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GABA-like and glycine-like immunoreactivities of the cochlear root nucleus in rat

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Summary

The cochlear root nucleus is part of the cochlear nuclear complex in small rodents. Its cells, the large root neurons, have a superficial resemblance to the globular neurons of the ventral cochlear nucleus. It has been a matter of debate, therefore, whether the root neurons and globular neurons represent the same or different types of cell. In the present study the two cell types with adjacent neuropil structures were compared by light microscopic, postembedding immunocytochemistry. Pairs of 0.5 μm sections of resin-embedded, glutaraldehyde-fixed material were treated with purified antisera raised against GABA- and glycine-glutaraldehyde-protein conjugates, respectively. Both types of cell were found to be immunonegative. Striking differences, however, occurred in what was interpreted as afferent nerve terminals. The globular cells appeared to receive numerous afferents with GABA- or glycine-like immunoreactivity on their somata. Immunoreactive terminals on the root neurons, on the contrary, were mostly GABA-positive and located on the dendrites. Although of unknown origin, the immunoreactive afferents were clearly different from the primary fibres as demonstrated both by the immunonegativity of the latter and by the different size and distribution of the terminals labelled anterogradely after horseradish peroxidase injections into the spiral ganglion.

Introduction

In rat (Harrison & Warr, 1962; Harrison & Irving 1965, 1966a, b; Ross & Burkel, 1971), gerbil (Chamberlain, 1977), and mouse (Webster & Trune, 1982) the cochlear nerve root contains a particular type of large neuron classified by Harrison's group as 'b cells' and collectively referred to by them as 'the acoustic nerve nucleus'. A detailed anatomical study of these cells in rat, based on Golgi impregnation and electron microscopy, was recently published by Merchan and co-workers (1988); because of the location of the cells central to the glial-Schwann cell border, they changed the nomenclature to 'the cochlear root neurons' and 'the cochlear root nucleus', respectively. We have adopted their terminology.

Because of a superficial resemblance to the globular cells of the ventral cochlear nucleus (VCN), the root neurons have been classified as such by some authors (Webster & Trune, 1982). According to Merchan and co-workers (1988), however, the two cell types differ significantly both in soma size, dendritic pattern, and amount and types of axosomatic afferents. In rat, as in other mammals (e.g. cat, Tolbert & Morest, 1982b), the somata of globular cells are studded with various types of boutons containing rounded, flattened or pleomorphic vesicles. The root neurons are less densely covered with boutons, and amongst those present, only terminals with rounded synaptic vesicles have been reported.

The present study was undertaken in order to test by light microscopic, postembedding immunocytochemistry whether the root neurons and the globular neurons differ also with respect to putative GABA-ergic and glycinergic afferents. In addition the distribution of primary afferents on the two cell types was compared following anterograde horseradish peroxidase (HRP) labelling from the spiral ganglion. Preliminary results of the latter experiments have been published in abstract form (Lopez *et al.*, 1989).

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Materials and methods

Immunocytochemistry

Three female Wistar rats, 200–300 g body weight, were anaesthetized with pentobarbital 50 mg kg^{-1} intraperitoneally, and perfused through the heart with a brief flush of 2% dextran (MW 70 000) in 0.1 M sodium phosphate buffer (PB), pH 7.4, followed by 750 ml 2.5% glutaraldehyde and 1% paraformaldehyde in PB. The perfusion was started within a few sec after opening the thorax, and the neck was stiff within 2 min. Immediately after the perfusion, the tympanic bulla were opened on both sides and the cochlear nerves detached from the petrous bones by dissection of the cochlea. The brain with the cochlear nerves was then removed from the skull and left in 0.1% glutaraldehyde in PB overnight.

The cochlear nuclear complex with the cochlear nerve root was sliced by hand, either parallel to or across the long axis of the root, i.e. in a modified parasagittal or horizontal plane, respectively. The slices were rinsed in PB, treated with 1% OsO_4 in PB for 45 min, dehydrated in ethanol and propylenoxide, and embedded in epoxy resin (Durcupan ACM, Fluka).

Serial 0.5 μm sections were cut on an ultramicrotome and mounted on gelatinized glass slides. Root neurons and globular neurons occurred in the same parasagittal section, but not in the same horizontal section. To make possible a comparison of identically-treated samples of the two cell types, in the latter cases separate sections of the cochlear root and the VCN were mounted together on the same slide. On each slide was also mounted a 0.5 μm test section of a resin-embedded 'sandwich' composed of one layer of glutaraldehyde- rat brain macromolecular conjugate and six layers of different amino acid-glutaraldehyde-rat brain macromolecule conjugates, including conjugates prepared from GABA and glycine (Gly) (Ottersen, 1987).

One or more sections of each series were stained with 0.1% Toluidine Blue in 1% sodium borate. Other sections were treated according to an immunocytochemical procedure based on that of Somogyi and co-workers (1984) and described in detail elsewhere (Ottersen, 1987; Ottersen *et al.*, 1988). This involves immersion of the mounted sections in (1) sodium ethanolate to remove the resin; and (2) sodium periodate to alleviate the masking effect of osmium; followed by sequential incubations in (3) 20% normal swine serum (NSS) in 0.1 M Tris-phosphate-buffered saline, pH 7.4 (TPBS); (4) purified GABA antiserum 25 or Gly antiserum 31, diluted 1:100 and 1:30, respectively, in TPBS containing 1% NSS (overnight at 4° C); (5) swine anti-rabbit IgG (Dakopatts) diluted 1:100 in TPBS with 1% NSS; (6) rabbit peroxidase-antiperoxidase complex (Dakopatts) diluted 1:500 in TPBS with 1% NSS; and (7) 0.05% diaminobenzidine/0.01% H_2O_2. The staining was intensified by treating the sections with 0.003% OsO_4 in PB for 1–7 min. The sections were thoroughly rinsed between each step.

The primary antisera had been raised in rabbit against GABA- or Gly-glutaraldehyde-protein conjugates and purified as described in previous reports (Storm-Mathisen *et al.*, 1983; Ottersen & Storm-Mathisen, 1984b; Dale *et al.*, 1986; Ottersen *et al.*, 1988). The test sandwich revealed no significant signs of cross-reactivities of the antisera with other amino acid-glutaraldehyde-brain protein conjugates. A slight reduction in background staining was nevertheless obtained by the addition, in step 4, of soluble glutaraldehyde complexes of amino acids other than that against which the serum was raised [of glutamate (Glu-G) and Gly (Gly-G) in the case of GABA antiserum, and of β-alanine (β-Ala-G) and GABA (GABA-G) in the case of Gly antiserum]. The addition of similar complexes of the amino acid against which the serum was raised (GABA-G and Gly-G, respectively) was used for adsorption control. In order to examine the GABA-like and Gly-like immunoreactivities (GABA-LI and Gly-LI) of the same neuronal elements and to relate the findings to the normal fibre- and cytoarchitecture, series of consecutive sections were treated as follows: (1) Toluidine Blue; (2) GABA antiserum + 300 μM Glu-G; (3) Gly antiserum + 300 μM β-Ala-G; (4) As 2 + 300 μM Gly-G; (5) as 3 + 300 μM GABA-G; (6) as 2 + 300 μM GABA-G; (7) as 3 + 300 μM Gly-G. The adsorption controls (6 and 7) showed no immunoreaction in tissue elements or conjugates.

HRP-injections

Nine female Wistar rats, 120–150 g body weight, were anaesthetized with a mixture of 10% ketamine hydrochloride (4 parts) and Rompun (Bayer, 3 parts), 1 ml kg^{-1}. After opening the tympanic bulla, a small orifice was made in the lateral wall of the cochlea and the underlying bony spiral lamina at various apicobasal levels. A 20% aqueous solution of HRP (type Sigma VI) was injected into the spiral ganglion through a glass micropipette, using a microinjector (Eppendorf 5242, Zeiss). To avoid reflux and dilution of the tracer, a second micropipette was placed in the oval window for removal of fluid. The injection lasted 20–30 min. In four animals the HRP was injected into the spiral ganglion in the basal coil, in two in the middle coil, and in three in the apical coil.

After 48 h survival the animals were perfused with pentobarbital anaesthesia in 200 ml saline followed by 500 ml 1.25% glutaraldehyde and 1% paraformaldehyde in PB and then with 10% sucrose in PB. The cochlear nuclear complex with the cochlear nerve root was sectioned parasagittally on a freezing microtome at 40 μm and recovered in PB. To visualize the HRP the sections were reacted for 20–60 min in 0.015% diaminobenzidine, 0.4% nickel ammonium sulphate, and 0.006% H_2O_2 in 0.1 M Tris buffer, pH 8 (modified after Hancock, 1984). The sections were washed in Tris buffer, dehydrated, cleared and mounted in Entellan (Merck).

Results

Toluidine Blue staining

The root neurons and globular neurons were easily distinguished in Toluidine Blue sections because of their typical location and structure. As indicated in Fig. 1, the two cell types were found to occupy adjacent but separate territories of the cochlear nuclear complex. The globular neurons were situated in the central region of the VCN, including the proximal (intranuclear) part of the nerve root. In the latter site they formed small, separate cell clusters. The cell

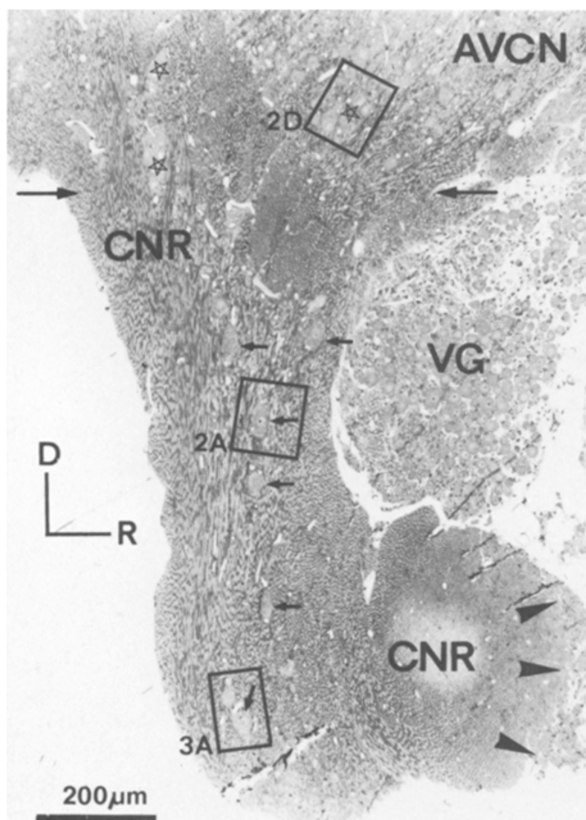

Fig. 1. Survey picture of Toluidine Blue-stained parasagittal section through the cochlear nerve root (CNR) with the glial-Schwann cell border (arrowheads), the AVCN, and the vestibular ganglion (VG). The two large horizontal arrows indicate the border between the intra- and extranuclear parts of the root. Root neurons are indicated by small arrows, groups of globular cells by open stars. The boxed areas are shown at higher magnification in Figs 2 and 3. D, dorsal; R, rostral.

bodies were medium-sized and ovoid with the longest diameter about 20 μm. They were characterized by a finely dispersed Nissl material and an eccentrically located, often round nucleus (Fig. 2D). They were surrounded by an abundant neuropil with numerous small myelinated fibres.

The root neurons formed incomplete rows in the relatively long extranuclear portion of the root. The most distal cells were situated near the glial-Schwann cell border, close to the base of the cochlea (Figs 1 and 4A). The perikarya were large and ovoid with the longest diameter about 35 μm. They contained distinct Nissl bodies and an eccentrically located, typically infolded nucleus (Figs 2A and 3A). At least in the middle part of the root, the cells were oriented with their long axes parallel to the cochlear fibres. The two sets of thick dendrites, parallel and perpendicular, were easily distinguished. Parallel dendrites emerged from the pointed ends of the cells and, as the name implies, coursed parallel to the cochlear fibres. Perpendicular dendrites emerged from the cell body across the direction of the cochlear fibres and were more easily appreciated in the most distal part of the root (Fig. 4A). Isolated segments of the dendrites could also be identified in between the cochlear fibres at a distance from the cell body (Fig. 2A). The root neurons were separated from the surrounding myelinated cochlear stem fibres by a narrow, but distinct rim of neuropil. This contained a few small myelinated fibres. Similar fibres appeared to climb the primary dendrites.

A small number of very small neurons, each with a deeply indented nucleus and a narrow rim of cytoplasm without distinct Nissl granules occurred scattered in between the other cells both in the root (Fig. 4A) and in the VCN proper (Fig. 2D). The most distal part of the root also contained occasional medium-sized cells that could be 'displaced' globular cells (Gl in Fig. 3A) (see below).

Immunostaining

The immunoreactions created highly selective staining patterns. The chemical specificity was confirmed by the staining of the test conjugates (Fig. 4B and C, insets) and by the adsorption controls. The globular neurons and root neurons appeared unstained with both antisera. A striking difference was found, however, with respect to the amount of immunoreactive puncta, presumably boutons, on the somata of the two cell types.

The somata of globular neurons were apposed by numerous GABA-LI- and Gly-LI-positive puncta (Fig. 2E and F). The two kinds of puncta had clearly different distributions, and possible examples of colocalization were found only occasionally (small arrows in Fig. 2E and F). Globular cell dendrites were not distinguishable, but the many puncta in the neuropil, if not fibres, could be terminals related to dendrites either of these or of intermingled multipolar and small cells.

In contrast to the globular neurons, the root neurons showed only occasional GABA-LI- and Gly-LI-positive puncta along their soma perimeter (Fig. 2B and C). A moderate number of mainly GABA-LI-positive puncta, however, could be observed on their dendrites (Fig. 4B and C). Judged by their location, some of the puncta might colocalize GABA-LI and Gly-LI (small arrows in Figs 3B and C, and 4B and C), but convincing evidence of colocalization was not found.

On the whole, the root contrasted sharply with the adjacent VCN due to its scarcity of GABA-LI- and Gly-LI-positive structures. Only a few stained fibres were seen in between the immunonegative cochlear

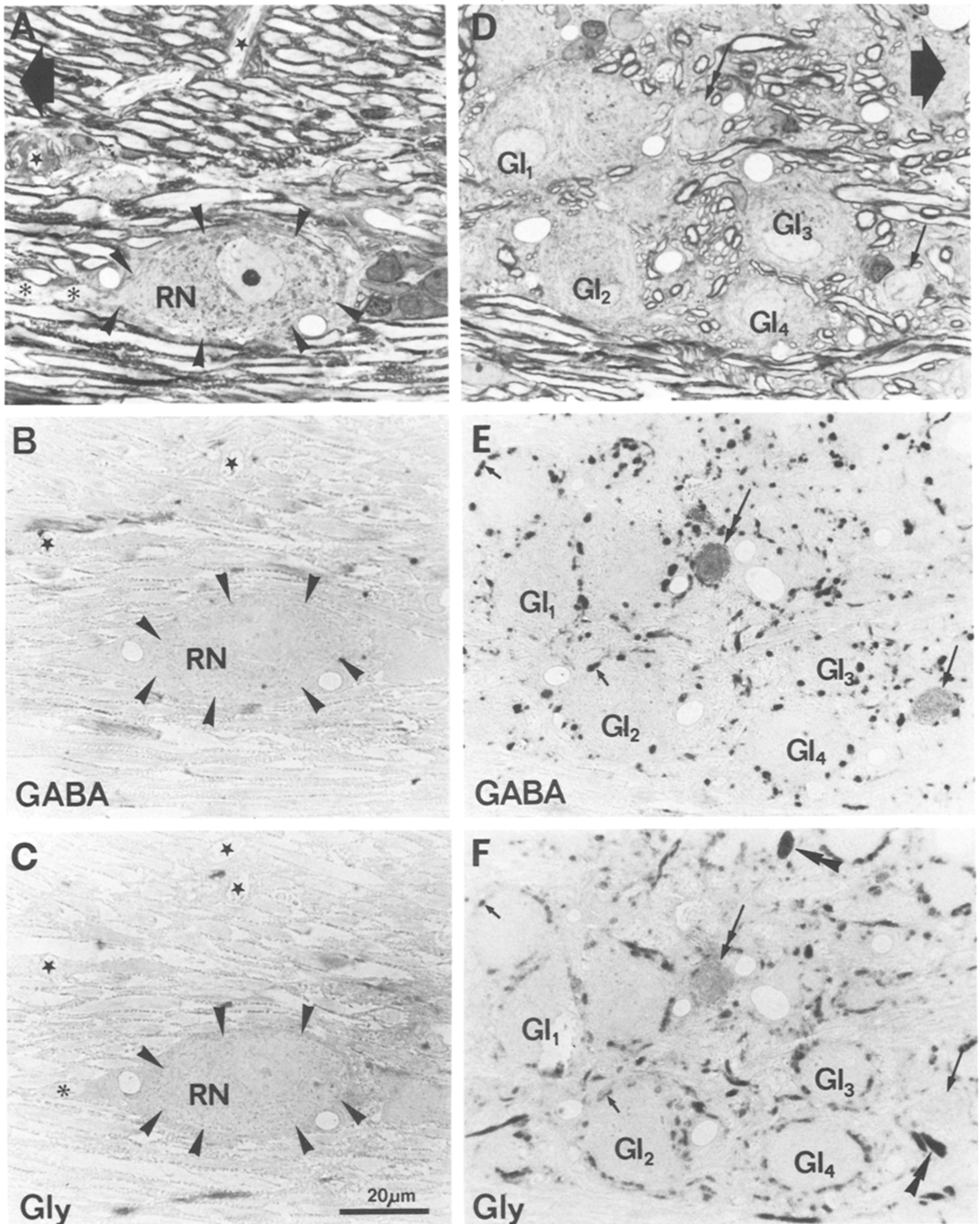

Fig. 2. Photomicrographs from three 0.5 μm parasagittal sections through the cochlear nerve root and the AVCN. (A) and (D) From the same Toluidine Blue-stained section as Fig. 1 (see boxed areas) show a root neuron (RN) and a group of globular neurons (Gl_{1-4}), respectively. (B) and (E) Corresponding cells from one section reacted with GABA antiserum plus Glu-G. (C) and (F) Another section reacted with Gly antiserum plus β-Ala-G. The GABA and Gly sections are adjacent to each other, the Toluidine Blue section is 3 μm apart. Note differences in soma size and in number of immunoreactive puncta around the two types of cell. Two small cells are intermingled with the globular cells, one (double arrow) shows colocalization of GABA-LI and Gly-LI, the other (single arrow) shows GABA-LI only. The soma perimeter of the root neuron is indicated by single arrowheads, parallel dendrites by asterisks, perpendicular dendrites by stars, possible double-labelled puncta by small arrows, and Gly-LI-positive axons by double arrowheads. Large arrows in A and D point dorsally. Scale bar in C is valid for A–F.

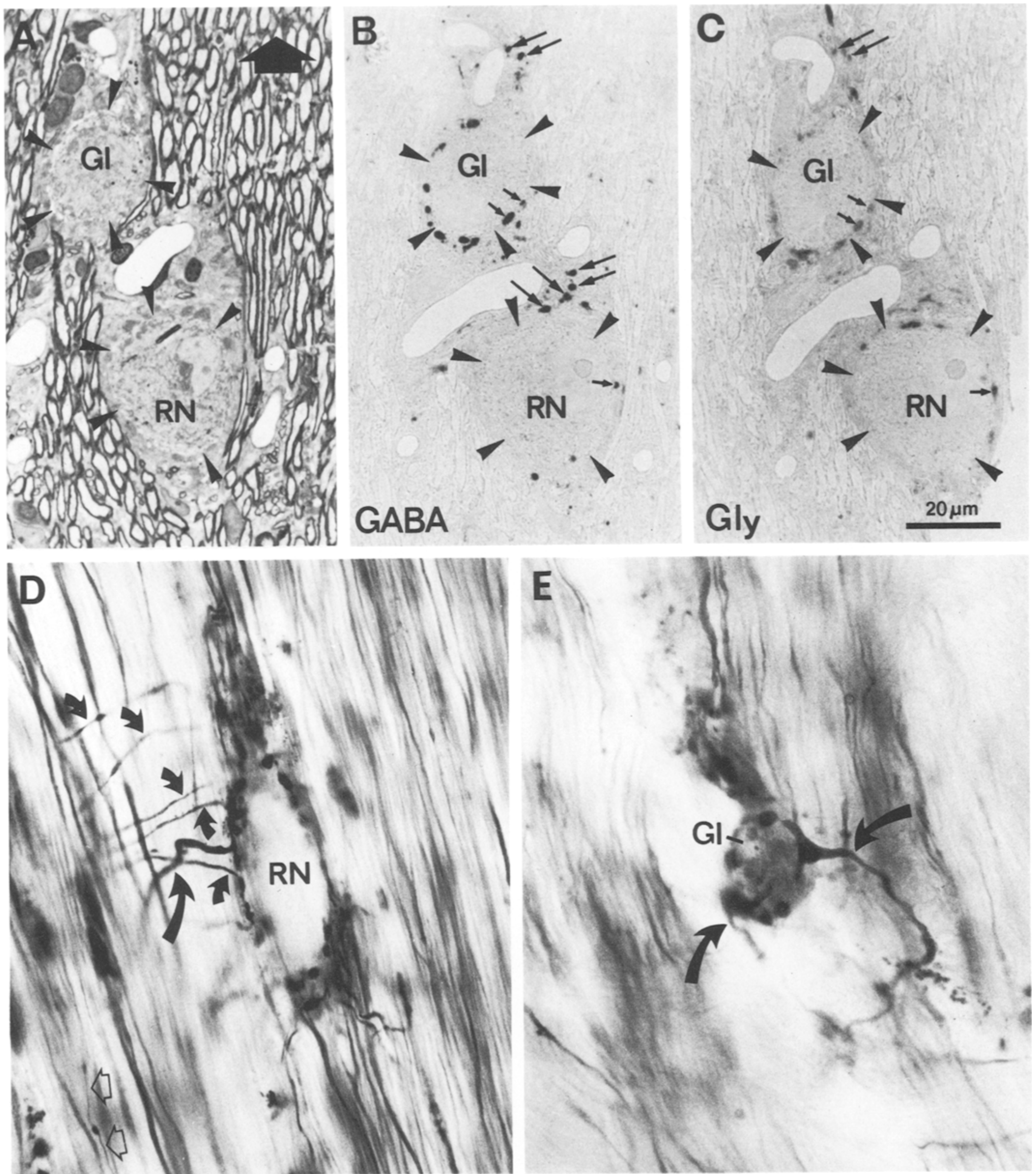

Fig. 3. (A–C) Photomicrographs of two cells in the distal part of the root (boxed area in Fig. 1) from the same three sections as Fig. 2. The larger cell (RN) is a typical root neuron with an infolded nuclear membrane and only occasional immunoreactive puncta close to the soma perimeter. The smaller cell (Gl), cut through its nucleus in B and C, has several immunoreactive puncta at the soma surface and may be a globular cell. Possible double-labelled puncta on the somata are marked by small arrows, puncta of any type in the neuropil by medium-sized arrows, and soma perimeters by arrowheads. Large arrow in A points dorsally. (D and E) From a 40 µm DAB/nickel-reacted section following HRP injections into the basal coil of the spiral ganglion. They show a root neuron (from the extranuclear part of the root) and a globular neuron (from the intranuclear part of the root), respectively. The labelled cochlear fibres give off both thick (large curved arrows) and thin (small curved arrows) collaterals. Note the different size of the terminals on the two types of cell. Open arrows point to a very thin beaded axon. Scale bar in C is valid for A–E.

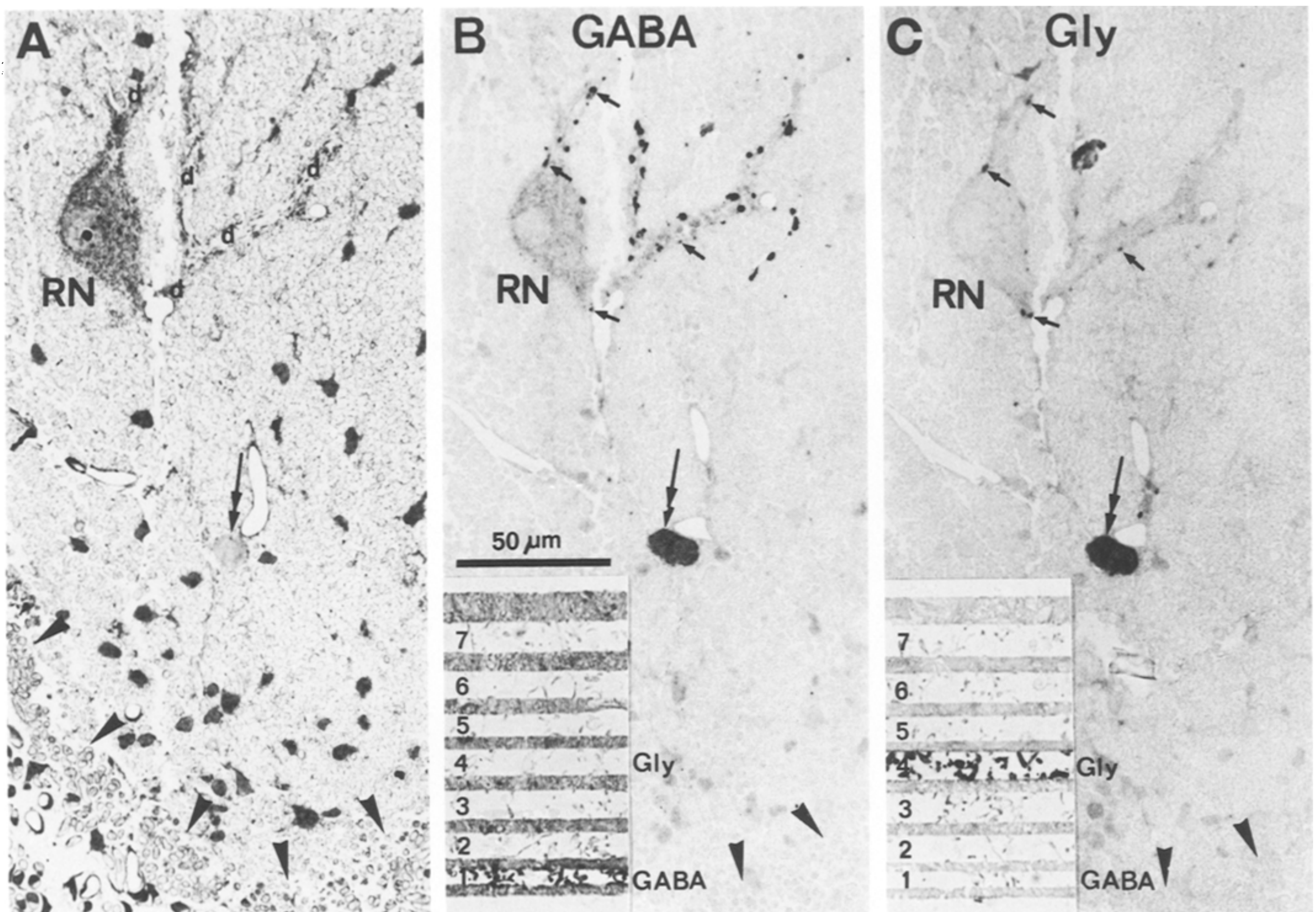

Fig. 4. Photomicrographs from three adjacent horizontal sections through the most distal part of the cochlear nerve root. (A) Stained with Toluidine Blue (after etching). (B) Treated with GABA antiserum. (C) With Gly antiserum. A root neuron (RN) and a small neuron (double arrow) are seen close to the glial-Schwann cell border (arrowheads). The root neuron has a number of GABA-LI- and a small number of Gly-LI-positive puncta on their dendrites (d). Only very few puncta of either type appear on the soma. Possible double-labelled puncta are marked by small arrows. The small neuron shows colocalization of GABA-LI and Gly-LI. Insets in B and C show the test 'sandwich' with conjugates of: 1, GABA; 2, glutamate; 3, taurine; 4, glycine; 5, amino acid-free control (*i.e.* glutaraldehyde-treated brain macromolecules); 6, aspartate; 7, glutamine, spaced by sections of brain tissue and showing positive reactions only in the GABA and Gly bands, respectively. Scale bar in B is valid for A–C.

fibres. Scattered GABA-LI- and Gly-LI-positive puncta in the rim of neuropil around the root neurons (medium-sized arrows in Fig. 3B and C) could perhaps be boutons supplying the dendritic plexus described by Merchan and co-workers (1988) in this location. Scattered puncta elsewhere in the root could relate to isolated dendritic segments. The few medium-sized neurons in the distal part of the root (Gl in Fig. 3B and C) resembled globular cells in having unstained somata surrounded by several GABA-LI- and Gly-LI-positive puncta. The somata of the small neurons in the root and the VCN stained either with both antisera (double arrow in Figs 2E and F, 4B and C) or with one of them (single medium-sized arrow in Fig. 2E and F). Occasional immunoreactive puncta were found at their surface.

HRP labelling

Following cochlear HRP injections, anterogradely labelled axosomatic terminals were found both on root neurons and globular neurons. The pattern of termination, however, was significantly different on the two cell types (Fig. 3D and E). Anterogradely labelled terminals on globular cells could always be found somewhere in the nucleus depending upon the site of injection in the spiral ganglion. Each cell appeared to receive a small number of relatively coarse fibres or fibre collaterals each giving rise to a number of large

axosomatic terminals (Fig. 3E). Labelled terminals on the root neurons, in contrast, were found only after labelling of the basal coil fibres. Collaterals (50–80 μm long) of various diameters were seen to emerge at right angles from the stem fibres and to end on the somata and the primary parallel dendrites as smaller terminal knobs (Fig. 3D). The somata of the root neurons were all confined to the region of the basal coil fibres. Although the perpendicular dendrites may span the entire cross-section of the root (Merchan *et al.*, 1988), no evidence was found for an input from the middle and apical coil fibres.

Discussion

The present study has revealed a striking difference between the globular neurons and the root neurons with respect to the number of GABA-LI- and Gly-LI-positive puncta along their soma surface. Although the fine structure of these puncta remains to be shown, it is reasonable to suggest that they are nerve terminals. The globular neurons, thus, appear to receive numerous GABA-LI- and Gly-LI-positive boutons on their somata. The immunoreactive terminals on the root neurons, on the other hand, are mainly GABA-LI-positive and located on the dendrites. These findings contribute additional evidence to the distinction of the root neurons as a particular type of cell, as advocated by Harrison and Warr (1962), Harrison and Irving (1965, 1966a, b), and Merchan and co-workers (1988).

Although the cochlear root nucleus does not merely represent a group of ectopic globular cells, as proposed by Webster and Trune (1982), a few cells of this category may actually occur in between the typical root neurons. This notion is compatible with Ross and Burkel's (1971) electron microscopic observation of two size categories of cell in the rat cochlear root, a large and a medium-sized. In their Plate 1, the latter category resembles globular cells in receiving very large axosomatic terminals that might represent primary afferents. The amount of neurons in the cochlear nerve root varies between species, but for instance in cat, which seems to lack root neurons, typical globular neurons are encountered close to the glial-Schwann cell border. In the following, the distinguishing criteria of globular neurons and root neurons, including their afferent and efferent connections, will be discussed.

The globular cells, also called globular bushy cells (Cant & Morest, 1984) because of their appearance in Golgi sections (Brawer *et al.*, 1974), have been observed in a variety of mammals including rat (Harrison & Warr, 1962) and cat (Osen, 1969; Brawer *et al.*, 1974; Tolbert & Morest, 1982a, b). The large number of GABA-LI- and Gly-LI-positive axosomatic boutons on these cells in rat corroborates previous immunocytochemical studies in other species using antibodies to glutamate decarboxylase (GAD) (Adams & Mugnaini, 1987; Saint Marie *et al.*, 1989), GABA (Ottersen & Storm-Mathisen, 1984a; Wenthold *et al.*, 1986), Gly (Wenthold *et al.*, 1987), and glycine receptor (Wenthold *et al.*, 1988).

Electron microscopically, GAD and GABA immunoreactivities have been demonstrated in certain VCN boutons containing pleomorphic synaptic vesicles (Wenthold *et al.*, 1986; Adams & Mugnaini, 1987; Oberdorfer *et al.*, 1988), while glycine receptor immunoreactivity has been found postsynaptic to boutons both with flattened and pleomorphic vesicles (Wenthold *et al.*, 1988). Although the relation between vesicle contents and shape is still disputed, it is tempting to believe that in the case of the VCN globular cells, the GABA-LI- and Gly-LI-positive puncta correspond at least to some of the numerous boutons with pleomorphic or flattened vesicles found by conventional electron microscopy (Tolbert & Morest, 1982b; Merchan *et al.*, 1988). The large size of the primary fibre terminals on the globular cells shown in the present HRP material also agrees with these investigations as well as with previous silver studies (Harrison & Warr, 1962).

The root neurons have so far been observed only in small rodents. In rat the somata are confined to the high frequency portion of the root. Judged by Chamberlain's (1977) illustrations (his fig. 4), this may be the case also in the Mongolian gerbil. In that species, however, the root neurons appear restricted to the most distal part of the root and to have perpendicular dendrites only. Also, in our material such dendrites seemed more prominent, or were more easily recognized in the distal part of the root where the basal coil fibres have a more spiraled course.

Merchan and co-workers (1988) found that only about 40% of the soma perimeter of the root neurons were covered with boutons, 30% of the perimeter with boutons resembling primary afferents. The corresponding values for globular cells were about 80% and 50%, respectively. The non-primary boutons on the root neurons, which together covered about 10% of the soma perimeter, were grouped in three classes, all with rounded synaptic vesicles. Although a minor population of boutons with non-round vesicles might have been overlooked, the findings are in close agreement with the presently demonstrated preponderance of primary afferents and the scarcity of GABA-LI- and Gly-LI-positive boutons on the somata of the root neurons. The small size of the primary terminals, as demonstrated in the present HRP materials, also accords with the original silver studies (Harrison & Warr, 1962).

GAD-immunoreactive puncta on the somata and proximal dendrites of 'isolated' cells in the cochlear nerve root of rat have been described by Moore and

Moore (1987, their fig. 2B). The somewhat higher frequency of stained terminals in their illustrations may be ascribed to the larger thickness of sections (30 μm against our 0.5 μm). According to Merchan and co-workers (1988), the smallest boutons on the root neurons are about 1 μm in diameter. In our sections, therefore, the number of boutons around the perimeter of each cell must be close to that registered in ultrathin sections.

The origin of the immunoreactive afferents remains unknown. It seems reasonable to suggest that the scattered immunoreactive fibres in the root are heading for the root neurons. Since the primary afferents are immunonegative, such fibres must originate either in the cochlear nuclear complex or in more central parts of the brainstem. Some fibres could originate locally in the single- or double-labelled small cells. The VCN, however, also receives a massive input of Gly-LI- and GABA-LI-positive fibres from the dorsal cochlear nucleus and the higher auditory centres (Potashner *et al.*, 1985; Adams & Wenthold, 1987; Godfrey *et al.*, 1988; Staatz-Benson & Potashner, 1988; Osen *et al.*, 1990). The specific sites of origin and termination of these fibres require further studies.

The central projections of the globular neurons and the root neurons also differ. The main target of the globular neurons is the contralateral nucleus of the trapezoid body (Harrison & Warr, 1962; Tolbert *et al.*, 1982), which in turn provides a strychnine-sensitive glycinergic input to the lateral superior olive (Moore & Caspary, 1983; Wenthold *et al.*, 1987). This pathway is presumably related to localization of sound in space.

The axons of the root neurons also project centrally along the trapezoid body (Harrison & Warr, 1962; Merchan *et al.*, 1988), but their exact site of termination remains to be defined. It may be somewhere at the midbrain level on the contralateral side (Cannon & Giesler, 1978; Willard & Ryugo, 1983). The distal location of the root neurons, their relation to high frequency cochlear fibres, their restricted number (rat: about 120 on each side according to Harrison & Warr, 1962; about 40–50 according to Merchan *et al.*, 1988; gerbil: on the average 22 according to Chamberlain, 1977), and their almost exclusively dendritic distribution of putative inhibitory synapses make these cells an interesting model for future morphological and physiological studies. The easy access to these cells in gerbil through the round window antrum (Sokolich & Smith, 1973; Chamberlain, 1977) seems to make this the animal of choice for such experiments.

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