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Ultrastructural Observations of Glutamatergic Synaptic Components in the Basilar Pontine Nuclei of the Dog

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개의 교핵내 glutamate성 연접 성분의 미세구조적 위치관찰

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요 약

개의 교핵내 glutamate를 함유한 연접 구조의 분포를 fixative-modified glutamate에 대한 monoclonal antibody를 사용하여 미세구조적 차원에서 조사하였다. 반응산물은 신경세포체의 핵주위 영역에 위치하였으며, 가지돌기내의 미세소관을 따라서 존재하였다. 반응산물을 함유하지 않은 한 개이상의 신경종말이 glutamate에 면역반응을 나타낸 가지돌기와 비대칭형 연접을 형성하였다. 그외에도 수초로 둘러싸인 축삭돌기나 신경종말내에서도 반응산물이 관찰되었다. 이들 신경종말은 표지된 또는 표지되지않은 가지돌기와 비대칭형 연접을 형성하였다. 이상의 관찰은 glutamate에 면역반응을 나타내는 흥분성 교핵 신경원이, glutamate를 함유한 교핵으로의 여러 수입계나, 교핵으로부터 소뇌로의 투사 신경세포, 그리고 소뇌피질내의 glutamate성 과립세포를 포괄하는 다연접 신경경로에서 어떤 역할을 수행하는지에 관한 형태학적 근거를 제시한다.

Key words : Basilar pons, Glutamate, Immunocytochemistry

INTRODUCTION

The basilar pontine nuclei have once been considered as a simple relay station in mammalian cerebro-cerebellar circuitry so that the animal might perform volitional motor activities. Recent morphological and electrophysiological studies, however, provided evidence for the basilar pons as a site of integration for a wide variety of ascending and descending information which was destined for the cerebellar cortex (Aas, 1989; Azizi *et al*., 1986; Border *et al*., 1986).

Previous studies have identified the neurochemical composition of the basilar pons and its afferent and efferent connections. Biochemical studies of the corticopontine system reveal a marked decrease in high affinity uptake of radiolabelled L-glutamate following transection of the

cerebral peduncles, thus suggesting that corticopontine afferents utilize the excitatory amino acid glutamate as a neurotransmitter (Thangnipon *et al.*, 1983). Serotonergic, noradrenergic and cholinergic fibers and axon terminals have also been identified as the neurotransmitters utilized by the various pontine afferent systems (Border and Mihailoff, 1991; Levitt and Moore, 1979; Steinbusch, 1981; Woolf and Butcher, 1989). On the other hand, the light microscopic and ultrastructural observations of gamma-aminobutyric acid (GABA)-immunoreactive neural elements suggested that GABAergic neurons might serve as intrinsic neurons and exert an inhibitory influence on pontocerebellar projection cells within the basilar pons of the dog (Lee, 1995[1]; 1995[2]). In regard to specific neurotransmitters, the possibility that certain basilar pontine projection neurons utilize acetylcholine as a transmitter has been suggested by acetylcholinesterase histochemistry of the rat cerebellar cortex (Ross *et al.*, 1983). In addition, following injection of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into the cerebellar cortex and glutamate immunocytochemistry, pontine neurons contain both retrogradely transported horseradish peroxidase and immunoperoxidase reaction products, indicating that many pontocerebellar projection neurons are glutamatergic in nature (Somogyi *et al.*, 1986).

The present study was, thus, conducted to investigate ultrastructural features of glutamatergic neuronal components in the basilar pons of the dog using a monoclonal antiserum raised against fixative-modified glutamate (Clements *et al.*, 1987). Fine structures of glutamate-immunoreactive elements including neuronal somata, dendrites, myelinated axons, and synaptic boutons were examined. The synaptic composition of glutamatergic structures within the pontine neuropil would reveal the mechanism of how the excitatory neurotransmitter might function in a synaptic circuitry involving cerebro-ponto-cerebellar communication system.

MATERIALS AND METHODS

A total of fourteen dogs of both sexes, ranging in weight from 1.5~2.0 kg, were used in these studies.

1. Perfusion and fixation

Under deep anesthesia (3.5% chloral hydrate, 10 ml/kg body weight), animals were artificially ventilated using a respirator and perfused through the left ventricle with a brief flush of saline (100~200 ml) followed by 200 ml of fixative containing 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 M 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 0.05 M Tris-buffered saline (TBS, pH 7.6) overnight at 4°C.

2. Pre-embedding immunocytochemistry

Tissue blocks involving the basilar pons and other brain regions for control experiments were sectioned in the transverse plane on a vibratome at 70 μm and collected in tissue culture wells containing TBS. The unlabelled antibody peroxidase-antiperoxidase (PAP) method of Sternberger (1986) was utilized in order to visualize immunolabelled neural elements in the basilar pons. Sections were washed twice with TBS and incubated for 5 min in 3% H_2O_2/10% methanol to block endogenous peroxidase activity. Sections were transferred to 10% normal goat serum and eventually incubated in a 1:1000 dilution of the primary antibody solution containing 2% normal goat serum. The rabbit anti-glutamate mono-

clonal antibodies raised against fixative-modified glutamate were utilized as the primary antiserum (Clements *et al.*, 1987). Tissue was incubated for a period of 16~24 hrs at room temperature followed by incubation in the secondary antiserum solution (1 : 100, 45 min) containing 2% normal goat serum and goat anti-rabbit IgG. After several rinses with TBS, sections were finally incubated in the rabbit PAP solution (1 : 100, 60 min) containing 2% normal goat serum. The detergent, Triton X-100, has not been included in any of these solutions. In order to visualize the resulting antigen-antibody complex, sections were rinsed with TBS and subsequently incubated in cold (4°C) TBS solution containing 0.05% DAB/0.01% H_2O_2 for 6~10 mins. Sections were then thoroughly washed with TBS and observed under a light microscope to identify the reaction product. Positive and negative control experiments for immunocytochemical methods were performed in parallel with these procedures and described in detail elsewhere (Lee, 1995[1]; 1995[2]).

3. Electron microscopy

Glutamate-immunolabelled regions within the basilar pons were isolated with the aid of a dissecting microscope. Sections were rinsed in PBS 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.

RESULTS

Tissue morphology was somewhat poorly preserved because the relatively low concentration (0.2%) of glutaraldehyde was utilized in the fixative in order to maximize the immunocytochemical staining. In addition, only the superficial layers of the tissue section were collected over grids because immunostaining might not penetrate deep enough. Despite these disadvantages, immunolabelled reaction product was localized within specific neuronal components in basilar pontine sections.

1. Neuronal somata

The majority of neuronal somata throughout the rostrocaudal extent and within all subdivisions of the basilar pons were immunoperoxidase labelled (Fig. 1a-c). Glutamate-immunolabelled neuronal somata in the pontine nuclei often appeared multipolar in shape when observed at the electron microscopic level, and the nuclei of the labelled somata were infolded, although not extensively so (Fig. 1a and b). Electron-dense reaction product was dispersed within the cytoplasm in the vicinity of the nucleus with very little chromatin aggregation (Fig. 1a-c). Electron-dense accumulations of peroxidase reaction product were distributed along various intracellular organelles including vesicular and mitochondrial membranes (Fig. 1a-c). Reaction product, however, was neither associated with Golgi apparatus nor observed within the nucleus (Fig. 1a).

2. Dendritic processes

Reaction product was occasionally observed in microtubule-containing, proximal dendritic profiles with the electron-dense label being specifically localized along the microtubules (Fig. 2a and b). Unlabelled axon terminals often made asymmetric synaptic contact with the immunoreactive dendritic profiles (Fig. 2a). Synaptic vesicles within these axon terminals were relatively

consistent in size and round in morphology. In addition to proximal dendritic profiles, a large number of small-sized (0.5~1.5 μm in diameter), distal dendritic processes were immunoreactive and located postsynaptic to one or more unlabelled axon terminals (Fig. 3a-f).

Reaction product in the dendritic profiles exhibited either dispersed (Fig. 3c and d) or spotty (Fig. 3b and e) appearance and was often associated with mitochondrial membranes (Fig. 3c). The majority of presynaptic boutons contained consistent sizes of clear and round synaptic vesicles (a-c), although some included a mixed population of clear and dense-core vesicles (d-f).

3. Axonal fibers and vesicle-containing boutons

Spotty (Fig. 4a and b) and dispersed (Fig. 4c) reaction products were observed within a large number of axonal processes surrounded by myelin sheath. A large number of synaptic boutons in the pontine neuropil were also glutamate-immunoreactive following application of the glutamate antiserum. These immunoreactive axon terminals made asymmetric synaptic contact with either unlabelled (Fig. 5a-d) or labelled (Fig. 6a-d) dendritic profiles. The majority of boutons contained consistent sizes of clear and round synaptic vesicles. Both immunoreactive and non-immunoreactive axon terminals often made simultaneous synaptic contact with immunoreactive dendritic processes (Fig. 6b).

DISCUSSION

Glutamate has been identified as a major excitatory neurotransmitter in the mammalian central nervous system (CNS) since the excitatory effect of glutamate on spinal neurons was demonstrated iontophoretically (Fagg and Foster, 1983). The recent availability of several highly specific antisera raised against glutamate has allowed the visualization of putative glutamatergic neural elements throughout the CNS (Beitz *et al.*, 1986; Clements *et al.*, 1987).

The present study identified ultrastructural features of glutamatergic neuronal components in the basilar pons of the dog using a monoclonal antiserum raised against fixative-modified glutamate. Distribution patterns of glutamatergic synaptic components were in a general accordance with those reported in the rat (Border and Mihailoff. 1991).

The present electron microscopic examination of glutamate immunostaining in the dog basilar pons has revealed immunochemical label in neuronal cell bodies, dendrites, myelinated axons, and axon terminals, thereby confirming light microscopic observations (Border and Mihailoff, 1991). A large number of glutamate-positive neuronal somata have been observed in the dog basilar pons (Fig. 1a-c). These immunoreactive neuronal somata often appeared multipolar in shape and the nuclei of the labelled somata were scanty of chromatin aggregation and infolded (Fig. 1a and b). Basilar pontine neurons with this morphology were generally considered to be a component of the pontocerebellar projection system (Mihailoff, 1978). Electron-dense accumulations of peroxidase reaction product were distributed throughout the cytoplasm and often associated with various intracellular organelles including polyribosomes and mitochondrial membranes. Reaction product, however, was not localized within the nucleus, thereby confirming the light microscopic observation that glutamate was specifically localized within the cytoplasm outside of the nucleus when the sectioning was performed right at the level of the nucleus (Border and Mihailoff, 1991). Portions of the unlabelled neuronal somata could be considered

as GABAergic and local-circuit type neurons (Lee, 1995[1]; 1995[2]).

Reaction product was occasionally localized within microtubule-containing, proximal dendritic profiles with the electron-dense label being specifically localized along the microtubules (Fig. 2a and b). The unlabelled bouton presynaptic to the glutamatergic dendrites contained round and clear synaptic vesicles and formed an asymmetric synapse, indicating that the synaptic complex was involved in the excitatory synaptic transmission (Fig. 2a). In addition to proximal dendritic profiles, a large number of small-sized (0.5~1.5 μm in diameter) distal dendritic processes were immunoreactive (Fig. 3). One or more unlabelled axon terminals made asymmetric synaptic contact with these immunoreactive dendritic profiles. Although the majority of unlabelled boutons contained round and clear vesicles (Fig. 3a-c), some exhibited dense-core vesicles as well as clear and round ones (Fig. 3d-f). Unlabelled boutons with clear and round synaptic vesicles might represent those of pontine afferent fibers using other excitatory neurotransmitters than glutamate. Axon terminals with dense-core vesicles might represent catecholaminergic pontine afferent systems or imply boutons with two different neurotransmitters (Levitt and Moore, 1979; Steinbusch, 1981). The confocal immunofluorescence study employing antibodies against two different excitatory neurotransmitters would identify the mechanism of how those boutons might have work in the synaptic circuitry involving the basilar pons.

The immunochemical label was also identified within the myelinated axons (Fig. 4) and axon terminals (Fig. 5 and 6). These immunoreactive axonal fibers might represent either a population of axon terminals of pontine afferent systems or axonal processes of pontocerebellar projection neurons on their way to the brachium pontis. Previous double-label study employing HRP injections into the basilar pons and glutamate-immunocytochemistry of various brain regions indicated that glutamatergic pontine afferent systems included those originating from the cerebral cortex, zona incerta, cerebellar nuclei, dorsal column nuclei, and medullary reticular formation (Border and Mihailoff, 1991; Clements *et al.*, 1987). On the other hand, the evidence supporting the existence of glutamatergic pontocerebellar projection neurons was also implicated by the double-label study involving HRP injections into the cerebellar cortex followed by glutamate immunocytochemistry of the rat basilar pons (Beitz *et al.*, 1986). However, the pontocerebellar projection is not the only afferent system to provide glutamatergic mossy fiber input to the cerebellar cortex. Other areas of the CNS that provide contingents of mossy fibers to the granule cell layer in the cerebellar cortex include the vestibular apparatus, the spinal trigeminal and principal sensory trigeminal nuclei, the dorsal column nuclei, and the pontine and medullary reticular formations (Kojima and Kanazawa, 1987; Matsushita *et al.*, 1982; Ottersen and Storm-Mathisen, 1984; Raymond *et al.*, 1984). A third possibility about the origin of glutamate-positive axonal fibers observed in the pontine neuropil might be the local collaterals of the pontocerebellar projection neurons (Mihailoff, 1978).

Glutamate-immunoreactive axon terminals made asymmetric synaptic contact with either unlabelled (Fig. 5) or labelled (Fig. 6) dendritic profiles. Presynaptic boutons at both cases contained round and clear synaptic vesicles, consistent with previous reports concerning glutamate-immunoreactive boutons observed in other CNS

regions including the spinal cord and cerebellar cortex (Madl *et al.*, 1986; Miller *et al.*, 1988; Somogyi *et al.*, 1986). In contrast, GABA-labelled axon terminals in the rat basilar pons generally contained pleomorphic synaptic vesicles and formed symmetric membrane specializations with the majority of postsynaptic dendritic profiles (Border and Mihaloff, 1990). Unlabelled axonal fibers with glutamatergic dendritic profiles (Fig. 3a-c) or glutamate-immunoreactive boutons with unlabelled dendritic processes (Fig. 5a-d) represented that pre- or postsynaptic structures at respective case might utilize other excitatory neurotransmitters than glutamate. In addition, both immunoreactive and non-immunoreactive axon terminals often made simultaneous synaptic contact with labelled dendritic processes (Fig. 6b). This synaptic profile implied that glutamate-immunoreactive pontocerebellar projection neurons received two different inputs, one of which was glutamatergic. An interesting point in the examination of neurotransmitter-specific pontine afferent systems regards the finding that certain regions, namely zona incerta, the dentate nucleus of the cerebellum and nucleus paragigantocellularis, provide both GABAergic and glutamatergic inputs to the rat basilar pons (Border *et al.*, 1986).

Although the significance of these findings is unclear, these cell groups apparently provide a dual input to the basilar pons which seems to involve neurotransmitters with opposing actions.

The present observation might provide the morphological evidence for the possible role of glutamate-immunoreactive neuronal elements in the synaptic circuitry of the dog basilar pons. It might be concluded that glutamatergic afferent projections to the basilar pons provide a substrate for at least a portion of the excitatory drive responsible for the activation of basilar pontine neurons. In addition, it was reaffirmed that glutamate might function as a neurotransmitter in a majority of basilar pontine projection neurons, in turn implying that this mossy fiber system provided a substantial glutamatergic excitatory input to the cerebellar granule cells.

ABSTRACT

The distribution of glutamatergic synaptic structures in the dog basilar pons was investigated at the ultrastructural level using monoclonal antibodies against fixative-modified glutamate. Electron-dense reaction product was densely localized at the perinuclear region in the neuronal somata and often observed along the microtubules located within the dendritic processes. One or more unlabelled axon terminals made asymmetric synaptic contacts with glutamate-immunoreactive dendritic profiles. In addition, reaction product was observed either within axonal processes surrounded by myelin sheath or axon terminals. Immunoreactive axon terminals made asymmetric synaptic contact either with unlabelled or labelled dendritic profiles. These observations provided an anatomic evidence of how this excitatory neural element might perform its function in a multisynaptic pathway involving glutamatergic afferents to the basilar pons, glutamate-immunoreactive pontocerebellar projection neurons, and the glutamate-positive granule cells of the cerebellar cortex.

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FIGURE LEGENDS

Fig. 1. Glutamatergic reaction product (arrowheads) was dispersed within the somatic profiles (So) in the vicinity of the nucleus (Nu). Electron-dense reaction product was distributed along various intracellular organelles including vesicular and mitochondrial membranes (a-c). It was neither associated with Golgi complex (Go) nor observed within the nucleus. Bars=500 nm.

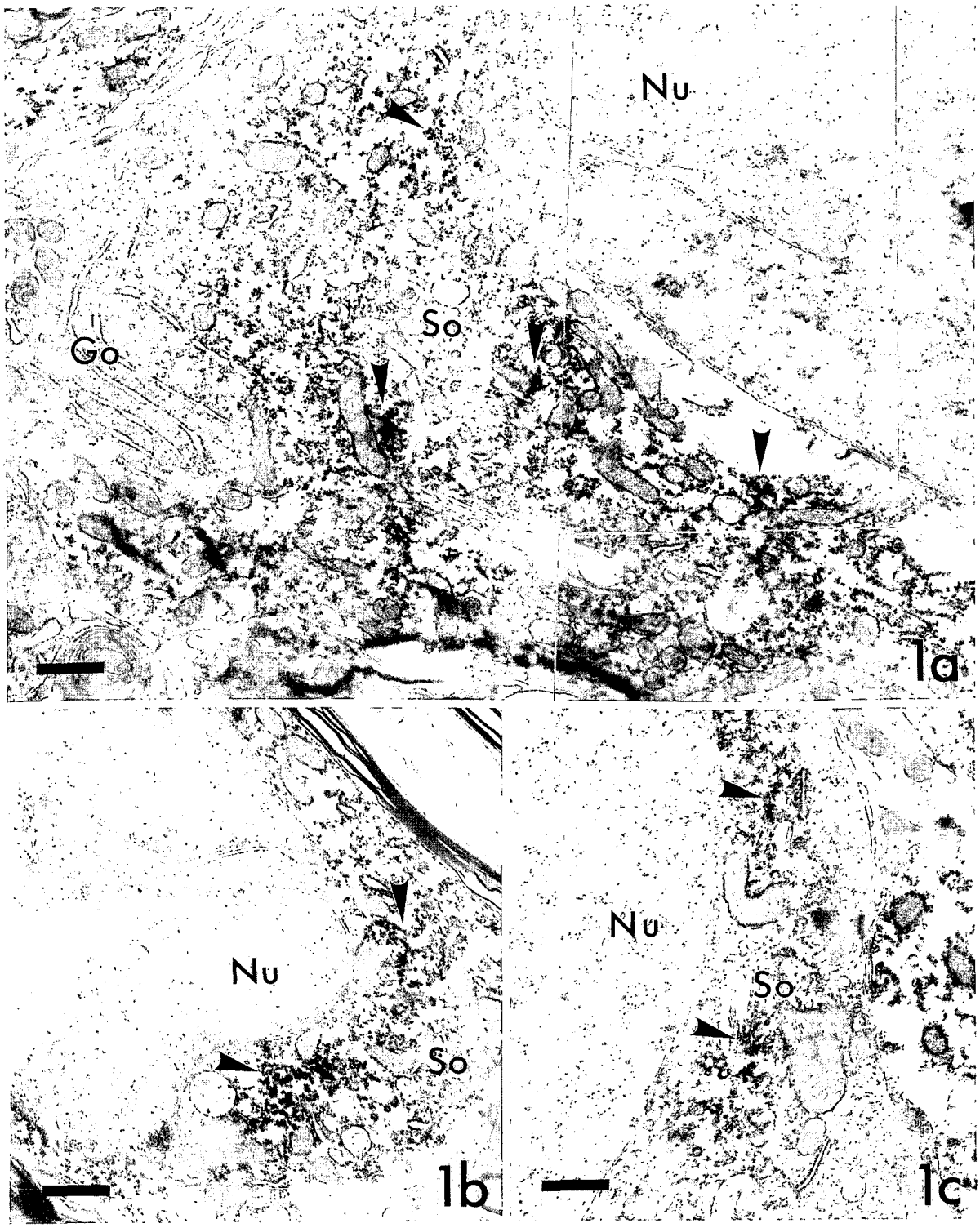
Fig. 2. Further evidence for the presence of glutamatergic neurons within the basilar pons was demonstrated by the fact that some dendritic processes (Dd) were immunoreactive (a and b). Reaction product (arrowheads) was specifically localized along the microtubules(mt). Unlabelled axon terminals (Ax) made asymmetric synaptic contact with the dendritic structures(a). The arrow indicated the presumed polarity of synaptic activity, which was determined based on the fact that postsynaptic density was thicker at Gray's type I synapse. Bars=500 nm.

Fig. 3. A large number of distal dendritic processes (Dd) were immunoreactive (arrowheads) and located postsynaptic to the unlabelled axon terminals (Ax). The majority of boutons contained consistent sizes of clear and round synaptic vesicles (a-c), although some included a mixed population of clear and dense-core (asterisks) vesicles (d-f). The arrows represented the presumed polarity of synaptic transmission. Bars=500 nm.

Fig. 4. Reaction product (arrowheads) was observed within the axonal processes (Ax) surrounded by myelin sheath (my). Bars=500 nm.

Fig. 5. Immunoreactive (arrowheads) axon terminals (Ax) made asymmetric synaptic contact with unlabelled dendritic profiles (Dd). Boutons contained consistent sizes of clear and round synaptic vesicles. The arrows indicated the direction of synaptic transmission. Bars=500 nm.

Fig. 6. Glutamatergic axon terminals (Ax) made synaptic contact with immunoreactive (arrowheads) dendritic processes (Dd). Both immunoreactive and non-immunoreactive axon terminals often made simultaneous synaptic contact with immunoreactive dendritic processes (b). The arrows represented the polarity of synaptic activity. Bars=500 nm.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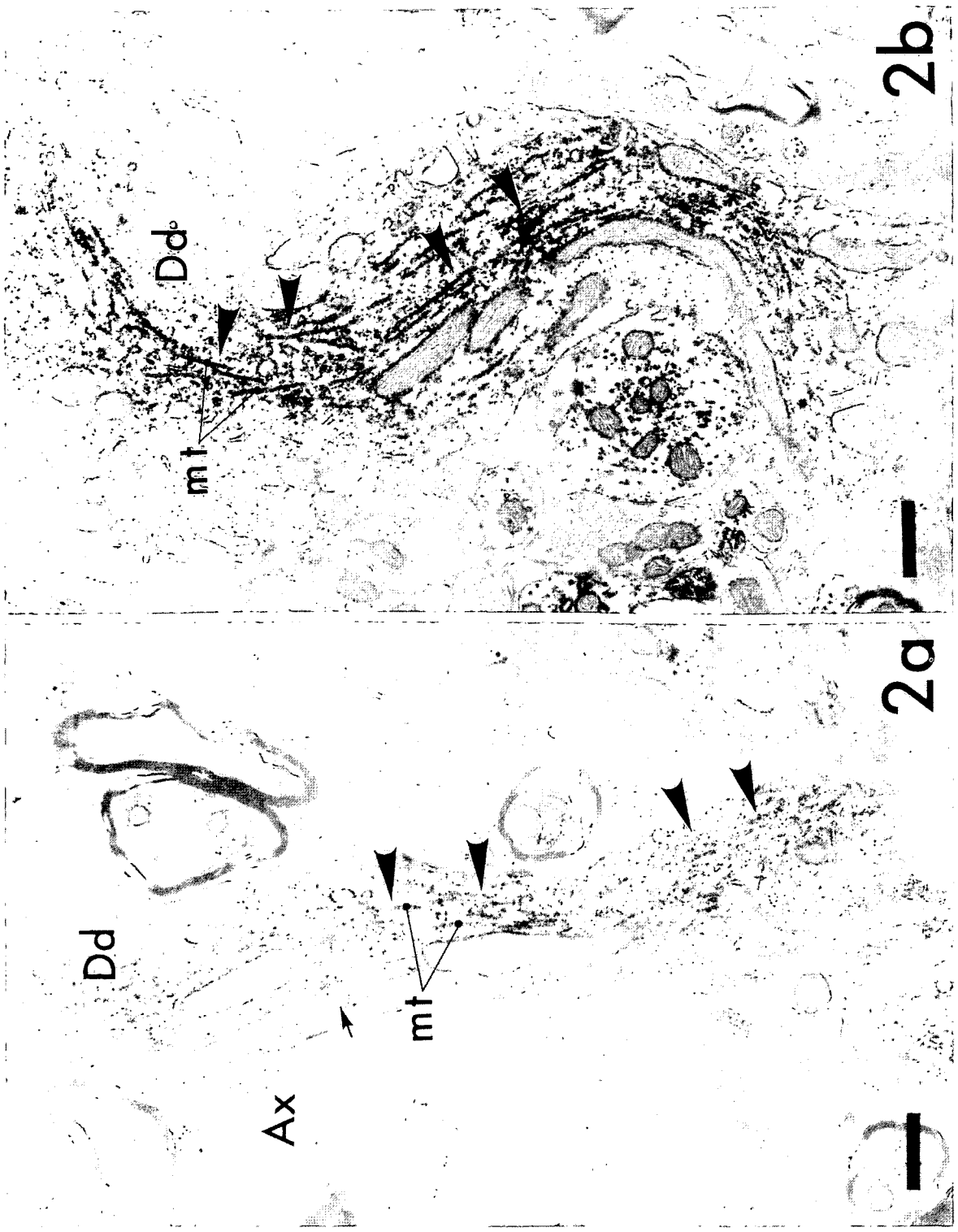
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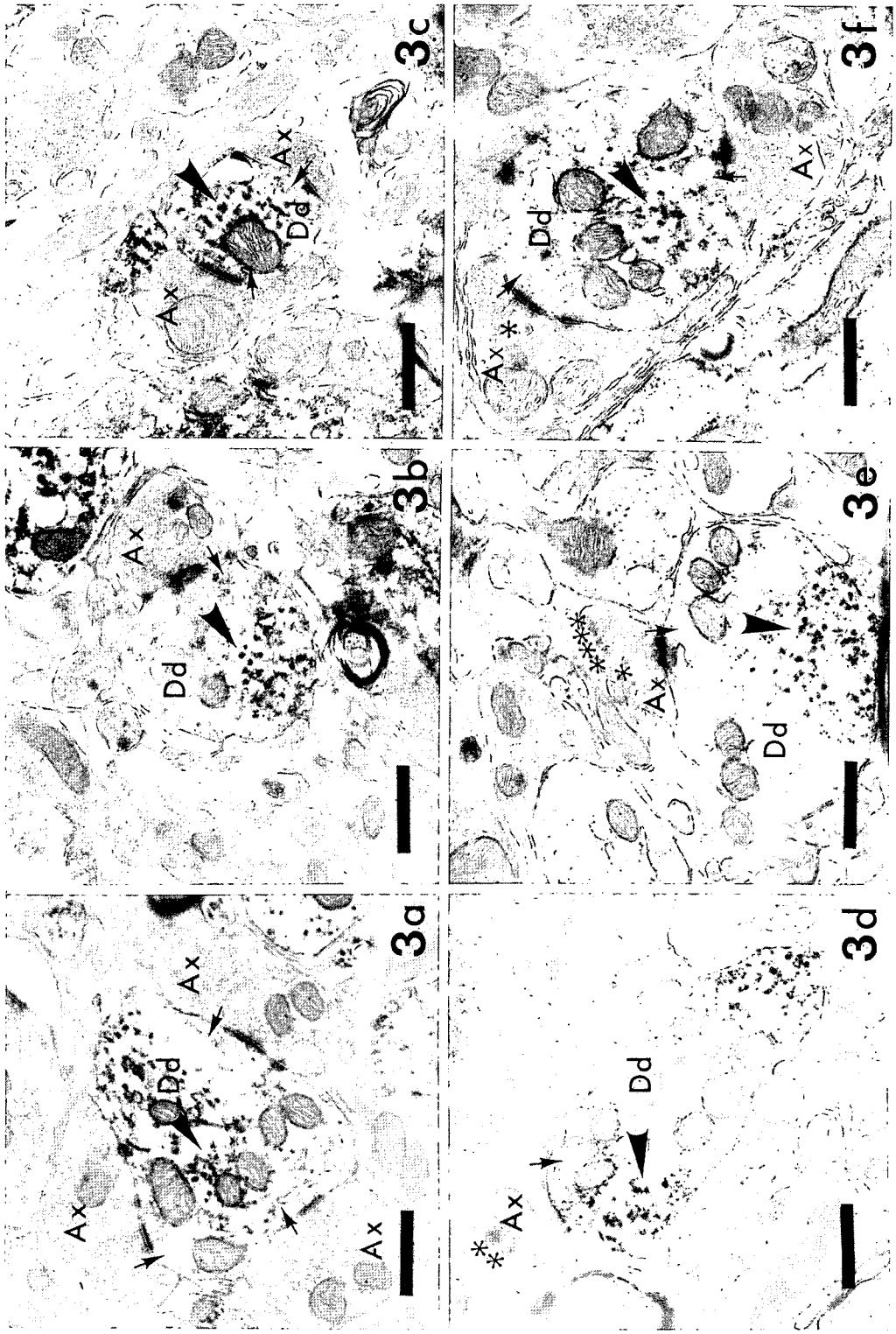
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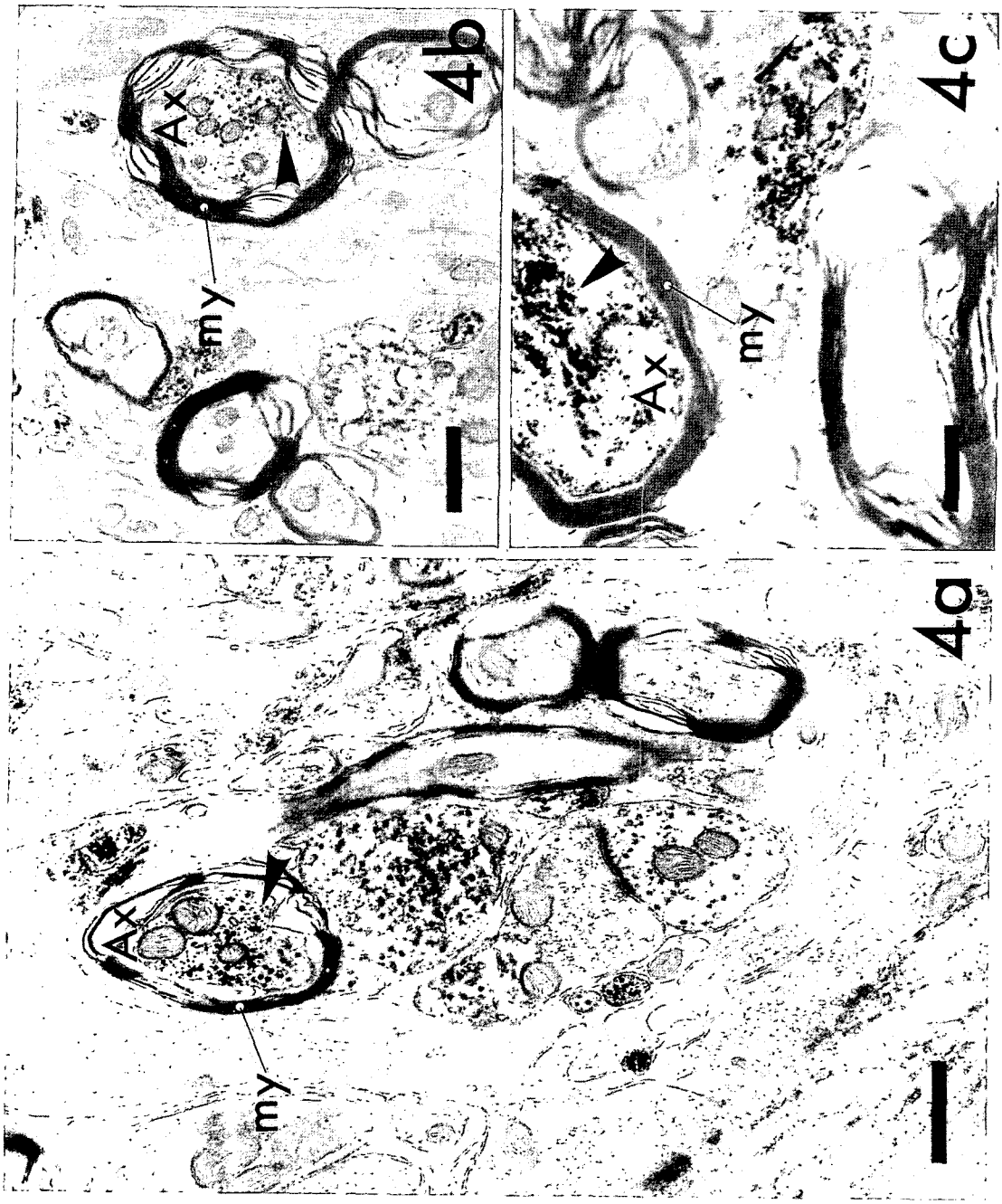
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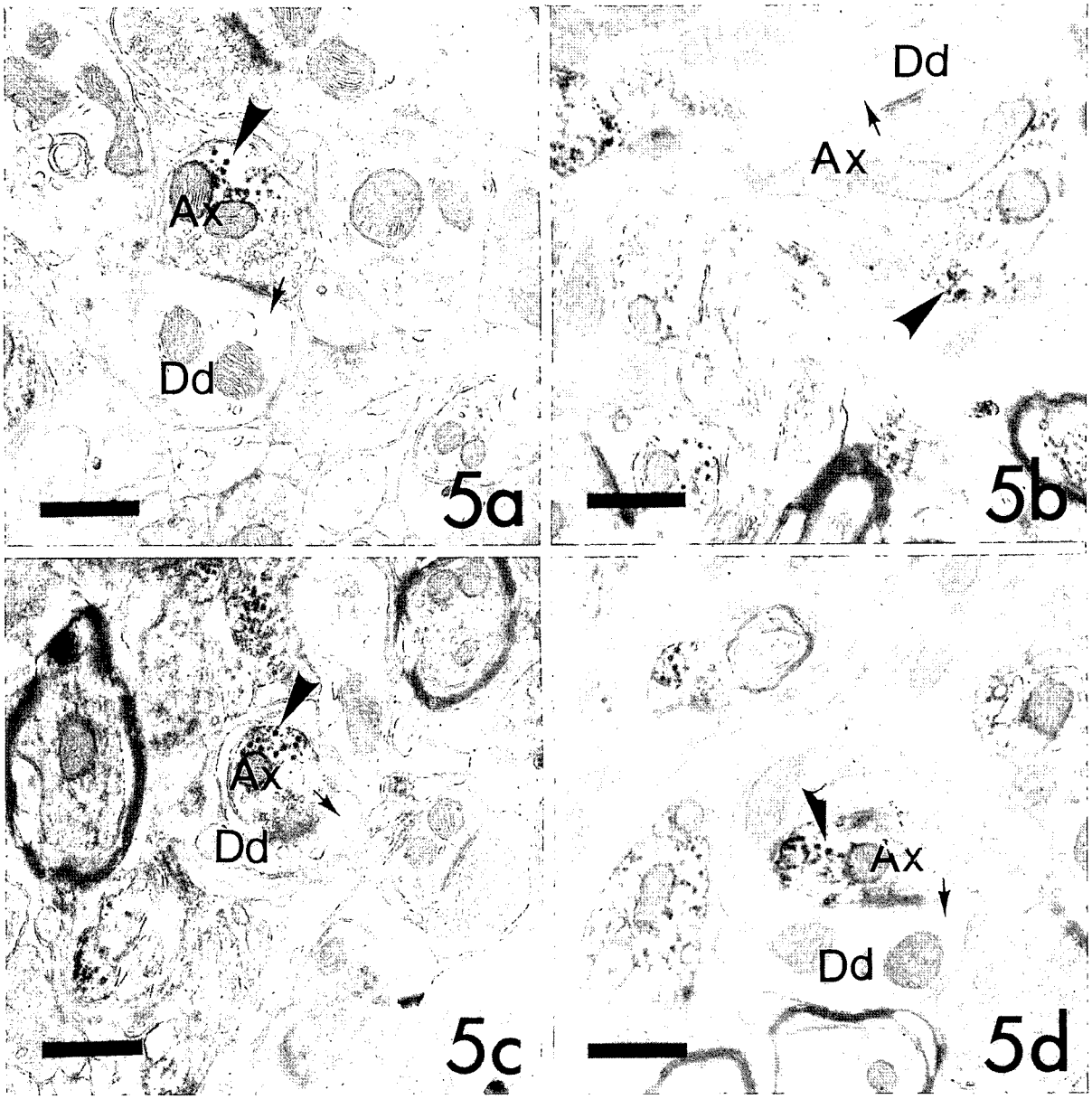
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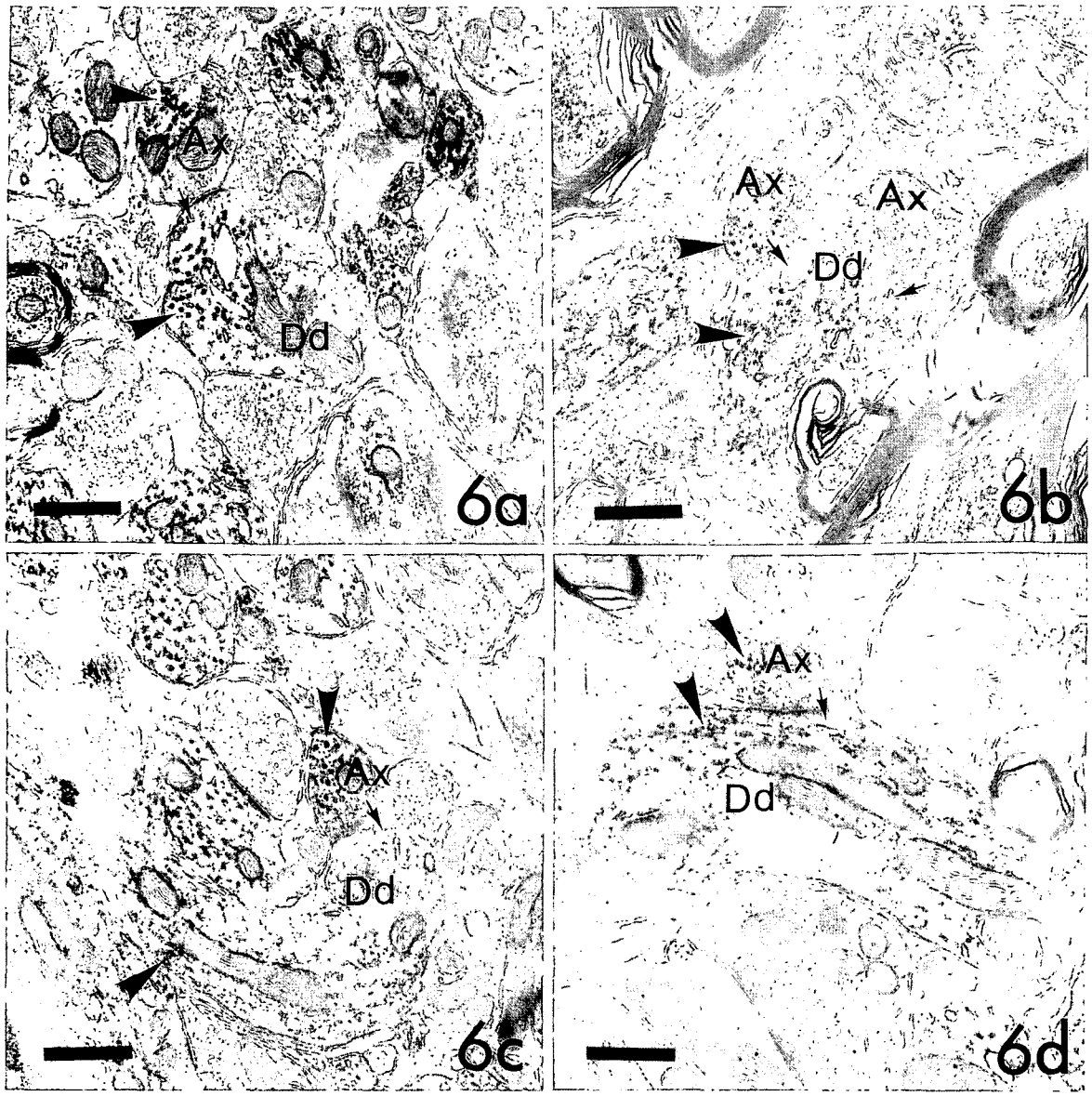
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