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Ultrastructural Localization of GABAergic Neuronal Components in the Dog Basilar Pons

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개의 교핵내 GABA성 신경세포 성분의 미세구조적 위치관찰

이 현 숙

적 요

개의 교핵내의 GABA를 함유한 억제성 신경세포의 연접부위를 전자현미경을 이용한 면역세포화학적 방법으로 조사하였다. 반응산물은 신경원의 핵부위나 가지돌기에서 관찰되었으며, 반응산물을 포함하고 있지 않은 신경종말이 이들과 비대칭형 연접을 형성하였다. 그 외에도 많은 수의 GABA성 신경종말이 관찰되었으며, 이들은 반응산물을 함유하지 않은 가지돌기와 대칭형 또는 비대칭형 연접을 형성하였다. 한편 축삭돌기-축삭돌기 연접에서는 연접후 유축삭돌기(axon-like processes)가 GABA-양성이었다. 이와 같은 관찰은 개의 교핵에 존재하는 억제성 개재신경원이 여러 수입계로부터의 정보를 통합하고, 이를 소뇌피질이나 소뇌핵에 전달함으로써, 대뇌-교핵-소뇌계의 신경경로에서 조절기능을 담당함을 뒷받침하는 것이다.

INTRODUCTION

The basilar pons has been considered as one of the important precerebellar nuclei, which performs an important role in preprogramming and execution of animal's volitional movement. Although the basilar pons receives a major input from the cerebral cortex, various subcortical pontine afferent projections have been identified (Mihailoff *et al.*, 1989b). In addition, potential feedback circuits from the deep cerebellar nuclei to the basilar pons have also been reported (Lee *et al.*, 1989).

One question of interest is whether there exist inhibitory processes superimposed on or intercalated between pontine afferent and efferent elements. Anatomically, local circuit neurons fulfilling the criteria of Golgi type II have been observed in Golgi impregnated pontine sections (Brodal *et al.*, 1988). Hyperpolarization of pontine cells occurring after the early electrophysiological excitation evoked by cerebrocortical stimulation has also been considered as an evidence of inhibition (Azizi *et al.*, 1986). The suggestion that inhibitory processes may indeed occur in the basilar pons of the rat was further supported by the autoradiographic demonstration of glutamate decarboxylase (GAD) activity and high affinity gamma-aminobutyric acid (GABA) uptake

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in the region (Thangnipon *et al.*, 1983).

An immunocytochemical study at the light microscopic level has also indicated the presence of GABAergic neuronal elements in the basilar pons of the rat, cat and monkey (Brodal *et al.*, 1988). These GABA-immunoreactive neurons represent a population of local circuit neurons, as evidenced by the fact that they are not double-labeled after the injection of wheatgerm agglutinin-conjugated horseradish peroxidase (WGA-HRP) into the cerebellar cortex in conjunction with the GABA immunocytochemistry in pontine sections.

Previous ultrastructural study of the dog basilar pons reported the presence of serial synaptic arrangements involving synapses between two vesicle-containing profiles similar to those observed in the other brain regions known to contain local circuit neurons (Lee, 1993). The light microscopic study identified the distribution of GABA-positive neurons and axon terminals in a rostro-caudal series of pontine sections in the dog (Lee, 1995). The present ultrastructural study was, therefore, performed to further examine the involvement of GABA-immunoreactive neuronal components in the synaptic circuitry of the dog basilar pons, including the axo-axonic synaptic complexes.

MATERIALS AND METHODS

Twelve dogs of both sexes, ranging in weight from 1.5~2.0 kg were used in the study.

1. Perfusion and fixation

Animals were anesthetized with the injection of 3.5% chloral hydrate (10 ml/kg body weight) into the peritoneal cavity. After tracheotomy, animals were artificially ventilated using a respirator and perfused intracardially with the following solutions delivered by means of a peristaltic pump. Physiological saline (100~200 ml) was infused until the cardiac auricular effluent became relatively clear. It was followed by 200 ml of fixative containing 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS, pH 7.4) and subsequently by 600 ml of 4% paraformaldehyde in the same buffer. The brain was removed and stored in 0.05M Tris-buffered saline (TBS, pH 7.6) overnight at 4℃.

2. Pre-embedding immunocytochemistry

Brain regions involving the basilar pons were sectioned in the transverse plane on a slicetome at 70 μm and collected in tissue culture wells containing TBS. Indirect peroxidase-antiperoxidase (PAP) method as described by Sternberger (1986) was used. The sections were treated with a fresh 0.5% solution of hydrogen peroxide in methanol for 10~30 mins to block free aldehyde groups and endogenous peroxidase. Subsequently, sections were incubated with 10% normal rabbit serum (NRS)/0.1M lysine in TBS for 60 mins to minimize non-specific binding of antibodies and then in mouse anti-GABA monoclonal antibody solution (an aliquot of 10 μg/100 μl PBS) at a dilution of 1:100 with 2% NRS at 4℃ for 24~48 hrs. After rinses with TBS, sections were incubated with rabbit anti-mouse IgG (an aliquot of 0.2 mg/100 μl PBS) at a dilution of 1:100 with 2% NRS for 30 mins. After rinses with TBS, sections were reacted with mouse PAP immune complexes (an aliquot of 1 mg/50 μl PBS) at a dilution of 1:200 with 2% NRS for 30~60 mins, washed with TBS and treated with 0.05% diaminobenzidine (DAB)/0.01% hydrogen peroxide solutions in TBS for 5~10 mins. Sections were then thoroughly washed with TBS and observed under a light microscope to identify the reaction product.

3. Electron microscopy

GABA-immunolabeled regions within the basilar pons were isolated with the aid of a dissecting microscope. Sections were rinsed in 0.2M cacodylate buffer (pH 7.4) and immersed in 1% osmium tetroxide for 2 hrs. Dehydration in ethanol series and Epon embedding followed. Ultrathin sections were mounted on copper grids, counterstained with uranyl

acetate and lead citrate and then examined under the JEOL 1200 EXII electron microscope. Positive and negative control experiments for immunocytochemical methods were performed in parallel with these procedures and described in detail elsewhere (Lee, 1995).

RESULTS

Although relatively low concentration (0.2%) of glutaraldehyde used in the fixative favored immunocytochemical staining, coarse tissue morphology was observed under the electron microscope. Immunostaining was limited to superficial layers of the sections, where tissue morphology was poorly preserved. Despite these disadvantages, immunolabeled reaction product was mainly localized within specific components of the neuronal elements in pontine sections.

1. Neuronal somata

A large number of GABA-immunoreactive neuronal somata have been observed. Dense reaction product was observed in the vicinity of the nucleus with very little chromatin aggregation (Fig. 1A and B). A number of mitochondria and endoplasmic reticula were dispersed in the region. One or more unlabeled axon terminals made asymmetric synaptic contacts with GABA-immunolabeled neuronal somata and contained a relatively consistent size of round synaptic vesicles (Fig. 1A and B).

2. Dendritic processes

Immunoperoxidase reaction product was occasionally observed in dendritic profiles with the electron-dense label being distributed in clumps throughout the cytoplasm(Fig. 2A-C). Dendritic profiles containing reaction product were localized postsynaptic to the unlabeled axon terminals. Synaptic vesicles in these axon terminals were relatively consistent in size and round in morphology. Asymmetric synapse was prominent between these unlabeled boutons and immunoreactive dendritic processes.

3. Synaptic boutons and other vesicle-containing profiles

A large number of immunoreactive axon terminals and vesicle-containing structures have been observed in the pontine neuropil. Although electron-dense reaction product tended to obscure the ultrastructural morphology within the labeled axon terminals, most GABAergic boutons seemed to form symmetric synaptic contacts with unlabeled dendrites (Fig. 3A, C and D), whereas others exhibited rather asymmetric morphology (Fig. 3B). Immunolabeled axon terminals included round synaptic vesicles, whereas unlabeled, postsynaptic dendritic profiles did some swollen vesicles (Fig. 3B) and microtubules (Fig. 3C).

Glomerular (Fig. 4A) and non-glomerular (Fig. 4 B-D) types of axo-axonic synapses were observed in control pontine sections where immunocytochemistry had not been performed. With immunostaining, postsynaptic axon-like processes in these synaptic complexes were consistently GABA-positive (Fig. 4E-G). Some unlabeled presynaptic axon terminals made simultaneous synaptic contact with non-immunoreactive dendritic profiles (Fig. 4E). It had not been observed where glomerular-type boutons made synaptic contact with immunoreactive axon-like processes in the pontine sections. Most of unlabeled, presynaptic axon terminals in axo-axonic synaptic complexes seemed to form asymmetric synaptic contact with immunoreactive, postsynaptic axon-like processes (Fig. 4E and F).

DISCUSSION

Although the pre-embedding immunocytochemical procedures using the indirect PAP method of Sternberger (1986) were highly specific in localizing GABA-immunoreactive neuronal components, there had been several problems that limited the

interpretation of ultrastructural findings. As described in the result section, the limited tissue penetrance of the antibody restricted the existence of immunolabeled reaction product to the surface of the tissue block, where ultrastructural preservation was generally the poorest. In addition, heavy deposits of electron-dense reaction product often obscured certain morphological characteristics of labeled profiles.

The present ultrastructural study has confirmed the previous light microscopic study in which a large number of basilar pontine neurons are GABA-immunoreactive (Lee, 1995). Certain neuronal components in axo-axonic synaptic complexes observed in control ultrastructural specimen (Lee, 1993) were also proved to be GABA-immunoreactive (Fig. 4E-G). The double-labeling study reported that GABA-immunolabeled cells were not retrogradely labeled following WGA-HRP injections into the middle cerebellar peduncle or cerebellar cortex (Brodal *et al.*, 1988). Thus GABA-positive neuronal somata and dendritic structures observed in the present study might represent those of the local circuit neurons (Figs. 1 and 2). In addition unlabeled axon terminals made asymmetric synaptic contact with these immunoreactive neuronal somata and dendritic profiles (Figs. 1 and 2), indicating that non-GABAergic pontine afferent fibers made synapse with pontine local circuit neurons.

It was, however, reported that a few cells in the zona incerta, the perirubral area, cerebellar nuclei, anterior pretectal nuclei and medullary reticular formation sent GABAergic axon terminals into the basilar pons(Border *et al.*, 1986). GABA-immunoreactive axon terminals observed in the present study might, therefore, represent those of either pontine afferent systems or local circuit neurons (Fig. 3). GABA-positive boutons in the pontine neuropil exhibited either symmetric or asymmetric active sites, although symmetric ones were predominant (Fig. 3). Existence of these two populations of active sites involving GABAergic boutons has been reported in other brain regions involving the inferior olive, the lateral geniculate nucleus and the hypothalamus (Hendrickson *et al.*, 1983; Sotelo *et al.*, 1986). Since these brain regions also contained Golgi type II local circuit neurons, it was speculated that GABAergic axon terminals of extrinsic or intrinsic sources might represent either one of the two types of active sites (Tappaz *et al.*, 1985).

Glomerular (Fig. 4A) and non-glomerular (Fig. 4B-D) types of synaptic complexes formed between the two adjacent vesicle-containing profiles were observed in control non-immunolabeled pontine sections. Ultrastructural observation of control pontine sections indicated that the two vesicle-containing structures made serial synapses with dendritic profiles (Lee, 1993). Difficulty of observing the triadic synaptic complexes in the present study might be simply due to plane-of-section problem. With immunostaining, it seemed that postsynaptic axon-like processes in axo-axonic synapses were GABA-positive (Fig. 4E-G). It was postulated that postsynaptic structures in axo-axonic synaptic complexes might represent axon-like processes of the local circuit neurons, based on Golgi and ultrastructural studies(Lee, 1993; Mihailoff *et al.*, 1981). Thus GABA-immunoreactive, postsynaptic axon-like processes observed in the present study might represent those of Golgi type II local circuit neurons.

Based on these ultrastructural and immunocytochemical observations, existence of basilar pontine local circuit neurons in cerebro-ponto-cerebellar neuronal circuitry was diagramed in Fig. 5. Existence of synapses between the two adjacent vesicle-containing profiles and GABA-immunoreactivity in postsynaptic axon-like processes implied some integrative function performed by the inhibitory local circuit neurons in the basilar pons. Precise functional mechanism of inhibitory local circuit neurons in these triadic synaptic complexes might require further electrophysiological evaluations. It might, however, be suggested in a couple of ways how this neuronal network performed its function; one might

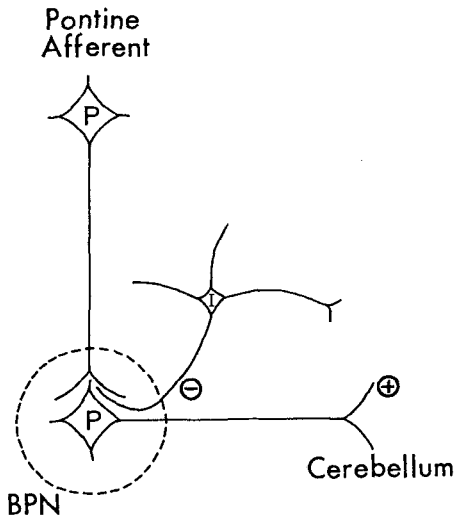

Fig. 5. A schematic diagram illustrating how intrinsic(I) neurons form a serial synapse between pontine afferent and pontine efferent projection(P) neurons, thus performing controlling and integrating functions in this neuronal circuitry. BPN denotes the basilar pontine nuclei, whereas the plus (+) or minus (−) signs represent excitatory or inhibitory neural transmission.

be that after the pontine afferent fibers conveyed synaptic input into the axon-like processes of the local circuit neurons, the information was once transfered to the somata of intrinsic neurons and integrative message subsequently transmitted into the pontine projection neurons. In the case the time lag might, therefore, exist between the axo-axonic synapses and serial synapses with dendritic profiles. The other might be the continuous neural transmission among the serial synaptic complexes, thus the neural signals between the two vesicle-containing structures could be directly transmitted to the postsynaptic dendritic components.

The present ultrastructural observations of GABA-immunoreactive neuronal elements in pontine sections implied that the inhibitory synaptic transmission might be a part of the functional circuitry of the basilar pons. The electrophysiological study also suggested that inhibitory postsynaptic potentials were consistently observed in the basilar pons following either orthodromic or antidromic activation of pontine neurons (Sasaki *et al.*, 1970). In addition, single-unit recording studies in the rat basilar pons indicated that a suppression of firing occurred in pontine neurons following the initial excitatory response to the sensorimotor cortical stimulation and further, such inhibition could also be observed after ablation of sensorimotor cortex followed by stimulation of the underlying cortical white matter(Mihailoff *et al.*, 1989a). Although these observations need to be investigated in greater detail with intracellular recording methods, such electrophysiological findings correlate well with the present ultrastructural study indicating that inhibitory local circuit neurons exist in the basilar pons of the dog. Existence of GABAergic components in axo-axonic and serial synaptic complexes also supports the notion that inhibitory local circuit neurons integrate various pontine afferent informations and convey them into the cerebellar cortex and the deep cerebellar nuclei, so that the animal may preprogram and perform volitional movement in a precise manner.

ABSTRACT

An immunocytochemical study of GABA-positive neuronal elements was performed at the electron microscopic level to examine subcellular distribution of the inhibitory neurotransmitter in the dog basilar pons. Electron-dense reaction product was observed in neuronal somata and dendritic processes. One or more unlabeled axon terminals made asymmetric synaptic contacts with these GABAergic somatic and dendritic profiles. A large number of GABA-positive axon terminals were also observed. They made symmetric as well as asymmetric synaptic contacts with unlabeled dendritic profiles. In axo-axonic synapses, postsynaptic axon-like processes were consistently GABA-immunor-

eactive. These observations suggest that the inhibitory local circuit neurons in the dog basilar pons play a major role in cerebro-ponto-cerebellar circuitry by integrating various afferent inputs and conveying them into the cerebellar cortex and the deep cerebellar nuclei.

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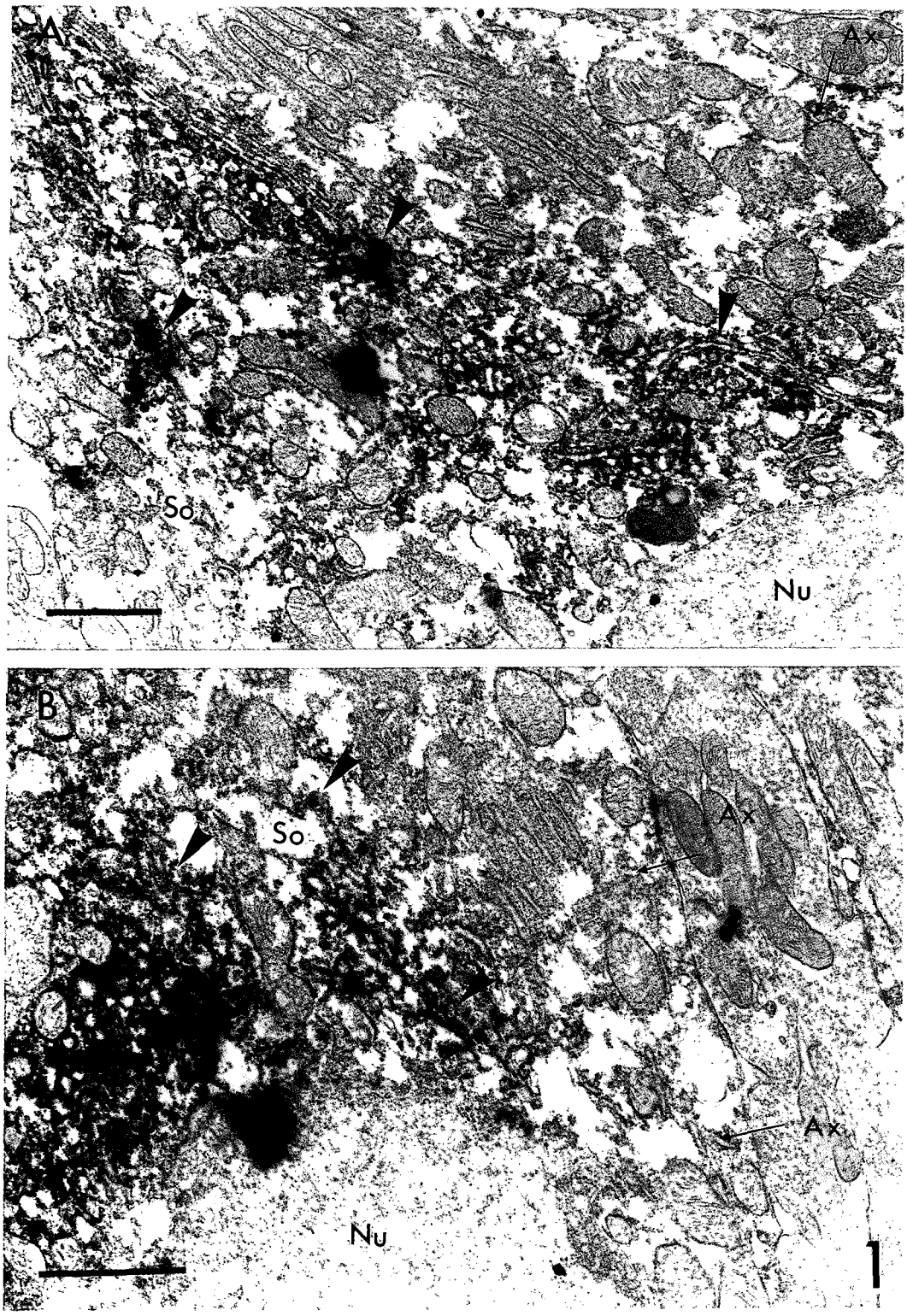
FIGURE LEGENDS

Fig. 1. GABA-immunolabeled neuronal cell bodies around the nucleus (Nu) were shown in A and B. The immunoperoxidase reaction product (arrowheads) within the neuron was associated with various subcellular organelles such as the endoplasmic reticula and the mitochondrial membranes. Unlabeled axon terminals (Ax) made synaptic contacts with immunoreactive somatic profiles (So). The arrows indicated the presumed polarity of synaptic activity, which was determined based on the fact that postsynaptic densities were thicker at Gray's type I asymmetric synapses. Bars=1 μm.

Fig. 2. Further evidence for the presence of GABAergic neurons within the basilar pons was demonstrated by the fact that some dendritic profiles (Dd) also contained reaction product (arrowheads) as shown in A-C. Unlabeled axon terminals (Ax) made asymmetric synaptic contacts with the dendritic structures. The arrows represented the presumed polarity of synaptic transmission. Bars=500 nm.

Fig. 3. Immunolabeled boutons (arrowheads) formed synaptic contacts with unlabeled dendritic profiles (Dd). The majority of labeled axon terminals contained a relatively consistent size of round synaptic vesicles. The arrows represented the presumed polarity of synaptic activity. Bars=500 nm.

Fig. 4. Axo-axonic synaptic complexes were observed in control pontine sections (A-D). Presynaptic axon terminals (Ax) made asymmetric synaptic contact with postsynaptic vesicle-containing structures (asterisks) as well as dendritic profiles (Dd). With immunostaining postsynaptic axon-like processes (arrowheads) were consistently GABA-positive (E-G). The arrows indicated the polarity of synaptic transmission. Bars=500 nm.



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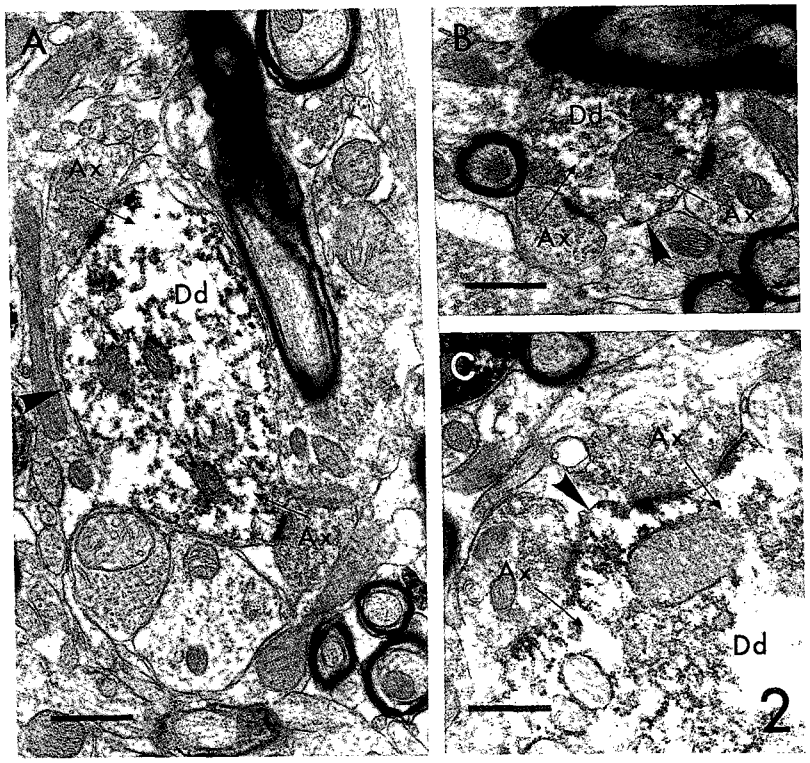
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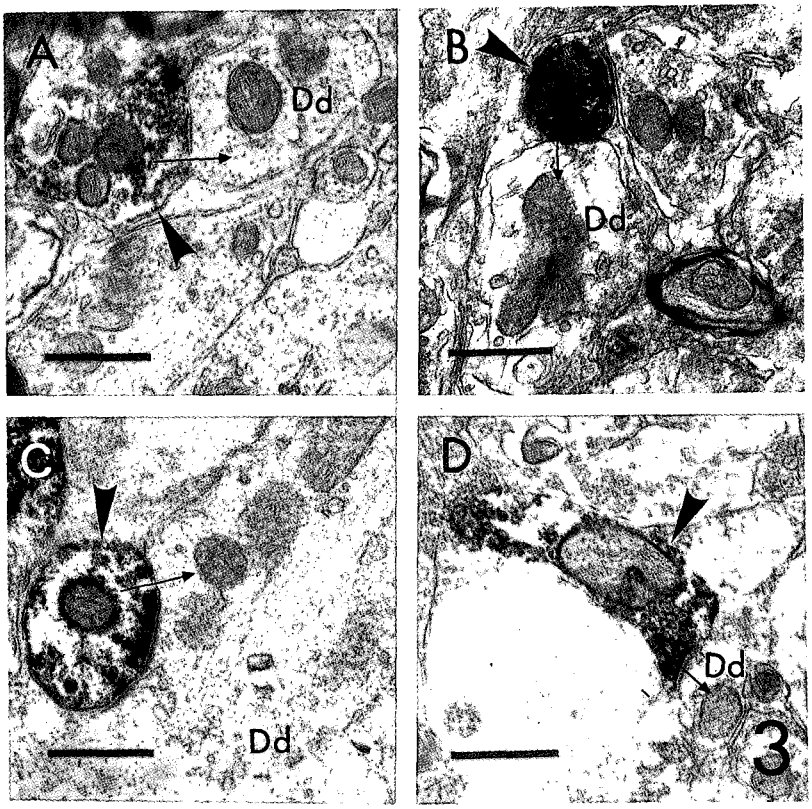
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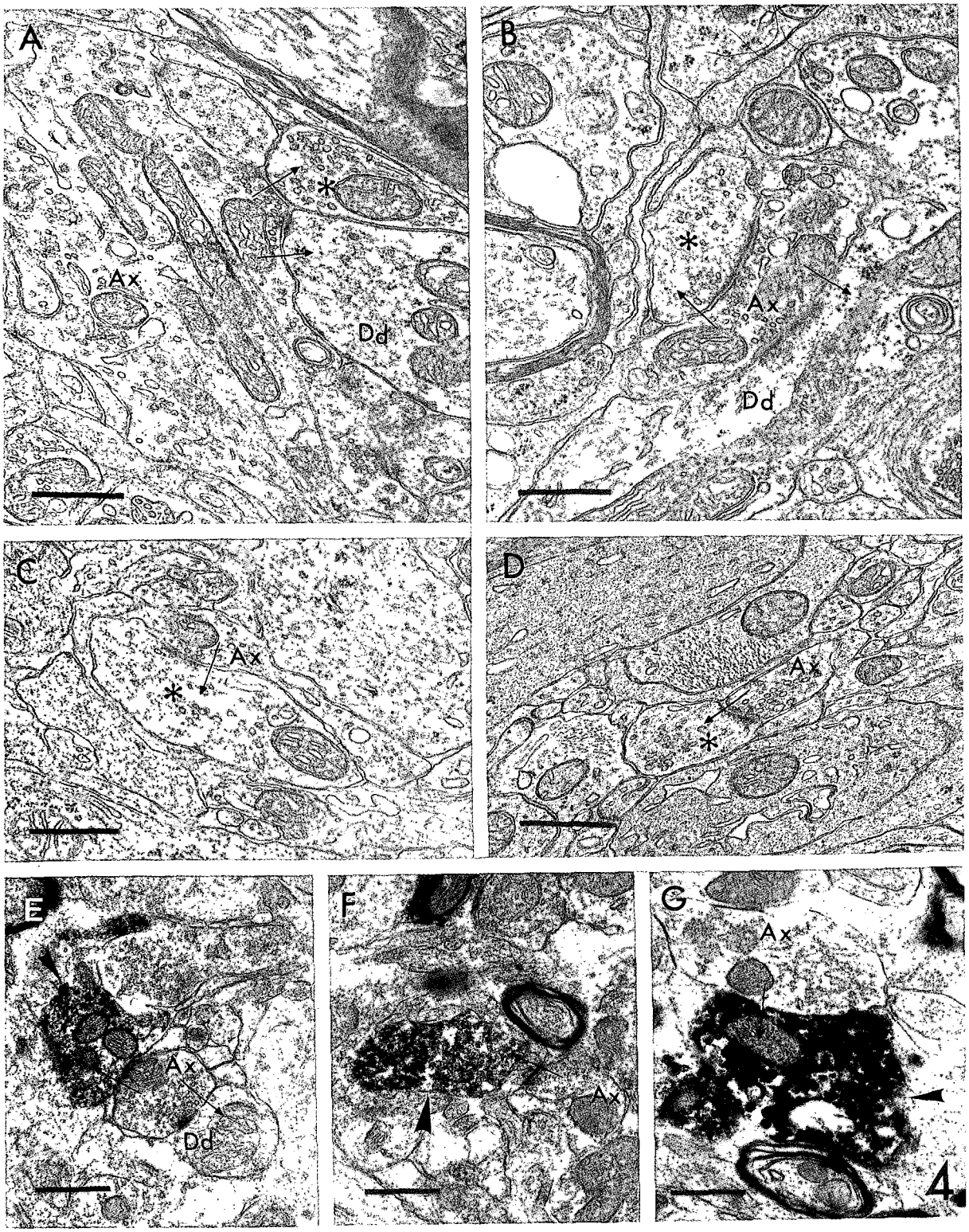
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