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Zhou, J
Ribak, CE
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et al.

Publication Date

DOI

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Peer reviewed

Research report

Synaptic and neurochemical features of calcitonin gene-related peptide containing neurons in the rat accessory optic nuclei

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Abstract

Within the rodent visual system, calcitonin gene-related peptide (CGRP) is selectively expressed in neurons in the accessory optic nuclei (AON), including the dorsal terminal nucleus (DTN), lateral terminal nucleus (LTN) and medial terminal nucleus (MTN). To determine whether CGRP-immunoreactive neurons are involved in visual circuitry, electron microscopic preparations were analyzed from normal rats and rats with optic nerve transections. A co-localization analysis was also made because CGRP-labeled neurons had features of GABAergic neurons. Thus, sections were prepared for light microscopy to determine whether CGRP-containing neurons also had glutamate decarboxylase (GAD) and other markers for GABAergic neurons, such as calcium binding proteins: calbindin (CB), calretinin (CR) and parvalbumin (PV). Electron microscopy of the DTN and LTN showed CGRP-labeled somata and dendrites that were postsynaptic to axon terminals forming asymmetric synapses. Many of these axon terminals degenerated following optic nerve transection indicating that retinal ganglion cells form synapses with CGRP-labeled neurons in the AON. In the DTN, LTN and MTN, CGRP-labeled axon terminals formed symmetric synapses with unlabeled somata as well as dendritic shafts and spines. Consistent with this type of synapse being GABAergic were the co-localization data showing that about 90% of the CGRP-labeled neurons co-localized GAD in the AON. Many CGRP-labeled neurons showed immunostaining for CR (40%) whereas only a few had labeling for CB (5%). No CGRP-labeled neurons had PV. These data show that CGRP-containing neurons receive direct retinal input and represent a subpopulation of GABAergic neurons which differentially co-express calcium-binding proteins. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Calcitonin gene-related peptide; GABA; Retinal axon terminal; Dorsal terminal nucleus; Lateral terminal nucleus; Medial terminal nucleus; Calcium binding protein

1. Introduction

Our previous paper analyzed the distribution of calcitonin gene-related peptide (CGRP)-containing somata and axons in the mammalian visual system [34]. It was shown that a specific part of the visual system contained these structures. Thus, CGRP-immunoreactivity was selectively localized to the accessory optic nuclei (AON) of the rat. Both immunolabeled somata and fibers were found in the lateral and dorsal terminal nuclei (LTN and DTN) as well as the interstitial nucleus of the superior fasciculus (posterior fibers). Immunolabeled fibers were also found in the medial terminal nucleus (MTN) but there were no labeled somata. Thus, all nuclear components of the rat accessory

optic system displayed CGRP-labeled structures whereas other regions of the visual system did not.

CGRP is found in other sensory systems, namely the somatosensory [3,5,18,38,39], gustatory [27], and olfactory systems [15,17]. The part of the somatosensory system that contains CGRP-immunolabeling is the nociceptive portion that mediates pain [14,18,38]. It is interesting to note that the portion of the visual system that contains CGRP is a primitive part dealing with the stabilization of the visual image on the retina. Thus, the significance of CGRP in these unrelated sensory systems may be similar in that they are involved with primitive functions [34].

The purpose of the present study is to analyze the electron microscopic features and synaptic circuitry of the CGRP-containing neurons in the accessory optic system because our previous study only analyzed the light microscopic features of CGRP-immunoreactive neurons in this

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system [34]. Thus, our initial goal is to determine the ultrastructural features of CGRP-immunolabeled somata and dendrites, the types of synapses found on these neurons, and the types of synapses formed by CGRP-immunopositive axon terminals in the DTN, LTN and MTN. As noted in previous investigations [12,16], retinal ganglion cells project to all nuclear components of the rodent accessory optic system. Therefore, it is of particular importance to determine whether retinal ganglion cell axons synapse with CGRP-immunolabeled neurons within the LTN and DTN. Moreover, the data for CGRP-immunolabeled axon terminals indicate that they may be GABAergic because they form symmetric synapses in the AON. Accordingly, in the last experiments in this study we determine whether CGRP-containing neurons co-localize GABA and other markers for GABA neurons (viz., the calcium-binding proteins, calretinin (CR), calbindin (CB) and parvalbumin (PV)). A preliminary report of this study has appeared [45].

2. Materials and methods

2.1. Animals and surgery

Experiments were performed on male and female adult albino rats (Sprague–Dawley) weighing 250–350 g. Eight non-operated rats were used for characterizing the normal ultrastructure of CGRP-containing neurons in the AON and to identify whether these cells co-express glutamate decarboxylase (GAD) and calcium binding proteins. To determine whether CGRP-containing AON neurons are directly innervated by retinal axons, an additional 10 rats had one optic nerve transected to induce degeneration of ganglion cell axon terminals in the contralateral AON. These latter rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) before their right eyeballs were separated from temporal orbital tissue using an operating microscope and microsurgical instruments. Then, the optic nerves were transected with scissors about 1–2 mm behind the posterior pole of the eyeball. All rats were returned to the vivarium after the surgery, and they were allowed to survive 4 and 7 days. All of the surgical procedures were approved by the animal research committee at the University of California at Irvine.

2.2. Tissue preparation

Control and experimental rats were deeply anesthetized with an over-dose of sodium pentobarbital and perfused transcardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4 followed by 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer PB, pH 7.4. All brains were removed and postfixed in the perfusion solu-

tion for 4–8 h at 4°C, and transferred to cold PBS for several hours to a few days. The midbrain and brainstem were blocked from these brains and the side ipsilateral to the nerve transection was marked. The brain blocks were then sectioned coronally at 30–50 μm with a vibratome, and sections containing the AON, at the diencephalic–mesencephalic junction, were collected in tissue-culture wells in PBS and processed for immunocytochemistry.

2.3. Immunocytochemistry for CGRP

Free-floating sections were immunostained for CGRP using the standard avidin–biotin complex (ABC) method as described previously [34]. Briefly, endogenous peroxidase activity was bleached with a 20-min rinse in 0.05% hydrogen peroxide. Non-specific background staining was blocked by a 2-h incubation in 3% normal goat serum (NGS) at room temperature, after which the sections were incubated with a rabbit anti-CGRP serum (Sigma) at 1:10,000 for 18 h at 4°C and with agitation in a PBS solution containing 1.5% NGS and 0.3% Triton X-100. Following this incubation, the sections were further incubated in 1% goat anti-rabbit IgG (1:200) with 1.5% NGS in PBS for 2 h at room temperature, followed by a 1-h incubation in 1% ABC solution (Vector Laboratories). The immunoreaction product was visualized with 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide. Three 10-min rinses with PBS were conducted between all incubations.

2.4. Electron microscopy

Pre-embedding immunocytochemistry was used to study the ultrastructure and circuitry of CGRP-containing neurons in the normal and deafferented AON. Briefly, 50-μm thick sections were first immunostained for CGRP as described in Section 2.3, except that Triton X-100 was omitted to preserve the ultrastructure of these sections. Also, the incubation time with primary antibody was increased to 36–48 h. After immunostaining, selected sections were post-stained with osmium tetroxide, dehydrated in a graded series of ethanol, placed in acetone and Epon–acetone solutions, and flat-embedded in Epon. Thin sections were obtained from the AON that were identified in semithin, 2-μm sections. After staining with uranyl acetate and lead citrate, these sections were examined with a Philips CM10 transmission electron microscope.

2.5. Double-labeling procedures

A modification of a previously described dual-chromogen procedure [20] was used for concurrent immunolabeling of CGRP and other markers. Briefly, sections were first immunostained for GAD-67, GAD-65, CB, CR and

PV using DAB as the chromogen to yield a homogeneous brown reaction product within the cytoplasm of the labeled neurons. The sources and concentrations of these antibodies were the following: GAD-67, polyclonal rabbit antiserum (Chemicon), 1:5000; GAD-65, PV and CB, monoclonal mouse antisera (Sigma), all at 1:8000; CR, polyclonal rabbit antiserum (Chemicon), 1:5000. Normal horse serum was added to the preincubation and primary antibody solutions to block non-specific labeling. The second antibody for these markers was an universal anti-rat/rabbit IgG made in horse (Vector Laboratories). After the reaction for these markers, sections were washed with PBS, and immunostained for CGRP following the same steps as described above until the ABC reaction. Instead, the sections for this antibody were processed through several changes of 0.02 M PB, pH, 6.5, to lower the pH and ionic strength. Then, the sections were incubated with 0.005% benzidine dihydrochloride (BDHC) and 0.001% hydrogen peroxide to yield granular blue-black deposits.

For a control of the immunostaining for these markers, the primary antibodies were replaced by PBS or normal goat and horse sera. Sections processed in this way displayed no specific immunolabeling.

2.6. Morphometric analysis

Double-labeled sections were first examined to verify any co-localization of immunoreactivities for CGRP and one of the other neurochemicals within individual neurons in the AON. Then, to measure the frequency of co-localization, cell counts were performed at $20 \times$ to $100 \times$ primary magnifications in DTN and LTN. CGRP-immunostained neurons and those co-labeled with other markers were examined and recorded based upon their morphology and staining features (single or double-labeled), and the percentage of co-localization calculated, without attempting to obtain their numbers per unit area.

3. Results

3.1. Ultrastructural features of CGRP-containing neurons in the AON

CGRP-immunolabeled somata were examined within the LTN and DTN. They had oval or round nuclei and were small- to medium-sized (Fig. 1). The nuclei were smooth but occasionally had a notch or infolding (Fig. 1B). The perikaryal cytoplasm had many organelles although some were difficult to discern because the immunoreaction product obscured them. The organelles that were visible included cisternae of the granular endoplasmic reticulum and mitochondria (Fig. 1). Only a few axon terminals (1–3 per thin section) formed axosomatic

synapses with these labeled somata (Fig. 2A). Somatic spines were not observed.

The CGRP-immunolabeled dendrites varied in size (Fig. 2) and their size correlated with their distance from labeled somata. The larger dendrites (1–3 μm in diameter) were continuous with or located close to labeled somata. In contrast, small caliber dendrites (< 1 μm) were found further away from somata. Thus, these dendrites were considered proximal and distal dendrites, respectively. Both types of dendrites were sparsely-spinous (Fig. 2B). Unlabeled axon terminals formed synapses with CGRP-immunolabeled dendritic shafts and spines (Fig. 2B–E). Most of these synapses were of the asymmetric type but this identification was difficult to make in all cases because the immunoreaction product was highly concentrated in both labeled dendrites and spines. However, when the reaction product was lighter within dendrites and spines, the prominent postsynaptic density of these asymmetric synapses was clearly visible (Fig. 2B,C). The presynaptic profile in such synapses was large and contained many round, clear synaptic vesicles. The axoplasm of these axon terminals was lucid. In many cases, axon terminals formed multiple active zones at their synapses (Fig. 2C).

CGRP-immunolabeled axon terminals were analyzed within three of the AON: the LTN, DTN and the ventral division of the MTN, the MTNv (Figs. 2F–H and 3A–D). As the features of these axon terminals were similar in the AON, they will be described together. CGRP-immunopositive axon terminals were small- to medium-sized and had synaptic vesicles and 0–2 mitochondria per thin section. It was difficult to determine the shape of the synaptic vesicles in these terminals because the reaction product was too electron dense. Because labeled axon terminals were mainly apposed to unlabeled profiles, it was easy to identify the symmetric synapses these terminals formed with small dendrites (Figs. 2F,G and 3A,C,D) and spines (Figs. 2H and 3B). The synaptic cleft of these synapses had some electron dense material and the postsynaptic density at this location was typical for symmetric synapses.

3.2. Ultrastructure of CGRP-immunolabeled neurons in the AON following transection of the optic nerve

The ultrastructure of CGRP-labeled somata, dendrites and axon terminals in the AON was unaltered four days following optic nerve transection. The electron density of immunoreaction product also was similar to the normal preparations described above. Synapses were observed on CGRP-labeled somata and dendrites. However, many of the axon terminals forming these synapses had a different appearance than those found in normal preparations. Four days following optic nerve transection, many axon terminals were found to display degenerating features similar to those described in the AON following eye enucleation
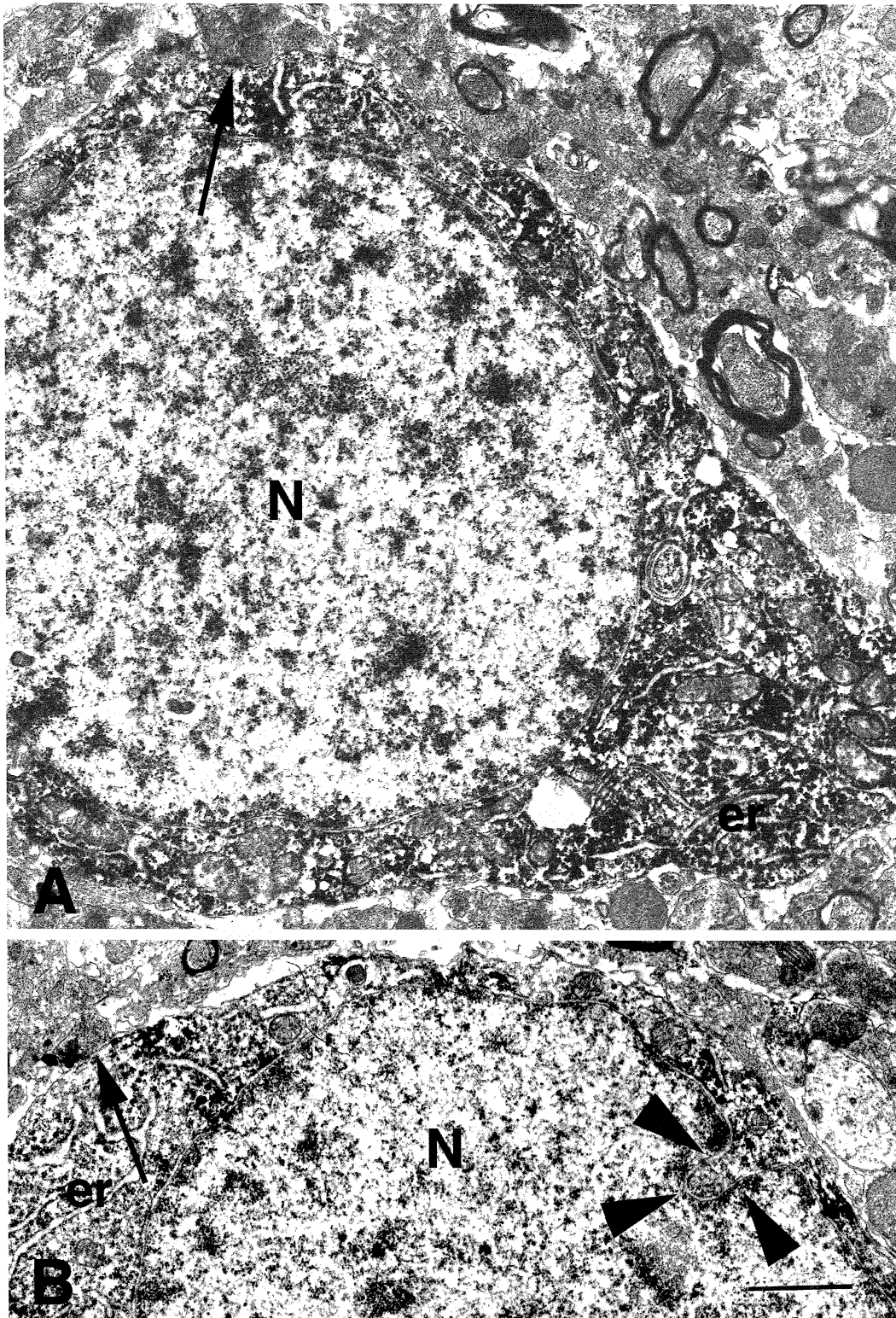
Fig. 1. Electron micrographs of CGRP-labeled somata in the LTN. (A) shows a small soma with immunoreaction product within the perikaryal cytoplasm. The nucleus (N) is large and occupies most of the cross-sectioned area. One axon terminal forms an axo-somatic synapse (arrow). (B) is another example of a CGRP-labeled soma. Its nucleus (N) displays a small infolding (arrowheads) and reaction product is patchy in the cytoplasm An axon terminal forms a symmetric synapse (arrow) with this soma. er: Granular endoplasmic reticulum. Scale bar = 1 μm.

[19]. These features included clustering of synaptic vesicles into a compact group and enhanced electron density of

the axoplasm (Figs. 4 and 5). Also, these axon terminals were smaller than the ones found in normal preparations.
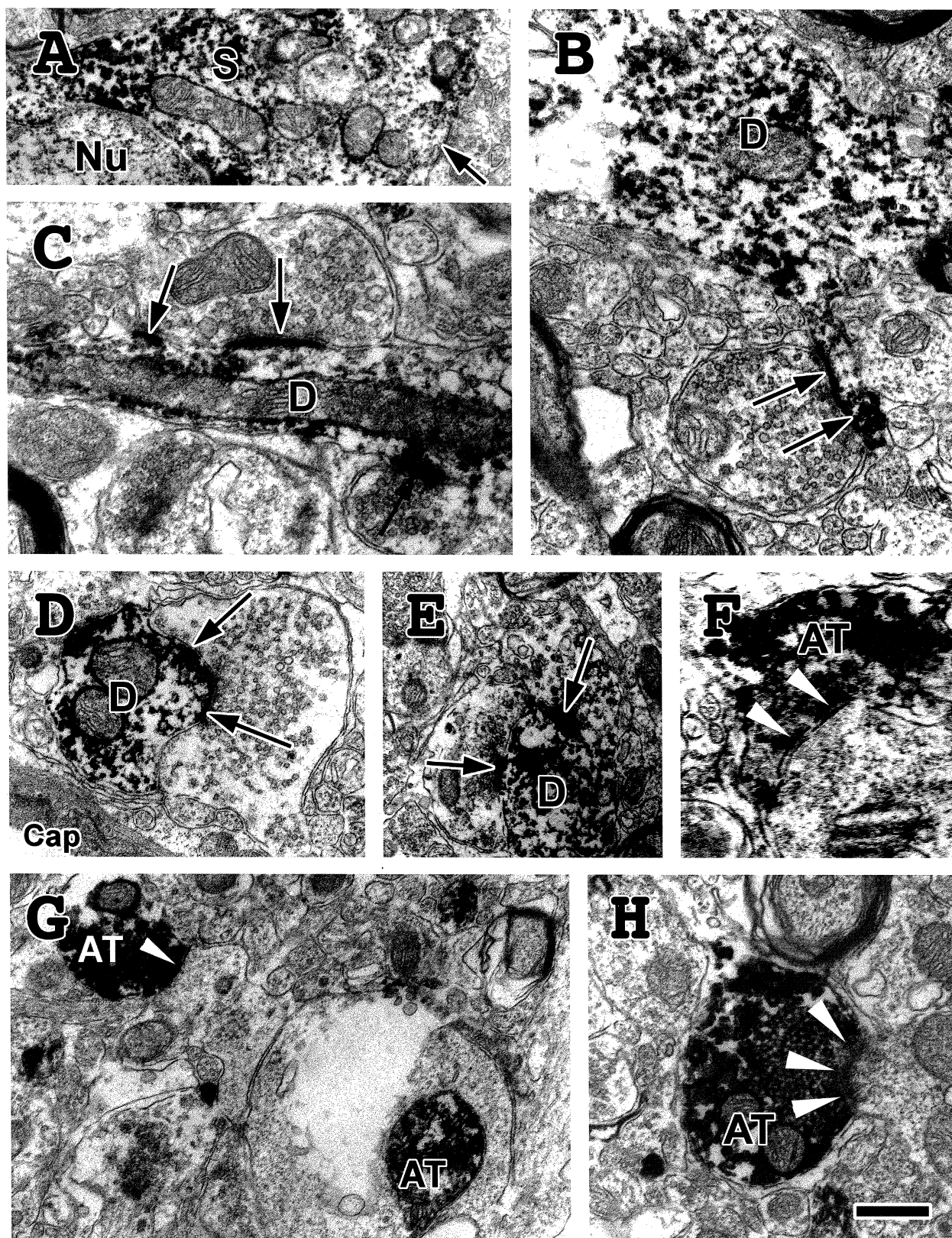
Fig. 2. Electron micrographs show unlabeled axon terminals synapsing (arrows) with a soma (A), a dendritic spine (B) and several distal dendrites (C–E) of CGRP-labeled neurons in the rat LTN. The axo-dendritic and axo-spinous synapses are of the asymmetrical type with distinct postsynaptic thickenings (B–E). Note that the presynaptic terminals are average in size and contain round, clear vesicles that are usually loosely-packed. In some cases (C, D, E), one terminal forms two or more active sites with CGRP-labeled dendrites. (F–H) show CGRP-labeled axon terminals that form symmetric synapses (white arrowheads) with unlabeled distal dendrites. Scale bar = 0.5 μm.
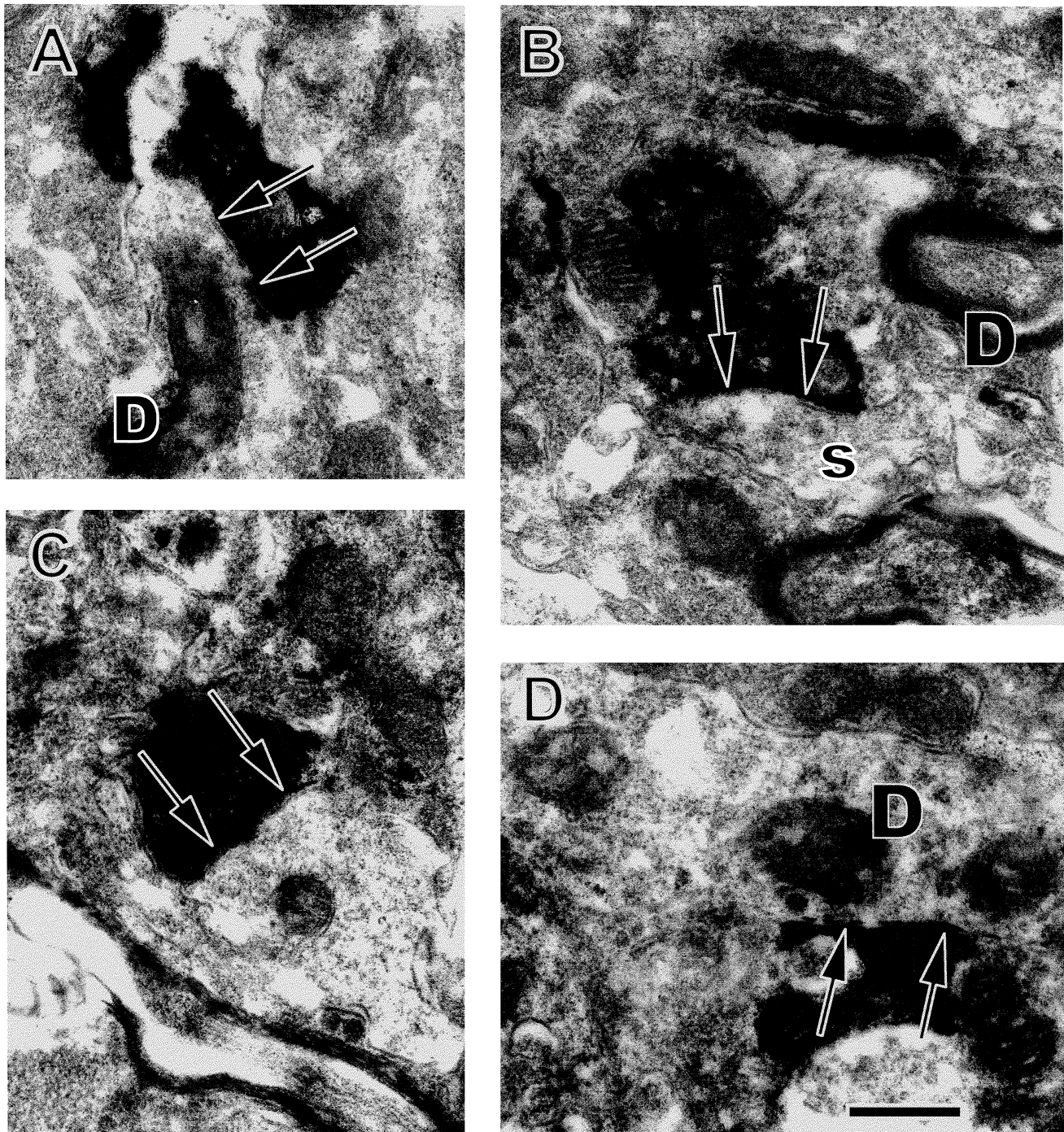
Fig. 3. CGRP-labeled axon terminals synapse with distal dendrites and a dendritic spine of neurons in the ventral division of the MTN. In (A), (C), and (D), densely immunolabeled axon terminals form symmetric synapses (arrows) with dendritic profiles seen to contain mitochondria. In (B), the postsynaptic profile displays features of a spine because mitochondria appear to be lacking. (A–D) = 0.5 μm.

In addition, small thin processes of astrocytes were sometimes found to wrap around many of these degenerating axon terminals (Fig. 5). The degenerating axon terminals were found in LTN and DTN where they mainly formed axodendritic and axospinous asymmetric synapses (Figs. 4 and 5). Because most CGRP-labeled dendrites that were targeted by these axon terminals were small, they are probably distal dendrites.

3.3. Do CGRP-labeled neurons co-localize GAD and other markers for GABAergic neurons?

Our previous study [34] showed CGRP-immunolabeled somata in LTN and DTN, but not in MTN. Initially, the co-localization analysis was limited to these two nuclei of the AON. However, during the course of these experiments, better preparations were obtained that clearly iden-
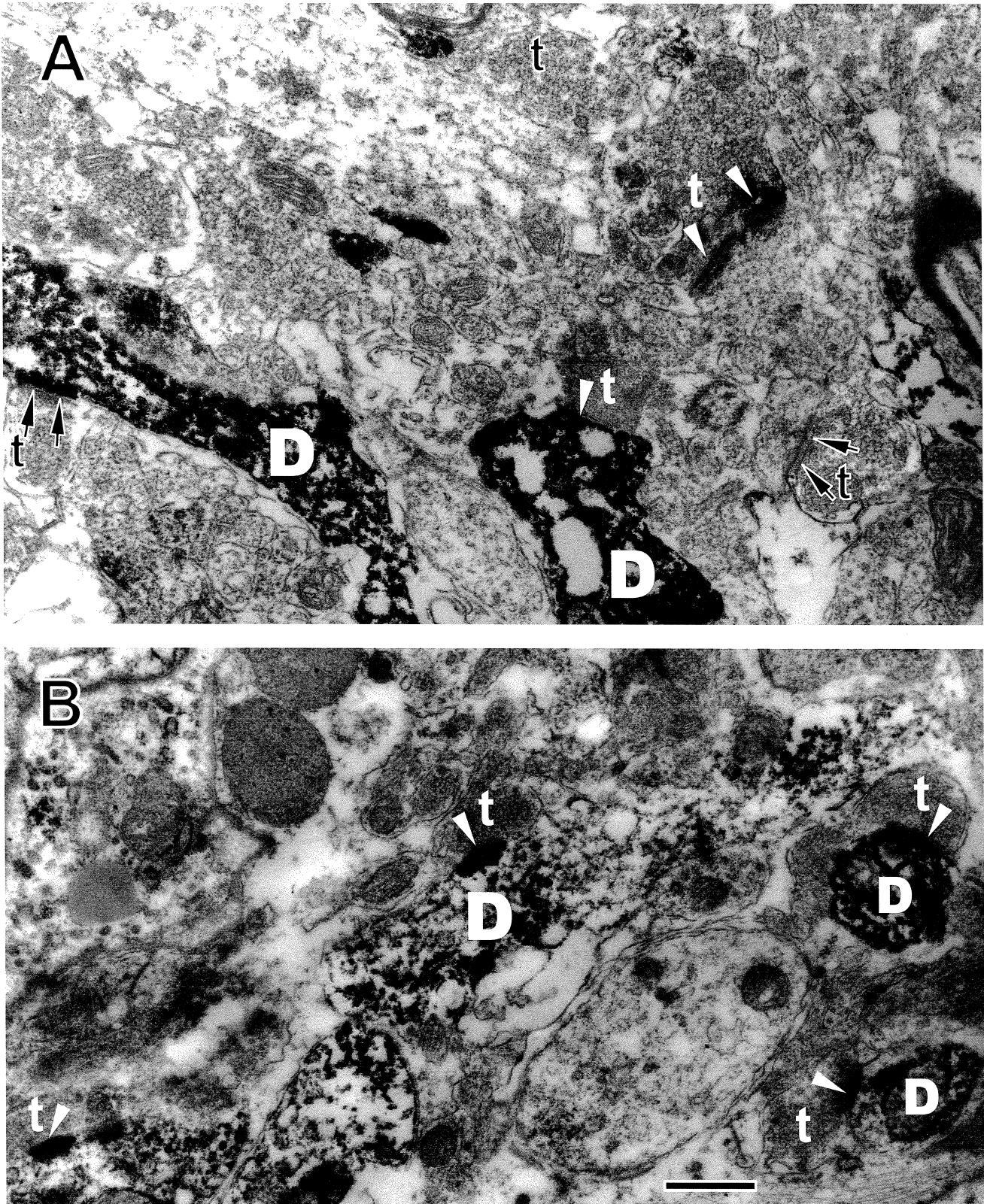
Fig. 4. Electron micrographs illustrate degenerating retinal axon terminals synapsing on CGRP-labeled distal dendrites in the LTN 4 days after contralateral optic nerve transection. In both (A) and (B), degenerating R-terminals (white t's), and in (A), normal axon terminals (black t) make synapses with, or are apposed to, CGRP-labeled distal dendrites (D). Compared with the normal terminals seen in (A), the degenerating terminals in (A) and (B) are denser, smaller in size, and exhibit a disintegration of their synaptic vesicles. (A and B) = 1 μm.
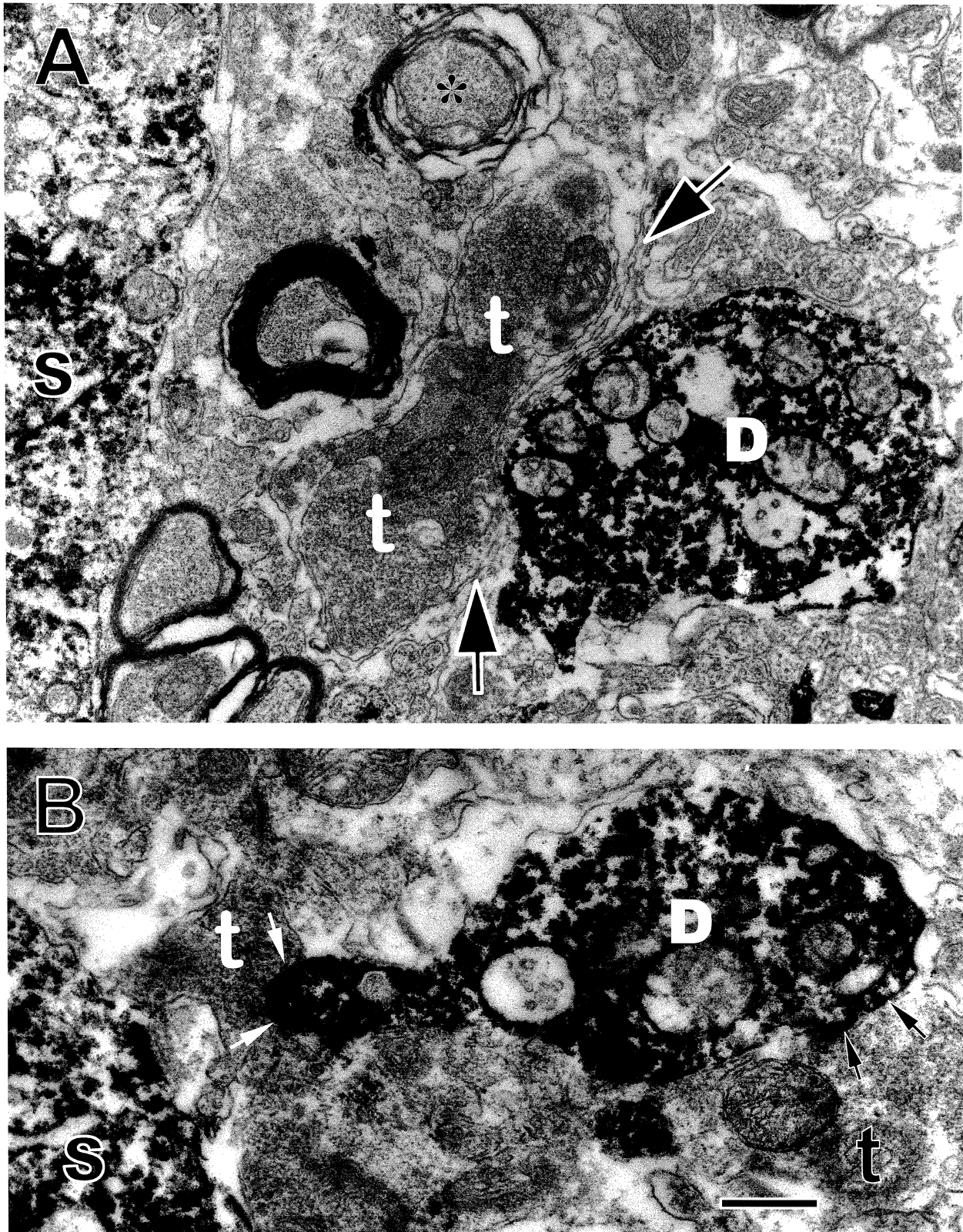
Fig. 5. Electron micrographs of CGRP-labeled dendrites that are next to labeled somata in the LTN four days following transection of the contralateral optic nerve. (A) shows a degenerating retinal axon terminal (white t) located between a labeled soma (S) and dendrite (D). The degenerating terminal exhibits a considerably enhanced electron density and contains densely packed, indistinct synaptic vesicles (to be compared with the normal synapses present in this field). Astrocytic processes (black arrows) surround the terminal and extend between it and the adjacent CGRP-labeled proximal dendrite. An axon (asterisk) shows disruption of its myelin sheath also resulting from the anterograde axonal degeneration. (B) the white arrows shows another example of a degenerating axon terminal lying between a labeled soma (S) and dendrite. This terminal forms a synapse with the spine of the CGRP-labeled dendrite (D). A normal axon terminal (black t) forms a synapse (black arrows) onto the shaft of this same CGRP-labeled dendrite. (A) and (B) = 0.5 μm.

tified CGRP-labeled somata in the dorsal part of the MTN (MTNd). Therefore, the following results were obtained from three AON, the LTN, DTN and MTNd.

In each of these nuclei, about 90% of the CGRP-immunolabeled neurons had immunoreaction product for GAD (see Fig. 6A–D). For the LTN and DTN, 124 out of 143 CGRP-immunopositive cells had GAD67 (86.77%). For MTNd, 32 CGRP-immunolabeled neurons were exam-

ined and all of them co-localized GAD65. Thus, our analysis involved antibodies to both GAD65 and GAD67. These double-labeled neuronal somata had similar features (sizes and shapes) as the GABAergic somata previously described in the AON of the rat and gerbil [8].

Several calcium-binding proteins are found in forebrain GABAergic neurons, and it was important to determine whether any were observed in CGRP-immunolabeled neu-
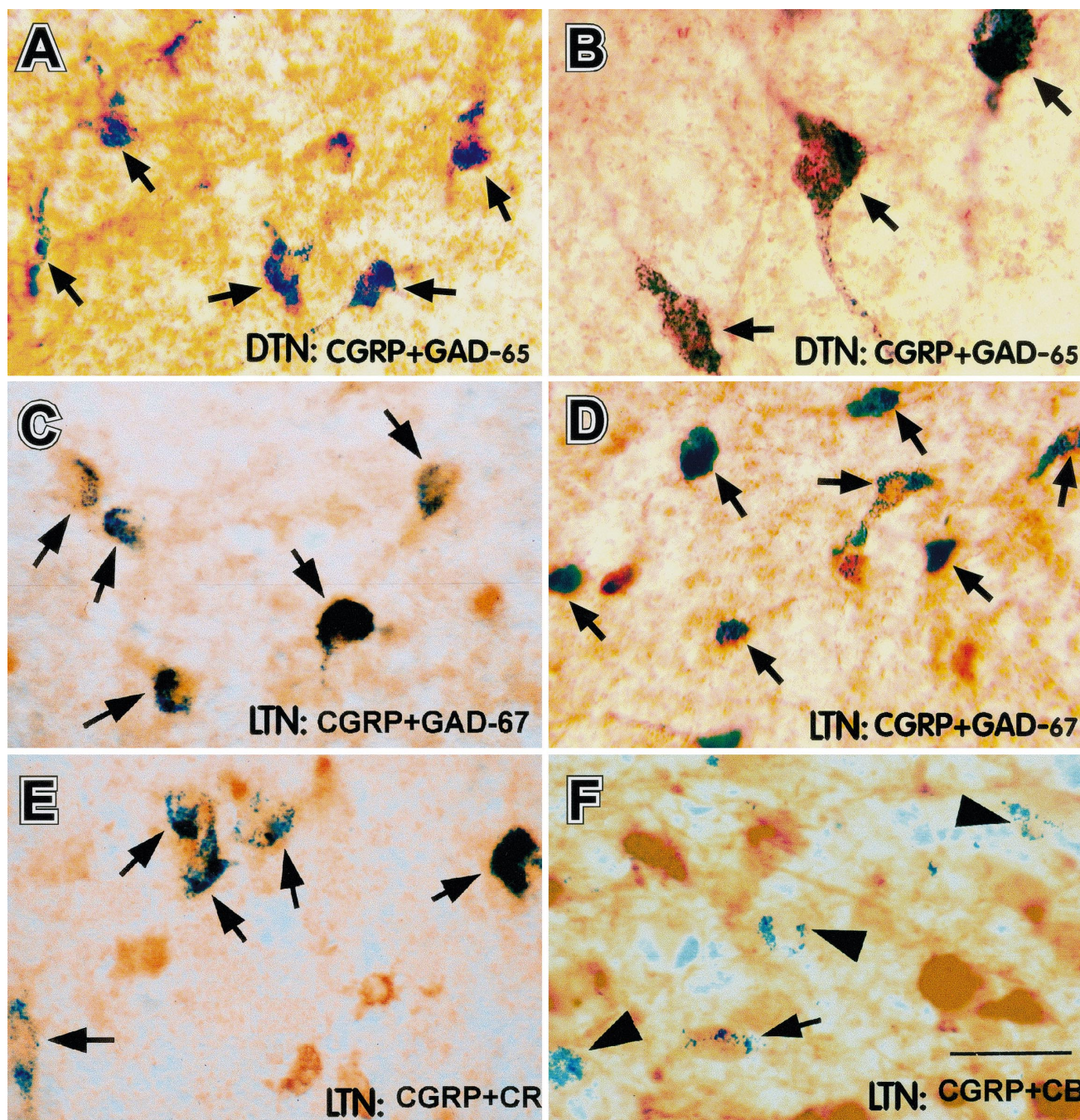

Fig. 6. Photomicrographs illustrating the co-expression of CGRP, GAD-65 and -67, and two calcium-binding proteins in neurons of the rat DTN and LTN. (A) and (B) show somata (arrows) that are double-labeled for CGRP and GAD65 in the DTN. (C) and (D) provide examples of double-labeling in somata (arrows) for CGRP and GAD67 in the LTN. (E) and (F) show the co-localization of CGRP with two calcium binding proteins, CR and CB in the LTN. (E) shows five CGRP-labeled somata (arrows) that co-localize CR. In contrast, (F) shows that CGRP co-localizes with only one CB-labeled neuron (arrow) in a field where several CB neurons (arrowheads) are found. Scale bar = A,C,D,E,F: 30 μm; B: 20 μm.

ronal somata within the AON. The data revealed that 40.9% of the CGRP immunolabeled somata in these AON contain CR-immunolabeling (54 out of 132) (see Fig. 6E). A small number (5%) of CGRP-labeled somata showed immunolabeling for CB (six out of 121) (Fig. 6F). The other calcium-binding protein that was analyzed, PV, was not found in any CGRP-labeled somata in the AON.

4. Discussion

The present findings show that CGRP-labeled somata and dendrites in the DTN and LTN are postsynaptic to axon terminals that make asymmetric synapses. Many of these axon terminals appear to be degenerating following contralateral optic nerve transection. Also, CGRP-labeled axon terminals form symmetric synapses with unlabeled somata, dendrites and spines in the DTN, LTN and MTNv. Finally, the co-localization studies show that most of the CGRP-labeled neurons contained GAD, many had CR and a few had CB; none had PV. Together, these data indicate that CGRP-containing neurons in the AON are involved in retinal/oculomotor circuitry and provide GABAergic inhibition to other AON neurons.

4.1. Technical considerations

All immunocytochemical studies that use the pre-embedding procedure are limited in the identification of neuronal processes when the immunoreaction product obscures the organelles within these labeled profiles. This problem is evident with the identification of small profiles less than 1 μm in diameter. They can be either axon terminals or cross-sectioned dendrites. For example, most of the labeled axon terminals contain dense amounts of reaction product that make it difficult to identify their synaptic vesicles. In contrast, the labeled dendrites in this study were not as densely labeled when cut longitudinally. In fact, it is possible to see the postsynaptic densities of many axon terminals forming asymmetric synapses with such dendrites. This identification is important for the examination of retinal/oculomotor circuitry (see below). The labeling within somata is not as dense as that found in dendrites and axon terminals, and thus, organelles and synapses are readily identified for CGRP-labeled somata.

4.2. CGRP-labeled neurons in AON

The electron microscopic features of CGRP-labeled somata in the DTN and LTN indicate that they are small- to medium-sized neurons. They lack a large number of axon terminals forming symmetric synapses with them. These features suggest that they are similar to the GABAergic neurons found in the neocortex and hippocampus [32,33]. The electron micrographic features of CGRP-immunolabeled somata were not examined in the AON prior to the

present investigation. GABAergic neurons were previously analyzed in the rabbit NOT/DTN [28,29] and the rat MTN [11,36,40–42] (for review, see Ref. [44]). Because the co-localization data show that CGRP-labeled somata are GABAergic (see below), the present data provide important features for this population of GABAergic neurons within the AON.

The CGRP-labeled axon terminals in the DTN, LTN and MTNv form symmetric synapses, the type formed by GABAergic axon terminals in the NOT/DTN complex arising from neurons in the MTN [40]. As discussed above, the labeled axon terminals are densely filled with immunoreaction product, and it is virtually impossible to identify the morphology of their synaptic vesicles. Previously, Lenn [19] described axon terminals in the MTN where they formed axo-somatic, axo-dendritic and axo-spinous synapses. His analysis of these axon terminals did not distinguish between the types of synapse formed; either symmetric or asymmetric synapses. It needs to be noted that axon terminals forming asymmetric synapses are not labeled for CGRP in the present study. The present data are in contrast to the data on the localization of CGRP in laminae I–III of the spinal cord where CGRP-labeled axon terminals form asymmetric synapses [21].

4.3. Degenerating axon terminals

Peters et al. [30] described two types of axon terminal degeneration in brain following transection of axons. One is the filamentous type and the other is the shrunken, electron-dense degeneration. The present study shows that axon terminals in the DTN and LTN after contralateral optic nerve transection display the features of the electron-dense degeneration type. It is interesting that Lenn [19] described the same type of degeneration for axon terminals in the rat MTN following contralateral optic nerve transection.

The degenerating axon terminals in the present study make asymmetric synapses with CGRP-labeled dendrites and dendritic spines. These axon terminals are numerous in the AON, as reported by Lenn [19] who stated that about one-fourth of the axon terminals in the MTN degenerated following contralateral optic nerve transection. The degenerating axon terminals in the present study display different stages of degeneration in that some are surrounded by astrocytic processes and are no longer apposed to dendrites and spines. Nevertheless, the presence of many degenerating optic axon terminals in the AON making synapses with CGRP-labeled dendrites indicates that CGRP-containing neurons in the DTN and LTN are retinal recipient neurons.

4.4. CGRP-containing neurons are GABAergic

The data from the present study indicate that most, if not all, of the CGRP-labeled neurons in the AON are GABAergic. Thus, these neurons represent an important

J. Zhou et al. / Brain Research 838 (1999) 119–130

subset of GABAergic neurons that co-localize a peptide with an unknown function in the AON. GABAergic neurons in the hippocampus co-localize different calcium binding proteins [4]. Therefore, the analysis of CGRP-labeled neurons in the AON was extended to determine the degree of co-localization with calcium binding proteins. The presence of CR in almost half of the CGRP-labeled neurons may indicate that they are protected against excitotoxicity as suggested by previous work [4]. The other two calcium binding proteins studied, CB and PV, are not prevalent in CGRP-containing neurons in the AON.

4.5. Functional significance

The extensive, strong interconnections existing between the accessory optic system and NOT is well-documented [1,2,6,7,9–11,40–43] (for reviews, see Refs. [37,44]) as is the fact that a large proportion of the interconnecting neurons are GABAergic [11,36,40–42] (for reviews, see Refs. [2,44]). Based upon their findings, Schmidt et al. [36] recently showed how the neurons in the MTN and NOT in rat interact in gaze stabilization (see their Fig. 8). Our findings now reveal that the population of CGRP immunolabeled neurons of the DTN, LTN and MTNd consists almost entirely (> 90%) of GABAergic neurons, whereas we have found no CGRP-containing neurons in the NOT (also, Ref. [34]), the functional ally of the AON. Because the AON and NOT act together to stabilize gaze [2,37,44], the question arises as to what special function the CGRP–GAD-ir neurons of the AON play in controlling compensatory eye movements which is not shared by NOT neurons.

Another issue involves the function of calcium-binding proteins in CGRP-containing neurons in the AON. According to Kruger et al. [17], it seems reasonable to assume that the calcitonin present in GABAergic neurons in the AON is concerned with the sequestering of calcium into the neuron as required in cellular mechanisms involving GABA action. The presence of calcium binding proteins, which we found in about 40% of CGRP-containing somata, is consistent with the suggestion that these neurons utilize large quantities of calcium, and could relate to their high neuronal activity.

The GABAergic neuronal populations within AON and the NOT have been investigated. Thus, studies have shown that retinal terminals synapse with non-GABAergic, but not GABAergic, somata in rabbit NOT [28,29] and in rat NOT and MTN [35,36,40–42]. In contrast, retinal terminals have been reported to synapse with GABAergic neurons in the dorsal lateral geniculate nucleus in cat [25,26] and the superior colliculus in the rat [13,31], cat [22] (for review, see Ref. [23]) and monkey [24] (for review, see Ref. [23]). Our findings indicate that a subset of GABAergic neurons in rat DTN and LTN containing CGRP receives direct input from retinal terminals. This population of CGRP immunolabeled neurons is unique in the visual

system because it is GABAergic and inhibitory, and is likely part of the elaborate GABAergic circuitry known to interconnect the AON and NOT (for review, see Ref. [44]).

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