Organization and Development of the Brain Stem Auditory Nuclei of the Chicken: Primary Afferent Projections [1]

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ABSTRACT The pattern of primary auditory projections to the brain stem of young chickens was investigated using terminal degeneration methods and orthograde transport of horseradish peroxidase (HRP) or tritiated amino acid. Of particular interest was the question of whether nucleus laminaris (NL) receives primary afferents. A study of silver-stained degeneration patterns in nucleus magnocellularis (NM) and NL at three intervals following unilateral interruption of the cochlear nerve revealed that by 48 hours after the lesion, degenerating terminals were found only in the ipsilateral nucleus angularis (NA), NM and lagenar projection areas but not in NL. Five- and eight-day survival times, however, also revealed degeneration bilaterally in NL. The appearance of terminal degeneration in NL at the longer survival times is attributed to the previously-reported severe and rapid transneuronal degeneration of neurons in NM following deafferentation and not to the presence of cochlear nerve terminals in NL. Injection of HRP or tritiated proline into the basilar papilla produced patterns of labeling similar to that seen in the 2-day degeneration material; HRP reaction product or autoradiographic label were seen only in the ipsilateral NA and NM and in the ipsilateral projection areas of the macula lagena but not in either NL. The patterns of primary auditory projections revealed by the three methods were quite similar to each other and to that previously reported for the pigeon and confirm the conclusion that the laminar nucleus of chickens does not receive primary afferents.

The existence of primary cochlear projections to the avian nucleus laminaris (NL) has been disputed for many years. This nucleus, which has been considered homologous to the medial superior olivary nucleus of mammals (Boord, '69; Rubel and Parks, '75) is thought not to receive such direct afferents by a majority of authors (Brandis, 1894; Wallenberg, 1898, 1900; Holmes, '03; Bok, '15; Levi-Montalcini, '49; Boord and Rasmussen, '63; Winter, '63; Rodriguez and Rebollo, '66). A few investigators, however, have contested this view (Winkler, '07; Sanders, '29; Wold and Hall, '75). Wold and Hall ('75) have revived the issue by reporting that section of the cochlear nerve in adult chickens produces silver-stained degenerating material bilaterally in NL after 4- to 7-day survival periods. These authors attribute their findings to primary cochlear fibers passing through the ipsilateral nucleus magnocellularis (NM) to innervate both NL via the dorsal cochlear tracts. Because the avian auditory system is of interest to students of developmental and comparative neurology (e.g., Schwartzkopff, '68; Konishi, '70; Gottlieb, '71; Rubel et al., '76) it seemed desirable to re-examine the primary auditory projection in chickens, with particular emphasis on the question of primary afferents in NL.

A previous study on Nissl-stained material has shown that NM undergoes severe transneuronal degeneration within a few days after interruption of the primary cochlear projection (Jackson and Rubel, '76). This finding

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suggested that the results of Wold and Hall ('75) might reflect a secondary degeneration of NM processes in NL rather than a primary cochlear projection to the laminar nuclei. This hypothesis seemed particularly likely since the 6- to 7-day survival times which gave Wold and Hall ('75) their best material were sufficient to produce at least a 25% loss of NM neurons in the chickens subjected to unilateral cochlea removal by Jackson and Rubel ('76). To directly evaluate this possibility of secondary transneuronal degeneration, the present study examined the pattern of silver-stained degeneration in the brain stem 2, 5, and 8 days following unilateral interruption of the cochlear nerve. Two other methods for tracing neural connections were also used to confirm the degeneration studies: (1) The pattern of horseradish peroxidase (HRP) staining in the brain stem was determined at three intervals following injection of the enzyme into one cochlea, and (2) The distribution of radioactivity in the brain stem was determined with autoradiographic (AR) methods at two intervals following injection of tritiated proline into one cochlea.

MATERIALS AND METHODS

Subjects and surgery

Five- to ten-day-old White Leghorn chicks hatched in the laboratory were used. The birds were anesthetized with a 2.0 ml/kg intraperitoneal injection of Equi-Thesin (Jensen-Salsbery Labs). Under a dissecting microscope, the tympanic membrane and columella of the left ear were removed to expose the oval window. In 11 animals, a fine forceps was then used to extract the basillar papilla through the oval window. This procedure reliably severs the distal processes of the cochlear and lagenar ganglion cells. Although the ganglion cell bodies themselves are not removed, they quickly degenerate, as do the cochlear and lagenar portions of the eighth nerve (Jackson and Rubel, '76; Rubel and Parks, unpublished). In a second group of five birds, a 30-ga. needle attached to a 100-μl or 10-μl (proline) Hamilton syringe was inserted into the basilar papilla through the oval window and 10 μl of a 10% HRP (Sigma Chemical Corp., Type VI) solution in sterile water was injected. Another four birds received injections of 2 μCi (in 2.0 μl distilled water) of tritiated proline (NEN; sp. ac. = 60 Ci/mM) into the left basilar papilla. Following all of these procedures, a piece of Gelfoam was packed into the middle ear cavity and the external auditory meatus was sealed with cyanoacrylate adhesive (Aron Alpha, Vigor Corp.).

Histology

The subjects which sustained basilar papilla removals survived for 2 (n = 3), 5 (n = 4), or 8 (n = 4) days. The animals were anesthetized with sodium pentobarbital and perfused transcardially with 10% formalin. The brains were removed and placed in a solution of 10% formalin and 30% sucrose for two weeks, at which time they were sectioned at 25 μm on a freezing microtome. Alternate serial coronal sections were stained by the first method of Fink and Heimer ('67) for degenerating axon terminals; some sections were counterstained with cresyl violet.

For the animals which received HRP injections, survival times of 16 (n = 2), 24 (n = 2), or 36 (n = 1) hours were allowed. The animals were anesthetized and perfused transcardially for ten minutes with a fixative at 2°C containing 2% glutaraldehyde, 2% paraformaldehyde and 5% sucrose in 0.1 M phosphate buffer at pH 7.2. The brains were removed, post-fixed in the same solution at 4°C for four hours and washed overnight in the buffer with 30% sucrose. Coronal sections were cut on a freezing microtome at 30 μm and the serial sections processed for HRP by the method of Graham and Karnovsky ('66). Alternate sections were counterstained with thionin.

The animals which received tritiated proline injections survived for 6 (n = 2) or 24 (n = 2) hours. These animals were anesthetized with sodium pentobarbital, perfused transcardially for ten minutes with 10% formalin and then decapitated. The brains were removed and placed in Bouin's solution for 12 hours and then in several changes of 70% alcohol until the picric acid was removed from the tissues. The brains were embedded in paraffin and sectioned at 6 μm in the coronal plane. A 1-in-4 series of sections through the medulla was mounted on acid-cleaned gelatin-coated slides and processed for autoradiography as previously described (Rubel et al., '76). Briefly, the slides were dipped in NTB2 emulsion (Kodak), sealed in light-tight boxes at 4°C for eight weeks, developed in D19, fixed, washed and stained through the emulsion with thionin (pH 4.0-4.2).

The serial sections processed by the HRP and Fink-Heimer methods were traced at × 46 from an overhead projector and details

were added to these drawings by viewing the sections under a × 40 oil-immersion objective (NA = 1.3). For the HRP and AR material, every section was examined closely under both bright-field and dark-field illumination, to detect the presence of reaction product and silver grains, respectively.

RESULTS

Basilar papilla removals

Figure 1 shows section tracings through the medulla in an animal which survived for 48 hours following removal of its basilar papilla. This figure includes every 25-μm section (at 50-μm intervals) through the entire extent of NM. Silver-impregnated degenerating material is seen only in the ipsilateral cochlear nerve NA, NM and regions of the descending and lateral vestibular nuclei which, as Boord and Karten ('74) have shown, receive projections from the macula lagena. The only degeneration seen in NL on either side of the brain consists of degenerating cochlear and/or lagenar nerve fibers and light terminal degeneration in the extreme caudolateral edge of the ipsilateral NL; this region of NL abuts the ventral division of NA and the ventrolateral division of NM, which form part of the lagenar nucleus (see the third section from the bottom in fig. 1). In the Fink-Heimer stained sections, the large end-bulbs formed by primary fibers in NM are evidenced by the dense cluster of argyrophilic particls outlining the cell bodies. The degeneration is confined within the borders of NM; no fibers pass ventrad or mediad of this nucleus. Both the ipsilateral and contralateral NL are free of degeneration, with the minor exception noted above.

The pattern of degeneration seen in the cochlear nuclei five days after surgery is shown in figure 2. In addition to the degeneration seen in NA, NM and the lagenar projection areas, dense degeneration is seen in the dorsal cochlear tracts, the dorsal half of the ipsilateral NL and in the ventral half of the con-

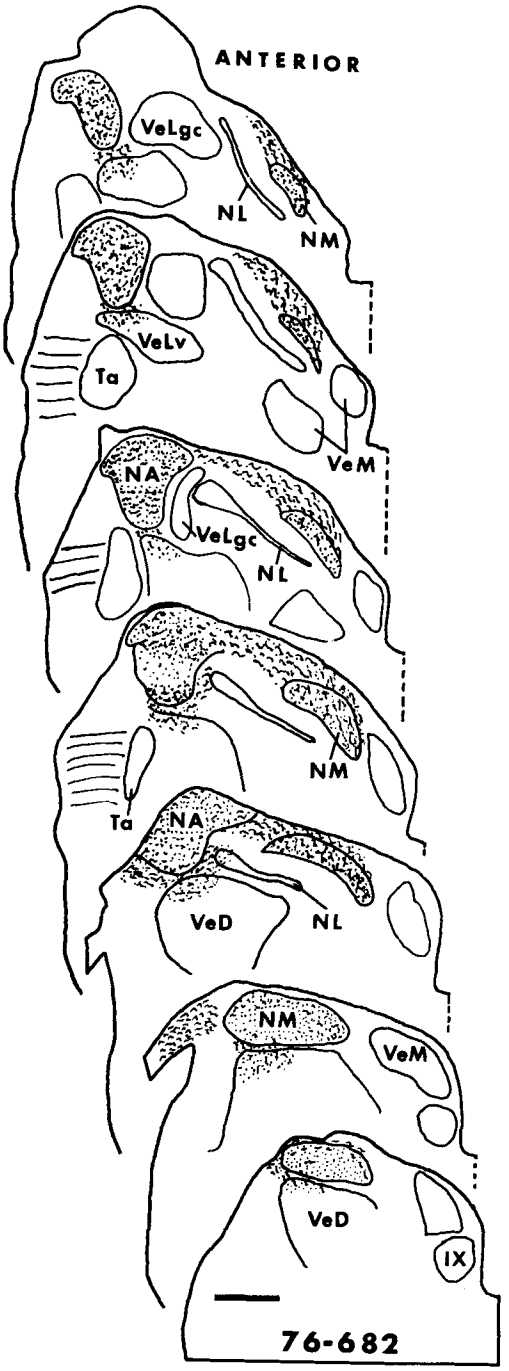

Figure 1

Fig. 1 A series of tracings through the left brain stem of animal 76-682 to show the pattern of silver-stained degeneration seen two days after removal of the cochlea. This figure includes every 25-μm section (at 50-μm intervals) through the entire extent of NM. Degenerated fibers are indicated by wavy lines, degenerated terminals by dots. Heavy degeneration is seen in all branches of the cochlear nerve, nucleus angularis (NA), nucleus magnocellularis (NM) and in restricted portions of the descending and lateral vestibular nuclei. Degeneration is seen only in the extreme caudolateral portion of nucleus laminaris (NL) where it borders on the lagenar nucleus; no degeneration is seen in the dorsal cochlear tracts ventrad and mediad of NM. Bar indicates 1.0 mm. FLM, fasciculus longitudinalis medialis; NIX, nucleus nervi glossopharyngei; Ta, nucleus vestibularis, pars gigantocellularis; VeLv, nucleus vestibularis lateralis ventralis; VeM, nucleus vestibularis medialis.

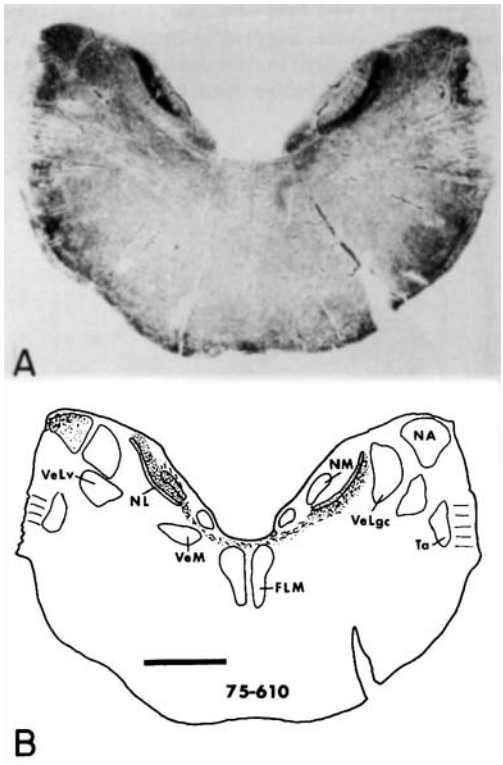

Fig. 2 To show the pattern of silver-stained degeneration seen in the brain stem of animal 75-610 five days after removal of the left cochlea. A, Low-power photomicrograph; Fink and Heimer ('67) method. B, Tracing of the same section to show detail. As in figure 1, degenerating fibers are seen in the cochlear nerve, nucleus angularis (NA) and nucleus magnocellularis (NM). In this animal however, degenerating fibers are seen passing in the two dorsal cochlear tracts ventrad and mediad of the left NM. The ipsilateral pathway ends in dense terminal degeneration along the dorsal surface of nucleus laminaris (NL). The crossed dorsal cochlear tract passes between the fourth ventricle and the medial longitudinal fasciculus (FLM) to end in heavy terminal degeneration along the ventral surface of the contralateral NL. Bar indicates 1.0 mm; abbreviations as in figure 1.

tralateral NL. Figure 2A is a low-power photomicrograph showing this pattern of degeneration. Figure 2B is a tracing of the same section to show detail more clearly. There was little variability among the 5-day survival animals in the pattern of staining exhibited. The pattern of degeneration seen in animals surviving for eight days was essentially identical to that of the 5-day survival animals.

HRP injections

Figure 3 shows a set of serial tracings made through the brain stem in an animal which

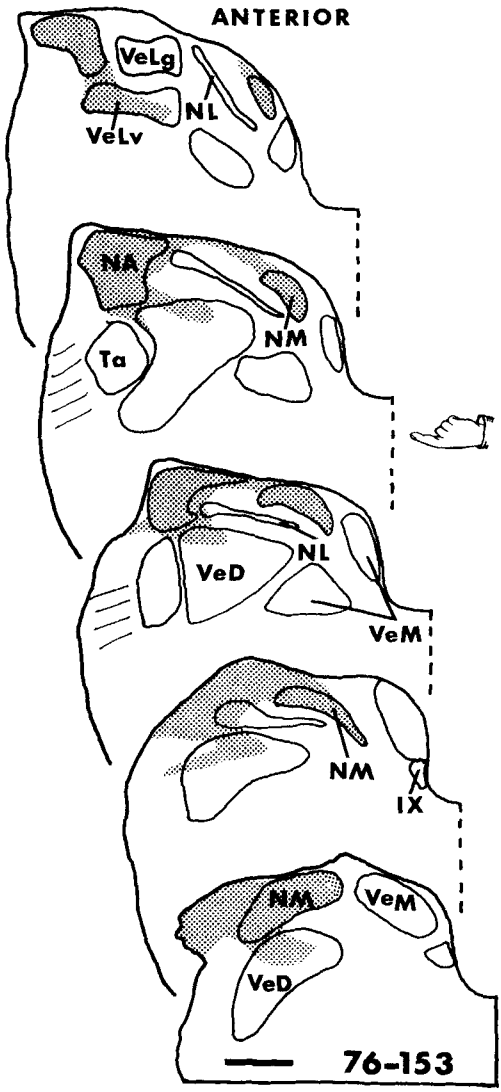

Fig. 3 A series of tracings through the left brain stem in animal 76-153 to show the pattern of horseradish peroxidase (HRP) staining seen 36 hours after injection of the enzyme into the left cochlea. Reaction product is seen in the lateral branch of the cochlear nerve passing to nucleus angularis (NA). The medial branch of the nerve, which innervates nucleus magnocellularis (NM) is also labeled. Heavy labeling appears in the lagenar nerve, which enters ventrad and rostrad to the cochlear nerve proper. All portions of NA and NM are labeled and very dense reaction product is also seen in restricted portions of the descending and lateral vestibular nuclei. No labeling is seen ventrad and mediad of NM in the dorsal cochlear tracts; labeling in nucleus laminaris (NL) is confined to the extreme caudolateral margin of the ipsilateral nucleus. Bar indicates 1.0 mm. Pointer indicates the level from which figures 4A and 4B were taken. Abbreviations as in figure 1.

had received an HRP injection 36 hours before sacrifice. This figure includes every 30-μm counterstained section through the entire extent of NM. HRP reaction product appeared throughout the extent of the cochlear nerve, nucleus angularis (NA), NM and in restricted portions of the descending and lateral vestibular nuclei. No reaction product was observed in NL on either side of the brain in any animal, with the exception of a small region of staining in the extreme caudolateral edge of the ipsilateral NL similar to what was observed in the silver-stained material after a 2-day post-lesion survival. In all cases, the reaction product in NM ended abruptly at the ventromedial boundary of the nucleus.

The absence of significant HRP reaction product in NL ipsilateral to the injection can be seen in the micrographs comprising figure 4. In figure 4A, a low-power view of the left side of the brain stem of animal 76-153, the pattern of HRP staining described above can be seen. In figure 4B, a higher-power view of the same section, the HRP reaction product is clearly confined to the cochlear nerve and NM; staining is absent in both dorsal cochlear tracts and in NL. Not every animal showed such a complete and diffuse staining pattern. In two cases, individual groups of fibers entering a discrete area of the cochlear and/or lagenar nuclei were heavily labeled, which allowed tracing of their course and easy observation of individual endings. Figure 5 shows this pattern of staining. A discretely-stained cochlear nerve fiber can be seen passing through NM, where it forms a characteristic endbulb on a large spherical NM neuron. Although there was variability between animals in the extent of HRP staining, the pattern of staining was essentially identical to that shown in figure 3; HRP reaction product was seen only in the ipsilateral cochlear nerve, NA, NM, and the lagenar projection areas—never in either NL. There was no difference in the pattern of staining among the 16-, 24- and 36-hour survival groups, although with longer survival times the reaction product is less intensely colored.

Tritiated proline injections

In the autoradiographic material at both survival times, heavy labeling was confined to the ipsilateral cochlear nerve, NA, NM and the lagenar projection areas of the vestibular nuclei. Light labeling was sometimes seen in the vestibular nerve and nuclei as well. The

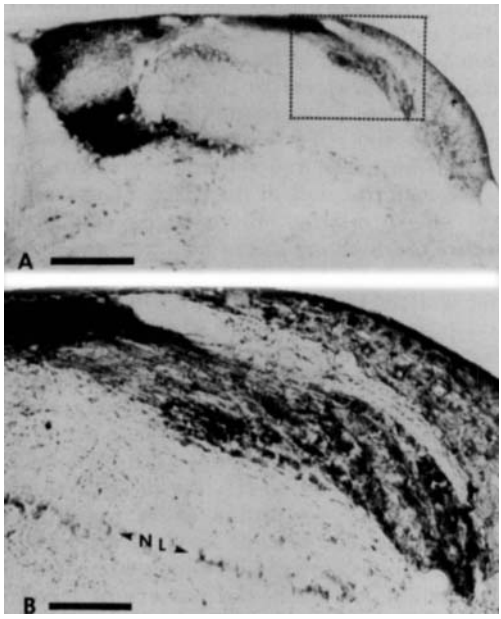

Fig. 4 Photomicrographs showing the distribution of HRP reaction product in the left brain stem of animal 76-153, 36 hours after injection of the enzyme into the left cochlea. A, low-power view of the section indicated by the pointer in figure 3. The dashed line shows the area enlarged in figure 4B. Lateral is to the left and the bar indicates 0.3 mm. B, higher-power view of the same section. The HRP reaction product follows the path of the medial branch of the cochlear nerve immediately beneath the fourth ventricle toward nucleus magnocellularis (NM). Note the absence of reaction product ventrad or mediad to NM or in nucleus laminaris (NL). Lateral is to the left and the bar indicates 0.1 mm. Graham and Karnovsky ('66) method; thionin counterstain.

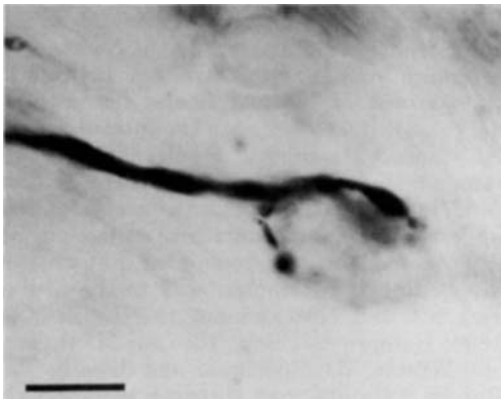

Fig. 5 High-power photomicrograph of nucleus magnocellularis in animal 76-154 to show a HRP-stained primary cochlear fiber forming a characteristic endbulb on a NM neuron. Lateral is to the left and the bar indicates 10 μm. Graham and Karnovsky ('66) method; thionin counterstain.

density of silver grains seen in NL at both survival times was determined by visual inspection to be much lower than that seen in NA and NM ipsilateral to the injection and not higher than "background" labeling. At these survival times there were no differences between the dorsal and ventral neuropil regions of NL on either side of the brain. These results are shown in the micrographs comprising figure 6, which are taken from representative sections in an animal which survived for 24 hours after the injection. Figure 6A shows a low-power dark-field view of a coronal section through the medulla and figures 6B-E give higher-power views of NA, NM and the ipsilateral and contralateral NL respectively. Heavy labeling is confined to the cochlear nerve and NM and does not extend into the dorsal cochlear tracts or to NL. Aside from the labeling in the vestibular nerve and nuclei, which probably results from labeled amino acid moving through the endolymph into the vestibular labyrinth, the pattern of labeling seen is very similar to that shown in figures 1 and 3.

DISCUSSION

A convincing demonstration that one structure is *not* innervated by another is difficult to achieve in any region of the central nervous system. The problem is further complicated in the avian brain stem auditory nuclei by two factors: (1) The proximity of the cochlear nerve, NM, and NL to each other, and (2) The potent "trophic" interactions which occur between the neurons in this system. It is probably the proximity of these auditory structures to one another which led Sanders ('29) to conclude from normal material that NL receives afferents from the ipsilateral nerve. In material stained for normal fibers, the cochlear nerve does pass very near the lateral edge of NL; indeed, the present results indicate that it may distribute a few endings to the extreme caudolateral margin of the ipsilateral laminar nucleus. Most investigators using normal fiber stains, however, concluded that primary afferents are distributed only to the ipsilateral NA and NM and not to NL (Brandis, 1894; Holmes, '03; Bok, '15; Levi-Montalcini, '49; Winter, '63; Rodriguez and Rebollo, '63). Ramón y Cajal's long research paper on the connections of the cochlear nuclei in birds (Ramón y Cajal, '08) stated clearly that NL does not receive direct cochlear afferents, although a later discussion of the same topic was more equivocal (Ramón y Cajal, '71, particularly fig. 363). Wallenberg (1898, 1900) and Boord and Rasmussen ('63), using experimental anatomical methods, concluded that the pigeon NL does not receive primary afferents. In the study of Wold and Hall ('75), 8 of 13 adult chickens which received unilateral cochlear nerve lesions displayed terminal degeneration bilaterally in the rostral two-thirds of NL (as well as in NM and NA) within four to seven days after surgery; the best material was provided by 6- to 7-day survival periods (their cases "H-12" and "H-20"). The authors interpreted these eight cases as being due to interruption of a primary cochlear projection which passes through NM to join both dorsal cochlear tracts in innervating the laminar nuclei. To account for the five cases (with postoperative survival times of 3-8 days) in which degeneration was confined to the ipsilateral NA and NM, Wold and Hall ('75) suggest that

> "the cochlear ganglion cells projecting to the nucleus laminaris are apparently situated in other parts of he ganglion than the cells projecting to the nucleus angularis and magnocellularis." (p. 75).

In the five negative cases, it was supposed by these authors, the "special" ganglion cells innervating NL were not destroyed. Wold and Hall ('75) went on to suggest that these "special" ganglion cells might lie with those of the lagenar ganglion or indeed might be the lagenar ganglion cells themselves. Wold and Hall specifically denied the possibility that their results were due to accidental lesions of the brain stem or to transneuronal degeneration. Because the evidence for the hypothe-

Fig. 6 To show the pattern of autoradiographic labeling seen in the brain stem 24 hours after an injection of 2 μCi of tritiated proline into the left basilar papilla.

A Low-power darkfield view of a 6μm coronal section. Heavy labeling is seen in the ipsilateral nucleus angularis (B) and nucleus magnocellularis (C), but not in the ipsilateral (D) or contralateral (E) laminar nuclei. Light labeling is seen in the ipsilateral vestibular nuclei. Bar indicates 0.5 mm.

B High-power lightfield view of nucleus angularis ipsilateral to the proline injection. Dense autoradiographic silver grains appear around the angularis neurons. Bar indicates 20 μm; thionin counterstain.

C High-power lightfield view of nucleus magnocellularis ipsilateral to the proline injection. Dense autoradiographic silver grains appear around the neurons. Magnification as in figure 6B; thionin counterstain.

D High-power lightfield view of nucleus laminaris ipsilateral to the proline injection. No dense silver grains are seen on either side of the cell lamina. Magnification as in figure 6B; thionin counterstain.

E High-power lightfield view of nucleus laminaris contralateral to the injection. No dense silver grains are seen on either side of the cell lamina. Magnification as in figure B; thionin counterstain.

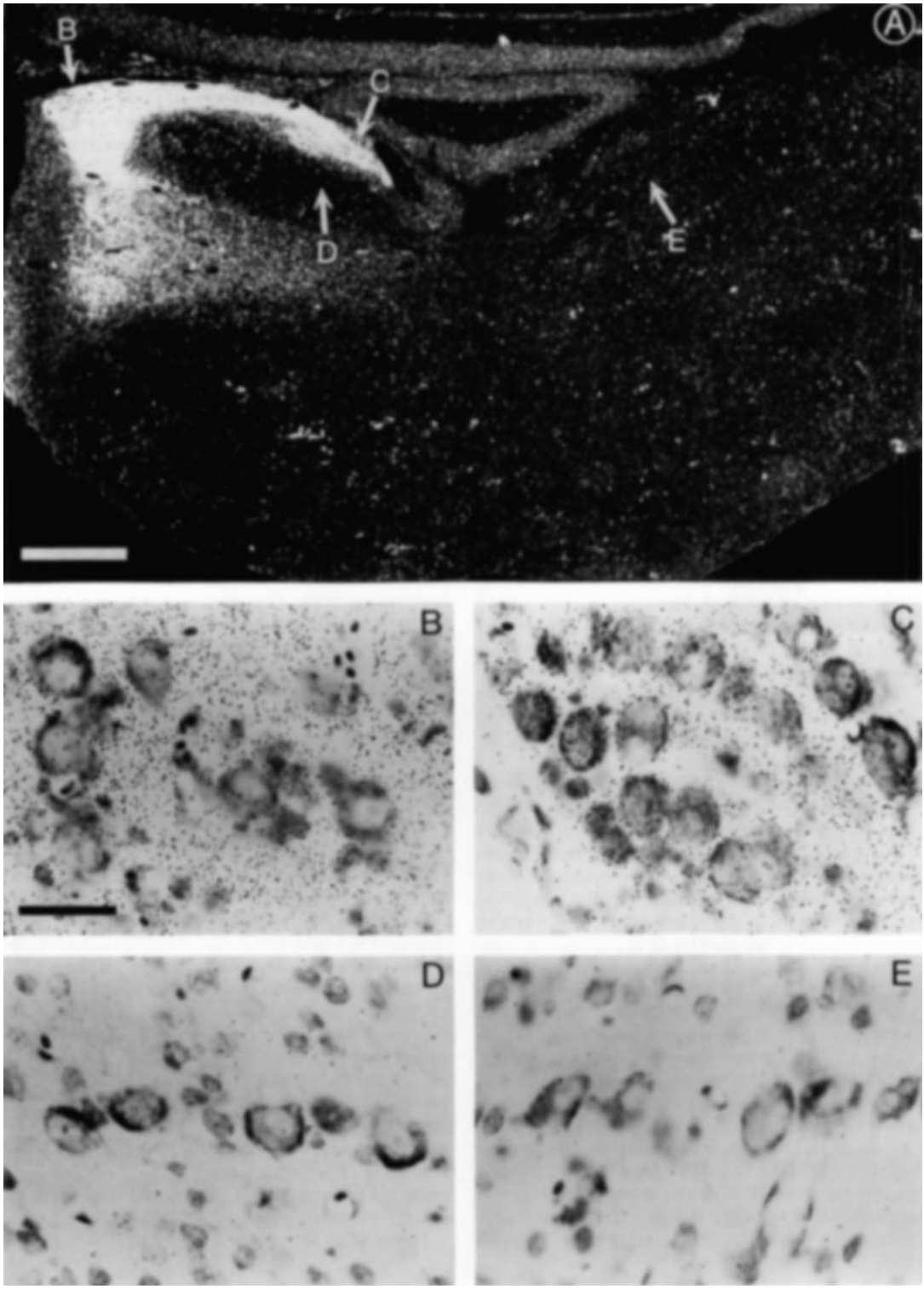

Figure 6

sized "special" ganglion cells is entirely indirect, it is only other indirect evidence that can be offered to refute their existence. And, of course, the characteristics of these hypothetical "special" cells could be altered in an *ad hoc* fashion to counter any such attempt at refutation. Nevertheless, several plausible lines of evidence appear to contradict the conclusions of Wold and Hall ('75).

Boord and Karten ('74) have described degeneration patterns in the brain stem following destruction of the lagenar nerve in pigeons; no degeneration was seen in NL. If there are "special" ganglion cells innervating NL, they most probably do not lie with the lagenar ganglion. If the cells projecting to NL were located among the other neurons innervating the cochlea, they would be expected to be organized in a topographic fashion, since Boord and Rasmussen ('63) have reported a topographic cochlear projection onto NA and NM in the pigeon. Moreover, since Parks and Rubel ('75) have shown a strict topographic projection of NM onto each NL, the supposed special ganglion cells would be expected to have their proximal and distal processes and cell bodies organized topographically along the cochlea and eighth nerve. If this were the case, it is difficult to imagine why the HRP and tritiated proline injections with the survival times used here would fail to produce any transport of enzyme or amino acid to NL, even though all portions of NA and NM are labeled in some animals.

The findings of both Winkler ('07) and Wold and Hall ('75) are probably best explained as the result of transneuronal degeneration of neurons in NM and their processes in NL. This conclusion is supported by the results of Jackson and Rubel ('76), who showed that removal of the cochlea in chickens from five days to three years of age results in a 25% loss of neurons and severe cell shrinkage in the remaining NM neurons by five days following surgery. The findings in the present study that silver-stained degeneration was confined to NA and NM at two days following interruption of the cochlear nerve provide further corroboration. By the fifth post-operative day, however, degeneration in the present study was seen in both dorsal cochlear tracts and bilaterally in NL. It might be argued that the supposed primary fibers innervating NL are of significantly smaller caliber than those projecting to NA and NM. In that case, the primary projections to NL might be expected to degenerate later than other axons in the cochlear nerve (Guillery, '70). This argument has little support. Boord ('69) has reported that 98% of the cochlear nerve fibers in the pigeon have diameters of 1-3 μm, while only 1% have diameters under 1 μm. In addition, a previous study on young chickens (Parks and Rubel, '75) found that 24- to 48-hour survival times gave maximal Fink-Heimer staining of degenerating endings in NL following lesions in NM. As a final argument against the possibility of primary innervation of NL, it can be noted that in those mammals and reptiles where the primary auditory afferent projection has been studied, NL or its probable mammalian homologue (the medial superior olive) do not receive primary endings (e.g., van Noort, '69; Osen, '70; Webster, '71; Cohen, Brawer and Morest, '72; Moskowitz and Liu, '72; Leake, '74; DeFina and Webster, '74).

Several lines of evidence are thus seen to converge on the conclusion that primary auditory fibers do not innervate NL in the chicken, except for a very small projection to the extreme caudolateral division of the ipsilateral nucleus. The contrary opinion of one early student of the avian auditory system (Sanders, '29) may have been due to the presence of these few cochlear fibers in the lateral edge of the ipsilateral NL. The conclusions of both Winkler ('07) and Wold and Hall ('75) are probably best explained as the result of rapid transneuronal degeneration in NM and its projection to NL, as seen by Jackson and Rubel ('76).

Transport of tritiated proline and HRP

This is the first report that radioactive amino acid introduced into the inner ear is incorporated by cochlear ganglion cells and transported into the brain stem. This result is not surprising, however, since orthograde transport of labeled amino acids is widely used to trace connections in the central nervous system (Cowan and Cuénod, '75). It should be noted that while we see no sign of transport to NL at the survival times used here (6- and 24-hour), with longer survival times (36- to 72-hours), we have observed a marked increase in grain density over NM cell bodies and differential labeling of the dorsal and ventral neuropil regions of NL bilaterally (Smith and Rubel, unpublished observations).

In the only previous report of HRP injection

into a peripheral auditory structure (Warr, '75), the enzyme was taken up and transported in a retrograde fashion to the neurons in the brain stem which give rise to the olivocochlear tract. No retrograde transport of this type was observed in the present study, although efferent innervation of the avian basilar papilla has been established (Takasaka and Smith, '71). The reason for this discrepancy is unclear but may involve the manner in which HRP was incorporated into cochlear ganglion cells. Three types of HRP transport have been reported: granular retrograde (Winer, '77), granular orthograde (e.g., Colman et al., '76) and "diffuse" orthograde/retrograde (e.g., Adams and Warr, '76). The HRP reaction product observed in the present study is of this last type—a diffuse dense staining which, at magnifications up to × 1,250, appears agranular. According to current notions, this pattern of staining results from diffusion of HRP into damaged cells followed by transport of the enzyme throughout the cell. Thus, it may be that trauma associated with the injection procedure in this study damaged the cochlear ganglion cells or their processes and allowed the HRP to be incorporated and transported in an orthograde fashion. It is also possible, however, that the enzyme is taken up into the intact distal processes of the ganglion cells, transported back into the cell body and then into the brain. Resolution of this issue would require study of the cochlea after HRP injection.

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Note added in Proof: Since the time of this investigation and our initial report of these findings (T. N. Parks and E. W Rubel, 1977 Anat. Rec., *187:* 677 abstract), several reports have demonstrated similar orthograde transport and transneuronal transport of tritiated amino acids introduced into the mammalian cochlea (e.g., E. S. Kane, 1977 Am. J. Anat., *150:* 641-652; J. H. Cassedy and D. R. Jones, 1977 Soc.Neursci. Abs., *3:* 4; M. S. Silverman et al. 1977 Soc. Neurosci. Abs., *3:* 11).