to central regions of NL proper. However, this projection does not appear to account for the high density of GABAergic terminals observed (Code et al., '87, '89). The anterograde labeling in NL coincides only partly with the distribution of GABAergic terminals in NL. GABAergic terminals circumscribe the nerve cell bodies in NL and extend out along their dendrites (Code et al., '89). The anterograde label observed in the present study was found predominantly in the neuropil and not on neuronal somata in NL. Thus, the paraflocculus is probably not a major source of GABAergic terminals to NL.

Comparison with the mammalian auditory brainstem

Nucleus magnocellularis (NM) is homologous with the anterior division of the mammalian anteroventral cochlear nucleus (AVCN). While NL is sometimes considered homologous with the medial superior olive (MSO) (Parks and Rubel, '75; Jhaveri and Morest, '82), homologies of parts of the olivary complex in mammals to avian brainstem nuclei are not clear; new auditory nuclei (e.g., periolivary nuclei) are assumed to have evolved in mammals (Carr et al., '89). Both AVCN and MSO receive GABAergic afferents but contain relatively few GABA-positive cell bodies (Thompson et al., '85; Adams and Mugnaini, '87; Moore and Moore, '87; Roberts and Ribak, '87). There is no evidence that the cochlear nerve is a source of GABAergic input to AVCN (Cant and Morest, '79) or NM (Code et al., '88). GABAergic afferents to AVCN may originate from neurons in the SO complex (periolivary nuclei) bilaterally (Adams and Mugnaini, '87; Adams and Wenthold, '87; Roberts and Ribak, '87; Spangler et al., '87), from the ipsilateral dorsal cochlear nucleus (Wenthold et al., '86; Adams and Mugnaini, '87; Roberts and Ribak, '87), from the ventral nucleus of the trapezoid body (Adams and Wenthold, '87), and/or from neurons intrinsic to AVCN (Adams and Mugnaini, '87; Roberts and Ribak, '87). Little is known about the source of GABAergic input to the MSO (Roberts and Ribak, '87; Moore and Moore, '87).

How do the assumed sources of GABAergic input to AVCN/MSO in mammals compare with the present findings of NM/NL innervation in birds? Possibly, the lateral and central groups of GABAergic neurons in the NM/NL complex of the chick are comparable to the periolivary nuclei within the SO complex in mammals which may provide GABAergic afferents to AVCN/MSO. Tissue sections through NM/NL that are immunoreacted with the neuronal marker MAP-2 show the existence of small groups of neurons that do not belong to any of the well-established auditory or vestibular nuclei. These small cell groups are entirely (central group) or partly (lateral group) GABAergic. On the other hand, some neurons of the periolivary nuclei in mammals project to the organ of Corti (Warr et al., '86), while in birds, neurons with projections to the cochlea are located in the ventral medulla, not in the NM/NL area (Whitehead and Morest, '81). Thus, the avian brainstem may contain two separate populations of "periolivary" cell groups—a dorsal one surrounding the NM/NL complex and a ventral one surrounding the superior olivary nucleus. Conclusive demonstration of the sources of GABAergic input to the mammalian AVCN and MSO, however, and further studies of the characteristics of these small cell groups in NM/NL are necessary for definite comparisons of avian/mammalian brainstem homologies.

ACKNOWLEDGMENTS

The authors thank J. Deitch and D. Smith for the Golgi-impregnated material and M. Seftel for MAP-2-stained material. We gratefully acknowledge the donation of MAP-2 antibody from Anthony Frankfurter (Univ. of Virginia) and Richard Vallee (Worcester Foundation). R. Hyson assisted with the in vitro tracer preparation. We also thank R. Rodieck for help with tracer protocols, C. Carr and J. Conlee for information about unpublished material, H. Karten for not-unsubstantial suggestions and discussions, and N. Cant and R. Hyson for critical comments on the manuscript. Research was supported by a Max-Kade-Fellowship (C.S.v.B.), NIH postdoctoral grant HD07239 (R.A.C.), and NIH grant NS24522 (E.W.R.).

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