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Ultrastructural localization of GABA-immunoreactive terminals in the anteroventral cochlear nucleus of the guinea pig

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The immunocytochemical distribution of gamma-aminobutyric acid (GABA) was studied by electron microscopy in the anteroventral cochlear nucleus (AVCN) of the guinea pig using affinity-purified antibodies made against GABA conjugated to bovine serum albumin. Our observations confirm that spherical cells are the predominant cell type in the guinea pig AVCN and receive numerous axosomatic contacts (Schwartz and Gulley, (1978) J. Anat. 153, 489–508). Stellate cells receive few axosomatic contacts. Electron microscopic immunocytochemistry shows that GABA immunoreactivity is present in synaptic terminals in the AVCN. Of the several classes of presynaptic terminals present in the AVCN as characterized by vesicle type (large round; oval/pleomorphic; flat; small round) only those containing oval/pleomorphic vesicles were GABA-immunoreactive. However, GABA immunoreactivity may not be present in all these terminals because some oval/pleomorphic terminals are unlabelled. Immunoreactive terminals are widespread in the AVCN; they are abundant on spherical cell bodies, rarely seen on stellate cell bodies and are also found scattered throughout the neuropile.

Auditory system; Neurotransmitter; Immunocytochemistry; GABA

Introduction

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system. A number of studies suggest that GABA is a neurotransmitter in the mammalian cochlear nucleus. Physiological and pharmacological studies, reviewed by Caspary et al. (1985) and Wenthold and Martin (1984), showed that the activity of neurons in the cochlear nucleus is depressed by local application of GABA. GABA has been demonstrated biochemically in the cochlear nucleus, with the highest levels in the dorsal cochlear nucleus (DCN) and slightly lower levels in the ventral cochlear nucleus (VCN) (Godfrey, et al., 1978). Additional support for GABA as a neurotransmitter in the cochlear nucleus comes from studies which demonstrate calcium-dependent release of endogenous GABA from guinea pig cochlear nucleus slices (Wenthold, 1979). Lesion studies (Potashner et al., 1985), retrograde transport studies of ^{3}H-GABA (Ostapoff et al., 1985) and uptake of tritiated GABA into terminals (Schwartz, 1985) suggest that one or more of the centrifugal inputs to the anteroventral cochlear nucleus (AVCN) may be GABAergic. Recent immunocytochemical studies have localized glutamic acid decarboxylase (GAD) immunoreactivity in the cochlear nucleus of the cat (Adams and Mugnaini, 1984; Saint Marie et al., 1985) the rat (Moore and Moore, 1984; Mugnaini,

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1985; Shiraishi et al., 1985) and the gerbil (Roberts and Ribak, 1987), and GABA immunoreactivity has been localized in the cochlear nucleus by this laboratory (Altschuler et al., 1985; Wenthold et al., 1986) and by others (Otterson and Storm-Mathisen, 1984; Thompson et al., 1985). The purpose of the present study is to localize GABA-immunoreactive (GABA-IR) neural elements in the guinea pig AVCN at the ultrastructural level, with the aim of correlating these observations with known synaptic terminals in the AVCN.

Methods

For electron microscopic studies pigmented guinea pigs were anesthetized with chloral hydrate and perfused through the heart with 0.1 M sodium cacodylate buffer (pH 7.3) at room temperature. This was followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer. The tissue was post-fixed for 1 h at 4°C and then transferred to phosphate buffered saline (PBS) at pH 7.3. For immunoperoxidase staining procedures, after an overnight rinse in PBS, 50 μm thick vibratome sections were cut in the frontal plane through the cochlear nucleus. Sections were preincubated for 1 h in PBS containing 10% normal goat serum and 0.1 M lysine. Free-floating sections were incubated overnight in antibodies to GABA at dilutions from 1:1000 to 1:5000 in PBS. Sections were rinsed in PBS with two to three changes over 60–90 min. Subsequent incubation steps on free-floating sections followed the avidin-biotin peroxidase complex (ABC) method of Hsu (1981), Vector Labs, with PBS rinses of 60–90 min. After the final incubation the sections were reacted using diaminobenzidene HCl (50 mg in 100 mls PBS plus 25 μl of 30% H_2O_2) as a chromogen for 3–7 min at room temperature. The sections were then postfixed in a solution of 1.5% potassium ferricyanide and 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 15 min on ice. The sections were rinsed in buffer, dehydrated through a graded series of methanol and infiltrated and flat-embedded in Epon 812. Polymerization took place for 48 h at 60°C. Ultrathin sections were cut, stained with Reynold's lead citrate and examined in a JEOL 1200 transmission electron microscope.

Results

In order to characterize GABA-IR synaptic terminals in the guinea pig AVCN, it is first necessary to establish morphological criteria for the neurons receiving inputs in the AVCN. Ultrastructural observations of cat AVCN by Cant and Morest (1979) revealed two types of neurons with large cell bodies, distinguished by the presence or absence of a well defined ring of rough endoplasmic reticulum, called the nuclear cap, around the nucleus. In the cat, spherical or spherical bushy cells have the nuclear cap and stellate cells do not. Cant (1981) further described two classes of stellate cells in the cat AVCN. These are type I stellate cells that receive few synaptic contacts on their cell bodies, and type II stellate cells that receive numerous synaptic contacts. Our studies of the guinea pig AVCN are limited to electron microscopic observations, and these criteria can not be applied directly. None of the neurons in the guinea pig AVCN has the prominent and distinctive nuclear cap found in the cat. In defining the criteria for distinguishing the cell types in the guinea pig AVCN, we limited ourselves to considering the density of the axosomatic terminals, the size and shape of the cell and the shape of the nucleus. Using these criteria Cant's type II stellate cell would not be distinguished from spherical cells. The present study focuses on the rostral portion of the guinea pig AVCN which contains a high density of large neurons. We believe the majority of these cells in the guinea pig are similar to the spherical cells in the cat, and that cells corresponding to the stellate cells of the cat are much fewer.

Spherical cells in the guinea pig are large and round with a centrally placed, unindented nucleus containing a pale nucleolus (Fig. 1) as described by Schwartz and Gulley (1978). The surrounding cytoplasm contains scattered smooth endoplasmic reticulum and polyribosomes. The endoplasmic reticulum does not surround the nucleus forming a cap as it does in the cat, although cisternae of the Golgi apparatus tend to be localized near the nucleus in the adjacent cytoplasm. The surface of spherical cells is covered with numerous axosomatic synapses. The end bulbs of Held are the largest of these terminals, particularly in the

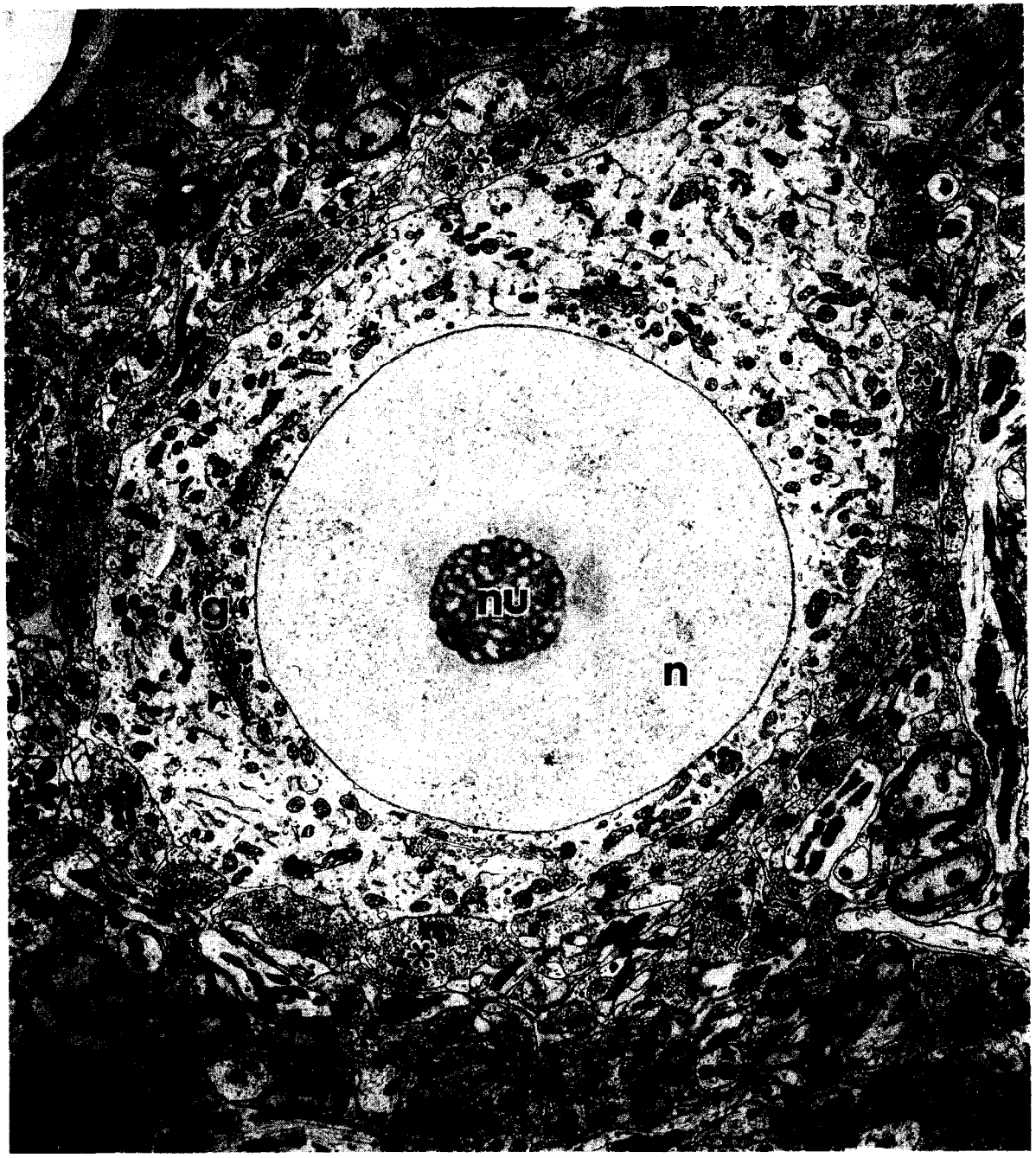

Fig. 1. A spherical cell in the rostral portion of the anterior division of the anteroventral cochlear nucleus. n, nucleus; nu, nucleolus; g, Golgi complex. Note that numerous axosomatic synapses (*) contact the cell surface. Bar = 1 μm.

most rostral parts of the AVCN. Thin astrocytic processes surround these terminals and fill the spaces between synapses.

Cells of the second type that we observed in the AVCN are smaller than spherical cells, tend to be ovoid in shape and have an eccentric nucleus

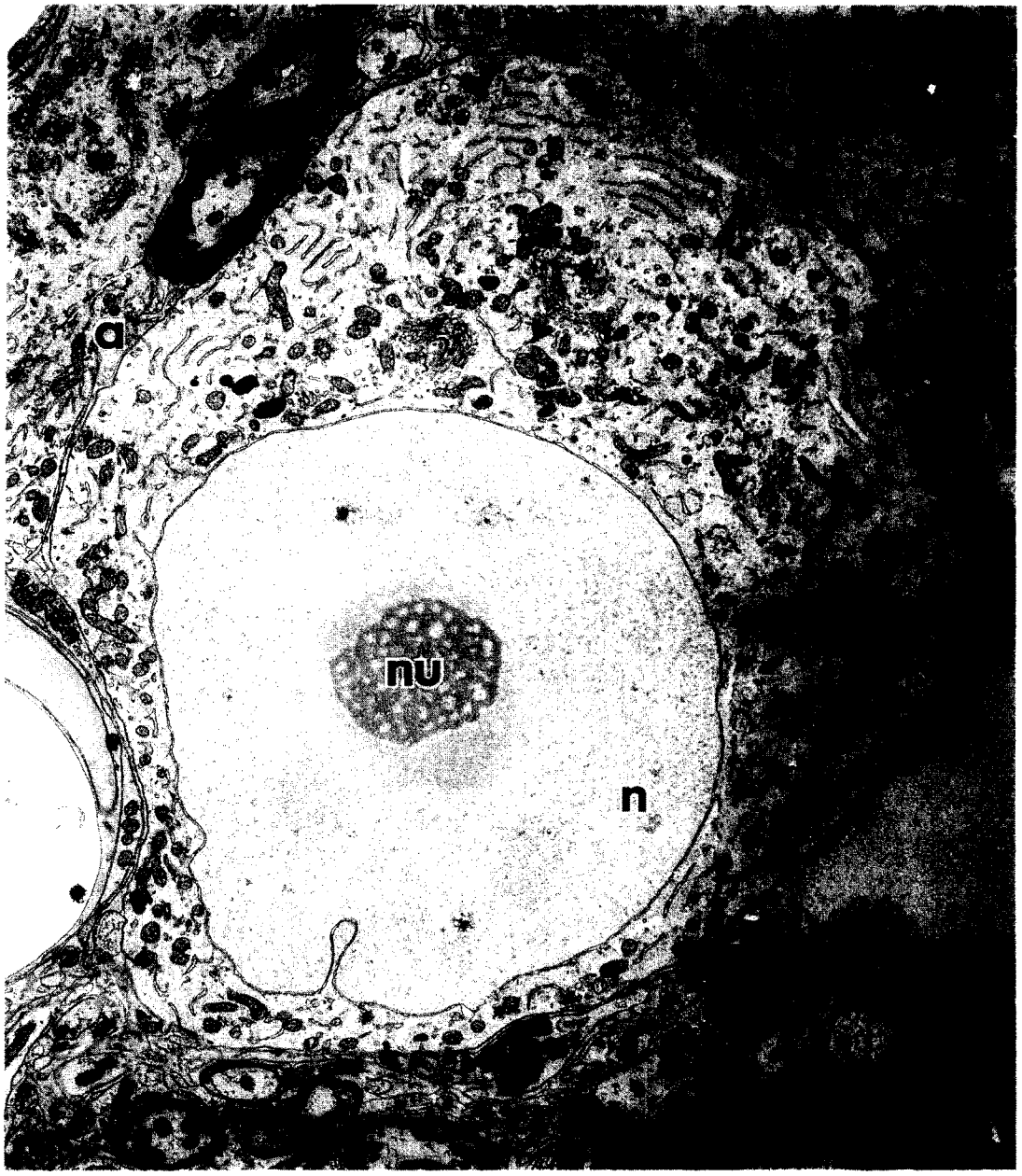

Fig. 2. A stellate cell in the rostral portion of the anteroventral cochlear nucleus. n, nucleus; nu, nucleolus; a, astrocytic processes. Very few synapses (*) contact the cell surface. Bar = 1 μm.

which is often indented, less prominent endoplasmic reticulum and few axosomatic terminals (Fig. 2). This cell type is similar to the type I stellate cell described in the cat by Cant (1981), these cells will be referred to as stellate cells. Stellate cells are rare in the rostral part of the guinea pig AVCN and are more common in the caudal portion of the AVCN near the interstitial zone.

The morphology of the synaptic terminals on these neurons has been well characterized in both the cat and the guinea pig (Brawer and Morest,

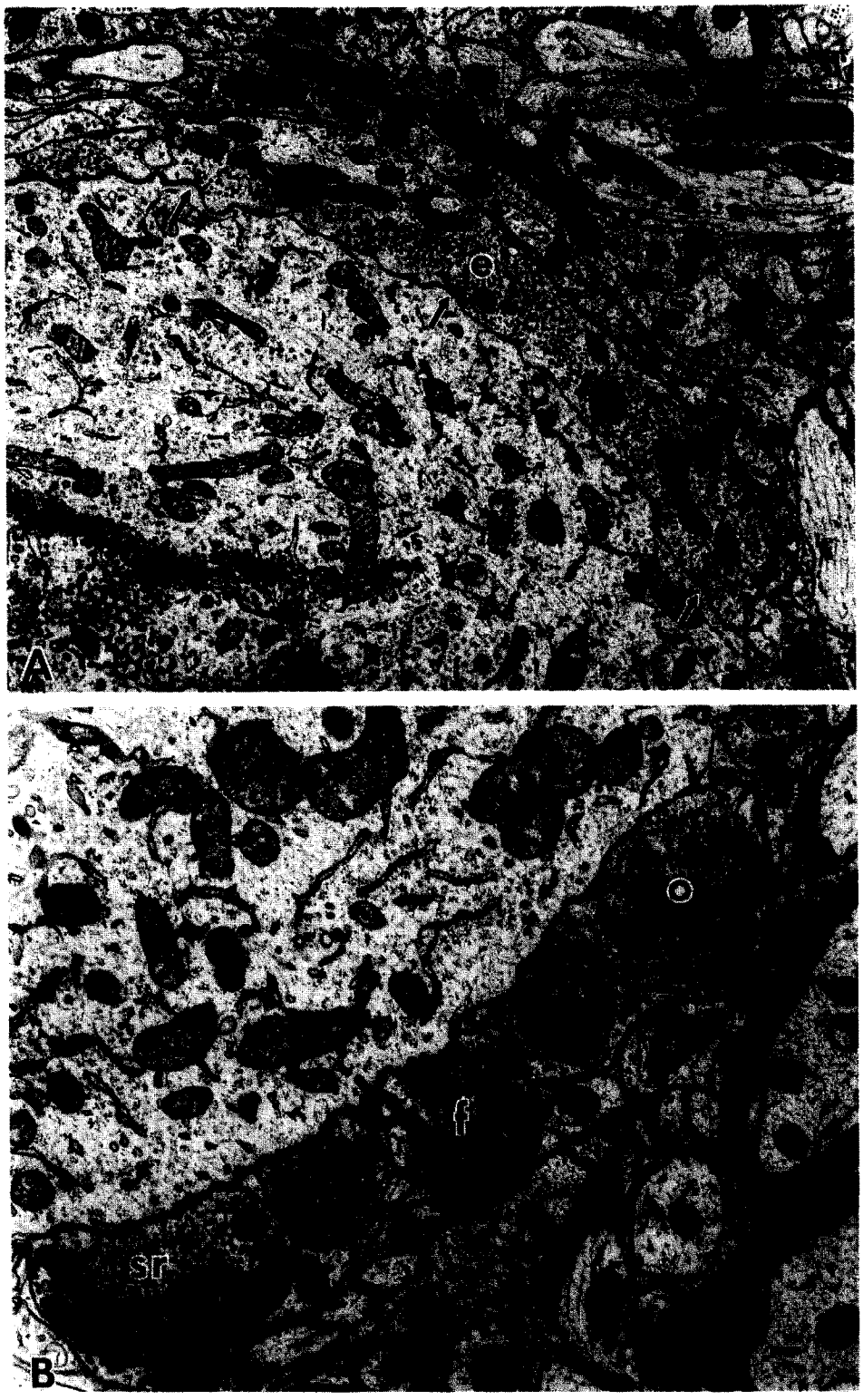

Fig. 3A. Section through a portion of a spherical cell contacted by an end bulb of Held (e) containing large, round synaptic vesicles. Note the multiple synaptic contacts (arrows). Bar = 4.5 μm.

Fig. 3B. Section through a portion of a spherical cell showing three additional types of synaptic terminals on the spherical cell soma. o, terminals containing oval/pleomorphic vesicles; f, terminals containing flattened vesicles; sr, terminals containing small, round vesicles. Bar = 3 μm.

1975; Cant, 1981; Cant and Morest, 1984; Gulley, 1978; Gulley et al., 1978; Ibata and Pappas, 1976; Lenn and Reese, 1966; Morest, 1973; Schwartz and Gulley, 1978; Wenthold et al., 1986). The surface of spherical cells is contacted by numerous axosomatic synapses described previously (Schwartz and Gulley, 1978) and reviewed briefly here. The most prominent are terminals which contain large round vesicles and make multiple asymmetric contacts often marked by pre-synaptic invaginations and post-synaptic evaginations (Fig. 3A). Ablation studies have shown these to be the primary afferent terminals, the end bulbs of Held, of the auditory nerve (Schwartz and Gulley, 1978). The second type are synaptic boutons which contain a mixed population of oval/pleomorphic vesicles and make symmetric contacts on the somatic surface singly or in small groups. The third type are flask-shaped synaptic boutons with densely packed flattened vesicles. Lastly, spherical cells are contacted by a small number of asymmetric terminals which contain small round vesicles (Fig. 3B). Synapses observed contacting stellate cell bodies are similar to those on spherical cell bodies except that synaptic inputs from the auditory nerve make single rather than multiple contacts.

At the ultrastructural level, GABA-IR in the AVCN is consistently localized to presynaptic terminals. Labelled synaptic profiles make axosomatic contacts with cells which are identified as spherical cells because of the dense axosomatic synaptic input (Fig. 4). Similar labelled axosomatic terminals are observed contacting stellate cells, identified by their sparse axosomatic synaptic input (Fig. 5A). Labelled axosomatic synaptic terminals are not seen on granule cells in the granule cell cap of the AVCN. Labelled axodendritic synapses are also common in the neuropile surrounding the cell bodies (Fig. 5B). The shape of such

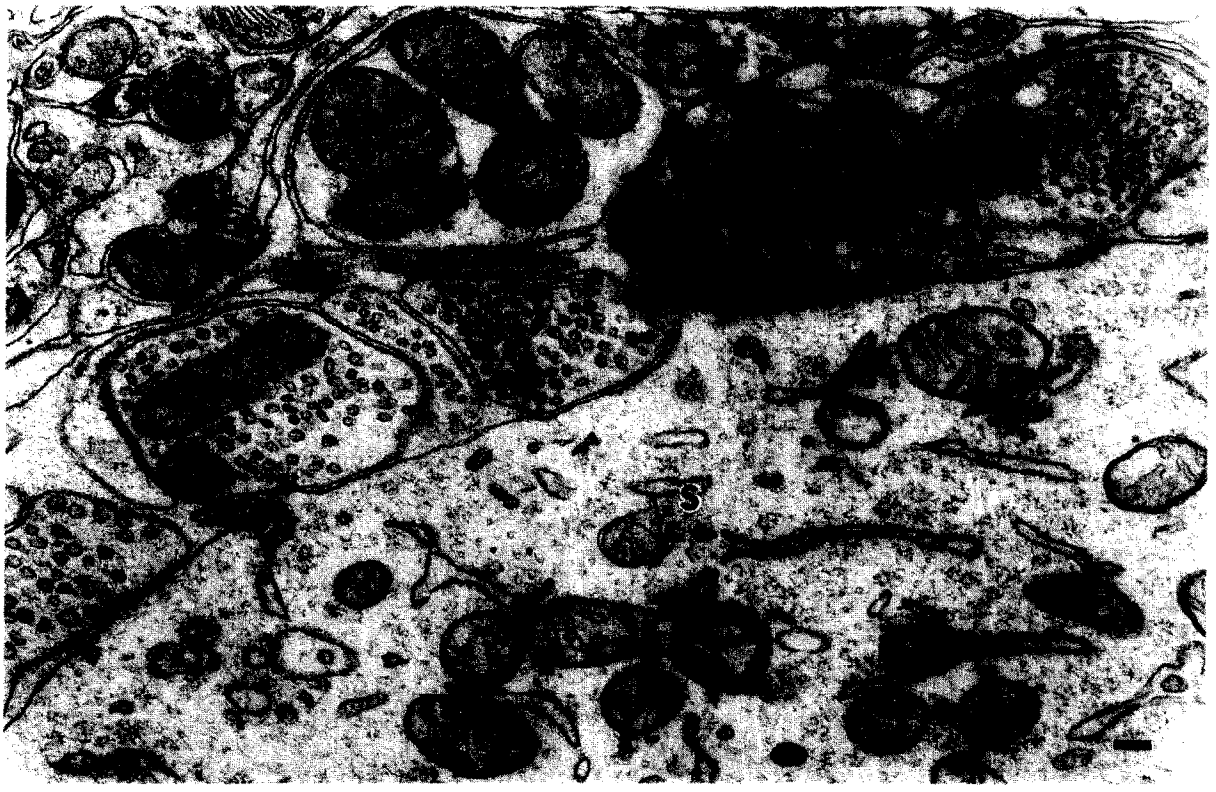

Fig. 4. A GABA-IR presynaptic terminal containing oval/pleomorphic vesicles (o) adjacent to an unlabelled terminal also containing oval/pleomorphic vesicles. Both are contacting the surface of a spherical cell (s). Note the high density of axosomatic synaptic contacts characteristic of spherical cells. Bar = 1.5 μm.

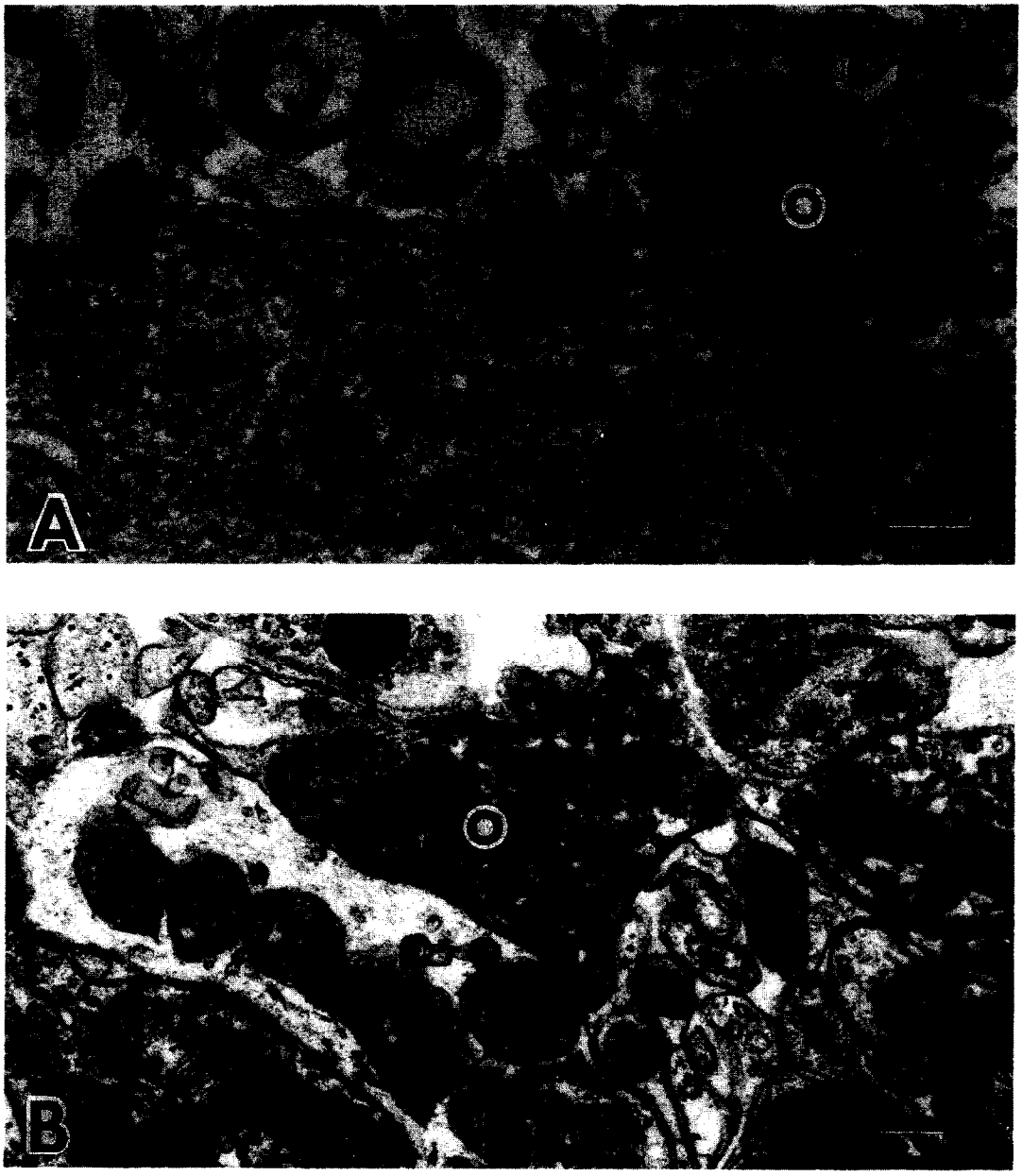

Fig. 5A. Heavily labelled GABA-IR terminal containing oval/pleomorphic vesicles (o) on the surface of a stellate cell. Note the low density of axosomatic contacts. Bar = 7 μm.

Fig. 5B. A labelled terminal containing oval/pleomorphic vesicles (o) adjacent to an unlabelled terminal containing flattened vesicles in the neuropile. Bar = 6 μm.

synaptic terminals and the density of the label may vary, but in all the cases where we could distinguish the vesicle type, the labelled profiles are presynaptic terminals containing oval/pleomorphic vesicles. Labelled synaptic terminals commonly contact the surface of spherical cells. The few stellate cells we observed in the rostral AVCN had no more than one immunolabelled

terminal contacting the cell surface. It is likely that many of immunolabelled terminals observed in the neuropile are on dendrites from nearby neurons, and therefore likely that the majority of GABA-IR inputs to stellate cells are via axodendritic contacts. Synaptic terminals containing oval/pleomorphic vesicles are common in the AVCN, but not all of these terminals are labelled, even in areas where immunolabelled terminals are evident (Fig. 4). The unlabelled synapses were not morphologically distinguishable from those containing label, since both contained oval/pleomorphic vesicles and contacted cell bodies and dendrites throughout the AVCN.

Discussion

The present study demonstrates that there are neurons in the guinea pig AVCN which receive GABA-IR input through presynaptic terminals containing oval/pleomorphic vesicles. Guinea pig spherical cells receive a dense axosomatic synaptic input as do the 'capped' bushy cells described in the cat (Cant, 1981; Cant and Morest, 1984). They are among the largest cells in the AVCN, and do not exhibit stacks of rough endoplasmic reticulum. A partial nuclear cap identified at the light microscopic level has been reported for guinea pig spherical cells (Hackney and Pick, 1986). Correspondingly, at the ultrastructural level we saw arrays of Golgi complexes located near the nucleus. Guinea pig stellate cells are smaller than spherical cells, often have an indented nucleus and receive few axosomatic synapses. The type I 'non-capped' stellate cell described by Cant (1981) in the cat AVCN is similar to guinea pig stellate cell. However, the cat has an additional stellate cell type, type II 'non-capped' stellate cells, which like the guinea pig spherical cells receive a dense axosomatic input (Cant, 1981). If this cell type exists in the guinea pig, it would not be distinguished from spherical cells.

Our results show that GABA-IR is localized in presynaptic terminals contacting the cell bodies of spherical cells and stellate cells and dendrites in the surrounding neuropile. This observation supports the idea that GABA acts as an inhibitory neurotransmitter acting on these neurons. Our results also show that the GABA-IR is restricted to terminals containing oval/pleomorphic synaptic vesicles. However, not all such terminals contain GABA-IR. Recent work (Wenthold et al., unpublished data), using a monoclonal antibody to the postsynaptic receptor for glycine in the guinea pig AVCN (Altschuler et al., 1986), has shown immunoreactivity for the glycine receptor postsynaptic to both terminals containing oval/pleomorphic vesicles and flat vesicles. There is also evidence that cells in the guinea pig dorsal cochlear nucleus co-contain GABA and glycine (Wenthold et al., 1987). Since such cells may project to the AVCN, there may be three different classes of synaptic terminals which contain oval/pleomorphic vesicles: those containing GABA, those containing glycine and those containing both GABA and glycine. The function of these neurotransmitter candidates awaits further study.

The present results are consistent with previous biochemical findings that GABA and GAD are present throughout the cochlear nucleus (Fisher and Davies, 1976; Godfrey et al., 1978; Tachibana and Kuriyama, 1974; Wenthold and Morest, 1976). While our results show GABA-IR inputs in the AVCN, quantitative studies will be necessary in order to determine if spherical and stellate cells receive different patterns of GABA-IR input in different regions of the AVCN. Work also needs to be done to determine the sources of these GABA-IR inputs. Previous studies indicate that most GABA-IR terminals in the cochlear nucleus arise from intrinsic neurons and descending pathways. Lesions of the auditory nerve (Wenthold and Morest, 1976) or the trapezoid body (Fisher and Davies, 1976) do not cause a decrease in the levels of GAD (Wenthold and Morest, 1976) or GABA release (Wenthold, 1979), and lesions of the dorsal acoustic stria cause small decreases in GAD levels in the cochlear nucleus (Davies, 1977). The uptake and release of GABA does not decrease in the AVCN after destruction of all descending inputs to the cochlear nucleus, but decreases 30% in the DCN and PVCN (Potashner et al., 1985). Recent evidence demonstrates GABA-IR cell bodies (Mugnaini, 1985; Otterson and Storm-Mathisen, 1984; Thompson et al., 1985; Wenthold et al., 1986) in the superficial layers of the DCN and only a few scattered immunoreactive cells in the VCN. Therefore, the DCN may be

a major source of GABA-IR fibers which may terminate in the AVCN.

In conclusion, this study and previous findings demonstrate that GABA is a neurotransmitter candidate in the AVCN that is localized in a specific class of synaptic terminals containing oval/pleomorphic vesicles.

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