Immunocytochemical localization of aspartate aminotransferase immunoreactivity in cochlear nucleus of the guinea pig

(aspartate/glutamate/afferent synapses/auditory nerve)

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ABSTRACT There is substantial evidence supporting the role of aspartate or glutamate as the neurotransmitter of the auditory nerve. The concentration of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), an enzyme associated with the metabolism of these amino acids, is high in axons and terminals of the auditory nerve. Antibodies were raised against aspartate aminotransferase and used in immunocytochemical studies to determine its localization in the cochlear nucleus of the guinea pig. Indirect immunofluorescence techniques were used for light microscopic localization of aspartate aminotransferase-like immunoreactivity in normal guinea pigs and guinea pigs with auditory nerve lesions. Fluorescent rings of aspartate aminotransferase-like immunoreactivity were seen around spherical cells in the anteroventral cochlear nucleus. In animals with auditory nerve lesions, rings were no longer seen in the ipsilateral cochlear nucleus. Immunoreactivity was also seen on cells in the posteroventral cochlear nucleus and in auditory nerve fibers. Ultrastructural studies were done in the rostral anteroventral cochlear nucleus, using the peroxidase-antiperoxidase technique. Aspartate aminotransferase-like immunoreactivity was seen at axosomatic synapses on large spherical cells in terminals with the morphological characteristics of auditory nerve terminals. Other classes of terminals on the soma of large spherical cells showed no immunoreactivity. It was concluded that aspartate aminotransferase-like immunoreactivity is present in axons and terminals of the auditory nerve. These findings indicate that aspartate aminotransferase-like immunoreactivity may serve as a marker at terminals where aspartate or glutamate is a neurotransmitter.

There is substantial evidence supporting a neurotransmitter role for glutamate and aspartate in the mammalian central nervous system (1, 2). The ubiquitous distribution of these amino acids, however, complicates the task of identifying synapses using glutamate or aspartate as neurotransmitters. Furthermore, there is little information on the metabolism of these substances where they may function as neurotransmitters. Biochemical (3–8) and pharmacological (9, 10) data support the role of glutamate or aspartate as a neurotransmitter for the auditory nerve. Two enzymes associated with the metabolism of these amino acids, glutaminase and aspartate aminotransferase (AATase; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) show high activity in auditory nerve fibers and decreased activity in the cochlear nucleus after creation of auditory nerve lesions (5). This suggests that these enzymes may be involved in the metabolism of glutamate or aspartate when they are carrying out a neurotransmitter role. If this is the case, one or both of these enzymes may serve as a marker for aspartergic or glutamergic neurons. To investigate this possibility and to further our understanding of the function of these amino acids in the central nervous system, we have carried out immunocytochemical localization of AATase in the cochlear nucleus.

MATERIALS AND METHODS

Antibody Preparation. Antibodies were raised against cytoplasmic AATase from pig heart, in three female New Zealand White rabbits. Commercially available AATase (Boehringer Mannheim) was purified on a Sephadex G-150 column. One milligram of AATase was emulsified in Freund's complete adjuvant and injected subcutaneously at four sites along the mammillary line of each rabbit. Four weeks later, 0.5 mg of AATase was injected with incomplete adjuvant in the same manner. One week later the rabbits were bled and the pooled antiserum was characterized. Analysis of the antiserum by the method of Ouchterlony (11) revealed a single precipitin band. When the antiserum was tested against AATase from guinea pig brain, a line of identity was seen to the commercially available AATase. Both preparations of AATase were shown by spectrophotometric analysis (12) to be enzymatically inactivated by the antiserum.

Analysis of the commercially available AATase by electrophoresis on sodium dodecyl sulfate/polyacrylamide gels (13) revealed several protein bands when the gels were stained with 0.25% Coomassie blue R-250 (Fig. 1A). Of these, a component of about 45,000 daltons is the major band and has been identified as the monomeric subunit of cytoplasmic AATase (14). Gel filtration, ion-exchange chromatography, affinity chromatography with immobilized pyridoxal phosphate, and preparative isoelectric focusing all resulted in the retention of the three major bands. The 45,000-dalton component was separated from the other major proteins with sodium dodecyl sulfate gel electrophoresis and cut out of the gel. Reelectrophoresis of this band showed a single major component at 45,000 daltons (Fig. 1A). The isolated 45,000-dalton component was used to prepare an affinity column to separate antibodies specific to the 45,000-dalton monomeric subunit and also to raise antibodies in the manner described against the 45,000-dalton subunit. Immunoprecipitation (15) of iodinated commercial AATase showed a precipitation of the 45,000-dalton subunit and higher molecular weight proteins by the original antiserum, by the affinity column-purified original antibody, and by the serum raised against the 45,000-dalton subunit (Fig. 1B). These results show that all three antisera are directed against the 45,000-dalton subunit. The fact that antibodies against this subunit also precipitate some higher molecular weight proteins suggests that these proteins are antigenically similar to the 45,000-dalton subunit and are, perhaps, complexes of AATase generated during purification. Only the 45,000-dalton subunit was determined to be present in the auditory nerve. [^{35}S]Methionine-labeled auditory nerve proteins were isolated from the cochlear nucleus and the auditory nerve (16). Immunoprecipitation of this preparation with the original antiserum showed a single labeled band corresponding to the 45,000-dalton subunit (Fig. 1C).
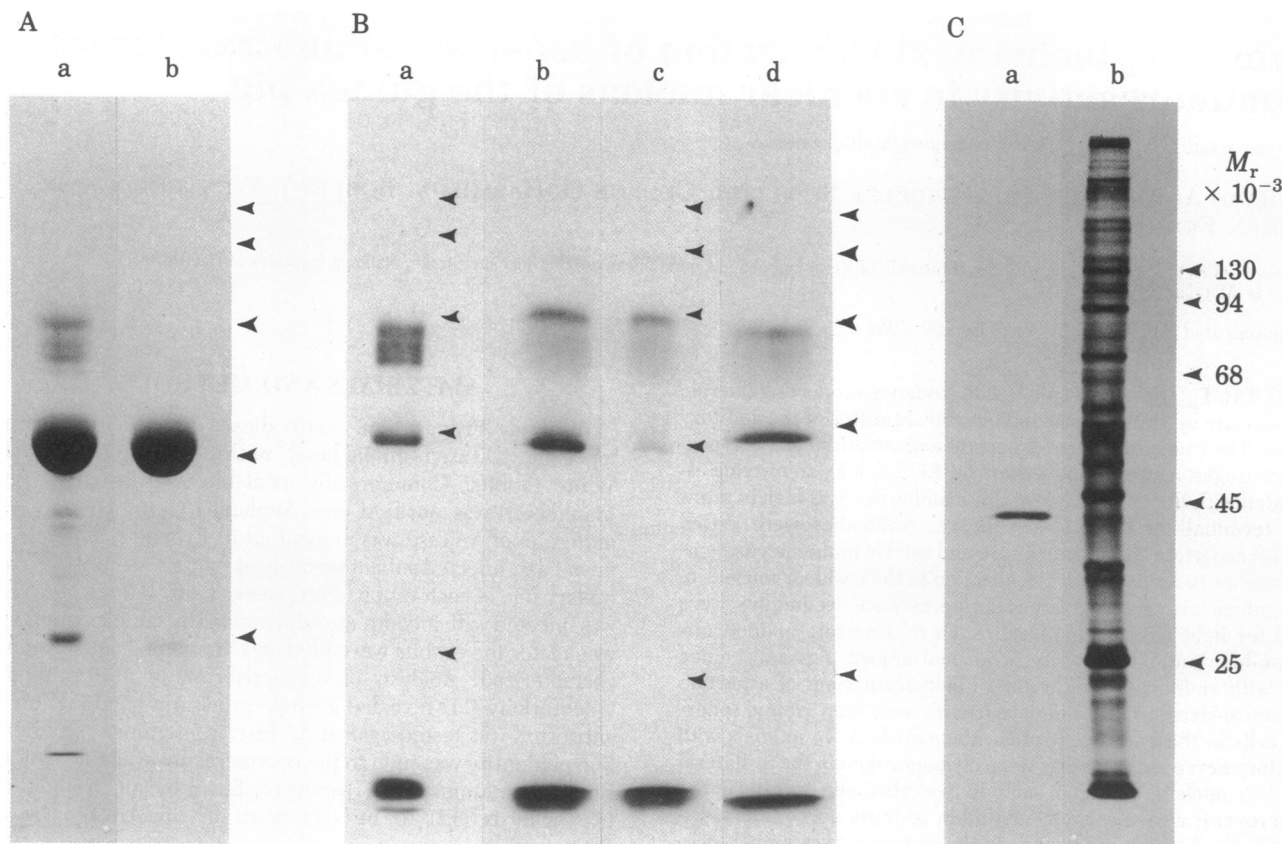
FIG. 1. Electrophoretic analysis of AATase. (*A*) Coomassie blue-stained gel. Lane a, commercial AATase; lane b, electrophoretically purified AATase. (*B*) Fluorography of immunoprecipitated commercial AATase that has been iodinated. Lane a, iodinated AATase; lane b, iodinated AATase precipitated by antiserum to commercial AATase; lane c, iodinated AATase precipitated by antiserum from commercial AATase that was retained by an affinity column of electrophoretically purified AATase; lane d, iodinated AATase precipitated by antiserum raised to electrophoretically purified AATase. The control rabbit serum (not shown) did not show any bands. (*C*) Fluorography of [^{35}S]methionine-labeled auditory nerve proteins from the cochlear nucleus and auditory nerve 36 hr after labeling by cochlear injection. Soluble proteins were extracted by homogenizing in 5 mM Tris·HCl (pH 8.1) and centrifuging at 100,000 × *g* for 1 hr. The soluble fraction was used for immunoprecipitation (15). Lane a, immunoprecipitate using antiserum from commercial AATase; lane b, total [^{35}S]methionine-labeled auditory nerve protein.

Light Microscopic Immunocytochemistry. NIH strain guinea pigs of both sexes were anesthetized with chloral hydrate and perfused through the heart with 50 ml of 0.1 M sodium caco-dylate buffer followed by 600 ml of 4% (wt/vol) paraformalde-hyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C. Ten-micrometer coronal cryostat sections were cut through the cochlear nucleus and the indirect immunofluorescence tech-nique of Coons (17) was applied to determine immunoreactivity. Antiserum to AATase diluted in phosphate-buffered saline P$_i$/NaCl, pH 7.2, with 0.3% Triton X-100, was applied to sections thaw mounted on slides. A 1:40 dilution with an incubation of 30 min at 37°C and a 1:400 dilution with an incubation of 16 hr at 4°C were used interchangeably. Affinity column-purified an-tibodies were used at a concentration of 20–25 μg/ml for 16 hr at 4°C. The antiserum raised against the 45,000-dalton subunit was used at a 1:5 dilution for 16 hr at 4°C. After the primary incubation, slides were rinsed in P$_i$/NaCl sections were then incubated with fluorescein isothiocyanate-labeled swine anti-serum to rabbit immunoglobulin (Bio-Rad) diluted 1:10 in P$_i$/NaCl with 0.3% Triton X-100 for 30 min at 37°C. The slides were rinsed in P$_i$/NaCl and coverslips were added, and then the slides were examined and photographed through a Zeiss microscope under epifluorescent illumination.

A lesion of the left auditory nerve was made by cochlear ablation (7) in six guinea pigs, and the cochlear nucleus was examined for AATase-like immunoreactivity at 2, 3, and 7 days after the lesion had been made. The right cochlear nucleus

served as a control.

Electron Microscopic Immunocytochemistry. NIH strain guinea pigs of both sexes were anesthetized with chloral hydrate and perfused through the heart with 50 ml of 0.1 M sodium acetate buffer (pH 6.0), followed by 600 ml of Zamboni fixative (18): 4% paraformaldehyde/1% glutaraldehyde/0.2% picric acid in 0.1 M sodium acetate buffer (pH 6.0) with 1.5% sucrose, at 4°C. Brains were removed and immersed in fixative for 2 hr at 4°C and then in 0.1 M sodium acetate buffer (pH 6.0) with 1.5% sucrose for 20 hr at 4°C. Fifty- to 70-μm coronal sections were cut in P$_i$/NaCl at pH 7.2, with a Vibratome (19). The sec-tions were then rinsed with agitation in P$_i$/NaCl for 1 hr at 4°C followed by a 1-hr rinse in a 10% solution of normal swine serum (Cappel Laboratories, Cochranville, PA) in P$_i$/NaCl. The un-labeled antibody enzyme method of Sternberger (20) as modi-fied for electron microscopy by Pickel *et al.* (21) was applied with minor changes. Free-floating sections were incubated in a 1:400 dilution of original antiserum to AATase in P$_i$/NaCl for 16 hr at 4°C. No Triton X-100 was used in this or subsequent incubations. After primary incubation, sections were rinsed for 3 hr with agitation in at least three changes of P$_i$/NaCl at 4°C. The sections were then incubated with swine antiserum to rab-bit immunoglobulin rinsed with agitation for 90 min in three changes of P$_i$/NaCl at 4°C. The sections were next incubated in rabbit peroxidase-antiperoxidase antiserum (E-Y Laborato-ries, San Mateo, CA) diluted 1:70 in P$_i$/NaCl for 25 min at 37°C and then rinsed with agitation for 90 min in three changes of

P_i/NaCl at 4°C. After the rinse the sections were allowed to react with 0.05% diaminobenzidine·HCl (Polysciences, Warrington, PA) with 0.005% H_2O_2 in P_i/NaCl for 6 min at 26°C. The sections were rinsed in P_i/NaCl for 1 hr with agitation and the rostral anteroventral cochlear nucleus was dissected out and placed into a solution of 2% OsO_4 in 0.1 M sodium cacodylate buffer for 1 hr at 4°C. Sections were rapidly dehydrated through a graded series of methanol and through propylene oxide and flat embedded in Epon 812. Ultrathin sections were cut from within the first 2 μm of each block and stained for 30 sec with lead citrate.

Controls. Absorption controls were used for light and electron microscopic studies as tests for specificity. Control antiserum was prepared by adding 100 μg of AATase to 50 μl of antiserum, bringing it to the appropriate dilution, and incubating for 20 hr at 4°C.

RESULTS

All three antisera used at the light microscopic level showed similar results. The affinity column-purified preparation of the original antiserum gave lower background fluorescence and was used in much of the immunofluorescence study. The antiserum raised against the 45,000-dalton component was of low titer requiring high concentration, and it was therefore used only in the confirmational studies. Absorption controls were used to establish specificity of immunoreactive staining. Structures that showed immunoreactivity after incubation with antiserum to AATase and showed none on sections incubated with absorption controls were considered to exhibit AATase-like immunoreactivity.

At the light microscopic level AATase-like immunoreactivity was seen as rings of fluorescence around the large spherical cells of the rostral anteroventral cochlear nucleus (Fig. 2A). These rings were not seen in absorption controls (Fig. 2B). At high magnification these rings were seen to be incomplete (Fig. 2C), although covering much of the outline of the cell body. Rings of AATase-like immunoreactivity could also be seen around cells of the caudal anteroventral cochlear nucleus and posteroventral cochlear nucleus (Fig. 2D), with an extent of contact on soma corresponding to primary afferent input. In the dorsal cochlear nucleus it was not possible to resolve any immunoreactive endings on cells. If such endings are present, ultrastructural localization will be needed to show them. A diffuse immunofluorescence, above the level in absorption controls, was seen throughout the cochlear nucleus; this was reduced in animals with auditory nerve lesion. AATase-like immunoreactivity was also evident in auditory nerve fibers in the interstitial region of the cochlear nucleus.

In animals with auditory nerve lesions, rings of immunoreactivity were no longer seen around cells of the ipsilateral cochlear nucleus (Fig. 2E). Small patches of immunofluorescence were all that remained. No changes were seen in the contralateral cochlear nucleus.

At the electron microscopic level, axosomatic synapses on large spherical cells in the anteroventral cochlear nucleus were examined. At low magnification a pattern of immunoreactivity similar to that seen in the light microscopic immunofluorescence studies was found (Fig. 3A) with much, but not all, of the soma apposed by immunoreactive terminals exhibiting morphological characteristics of auditory nerve endings: large round vesicles, asymmetrical active zones with a dome-shaped postsynaptic evagination and presynaptic invagination at the active zone, and enlarged areas of extra-cellular space between active zones, often filled with glia (22) (Fig. 3B–D). Other types of terminals on the large spherical cells, identified as nonprimary afferent (23), did not show AATase-like immunoreactivity.

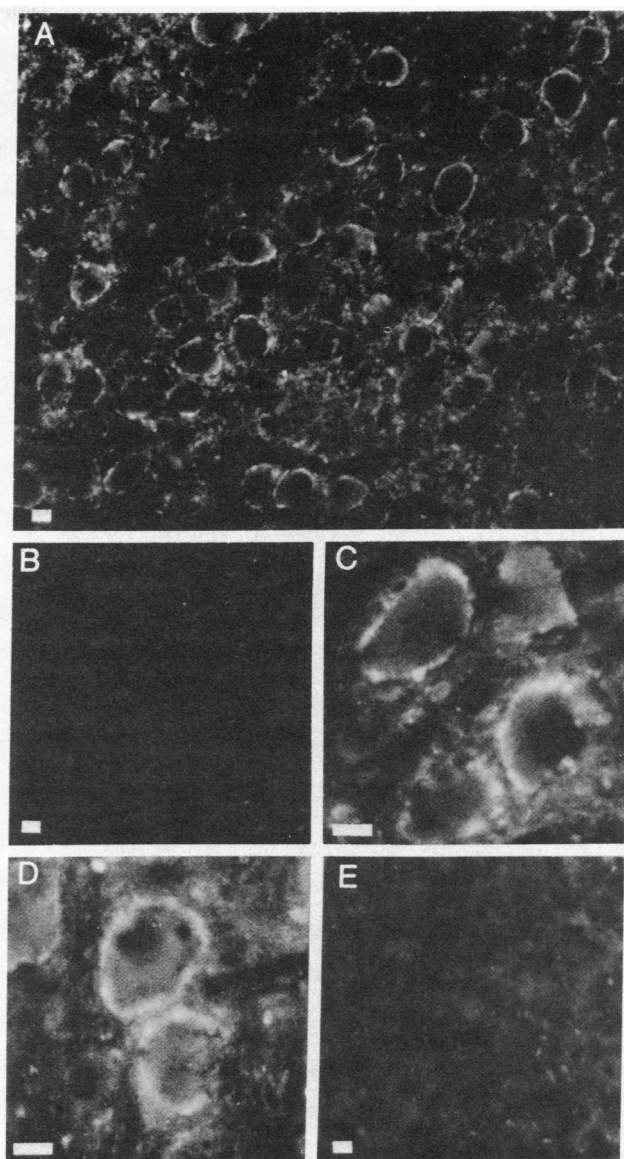AATase-like immunoreactivity was seen in glial elements at

FIG. 2. Fluorescence micrographs of cryostat sections through the guinea pig cochlear nucleus incubated with column-purified antiserum to AATase (A, C–E) or antiserum absorbed with AATase (B) (bars = 10 μm). (A) Immunofluorescent rings around large spherical cells in rostral anteroventral cochlear nucleus. (B) Section through the rostral anteroventral cochlear nucleus incubated with antiserum absorbed with AATase showing no specific immunofluorescence. (C) Immunofluorescent rings around large spherical cells in the rostral anteroventral cochlear nucleus. (D) Immunofluorescent rings around globular cells in the posteroventral cochlear nucleus. (E) Rings of AATase-like immunoreactivity are absent in this section through the anteroventral cochlear nucleus 7 days after the auditory nerve had been destroyed through ablation of the cochlea.

immunoreactive auditory nerve terminals (Fig. 3D) and in myelinated axons (Fig. 3B). Reaction product of variable intensity was often localized in the cell bodies and proximal dendrites of the large spherical cells.

DISCUSSION

This study has established the presence of AATase-like immunoreactivity in terminals and fibers of the auditory nerve. Light microscopic analysis showed rings of immunoreactivity around cell bodies in the anteroventral cochlear nucleus. These rings were not present after lesions had been made in the auditory nerve and thus appeared to be associated with terminals
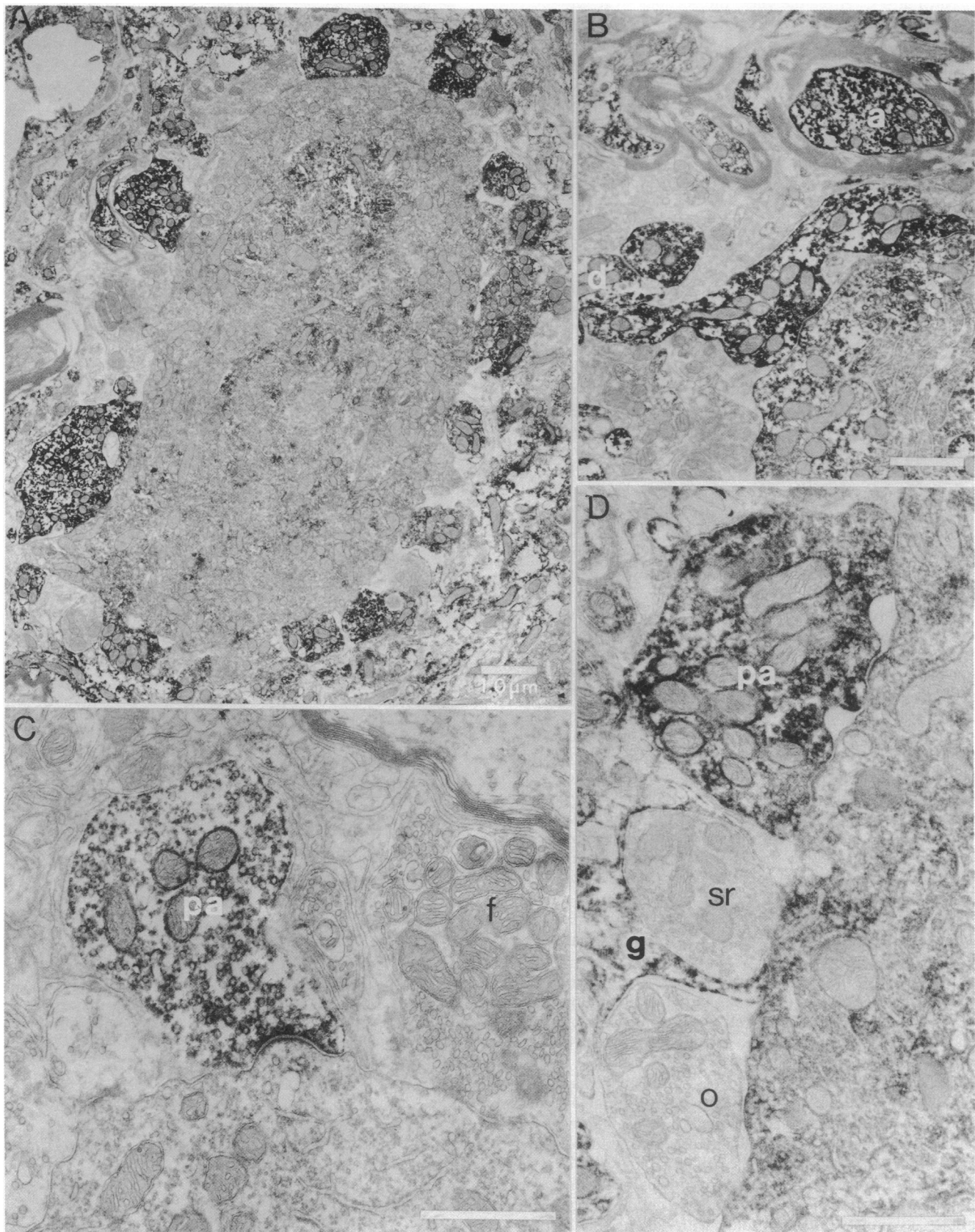
FIG. 3. (*A*) AATase-like immunoreactive terminals can be seen to appose much of the soma of a large spherical cell. (*B*) An AATase-like immunoreactive terminal contacting the soma of a large spherical cell and a proximal dendrite (d). An immunoreactive myelinated axon (a) is also seen. (*C*) An immunoreactive primary afferent terminal (pa) and a terminal with flattened vesicles (f) can be seen synapsing with a large spherical cell. (*D*) A primary afferent terminal (pa), a terminal containing small round vesicles (sr), and a terminal containing oval vesicles (o) are seen synapsing with a large spherical cell. AATase-like immunoreactivity is seen in the primary afferent terminal and in glia (g). Bars = 1 μm.

of the auditory nerve. Ultrastructural analysis confirmed the presence of immunoreactivity in axosomatic terminals with the morphology of auditory nerve terminals. Because of the possibility of antigen diffusion taking place inside the terminal and a resulting nonspecific adherence to membraneous structures, no conclusions can be made concerning the subcellular compartmentalization of immunoreactivity that appeared on synaptic vesicles and mitochondria.

Although rings around neurons in the anteroventral cochlear nucleus are the most prominent structures of immunofluorescence seen, a background of specific immunofluorescence was seen throughout the cochlear nucleus. Some of this may have been in auditory nerve fibers and dendritic terminals, but the fact that most of the fluorescence remained after a lesion had been made in the auditory nerve suggests that it was associated predominantly with non-auditory-nerve structures. Ultrastructural analysis of the rostral anteroventral cochlear nucleus showed that some glial cells and most neuronal cell bodies were labeled. Because AATase is involved in the general metabolism of glutamate and aspartate, it is likely to be present in all neurons and glial cells. This may account for some of the immunospecific background staining. Increased immunoreactive staining intensity reflects increased levels of AATase in particular cells, fibers, and terminals, which may in turn reflect a specialized function for this enzyme. Spiral ganglion cells are strongly labeled with AATase antiserum (unpublished observations). A high level of AATase in the cell body of a neuron may suggest an increased synthesis for axonal transport to the presynaptic terminal to be used in neurotransmitter metabolism. AATase may also have a neurotransmitter-related function in glial cells. Studies have suggested that the major mechanism of glutamate and aspartate inactivation after release is through uptake into synaptic terminals, neuronal cell bodies, and glia (24–26). AATase may play a role in metabolizing the newly sequestered amino acid. Glutamine synthetase is concentrated in glial cells, where it may play a role in the conversion of the neurotransmitter glutamate into glutamine for release into the extra-cellular fluid (27, 28).

An important finding of this study is that, whereas terminals of the auditory nerve are strongly immunoreactive, terminals in the anteroventral cochlear nucleus identified as nonprimary are not immunoreactive. Three types of nonprimary terminals on large spherical cells have been described (23). The neurotransmitter used at these synapses is unknown, but some of these synapses are inhibitory on the basis of morphological criteria. This finding indicates that a high concentration of AATase is not present in all presynaptic terminals. AATase may be present in auditory nerve terminals to carry out a specific function, such as neurotransmitter biosynthesis. For several other neurotransmitters, it has been shown that enzymes related to the neurotransmitter's synthesis are concentrated in presynaptic terminals from which the respective neurotransmitter is released. Immunocytochemical localization of these enzymes has been routinely used to identify neurons using a specific neurotransmitter including, for example, γ-aminobutyric acid and monoamines (29, 30).

Although evidence suggests that glutamate and aspartate may be major neurotransmitters in the mammalian central nervous system, progress in identifying pathways that use these amino acids as neurotransmitters has been impeded by lack of a specific marker. The present results suggest that the presence of AATase in the presynaptic terminal may be a characteristic of

a synapse in which glutamate or aspartate is the neurotransmitter. Although AATase may be concentrated in all glutamate/aspartate terminals, it may also be present in terminals in which other neurotransmitters are used. For example, γ-aminobutyrate (GABA) is synthesized from glutamate and AATase may be present in a GABAergic terminal and be involved in the production of glutamate. The hypothesis that the presence of AATase in the presynaptic terminal may be a characteristic of a glutamate/aspartate terminal should be tested on other proposed glutamate or aspartate synapses.

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