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Peripherin immunoreactivity labels small diameter vestibular 'bouton' afferents in rodents

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Abstract

Recent morphophysiological studies have described three different subpopulations of vestibular afferents. The purpose of this study was to determine whether peripherin, a 56-kDa type III intermediate filament protein present in small sensory neurons in dorsal root ganglion and spiral ganglion cells, would also label thin vestibular afferents. Peripherin immunohistochemistry was done on vestibular sensory organs (cristae ampullares, utriculi and sacculi) of chinchillas, rats, and mice. In these sensory organs, immunoreactivity was confined to the extrastriolar region of the utriculus and the peripheral region of the crista. The labelled terminals were all boutons, except for an occasional calyx. In vestibular ganglia, immunoreactivity was restricted to small vestibular ganglion cells with thin axons. The immunoreactive central axons of vestibular ganglion cells form narrow bundles as they pass through the caudal spinal trigeminal tract. As they exit this tract, several bundles coalesce to form a single, narrow bundle passing caudally through the ventral part of the lateral vestibular nucleus. Finally, we conclude that all labelled axons and terminals were vestibular afferents rather than efferents, as no immunoreactivity in the vestibular efferent nucleus of the brainstem was observed.

Keywords

Semicircular canal; Crista ampullaris; Macula; Otolith; Utriculus; Sacculus; Scarpa's ganglion; Chinchilla; Mouse; Rat

1. Introduction

Recent studies in chinchilla and squirrel monkey have elucidated the differential distribution of vestibular afferents (Baird et al., 1988; Fernández et al., 1988, 1990, 1995; Lysakowski et al., 1995). Three subpopulations have been described, based upon morphophysiological criteria: calyx, dimorphic, and bouton afferents. *Calyx* afferents are thick fibers ending with chalice-shaped terminals on type I hair cells in the central zone of the crista ampullaris and in the striola of the utricular macula. They exhibit an irregular spontaneous discharge pattern. *Dimorphic* afferents end with calyx terminals on type I hair cells and bouton endings on type II hair cells and are distributed throughout the sensory epithelium. They are thick and exhibit an irregular discharge pattern when located in the central zone, and are thinner with a more regular discharge pattern when located in the peripheral zone. *Bouton* afferents are thin fibers ending with bouton terminals on type II hair cells in the peripheral zone of the crista and in the extrastriolar zone of the utricular macula (Fernández et al., 1988, 1990, 1995). Bouton afferents are also physiologically distinct, i.e., they are regularly

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discharging, tonic afferents with slow conduction velocities (Baird et al., 1988; Lysakowski et al., 1995).

Peripherin is a 56-kDa type III intermediate filament protein (Portier et al., 1983; Parysek and Goldman, 1988). Recent studies have demonstrated that it is selectively expressed by small, thin sensory afferents, for example, in small dorsal root ganglion cells (Oblinger et al., 1989), in type II neurons of the human spiral ganglion and in boutons beneath the outer hair cells in the organ of Corti (Despres et al., 1994; Hafidi, 1998). We were interested in whether peripherin might also be a specific marker of small diameter vestibular afferents, i.e., bouton afferents. If so, it could serve as a tool to further investigate the function of these afferents and their connections with brainstem nuclei. Another use for this marker would be to examine the interplay of efferent subgroups with this particular class of afferents, by using it to detect whether specific efferent subgroups contact bouton afferents solely. Preliminary reports have been published in abstract form (Jacobson and Lysakowski, 1996; Alonto and Lysakowski, 1997).

2. Materials and methods

2.1. Tissue preparation

Eleven adult chinchillas (*Chinchilla laniger*, both sexes, 300–600 g), four rats (*Rattus norvegicus*) and two mice (*Mus musculus*) were anesthetized intraperitoneally with Dial (0.05% diallylbarbituric acid in urethane) and perfused transcardially with a heparinized (1000 IU/100 ml) saline solution followed by a fixative consisting of 3% paraformaldehyde, 0.02% glutaraldehyde, 2% acrolein, 0.1% picric acid and 5% sucrose in 0.1 M phosphate buffer (PB, pH 7.4). The brainstem from each animal, with the vestibular ganglia attached, was dissected out and postfixed in 30% sucrose-fixative for 2 h. They were first gelatin-embedded, then cryoprotected and rinsed by placing them in a 30% sucrose-0.1 M PB solution overnight. The vestibular organs and some dorsal root ganglia were dissected out individually and rinsed in phosphate-buffered saline (PBS) overnight. A brainstem block from the inferior colliculus to the end of the fourth ventricle was serially sectioned at 30 μm the following day on a freezing sliding microtome, and rinsed 5× in PBS.

2.2. Immunohistochemical procedures

To facilitate penetration of the antibody, we digested tissue with a Triton X-100 solution (0.4–1.0% for brain sections and 2–4% for end organs and ganglia for a period of 30 min to 3 h). Brain sections and end organs were rinsed and placed in a blocking solution of 5% normal goat serum and 0.4% Triton in PBS for 1 h. The tissue was incubated on a rotating platform overnight at 4°C in primary antiserum (rabbit anti-peripherin 1:2000, Chemicon). The following morning, tissue was rinsed in PBS and placed in a 1:5000 dilution of biotinylated goat anti-rabbit IgG (Chemicon) for 2 h. Tissue was rinsed and incubated with the ABC Elite kit (Vector Laboratory) for 2 h. A standard DAB reaction was then performed. Sections were preincubated for 15 min in 0.5% 3,3′-diaminobenzidine (DAB) in PB. After preincubation, 0.003% H_2O_2 was added. The reaction was monitored until stained neurons in the vestibular ganglion could be seen in the microscope, at which time the reaction was terminated with PB rinses.

Negative controls consisted of incubating sections without primary antibody and the substitution of normal serum for primary antiserum. No staining was observed under either of these conditions. Positive controls showed labelling in small dorsal root ganglion cells and in thin fibers of the spinal trigeminal tract.

2.3. Morphological analyses

Brainstem sections were mounted and dried overnight before dehydration and coverslipping with Permount. The end organs were osmicated (1% OsO_4 in PB), dehydrated in a graduated series of alcohols and propylene oxide, and finally embedded in liquid Araldite (Durcupan). Araldite capsules were hardened in a 60°C oven for 48 h. Serial sections of 2–20 μm were cut from the hardened Araldite capsules and mounted onto glass slides. Cell counts were done on three ganglia from three chinchillas in which the cell staining was well-differentiated. The percentage of labelled cells was obtained by dividing the number of labelled cells by the total number of ganglion cells. The disector method of counting was used to avoid double counting. Means ± S.E.M. are given. Most of the peripherin data shown are taken from chinchilla, since previous estimates of this afferent population were obtained in this species (Fernández et al., 1988, 1990, 1995). Mouse and rat data were obtained to determine whether peripherin was characteristic of bouton afferents in rodents, in general.

The care and use of animals were approved by the University of Illinois Institutional Animal Care and Use Committee in accordance with the guidelines of the Declaration of Helsinki. This work was supported by NIH (PHS R01 DC-02521).

3. Results

Peripherin-like immunoreactivity was confined to the peripheral zone of the crista in chinchillas (Fig. 1A), mice (Fig. 1B,C) and rats (data not shown) and to the extrastriolar zone of the otolith end organs in each species (data from chinchilla in Fig. 2). The lack of label in the central portions of the sensory epithelia of the crista (Fig. 1C) and utriculus suggests that peripherin is found in afferent, not efferent, processes and terminals. This conclusion is strengthened by the lack of labelling in the vestibular efferent nucleus (Fig. 3, which is taken from chinchilla). Similar data were obtained from mouse and rat (not shown).

Positive immunostaining for peripherin was also observed in the smallest cells in Scarpa's ganglion (Fig. 4). These cells measured 9–12 μm by 15–20 μm. Cell counts were done and showed that these cells constituted 5.2 ± 1.8% (mean ± S.E.M.) of the ganglion cell population, approximately 125 out of 2500 cells. Percentages from the three cases were 3.6%, 5.1% and 9.2% (Table 1). This is slightly lower than, but consistent with, prior estimates of the bouton afferent population in chinchilla which ranged from 1.5% in the utricular macula (Fernández et al., 1990) to 20% in the crista (Fernández et al., 1988). Vestibular ganglion cells also had very thin central and peripheral processes. The pattern of small diameter ganglion cells, very thin nerve fibers and small bouton terminals, is similar to that seen in other sensory systems, namely the dorsal root ganglion (Oblinger et al., 1989) and type II spiral ganglion cells and afferent boutons beneath the outer hair cells in the organ of Corti (Despres et al., 1994;Hafidi, 1998).

Occasionally, a calyx appeared to be lightly labelled (not shown). There were a total of three such calyces versus hundreds of boutons seen in our material from 17 animals. In each instance, the calyx was thin-walled and emerged from a thin fiber in the peripheral zone, indicating that it was probably part of a regularly discharging dimorphic unit (Baird et al., 1988; Goldberg et al., 1990).

Peripherin-labelled axons are dispersed throughout the vestibular nerve root and come together in a few small tight bundles (Fig. 5) as they pass through the caudal part of the spinal trigeminal tract. After they exit the spinal trigeminal tract, several bundles coalesce to form a single, narrow bundle (180 μm in diameter) passing caudally, initially through the ventral part of the lateral vestibular nucleus (Fig. 6).

4. Discussion

The terminals of bouton and regular dimorphic afferent fibers are confined to the periphery of vestibular end organs in chinchillas, while calyx and irregular dimorphic afferents occupy the central zone (Baird et al., 1988; Fernández et al., 1988, 1990; Goldberg et al., 1990). Efferent fibers, which terminate in boutons that are slightly smaller and more homogeneous in size than those of bouton afferents (Lysakowski and Goldberg, 1997), are distributed throughout the sensory epithelium, in both the peripheral and central zones.

Calretinin immunoreactivity has proven to be a useful marker for calyx afferents in mammals (Desmadryl and Dechesne, 1992; Zeh et al., 1999) and so the purpose of the present study was to determine whether peripherin could be used as another specific vestibular afferent marker.

Peripherin is a type III intermediate filament protein (Portier et al., 1983; Parysek and Goldman, 1988; see also review by Greene, 1989). At present, its presence merely serves as a marker for small afferents. As stated by Parysek and Goldman (1988) in an early description of peripherin, “This protein is distributed in only certain neuronal elements; these elements may be unified by an as yet unrecognized pattern of function in the nervous system”. Although little evidence is available on its specific function, structural differences between peripherin and other intermediate filament proteins may account for a unique, but as yet unknown, function of peripherin in small ganglion cells. Peripherin is phosphorylated, along with other type III IF proteins, such as vimentin, desmin and glial fibrillary acidic protein, on N-terminal serine or threonine residues. Peripherin is also constitutively phosphorylated on a C-terminal tyrosine residue, which is lacking on other IF proteins (Angelastro et al., 1998; Ho et al., 1998). This C-terminal phosphotyrosine is highly conserved in rodent and human peripherins, but, curiously, is not present in *Xenopus* (Sharpe et al., 1989). As an aside, it would be interesting to know whether peripherin is also a marker for small ganglion cells in *Xenopus*.

Phosphorylation inhibits the assembly of vimentin (Inagaki et al., 1987) and desmin (Geisler and Weber, 1988) into filaments, but it is not required for self-assembly of peripherin. It has been suggested that one potential function of the phosphotyrosine group on peripherin could be to modulate its interaction with other proteins (Angelastro et al., 1998). Another possibility is suggested by the evidence that neurofilament proteins, which are type IV intermediate filament proteins, are involved in determining axonal caliber through their interactions with microtubules (Xu et al., 1996). In this case, increasing levels of phosphorylation produce wider interfilament spacing. By analogy, we could speculate that peripherin is involved in determining the small axonal diameter of specific classes of sensory afferents.

One possible use of peripherin as a marker would be to track the central projections of bouton afferents, a task that has proven extremely difficult with intra-axonal electrophysiological recording and injection due to the very small diameter (0.2–1 μm) of their axons (Lysakowski, Fernández and Goldberg, unpublished observations). A marker that could label this population would be very powerful for determining the targets of regularly discharging thin afferents. Our central peripherin data show that the peripherin-labelled axons are dispersed throughout the vestibular nerve root and come together in a few small tight bundles as they pass through the caudal part of the spinal trigeminal tract. It is intriguing that several bundles coalesce to form a single, narrow bundle (180 μm diameter) passing caudally through the ventral part of the lateral vestibular nucleus as this suggests that they have a restricted target area. We are currently tracing the rostral course course of this bundle and its possible route to the cerebellum. We are also attempting to determine if it

targets a particular postsynaptic population, e.g., vestibulo-ocular or vestibulospinal neurons.

Two lines of evidence support our hypothesis that these thin labelled fibers are not efferents. First, in the chinchilla, rat and mouse, there are no labelled neurons in the e-group, although recent preliminary data in the gerbil indicate that this may not be true for all rodent species (Kevetter and Leonard, 1996). Second, the fiber pattern is restricted to the periphery of the end organs, while efferents are distributed throughout the sensory epithelium. A counter-hypothesis, that these fibers may represent a subpopulation of efferent fibers restricted to the peripheral zone, was considered, but the absence of labelled neurons in the e-group also argues against this hypothesis, at least for the rodent species in this study.

Thin peripherin-labelled fibers course through the vestibular nerve in small discrete bundles. This suggests that they have a common, as yet undetermined, target. Future studies will attempt to determine this target.

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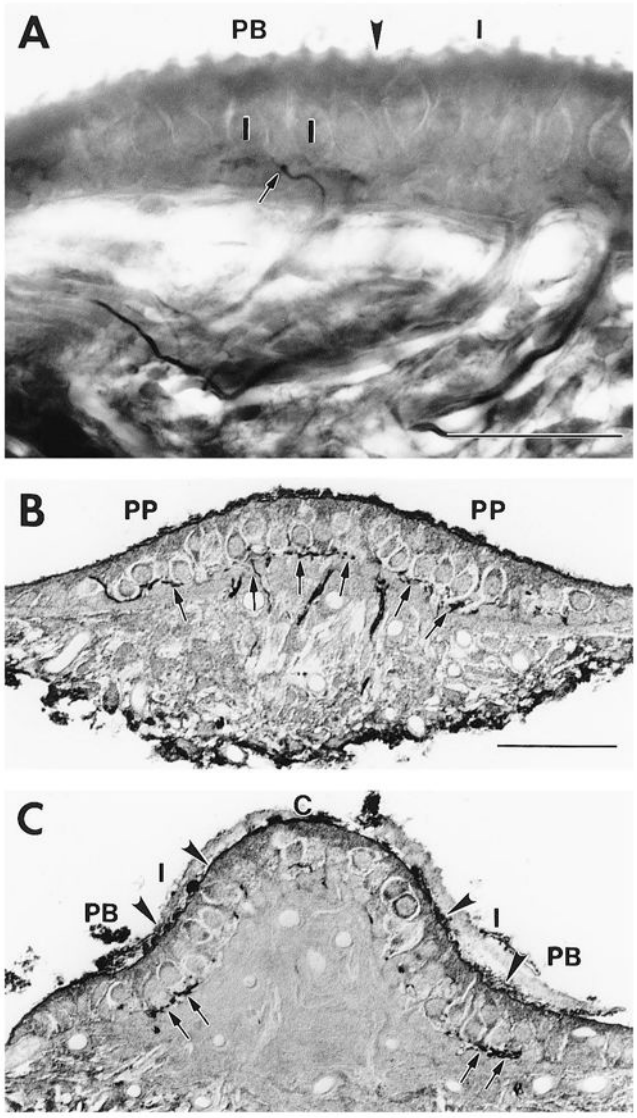

Fig. 1.

Vestibular sensory epithelium. A: Photomicrograph of 20 μm plastic-embedded, osmicated, transverse section from the peripheral (PB) region at the base of a chinchilla posterior crista. Peripherin-labelled bouton (arrow) making contact with the base of a type II hair cell whose outline can be distinguished between two adjacent type I (I) hair cells, enclosed by calyx endings. B: Low magnification photomicrograph of 2 μm plastic transverse section from the planum region of a mouse posterior crista. Note the fiber staining (small arrows) throughout the peripheral zone near the planum semilunatum (PP). C: Low magnification photomicrograph of 2 μm plastic transverse section from a region more central in a mouse posterior crista. Note the dense fiber staining (small arrows) in the peripheral region near the base of the crista (PB). I, intermediate zone; C, central zone. Regional boundaries defined as described in Lysakowski and Goldberg (1997). Scale bars in A–C = 50 μm.

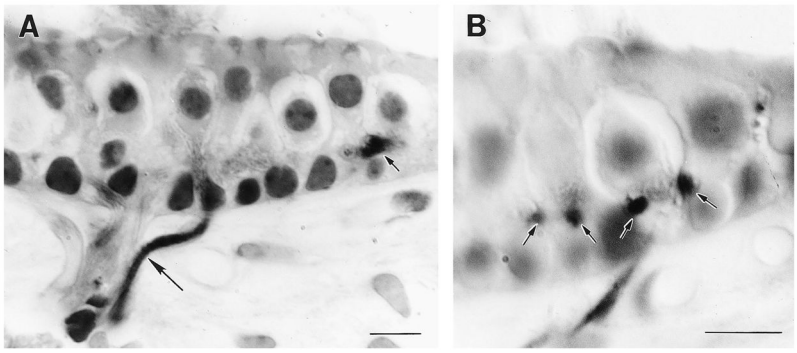

Fig. 2.

Extrastriolar utriculus. 5 μm semithin sections stained with Richardson's stain showing cell types and labelled fibers. A: Note the small diameter immunolabelled fiber (large arrow) entering the epithelium adjacent to larger unlabelled fibers. These fibers are very thin and myelinated. One labelled bouton (small arrow) is seen terminating at the base of a type II hair cell. B: Labelled boutons (small arrows) at the base of hair cells near the extreme periphery of the end organ (tapering to right). Note labelled thin fiber entering the epithelium from below. Scale bars in A,B = 10 μm.

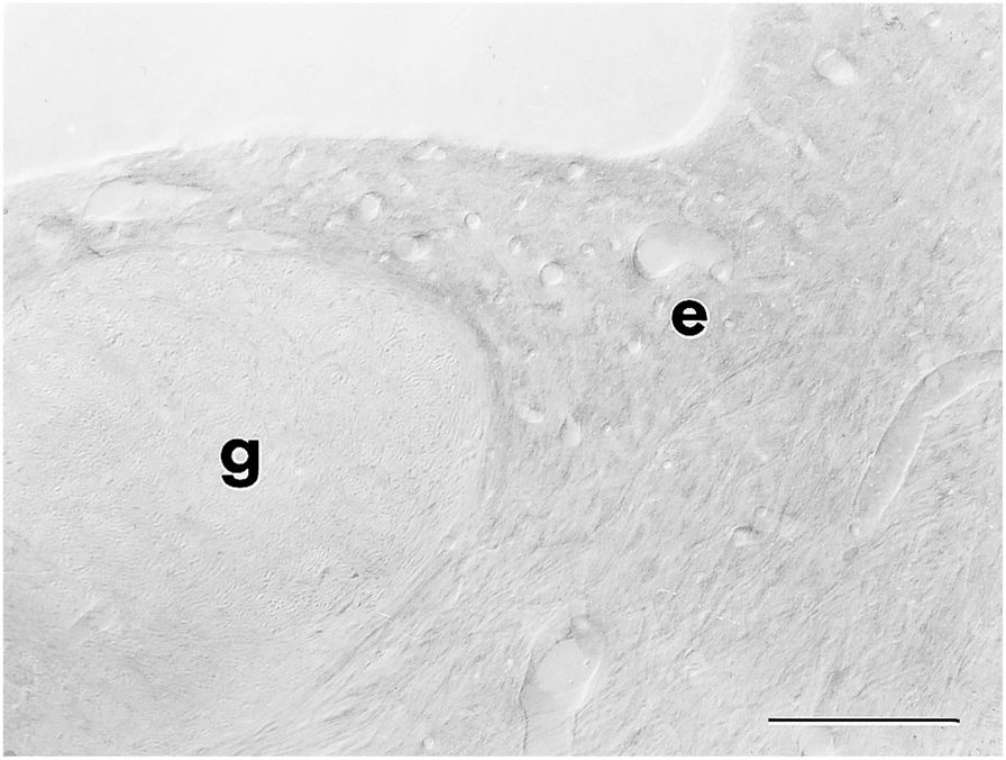

Fig. 3.

E-group. No labelled cells were observed in the e-group (e), located next to the genu of the facial nerve (g), indicating that the thin fibers and boutons observed in the sensory epithelium are not vestibular efferents. Scale bar = 200 μm.

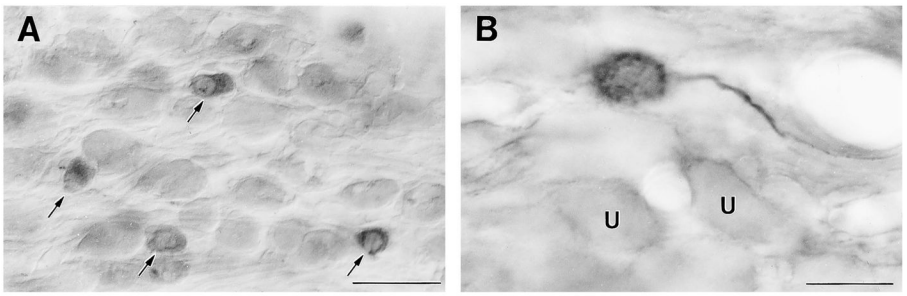

Fig. 4.

Vestibular ganglion. A: Four vestibular ganglion cells (arrows) are immuno-labelled with peripherin in this photomicrograph. Note their small size compared to the surrounding ganglion cells. It is reasonable to assume that these small ganglion cells give rise to small diameter fibers and thus are bouton afferents (or regularly-discharging afferents). B: This small peripherin-labelled ganglion cell can be seen giving rise to a small diameter fiber. There are two larger, unlabelled (U) ganglion cells below. Scale bar in A = 50 μm, in B = 20 μm.

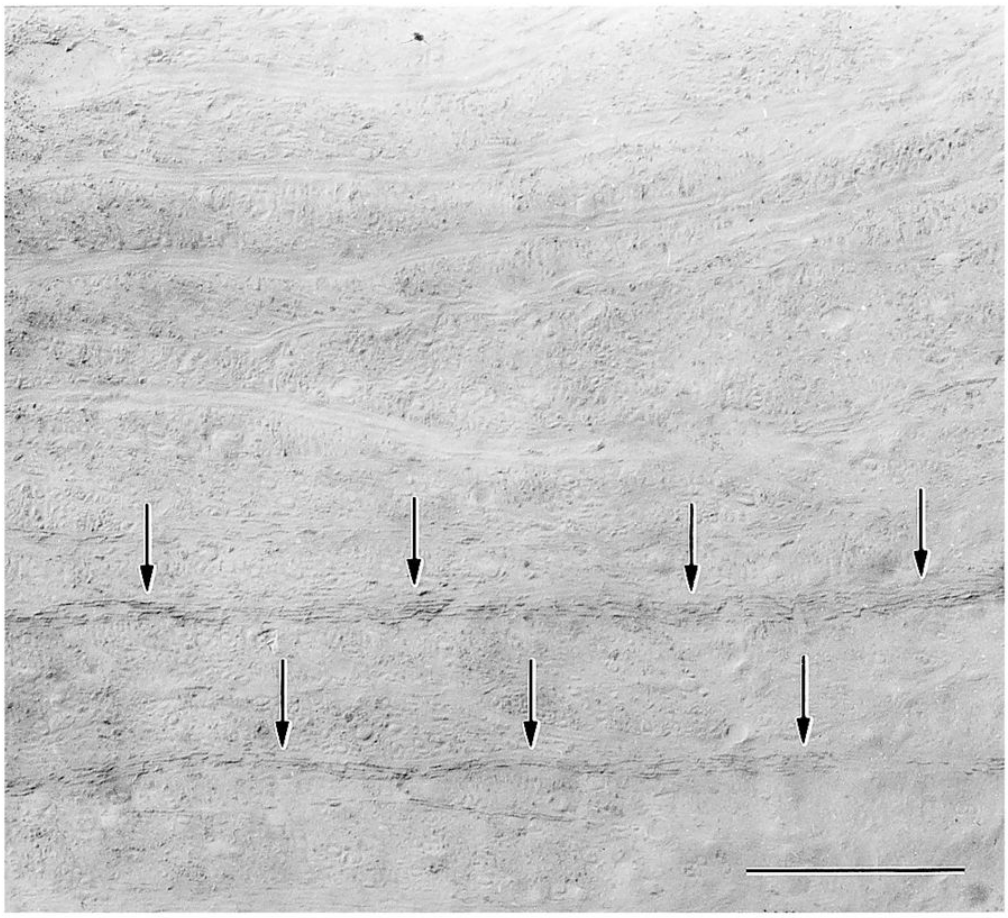

Fig. 5.

Vestibular nerve. Several fascicles from the vestibular nerve are seen coursing through the spinal trigeminal tract. Two of these bundles (thin arrows) are filled with peripherin-labelled fibers. Note that other bundles in the same field are not labelled, suggesting that these thin, presumably bouton afferents maintain segregated bundles and appear to run a distinct course in their central projections to the vestibular nuclei. The labelled fiber bundles were found only at caudal levels in the spinal trigeminal tract. Scale bar = 100 μm.

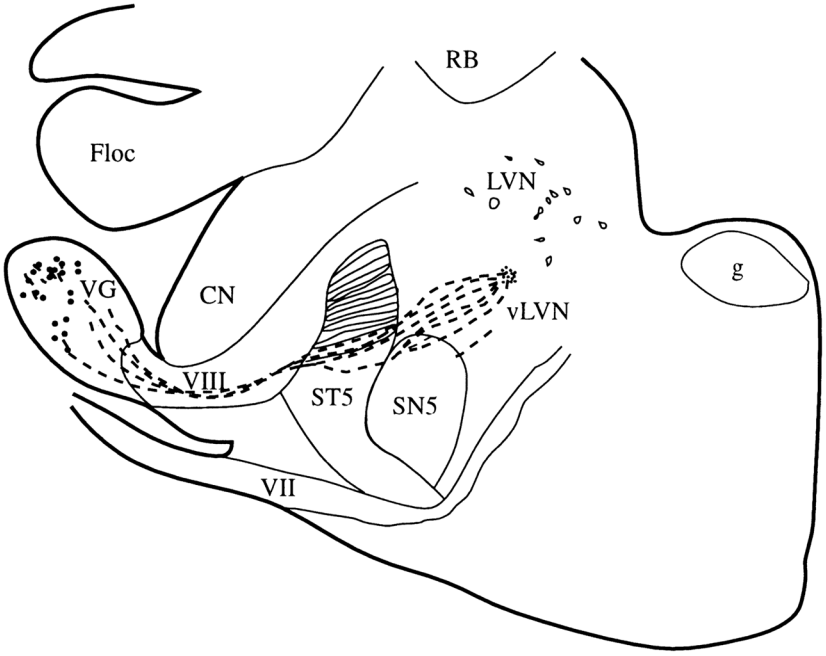

Fig. 6.

Camera lucida reconstruction of the chinchilla brainstem at the level of the genu of the facial nerve. This camera lucida drawing of the brainstem summarizes data from many sections (some examples are seen in Figs. 4 and 5) and shows peripherin-labelled small fibers from the vestibular ganglion cells (large dots) crossing through the spinal tract of the trigeminal nerve and progressing to the ventral part of the lateral vestibular nucleus. The dashed lines represent fascicles of fibers. These fascicles run in the coronal plane and then turn caudally to run in a small tight bundle (small dots) in the longitudinal plane through the ventral part of the lateral vestibular nucleus. Floc = flocculus; RB = restiform body; CN = cochlear nucleus; g = genu of facial nerve; LVN = lateral vestibular nucleus; vLVN = ventral part of the lateral vestibular nucleus; VG = vestibular ganglion; VIII = 8th nerve; ST5 = spinal tract of the trigeminal nerve; SN5 = spinal nucleus of the trigeminal nerve; VII = 7th nerve.

Table 1

Peripherin-labelled cells in the vestibular ganglion

Animal	Number of peripherin-labelled cells	Total number of cells	Percent of peripherin-labelled cells
86	123	3458	3.6
90	134	1460	9.2
91	123	2404	5.1
Total	380	7322	5.2
Average	126.7 ± 3.7	2440.7 ± 577.1	5.2 ± 1.8

Average values = Mean ± SEM