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GABA, glycine, aspartate, glutamate and taurine in the vestibular nuclei: an immunocytochemical investigation in the cat

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Summary. The distributions of five amino acids with well-established neuroexcitatory or neuroinhibitory properties were investigated in the feline vestibular complex. Consecutive semithin sections of plastic-embedded tissue were incubated with antisera raised against protein-glutaraldehyde conjugates of GABA, glycine, aspartate, glutamate and taurine. This approach allowed us to study the relative densities of the different immunoreactivities at the level of individual cell profiles. The results indicate that in the vestibular nuclei, neuronal colocalization of two or more neuroactive amino acids is the rule rather than an exception. Colocalization was found of immunoreactivities for GABA and glycine; glycine, aspartate and glutamate; glycine and aspartate, and glutamate and aspartate. GABA immunoreactive neurons were generally small and were found scattered throughout the vestibular complex. Glycine immunoreactive neurons were similarly distributed, except in the superior nucleus where the latter type of neuron could not be detected. Neuronal profiles colocalizing immunoreactivities for GABA and glycine occurred in all nuclei, but were most numerous in the lateral nucleus. The vast majority of the neurons showed noteworthy staining for glutamate and aspartate, although the level of immunoreactivities varied (e.g., the large neurons in the lateral and descending nuclei were more intensely aspartate immunoreactive than the smaller ones). Taurine-like immunoreactivity did not occur in neuronal cell bodies but appeared in Purkinje cell axons and in glial cell profiles. The functional significance of the complex pattern of amino acid colocalization remains to be clarified. In particular it needs to be distinguished between metabolic and transmitter pools of the different amino acids. The present results call for caution when attempts are made to conclude about transmitter identity on the basis of amino acid contents alone.

Key words: Neurotransmitter – Colocalization – Vestibular nuclei – Immunocytochemistry – Cat

Introduction

The experimental data accumulated during the last decades have provided a detailed insight in the anatomy and physiology of the vestibular complex (for reviews and references, see Wilson and Jones 1979; Goldberg and Fernandez 1982; Carpenter 1988; Shinoda et al. 1988). In comparison, knowledge about the chemoarchitecture of this complex has been slow to develop. This is particularly striking in view of the considerable advances that have been made over the last few years in our understanding of the chemoarchitecture of related structures, such as the cochlear nuclei, in which immunocytochemical methods, used alone or in combination with axonal tracing techniques, have assigned transmitter candidates to a number of different neuronal populations (Adams and Mugnaini 1987; Mugnaini 1985; Wenthold 1987; Wenthold et al. 1986, 1987).

In the present report, we describe the distributions of five different neuroactive amino acids in the vestibular nuclei. The amino acids were visualized by selective antisera that recognize amino acids fixed to brain protein by means of glutaraldehyde (Storm-Mathisen et al. 1983; review: Ottersen and Storm-Mathisen 1987). Two of the amino acids

Abbreviations: D: descending vestibular nucleus, L: lateral vestibular nucleus (Deiters' nucleus), M: medial vestibular nucleus, S: superior vestibular nucleus, N. VIII: eighth cranial nerve, V: spinal trigeminal tract

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studied (gamma-aminobutyric acid, GABA, and glycine, Gly) are thought to occur in high concentrations exclusively in nerve cells using them as transmitters. In contrast, glutamate (Glu), aspartate (Asp) and taurine (Tau), in addition to being putative transmitters, also serve many other functions in brain tissue (Fonnum 1984; Wright et al. 1986). Thus, the presence of e.g. Glu-like immunoreactivity (Glu–LI) in a particular cell compartment is often difficult to interpret in a functional context. In spite of this short-coming, a mapping of the distributions of Glu, Asp and Tau should prove valuable as a platform for further studies aimed at distinguishing transmitter pools from non-transmitter ("metabolic") pools.

Material and methods

Tissue preparation

Three normal adult cats were used in the present study. The animals were deeply anesthetized with pentobarbital (50 mg/kg), and perfused transcardially with a brief flush of 2% dextran (molecular weight 70 000) in 0.1 M sodium phosphate buffer (pH 7.4), followed by the same buffer containing 5% glutaraldehyde or a mixture of 1.5% depolymerized paraformaldehyde and 2.5% glutaraldehyde at room temperature. The brains were dissected free, cut in two halves and stored at 4° C in phosphate buffer with 0.5% glutaraldehyde until sectioning. In two of the cats one half of the medulla was cut in 40 µm transverse sections on a Vibratome (Oxford). From the other half, and from the medulla of the third cat, transverse thin slices through the vestibular complex were cut with a razor blade. To facilitate identification of the vestibular nuclei, the slices were post-fixed in OsO_4 (45 min, 1% in sodium phosphate buffer). After dehydration, blocks containing the various vestibular nuclei were isolated and embedded in Durcupan. Semithin (0.5 µm) serial sections were cut through the transversely oriented blocks. These sections contained parts of one or two vestibular nuclei.

Immunocytochemical procedures

Vibratome sections were processed free-floating according to the peroxidase-antiperoxidase procedure (Sternberger 1979) as previously described in detail (Storm-Mathisen et al. 1983; Ottersen and Storm-Mathisen 1984b). The antisera were used in the following dilutions: Glu antiserum 13 (Storm-Mathisen et al. 1983): 1:1000; Asp antiserum 18 (Ottersen and Storm-Mathisen 1985): 1:50; GABA antiserum 25 (Ottersen and Storm-Mathisen 1984b): 1:150; Gly antiserum 31 (Dale et al. 1986; Ottersen et al. 1986): 1:150.

Nitrocellulose filters carrying a series of different amino acid conjugates were placed in the same wells as the tissue sections and incubated together with these. This procedure allowed us to monitor the specificity of the immunoreaction in each experiment (Ottersen and Storm-Mathisen 1984b) (Fig. 1G,H).

Semithin sections were mounted on glass slides and processed according to the peroxidase-antiperoxidase procedure following removal of the resin by sodium-ethanolate (Lane and Europa 1965). The immunocytochemical procedure was based on that of Somogyi et al. (1984), and has been described in full detail previously (Ottersen 1988). The serum dilutions were the same as for Vibratome sections, except for the Gly antiserum which was diluted 1:50; the Tau antiserum (Madsen et al. 1985) was diluted 1:3000. Contiguous 0.5 µm semithin sections were treated with different antisera, permitting a detailed comparison of the different labelling patterns. A semithin section containing conjugates of the six most abundant amino acids in the brain was mounted in close proximity to the tissue sections, allowing it to be incubated in the same serum drops (Ottersen 1987). Absorption controls were made by pretreating the antisera with different amino acid-glutaraldehyde complexes (prepared from GABA, Gly, Asp, Glu, Tau or glutamine) (Dale et al. 1986; Ottersen et al. 1986).

Densitometrical analysis

In contrast to the situation in GABA- and Gly-immunostained preparations, where neurons could be classified as either immunonegative or immunopositive, sections stained with Glu- or Asp-antisera displayed a wide range of staining intensities in nerve cell bodies. It was therefore deemed necessary to supplement the visual evaluation of the immunolabelling pattern with densitometrical readings. This was performed in semithin sections, using a Nikon densitometer coupled to a Nikon light microscope with a constant current supply. All densitometrical data were obtained with a x20 objective lens, and a diaphragm diameter of 0.2 mm (corresponding to a measuring area of 7.8 µm²). The densitometrical values were fed directly into a computer, enabling histograms to be prepared on-line by a modified Phoscan program. Statistical evaluation of the data was carried out by exporting the primary data to a specially designed program (Phostatx, developed by T. Karagülle).

Terminology

The nomenclature and delineation of the vestibular nuclei follow that of Brodal et al. (1962). With regard to cell size, the terms "small", "medium-sized", "large" and "giant" will be used for cells with diameters in the ranges of 5–30 µm, 30–80 µm, 80–100 µm and 100–130 µm, respectively. Only the four main nuclei will be considered in this publication.

Results

Specificity of staining

Each antiserum reacted selectively as judged from the staining of the test conjugates (Fig. 1). Exchange of the primary antiserum with a preimmune serum, or preabsorption of the antisera with glutaraldehyde complexes of the amino acid used for immunization abolished staining of test conjugates and tissue sections (Fig. 1C). Other amino acid complexes had no effect on the immunostaining. It should be noted, however, that preincubation of the Glu antiserum with Asp-glutaraldehyde complexes or of the Asp antiserum with Glu-glutaraldehyde complexes led to a moderate decrease in the overall staining intensity without any detectable influence on the staining pattern. This phenomenon probably reflects low affinity, nonspecific interactions between immunoglobulins and amino acid-glutaraldehyde complexes.

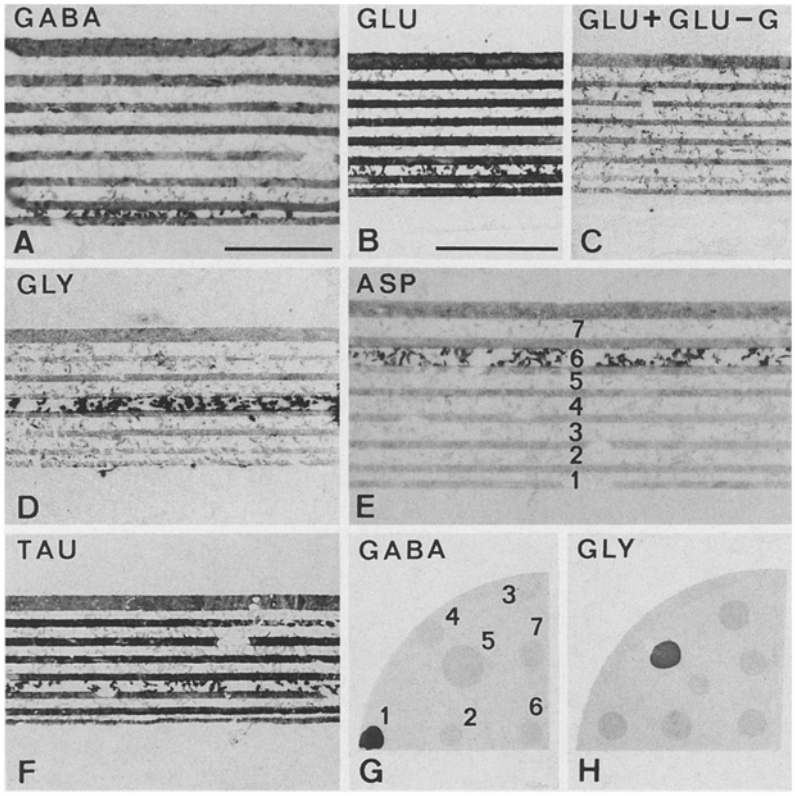

Fig. 1A–H. A series of different amino acid-glutaraldehyde-brain protein conjugates was embedded in Durcupan (**A–F**) or spotted on nitrocellulose filters (**G, H**) and incubated in the same drops of immunoreagents as the tissue sections for optimum specificity control. Code: 1, GABA; 2, Glu; 3, Tau; 4, Gly; 5, none (only glutaraldehyde-protein); 6, Asp; 7, glutamine (**A–F**) or β-alanine (**G, H**). Note that selective labelling was obtained with all antisera used. C shows an example of an absorption test, in which the specific staining for Glu (see **B**) was completely removed by preabsorption with Glu-glutaraldehyde complexes. Note that the dark zones between the conjugate-containing sections in E–F represent brain sections which only serve as spacers. Scale line: **A**, 30 μm (also valid for **E**); **B**, 40 μm (also valid for **C**, **D**, **F**). The radius of the nitrocellulose filters is 6 mm

Distribution of amino acid immunoreactivities

Unless stated otherwise, the following description refers collectively to the Vibratome and semithin sections, which produced essentially identical results with regard to the neuropil staining. The results were the same in all experimental animals and did not differ between the different fixation procedures. As for the cell body staining, some discrepancies were noted between the two types of sections (see below). This could usually be attributed to the occurrence of falsely immunonegative cells in the interior of the Vibratome sections, reflecting the limited penetration of the immunoreagents. The semithin sections naturally afforded the higher resolution and were therefore chosen as objects for most of the illustrative material. They were also indispensable for colocalization studies.

GABA immunoreactivity

GABA immunopositive (GABA(+)) cells lie scattered throughout the vestibular complex, comprising less than 10% of the total cell number in each nucleus as assessed by countings within a fixed ocular rectangular frame. (This is a conservative estimate since differences in cell size were not taken into account.) All of the immunopositive cells are small and intensely stained (Fig. 2A), except for a few medium-sized, moderately stained cells in the medial nucleus. In all nuclei immunopositive fibres of varying caliber and staining intensity course in various directions (Fig. 2A, 6A). In the medial nucleus, the majority of the coarser immunoreactive fibres pass medially towards the perihypoglossal nuclei. Furthermore, intensely GABA(+) Purkinje cell axons reach the dorsal part of the lateral vestibular nucleus (Fig. 5C), which has a higher number of positive fibres than its ventral part. GABA(+) bouton-like puncta are found in close apposition to dendritic profiles and to perikarya of all types of cells, GABA(+) as well as GABA(−), and are also dispersed in the neuropil (Fig. 2A, 6A, 9A). In the lateral nucleus, their concentration appears to be somewhat higher ventrally than dorsally. There is no detectable glial labelling.

Glycine immunoreactivity

The Gly(+) cells in the medial, descending and lateral vestibular nuclei are small or medium-sized, with varying staining intensities (Fig. 2B, 6C). They lie dispersed throughout the nuclei, and their concentration is similar to that of the GABA(+) cells.

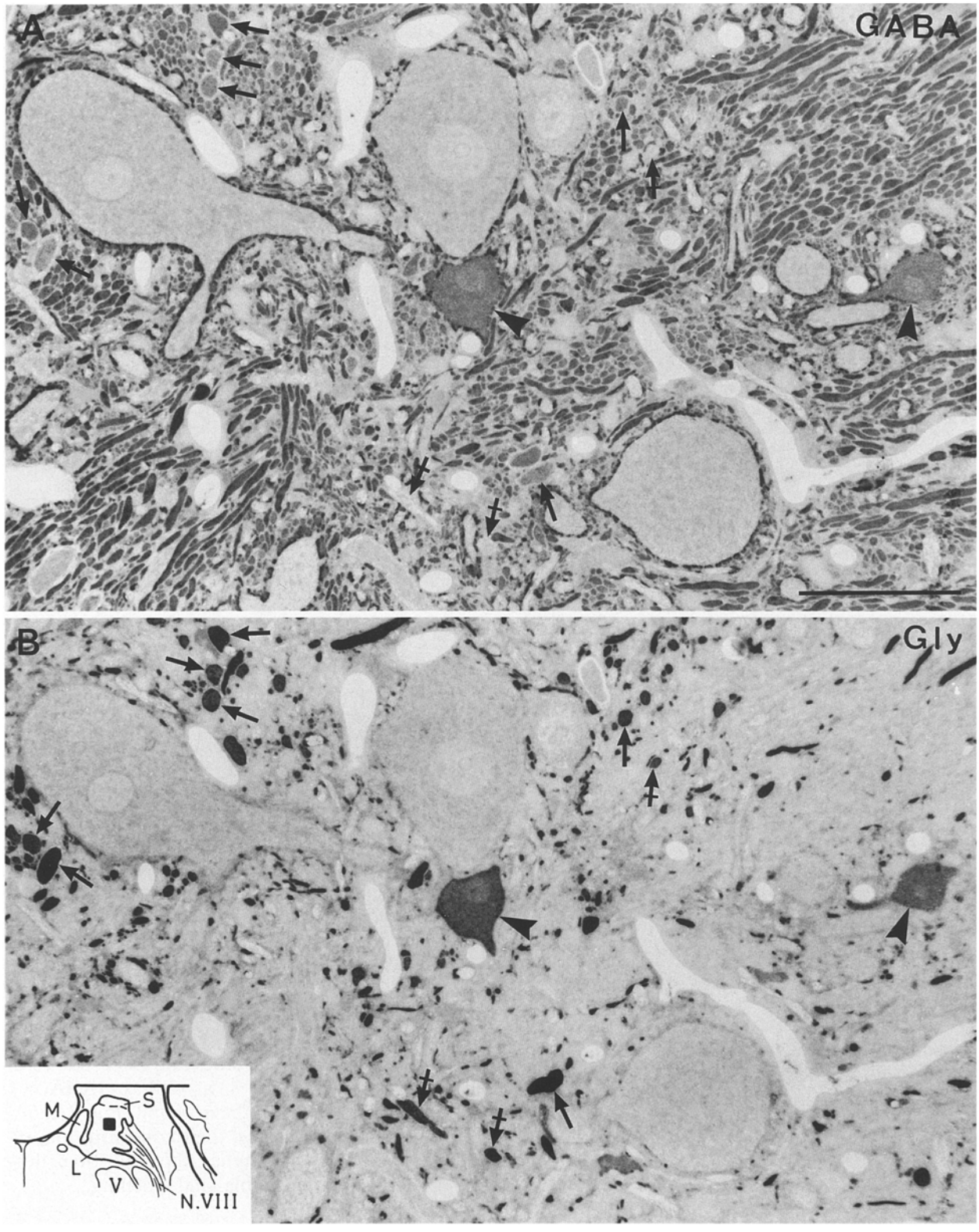

Fig. 2A, B. Photomicrographs of adjacent semithin sections cut transversely through the upper part of the lateral vestibular nucleus (see inset for the selected region). One section was treated with the GABA antiserum diluted 1:100 (**A**), the other with the Gly antiserum diluted 1:50 (**B**). Two small cells (arrowheads) are immunostained for both GABA and Gly. GABA immunoreactive Purkinje fibres are abundant, and GABA immunoreactive bouton-like puncta are found in close apposition to the GABA(+) as well as GABA(−) cells. In contrast, there are only scattered Gly(+) immunopositive fibres and puncta. Most of the transversely cut Gly(+) myelinated axons can be identified as moderately GABA immunoreactive (arrows). Only a few Gly(+) profiles are GABA(−) (crossed arrows). Strongly GABA(+) axons (such as the bundle in the upper right corner) generally appear as Gly(−). Scale line 50 μm. Cat 13787

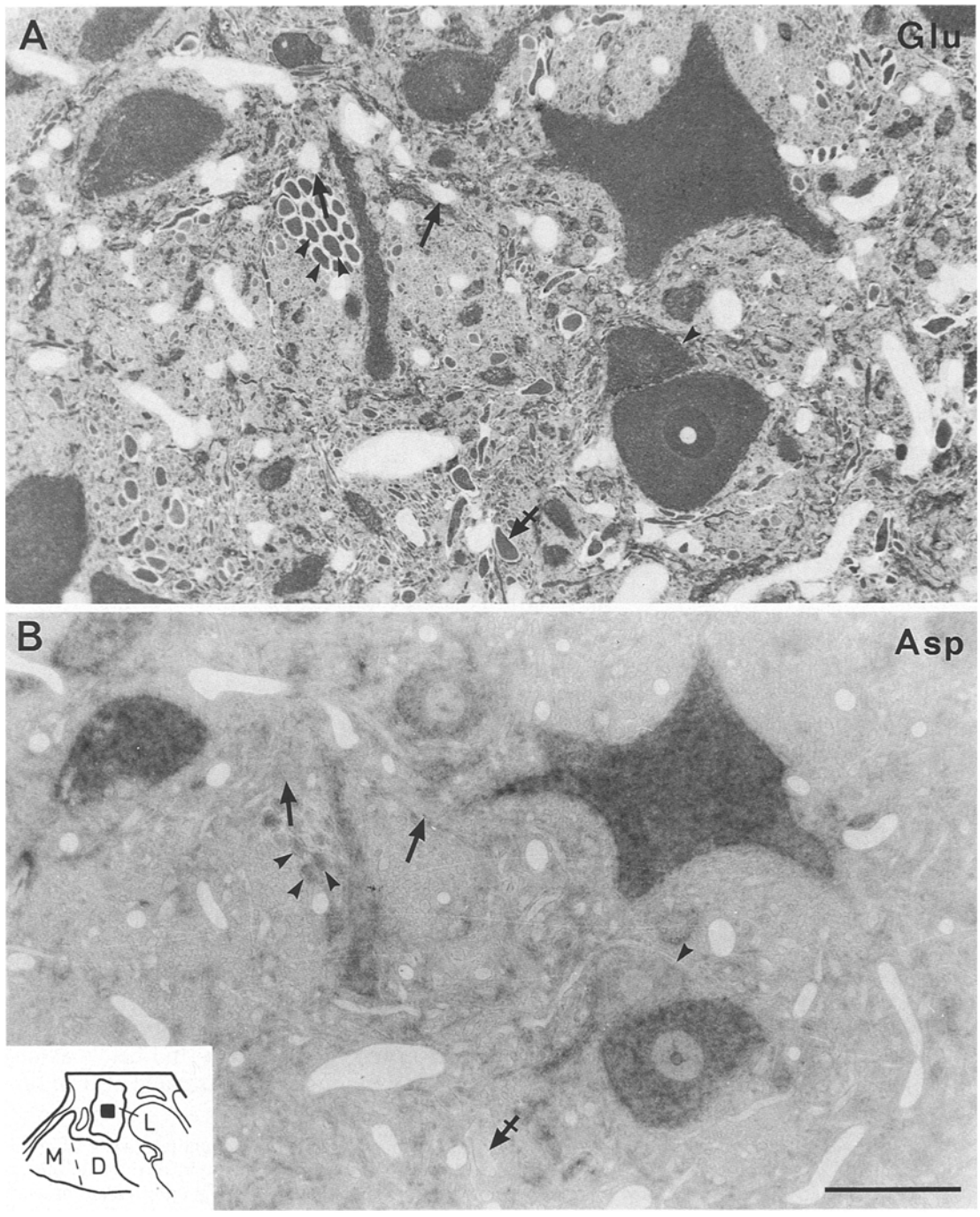

Fig. 3A, B. Photomicrographs of adjacent semithin sections through the lateral vestibular nucleus (see inset). All nerve cell bodies in this field are strongly labelled for Glu. There is, in contrast, a wide range of staining intensities for Asp. The staining intensity for the latter amino acid is to some degree correlated to neuronal size: the small neuron indicated by an arrowhead was subjectively considered as immunonegative (transmittance 62%), whereas the adjacent, somewhat larger neuron, was considered as immunopositive (transmittance 47%; see Fig. 4B). Small arrowheads: bundle of myelinated axons colocalizing Asp and Glu. Crossed arrow: Glu(+) but Asp(−) myelinated axon. Arrows: glial cells. Scale line 50 μm. Cat 13788

There are many Gly(+) fibres in the penetrating cross-sectioned bundles of the descending nucleus, and several in the hook-bundle traversing the lateral nucleus (Fig. 5A,B). Otherwise, only scattered moderately stained fibres are observed, the Purkinje axons being negative. Some Gly(+) puncta are found in close proximity to neuronal perikarya; and also occur freely distributed in the neuropil (Fig. 5B, 6C, 9C).

The superior vestibular nucleus is negative with

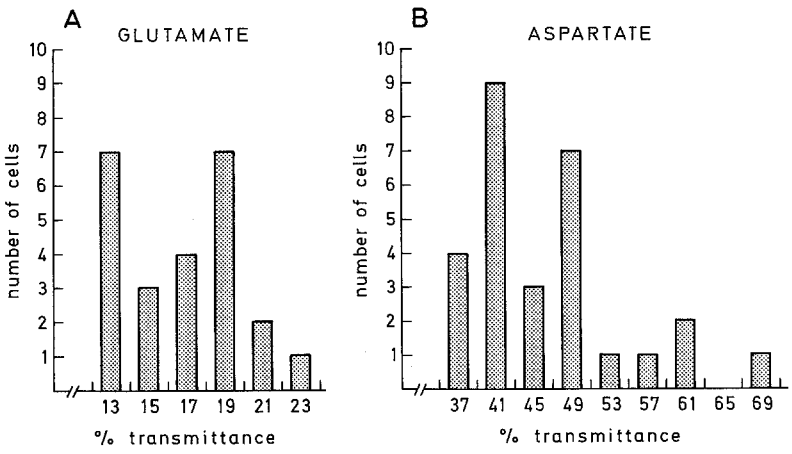

Fig. 4. Histograms showing the optical transmittance for Glu (**A**) and Asp (**B**) of all neuronal cell bodies present in the lateral nucleus in the sections represented in Fig. 3A, B. The ordinate indicates the number of cells in each bin (bin width corresponds to 2% in **A** and 4% in **B**). 100% transmittance was defined through glass outside the tissue section. The average transmittance in glial cells was 48.2% (range 44.1–61.3%; n=20) for Glu and 77.2% (range 69.8–87.4%; n=12) for Asp (data not included in the histograms)

respect to immunostained cells, but a moderate number of Gly(+) fibres of varying caliber and Gly(+) puncta are found throughout the nucleus.

Glutamate immunoreactivity

In semithin sections, almost all neurons appeared to contain a considerable level of Glu–LI (Fig. 3A, 6B, 9B). Only a very few neurons (e.g. see inset Fig. 6B) were subjectively considered as "immunonegative"[1]. This impression was substantiated by densitometrical readings in the lateral and descending nuclei (Fig. 4A, 7). In both nuclei, the recorded neurons (i.e., all neurons present in the section) formed a population that was clearly separate from the pale glial cells. Neurons subjectively interpreted as immunonegative were more numerous in the Vibratome sections, but this is probably due to incomplete labelling of the interior of the sections (see above). As judged from the latter type of sections, Glu "immuno-negative" neurons are more abundant in the superior than in the remaining nuclei of the vestibular complex.

In the neuropil, Glu(+) fibres of varying caliber cross in all directions, and numerous fibres in the bundles penetrating the descending nucleus are Glu(+) (Fig. 6B). The neuropil shows widespread stained puncta, and some cells, especially the medium sized neurons in the medial nucleus (Fig. 9B), have a prominent pericellular distribution of presumed en passant and terminal boutons.

Aspartate immunoreactivity

Similar to the Glu immunostained preparations, the sections incubated with the Asp antiserum showed noteworthy staining of most neurons. However, the range of staining intensities was wider, and the number of neurons subjectively interpreted as immunonegative was higher, than in the Glu immunostained preparations (Fig. 3, 6, 9). Again, this impression was corroborated by the densitometrical data (Fig. 4B, 8). In the section shown in Fig. 3 (from the lateral nucleus), neurons with a transmittance of about 60% or above were subjectively regarded as immunonegative; those with a transmittance about 45% and below as unequivocally immunopositive[2]. As could also be sur-

[1] The term "immunonegative" is given with quotation marks since it does not necessarily signify a total abscence of antigen (see discussion). In this context the term points to cell profiles that are poorly stained compared to the average tissue level of staining

[2] Whether a neuron is perceived as immunopositive or immunonegative depends on its staining intensity relative to the general staining intensity of the tissue. Thus the optical transmittance value at which a neuron becomes "immunopositive" is not fixed, but varies from section to section

Fig. 5A–F. Vibratome sections from the dorso-medial part of the lateral vestibular nucleus (see inset) immunostained for Gly. The immunopositive fibres of the hook bundle penetrating the nucleus are seen in the upper part of **A**. The boxed area in **A** is shown at higher magnification in **B**. Cat 13788. **C** Adjacent Vibratome section from the dorsolateral part of the same nucleus (see inset) showing GABA(+) Purkinje axons. **D** Is from a Tau-immunostained semithin section showing immunopositive fibres in the vestibular nerve just before entrance into the lateral vestibular nucleus (see inset). Cat 2916. **E, F** are from the same section through the lateral vestibular nucleus treated with the Tau antiserum. **E** Is from the dorsal half and shows the Tau(+) Purkinje axons. **F** Is from the ventral part which is devoid of Purkinje axons, but which contains immunostained fibres of other types. A moderate amount of thin fibrous processes and puncta surround neuronal perikarya in both parts of the nucleus. Scale line in **A** 200 μm; **B** 40 μm; same magnification for **C–F**

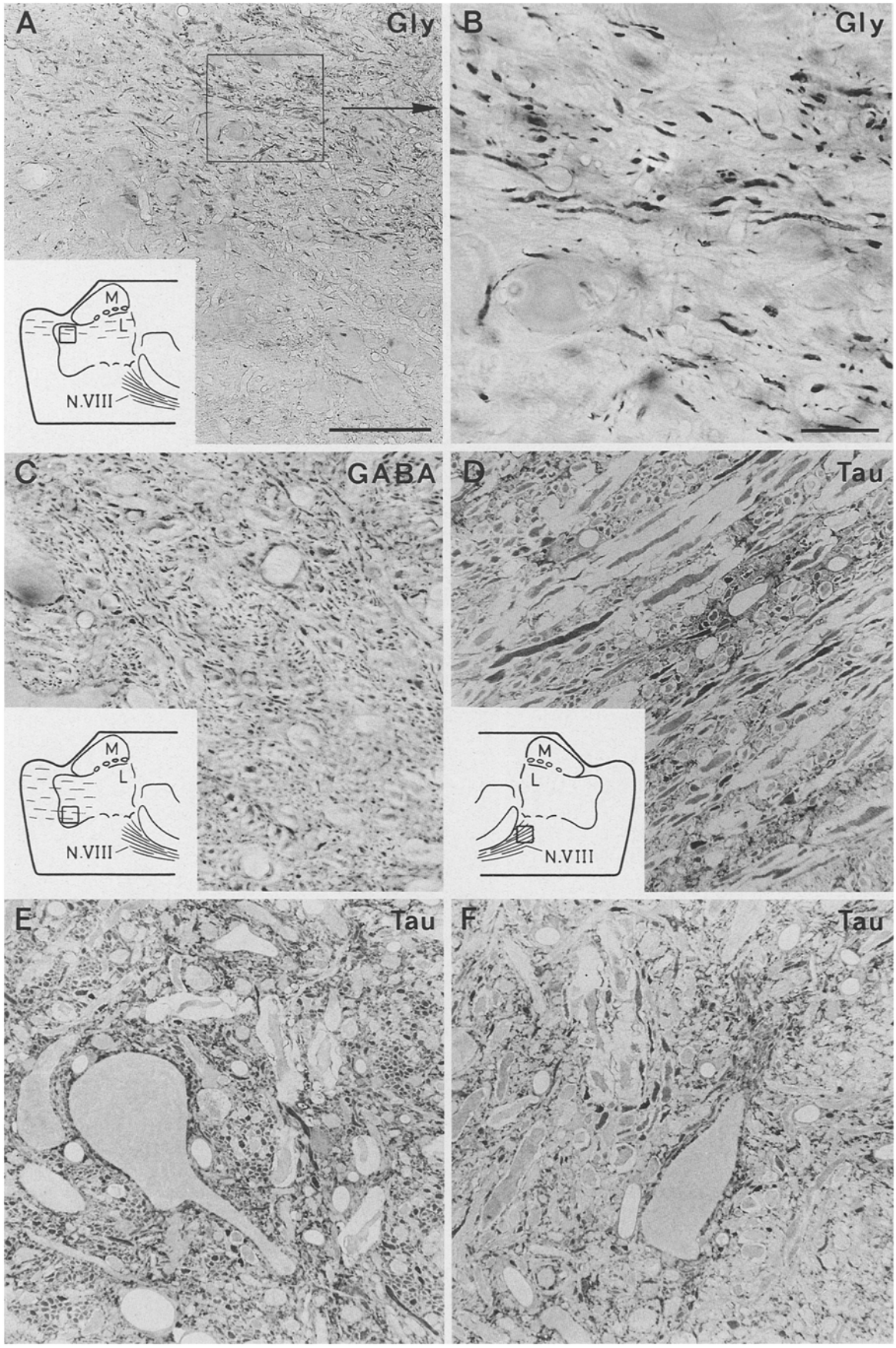

Fig. 5

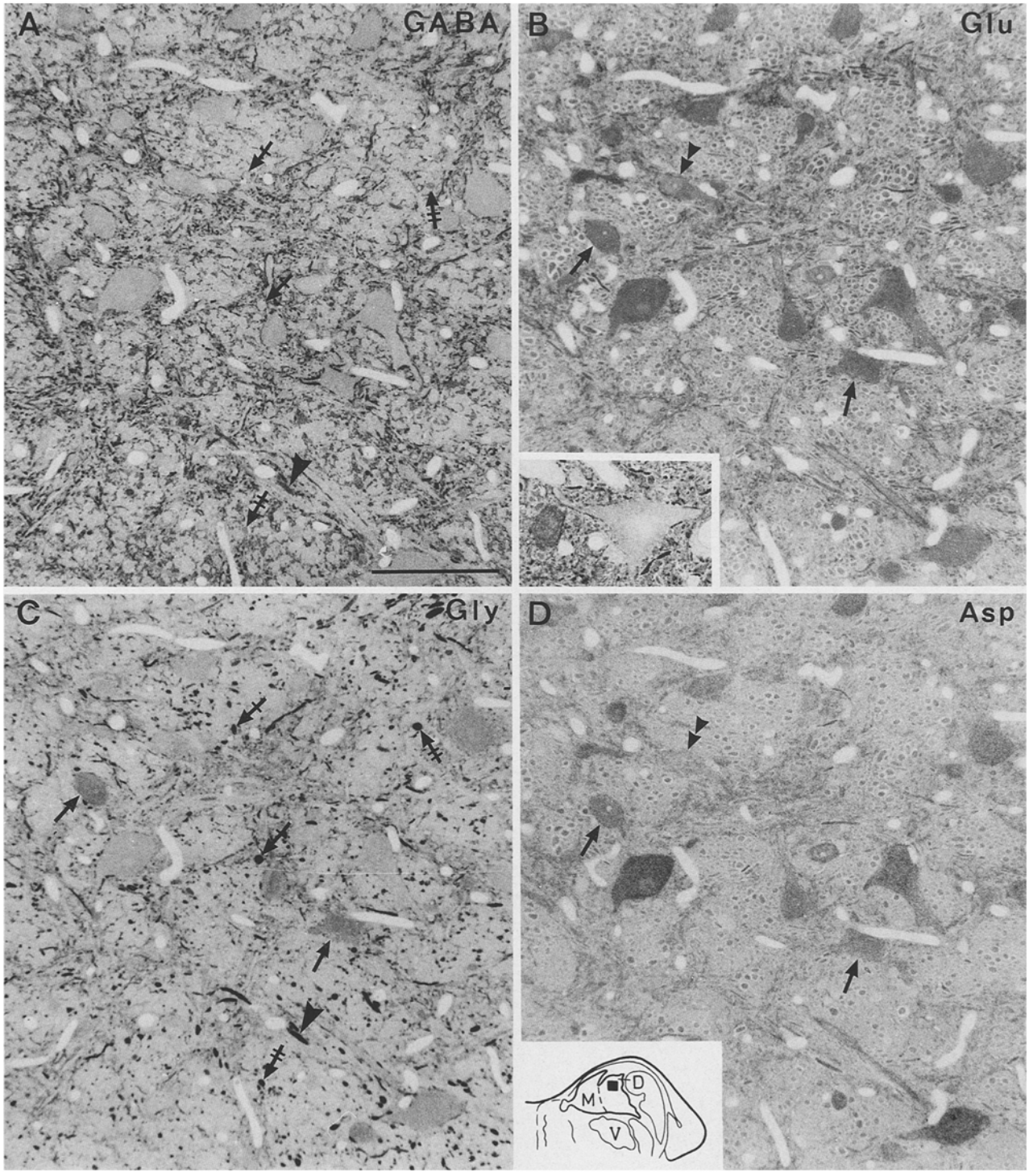

Fig. 6A–E. Photomicrographs of adjacent semithin sections cut transversely through the descending vestibular nucleus (see inset). Strongly Gly(+) profiles usually contain high (arrowhead) or moderate (double crossed arrows) levels of GABA immunoreactivity; only a few are GABA(−) (crossed arrows). Several cells (arrows) are immunostained for Glu, Gly as well as Asp. Although most neurons contain a considerable amount of Glu–LI, very weakly stained neurons are also found (inset in **B**). Only subtle differences exist between the Glu and Asp staining patterns in this particular view (e.g., cell indicated by double arrowhead is moderately labelled for Glu, but very weakly labelled for Asp). For details, see text. Scale line 100 μm. Cat 13787

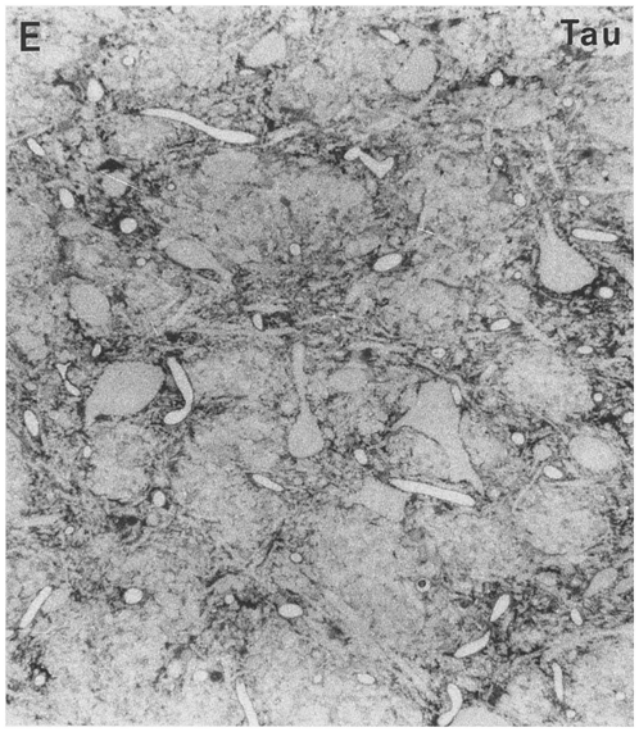

Fig. 6E

mised from the visual inspection of the sections, the densitometry revealed several neurons that had intermediate staining intensities, i.e., a transmittance between 45% and 60% (Fig. 4B).

As was the case for Glu–LI, Asp–LI was low in the glial cells. Although the difference in Asp immunostaining intensity between neurons and glia was less distinct than for Glu (compare Fig. 7 and 8), it was statistically significant (see legend to Fig. 8). The large neurons were richer in Asp–LI than the smaller ones (Fig. 3, 6, 9).

All nuclei contain Asp(+) fibres of varying caliber running in all directions, and several axons in the fibre bundles penetrating the descending nucleus are Asp(+) (Fig. 6D). A few Asp(+) nerve terminal-like puncta are also observed, but are rather inconspicuous since the level of immunoreactivity that they contain is only slightly higher than the average tissue level and lower than that of the immunopositive perikarya and dendrites.

Taurine immunoreactivity

There are no Tau(+) nerve cell bodies in the vestibular complex (Fig. 5, 6, 9). The pattern of Tau

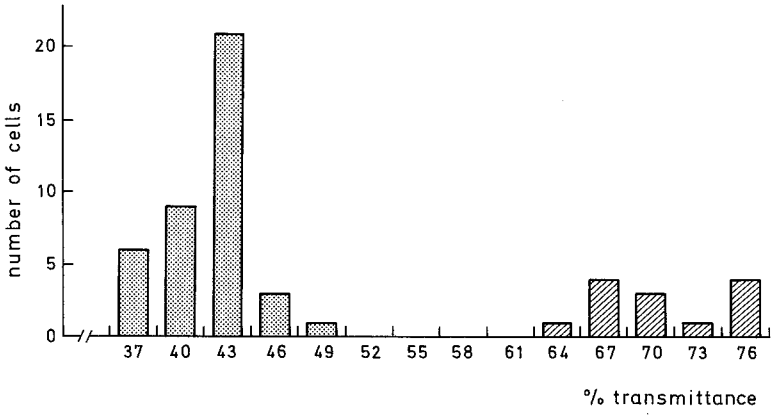

Fig. 7. Histogram showing the optical transmittance in neuronal (stippled columns) and glial cell bodies (hatched columns) in a Glu immunostained section through the descending nucleus (all neurons included). Bin width = 3%. Note that the glial cells are much less intensely stained than the neurons

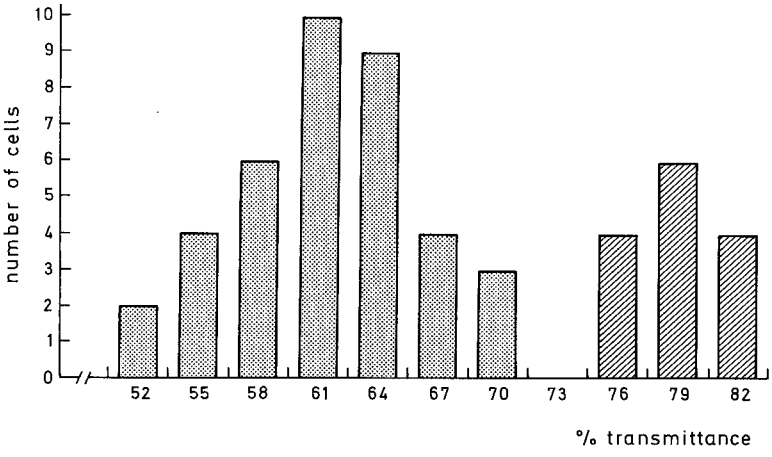

Fig. 8. Histogram showing the optical transmittance in neuronal cell bodies (stippled columns) and glial cell bodies (hatched columns) in an Asp immunostained section through the descending nucleus (all neurons included). Bin width = 3%. The difference in transmittance between neuronal and glial cells is less than for glutamate (see Fig. 7), but is highly significant statistically ($p < 0.001$, Student's t-test)

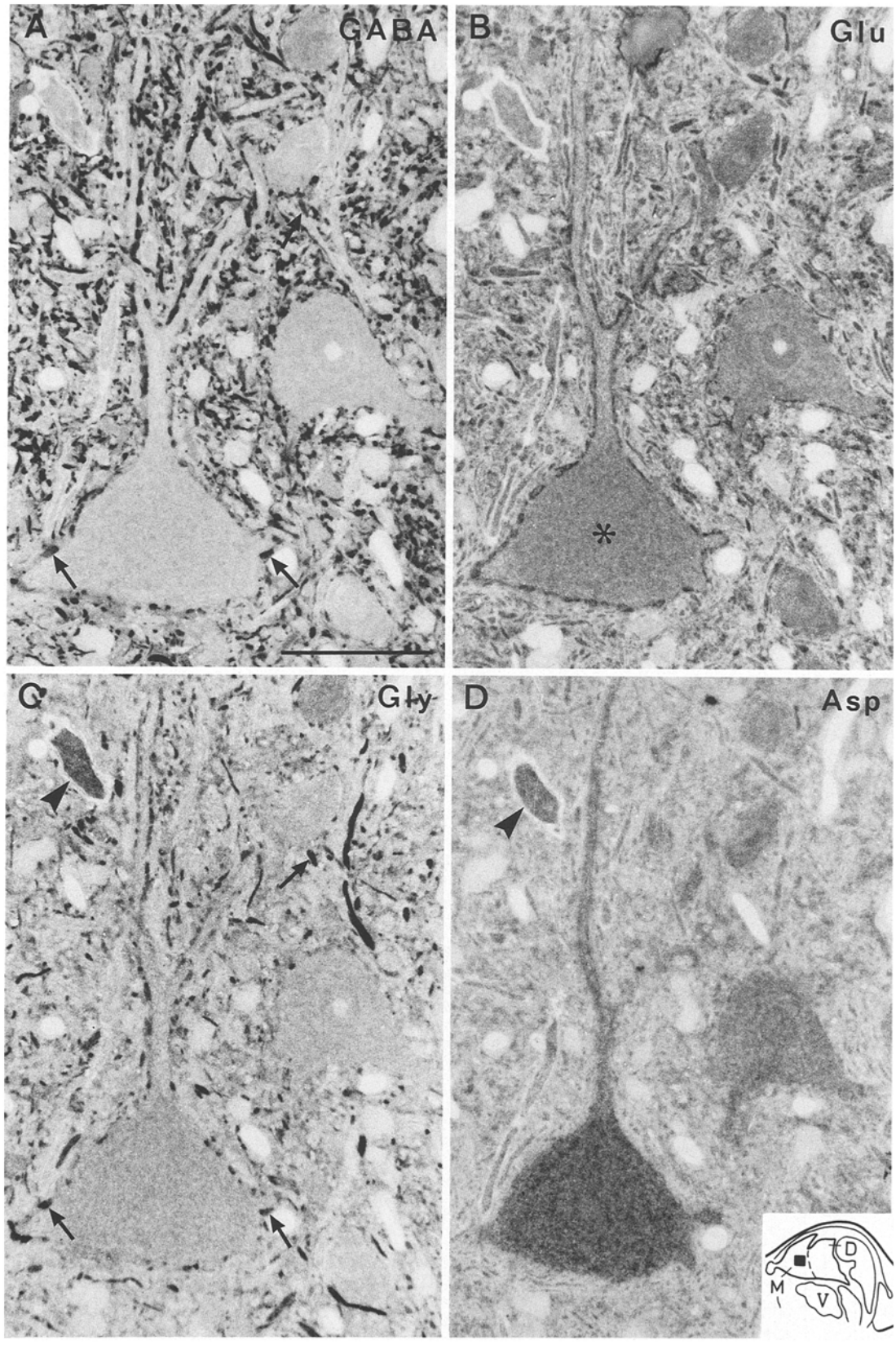

Fig. 9A–E. Photomicrographs of adjacent semithin sections cut transversely through the medial vestibular nucleus (see inset). In **B**, note that one of the Glu(+) neuronal cell bodies (asterisk) appears to be contacted by a large number of Glu(+) bouton-like profiles. The latter profiles are not labelled for Asp, although Asp–LI coexists with Glu–LI in the cell bodies. Arrowhead, thick myelinated axon colocalizing Gly-LI and Asp–LI. Arrows, nerve profiles colocalizing Gly-LI and GABA–LI. Scale line 50 μm. Cat 13787

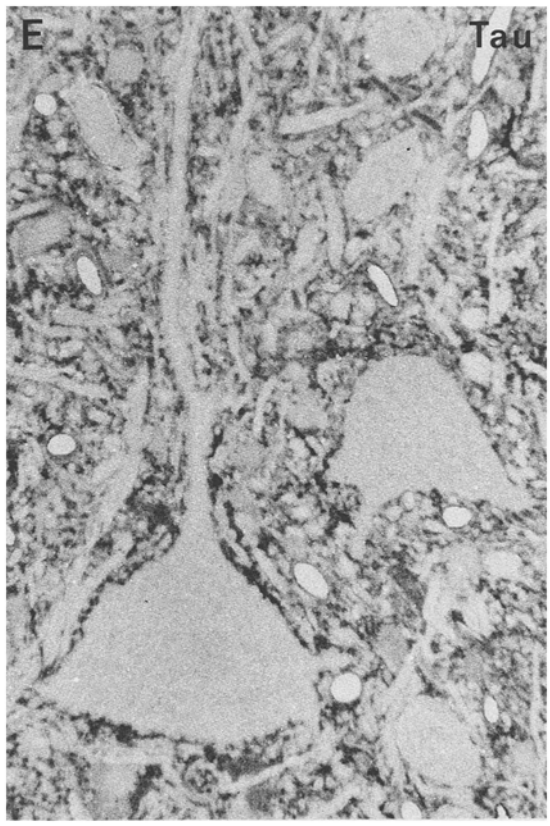

Fig. 9E

immunoreactivity varies considerably among the different nuclei. The dorsal part of the lateral nucleus contains numerous labelled fibres (Fig. 5E), probably representing Purkinje cell axons, and many cell bodies and major dendrites are outlined by labelled bouton-like structures. The perisomatic labelling is also quite extensive in the ventral part of the lateral nucleus, which however, shows much fewer Tau(+) axons than the dorsal part (Fig. 5F). In the descending nucleus (Fig. 6E), labelling of glial cells and profiles predominates, whereas the immunopositive structures in the medial nucleus (Fig. 9E), appear to be partly neuronal, partly glial in origin. However, in all nuclei, the immunolabelling pattern is such that the exact ratio between the two types of labelled profiles (glial or neuronal) can only be determined by electron microscopy. Several of the fibres in the vestibular nerve are Tau(+) (Fig. 5D).

Comparison of immunostaining patterns

The use of serial 0.5 μm thin sections permitted a detailed comparison between the different staining patterns. The question of amino acid colocalization could easily be resolved for cell bodies and transversely or obliquely cut axons and dendrites. In contrast, the small size of the nerve terminals often deterred any definitive conclusions about colocalization at this level.

GABA versus glycine

The most extensive colocalization between GABA and Gly was found in the lateral vestibular nucleus. The vast majority of Gly(+) profiles that could be identified in the adjacent GABA-immunostained section showed colocalization of these two amino acids (Fig. 2). This was true for nerve cell bodies as well as for transversely cut myelinated axons. Only a very few of the Gly(+) profiles could be identified as GABA(−).

Whereas a majority of the Gly(+) immunopositive profiles in the lateral nucleus also contain GABA, the converse is not true. Large bundles of GABA(+) axons probably representing Purkinje cell fibres were devoid of Gly-LI as was also the case for numerous fibres of smaller calibre. Most, if not all, of the perisomatic nerve terminal-like profiles were only GABA immunostained, as judged from the extremely sparse perisomatic labelling for Gly (Fig. 2).

In the descending nucleus (Fig. 6), some axons were found to colocalize GABA and Gly. However, such double-labelled structures were considerably less common here than in the lateral nucleus, and profiles containing Gly immunoreactivity only were frequently seen. Similar to the situation in the lateral vestibular nucleus, GABA immunostained profiles were much more numerous than Gly immunostained ones. The greatest differences between the two amino acid immunoreactivities pertain to the fine nerve terminal-like profiles occurring freely in the neuropil and in contact with cell bodies (Fig. 6A,C).

The relative distribution of GABA and Gly immunoreactive elements in the medial nucleus was similar to that in the descending nucleus. Some of the terminal-like structures that were large enough to be identifiable in adjacent sections appeared to colocalize GABA and Gly (Fig. 9).

GABA versus glutamate and aspartate

The GABA and Glu immunostaining patterns were dissimilar and in many areas roughly complementary. This is particularly well illustrated in Fig. 6A and B: the cell bodies in this field are uniformly GABA(−), but Glu(+), and the great majority of the transversely cut myelinated axons are stained for Glu but not for GABA. GABA-LI and Asp-LI

similarly appeared to be concentrated in separate profile types.

Glycine versus glutamate and aspartate

Some Gly immunopositive nerve cell bodies, most notably in the descending nucleus, also showed an enrichment of aspartate immunoreactivity (Fig. 6). Most of the neurons that were found to colocalize Gly and Asp were also immunostained for Glu, but none of these neurons contained detectable levels of GABA–LI. A coexistence of Gly and Asp was also found in some myelinated axons in the medial vestibular nucleus (Fig. 9).

Glutamate versus aspartate

Glutamate- and Asp–LI showed extensive overlap in nerve cell bodies. However, the exact staining intensities for the two amino acids were neither positively nor negatively correlated: as shown in Fig. 3, neurons with comparable levels of Glu–LI showed great variation in Asp immunostaining. The neuropil labelling was strikingly different with the two antisera. Whereas the Glu antiserum produced distinct labelling of nerve terminal-like profiles in the neuropil as well as in perisomatic positions, especially in the medial nucleus (Fig. 9B), incubations with the Asp antiserum led to a relatively weak and homogeneous neuropil labelling. Aspartate(+) perisomatic terminals were extremely rare (see Fig. 9D). In the fibre bundles traversing the descending nucleus, a substantial proportion of the fibres showed colocalization of Glu and Asp (Fig. 6). However, the colocalization appeared to be restricted to the thick myelinated axons; most of the thinner axons were singularly labelled for Glu. In the lateral nucleus, myelinated fibres were either double labelled for Glu or Asp or labelled for glutamate only (Fig. 3).

Taurine versus the other amino acids

As stated above, the distribution of Tau differs from that of all of the other amino acids by its occurrence in glial as well as in neuronal elements. In the lateral nucleus (Fig. 5), the distribution of Tau is in many respects similar to that of GABA: both immunoreactivities occur in Purkinje cell axons, and both immunoreactivities outline nerve cell bodies and major dendrites. At least part of the perisomatic and peridendritic Tau–LI is likely to represent axon terminals. In the descending and medial nuclei (Figs. 6, 9), the patterns of GABA–LI and Tau–LI are less similar than in the lateral nucleus. Taurine(+) axons are rare, and the perisomatic Tau labelling has a scalloped appearance, quite unlike the punctate appearance of the GABA immunostaining. Although electron microscopy is required to resolve this point, the staining pattern strongly suggests that Tau is localized in glial lamellae between the perisomatic terminals (see Fig. 9E). Blood vessels, particularly those in the descending nucleus (Fig. 6E), are outlined by Tau–LI which probably resides in the perivascular glial processes. None of the other amino acid-like immunoreactivities are similarly concentrated around blood vessels.

Discussion

Interpretation of immunolabelling patterns

All data presented in this article were obtained from tissue sections that had been incubated together with a series of test conjugates for continuous monitoring of the selectivity of the immunocytochemical reaction. The test results suggest that the different antisera recognize selectively the homologous amino acid conjugates under the conditions used. All sera have previously been subjected to more extensive specificity testing with satisfactory outcome (Ottersen and Storm-Mathisen 1984b, 1985; Storm-Mathisen and Ottersen 1986; Ottersen et al. 1986, 1988; Ottersen 1988).

The GABA antiserum presently used has been shown in a large number of studies to label selectively neuronal populations that are thought to be GABA-ergic on other grounds (e.g., Ottersen and Storm-Mathisen 1984b; Rinvik et al. 1987). This fits with the notion that GABA is concentrated exclusively in neurons that use GABA as a transmitter. In fact, the demonstration that GABA synthetic capacity (Fonnum et al. 1970) is localized in the Purkinje axon terminals in the lateral vestibular nucleus was the first demonstration that GABA is specifically produced in GABA-ergic neurons in brain. By inference, there are good reasons to assume that all of the GABA-immunostained profiles in the vestibular nuclei belong to GABA-ergic neurons.

Glycine is, in contrast to GABA, used as a metabolic intermediate, and not only as a transmitter. However, in a simple vertebrate nervous system significant Gly–LI is confined to the single type of neuron that is glycinergic (Dale et al. 1986). Furthermore, semiquantitative immunocytochemical studies based on the post-embedding immunogold method have suggested that the level of Gly is very much lower in metabolic pools than in presumed transmitter pools in mammalian brain

(Ottersen et al. 1988, 1990; Ottersen 1989b; also see van den Pol and Gorcs 1988).

For Glu, in contrast, the size of the metabolic pool is large enough to severely interfere with the interpretation of Glu immunostained preparations (Fonnum 1984). Taking into account the multifarious roles of Glu in general cell metabolism, Glu would be expected to occur in varying amounts in all cellular compartments in the brain. This implies that the term "Glu immunonegative" must be used with due caution: it does not necessarily indicate that the profile in question is devoid of Glu, but merely signals that the profile contains a much lower concentration of Glu than tissue average.

These considerations raise the question whether it is possible to distinguish between metabolic and transmitter pools of Glu. Quantitative immunogold studies suggest that such a distinction can indeed be made at the level of the nerve terminals. Thus, nerve terminals of presumed glutamatergic fibres contain a several fold higher concentration of Glu immunoreactivity, as assessed by gold particle densities, than nerve terminals with other transmitter identities (Somogyi et al. 1986; Ottersen and Bramham 1988; Ottersen 1989a,b). A similar conclusion was drawn by Liu et al. (1989), based on the use of a monoclonal Glu antibody and a highly sensitive light microscopic staining method. Moreover, the immunoreactivity in the presumed glutamatergic terminals can be partially removed in a Ca^{++}-dependent manner by depolarizing the tissue in vitro (Storm-Mathisen et al. 1986; Ottersen and Laake 1989). The possibility should thus be considered that the strongly Glu(+) terminals encountered in the present material indeed release Glu as a transmitter. In contrast, the paucity of distinctly Asp(+) terminals argues against a major transmitter role for the latter amino acid in the vestibular complex.

The nerve cell bodies are far removed from the site of transmitter release; consequently, their Glu contents may not closely reflect the size of the transmitter pool but rather depend on the level of metabolic Glu. This notion receives support from previous findings of a significant level of Glu–LI in cell bodies of certain populations of neurons assumed to use GABA, dopamine or serotonin as transmitters (Ottersen and Storm Mathisen 1984b). The present densitometrical data suggest that most, if not all, of the neuronal somata contain noteworthy concentrations of Glu, and that the glial cells are comparatively poor in Glu. The limited number of observations provided no clues for singling out a subpopulation of putative glutamatergic neurons in the vestibular complex.

Similarly, the densitometrical data indicated that Asp–LI is present in varying amounts in all neuronal cell bodies, whereas the glial cells are generally weakly stained. When interpreting the cell body staining for Asp, it should be recalled that substantial levels of Asp–LI have been observed in non-principal neurons in hippocampus and fascia dentata, most of which are believed to use GABA as transmitter (Ottersen and Storm-Mathisen 1985), and in the cholinergic neurons of the hypoglossal nucleus (Yingcharoen et al. 1989). It seems generally difficult to draw firm conclusions about transmitter identity on the basis of the cell body staining for Glu and Asp.

With regard to the densitometrical data presented here it should be emphasized that the relationship between staining intensity and antigen concentration is not known, although we have previously shown in a model system that the staining (expressed by optical density) and antigen concentration (expressed on a logarithmic scale) may be roughly linearly correlated (Ottersen et al. 1986). A direct correlation between immunolabelling intensity and absolute concentrations of fixed amino acids can be established with the postembedding immunogold procedure at the electron microscopic level (Ottersen, 1989a,b). Work is in progress to adapt a similar strategy for light microscopic studies.

Amino acid colocalization

A major objective of this study was to compare the distributions of the five different amino acid immunoreactivities down to the level of individual cell profiles. This was made possible by the use of serial semithin sections. Conditions were selected so as to achieve the best possible correlation between the immunolabelling patterns and the in vivo distributions of amino acids. Notably, the animals were fixed very quickly after thoracotomy, to minimize amino acid redistribution due to hypoxia or acidosis, and with a relatively high concentration of glutaraldehyde, to retain as much of the free amino acids as possible. Model experiments based on animals preloaded with radiolabelled Tau suggest that about 80% of the free amino acid pool is fixed under the selected conditions (S. Madsen and O.P. Ottersen, unpublished). The results obtained in the present study have general interest, in as much as they show that neuroactive amino acids may coexist in many different combinations within individual neurons. An important task of future research will be to resolve the biological significance of this complex pattern of amino acid

colocalization. The present stage of knowledge permits only some speculative comments on this issue.

The findings of 1: GABA and Gly, 2: Gly, Asp and Glu, 3: Gly and Asp, or 4: Glu and Asp in the same cell profile naturally raise the question whether only one of the amino acids is released, or whether co-release occurs. Colocalization of GABA and Gly has previously been described in the cerebellum (Ottersen et al. 1988), cochlear nuclei (Wenthold et al. 1987), and retina (Yazulla and Yang 1988), and our own observations (Ottersen et al. 1990) suggest that both amino acids may be released from the same neuron, at least as far as the cerebellar Golgi cell terminals are concerned. In cases where Glu and Asp occur in cells that are also enriched in Gly, the former two amino acids may play metabolic roles only (see above). It should be noted, however, that recent studies by Docherty et al. (1987) have suggested that release of an inhibitory and an excitatory amino acid (GABA and Glu) may occur from the same nerve terminals. Possibly, corelease of several "classical" transmitter candidates is more common than previously expected. In cases of corelease, an amino acid does not need to be transmitter in the restricted sense, but rather serve to modulate the receptor-mediated response of the true transmitter (e.g., Werman 1980; Johnson and Ascher 1987). The extensive neuronal colocalization of Glu and Asp may reflect the tight metabolic coupling of the two amino acids, through the enzyme aspartate aminotransferase (Schousboe and Hertz 1983).

Comparison with other immunocytochemical studies

The previous immunocytochemical studies on the vestibular complex have primarily dealt with the cell body labelling and have, unlike the present report, not focused on the detailed neuropil staining pattern. Neither have any attempts previously been made to compare the fine distributions of the different neuroactive amino acids.

Ottersen and Storm-Mathisen (1984a, mouse) described that GABA(+) cells lay scattered throughout all vestibular nuclei. A scattered distribution of small GABA(+) neurons, with their highest concentration in the medial nucleus, was also observed by Kumoi et al. (1987, guinea pig). Likewise, Mugnaini and Oertel (1985, rat) described GAD immunolabelled cells to be present within all four nuclei, but with the highest concentration in the medial and the caudal part of the descending nucleus. On the other hand, Nomura et al. (1984, rat) found GABA(+) cells to be confined to the medial and descending nucleus. The positive cells were small in the former, and small and medium-sized in the latter. Our observations show that in the cat all the GABA–LI positive cells are small and lie scattered throughout the entire vestibular complex. It is at present unclear to what extent the divergent results obtained by the various students reflect species differences or differences in methodology (GAD or GABA immunocytochemistry, with or without colchicine). Of the mentioned studies, it is only that by Mugnaini and Oertel (1985) that contains quantitative estimates. Their observations give evidence for a concentration of GAD-containing cells exceeding by far that of GABA-immunoreactive cells in the present material. In the present studies the use of colchicine would possibly have increased the number of cell bodies with detectable levels of GABA immunoreactivity, but was deliberately avoided since its intended effect is to produce a redistribution of the amino acids. This effect is desirable when the primary aim is restricted to identifying the cell bodies of putative GABA-ergic neurons, but not when the objective is to obtain immunocytochemical staining patterns that mimic as closely as possible the in vivo distribution of free amino acids (see above). It is well known that certain populations of presumed GABA-ergic neurons contain very low levels of GABA–LI in their perikarya, despite the presence of intense immunolabelling at the level of the nerve terminals. This seems, e.g. to be the case for the Purkinje cells (Ottersen et al. 1988). The discrepancy between nerve terminal and cell body labelling may reflect a sparse GABA synthesis in the cell bodies due to rapid transport of newly synthesized GAD into the axons.

Turning now to the distribution of the GABA(+) fibres, our material shows that these are amply distributed to all parts of the vestibular complex, a finding in agreement with that of Nomura et al. (1984). Two fiber contingents should especially be mentioned. One is the bundles penetrating the descending nucleus. The majority of these fibres are positive. The other contingent is the coarse Purkinje axons which terminate within the dorsal part of the lateral nucleus (Walberg and Jansen 1961). Most of these fibres are intensely GABA(+), an observation also made by Nomura et al. (1984).

Previous studies have shown that GABA(+) beaded terminal fibres and puncta (the presumed en passant and terminal boutons) occur within all parts of the vestibular complex (Nomura et al. 1984; Mugnaini and Oertel 1985; Kumoi et al. 1987, see also Houser et al. 1984). Our semithin sections confirm these findings. In this context it should be recalled that Ottersen and Storm-Math-

isen (1984a), based on their Vibratome sections, described a higher concentration of GABA–LI in the dorsal part of the lateral nucleus, the region for termination of the inhibitory Purkinje cell axons (Walberg and Jansen 1961), than in the ventral part of the same nucleus. Our semithin sections show, however, that the GABA(+) nerve terminal-like puncta are distributed almost evenly throughout the lateral nucleus with a somewhat higher concentration ventrally[3]. This observation, therefore, demonstrates that the high density of GABA–LI observed by the former authors must be related to the content of GABA(+) Purkinje cell axons within the dorsal part of the lateral nucleus and not to the concentration of axonal terminals.

To our knowledge this is the first detailed description of the distribution of Glu–LI, Gly–LI and Tau–LI within the vestibular complex. With regard to the distribution of Asp–LI our findings agree with those of Kumoi et al. (1987, guinea pig), who found a high percentage of nerve cells of all sizes to be Asp(+), with the highest concentration of such cells in the lateral nucleus, where almost all giant cells were intensely stained. Furthermore, as in the guinea pig, there is also in the cat a very modest pericellular labelling. In contrast to Kumoi et al. (1987) we find that many of the fibres in the bundles penetrating the descending nucleus are Asp positive.

Fibre connections and functional aspects

The intrinsic and extrinsic connections of the vestibular complex are well known (see especially Brodal et al. 1962 and Hauglie-Hanssen 1968). The most comprehensive study of the intrinsic organization is that by Hauglie-Hanssen (1968). In the kitten he failed to observe interneurons with heavily branching short axons, but found that in the medial and descending nuclei some of the smaller cells had axons dividing close to their soma, an observation in accordance with those by Cajal (1909–11), Lorente de Nó (1933) and Grant et al. (1980). We do not know if the negative observation concerning possible interneurons in the lateral and superior nuclei is related to the well known difficulties in demonstrating axons in Golgi sections[4], and it can therefore not be excluded that some of the immunopositive cells also in these two vestibular nuclei are Golgi type II cells. However, it should be recalled that all cell types, also those belonging to the small category, are projecting neurons (see Brodal et al. 1962).

It is well known that within all vestibular nuclei there is an intermingling of cells giving rise to the various efferent fibre projections. For example, the cells within the lateral, medial and descending nuclei that emit the three vestibulospinal fibre tracts lie adjacent to cells which project to the cerebellum. Labelling experiments with retrograde tracers combined with immunocytochemistry are therefore needed to localize the cells belonging to the various vestibulofugal tracts, and at the same time identify the type of amino acid(s) in the projecting cells. Using this approach, Blessing et al. (1987, rabbit) demonstrated GAD–LI in vestibular neurons that had transported Fast Blue retrogradely from the spinal cord. Fifty per cent of the retrogradely labelled cells in the medial and descending nuclei were GAD(+) and these cells were of varying sizes. (All animals were given colchicine injections into the vestibular nuclei.) Their description of a possible GABA-ergic vestibulospinal projection is of great functional importance since the classical concept is that the vestibulospinal fibres are excitatory (Akaike et al. 1973; Bach and Magoun 1947; Rapoport et al. 1977; Wilson and Yoshida 1968).

Experimental studies have given evidence that there is a somatotopical arrangement in the spinal projection from the lateral nucleus, with the cells sending their axons to the lumbar cord lying dorsally, those projecting to the cervical cord ventrally, and those to the thoracic cord in an intermediate position (Pompeiano and Brodal 1957a). Since, however, GABA(+) terminals are distributed to the entire lateral nucleus[5], this shows that GABA exerts its activity not only on vestibular cells sending their axons to motoneurons influencing hindlimb muscles as suggested by Nomura et al. (1984); also vestibular cells projecting to motoneurons in the cervical and thoracic cord must be influenced by GABA-ergic terminals. However, the latter terminals cannot belong to Purkinje axons since these are restricted to the dorsal part of the lateral nucleus (Walberg and Jansen 1961). The ventral half of the lateral nucleus receives afferents from the vestibular nerve (Walberg et al. 1958), the caudal third of the fastigial nucleus (Walberg et al. 1962)

[3] Electron micrographs show that whereas the highest concentration of axons is found dorsally, the highest concentration of boutons is found ventrally in the lateral nucleus (Fonnum et al. 1970, Table 3)

[4] Pompeiano and Brodal (1957a) are of the opinion that the lateral nucleus does not contain internuncial cells. This conclusion is based on observations of retrograde cellular changes in the nucleus following sections of the lateral vestibulospinal tract

[5] Previous studies (Table 1 in Fonnum et al. 1970) have shown that although it was higher dorsally, the content of GAD was not restricted to this part of the nucleus

and the spinal cord (Pompeiano and Brodal 1957b). Our material shows that in the cat the fibres of the vestibular nerve are GABA(−), but nothing is known about the amino acid content of the two other afferent pathways. Likewise the pericellular Glu(+) puncta, which are particularly numerous around the large cells in the medial vestibular nucleus, cannot be ascribed to a specific afferent pathway. The medial nucleus receives fibres from the labyrinth, the fastigial nucleus and the interstitial nucleus of Cajal (see Brodal et al. 1962 for details). Again, a combination of various techniques would be necessary for an analysis of the amino acid content in these and the other pathways terminating in the vestibular complex.

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