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Akiteru Takagi · Kenichiro Oda · Tateki Kikuchi
Hiroki Kajihara

Fine structural changes of muscle spindles in the gracile axonal dystrophy mutant mouse

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Abstract Fine structural changes of muscle spindles in the extensor digitorum longus of the gracile axonal dystrophy mutant mouse were studied from 20 to 120 postnatal days. Degenerative nerve endings in muscle spindles were first recognized at 20 postnatal days. The sensory nerve endings were usually swollen with decrease of cell organelles, and the cytoplasm was electron-lucent. At 50 postnatal days, atrophic nerve endings were frequently observed in the narrow spaces between the indented cell membrane of intrafusal muscle cells and the basement membrane. In addition to degenerative and atrophic changes, regenerative axons showing fine sprouts (with or without Schwann cell projections) appeared in the sensory nerve endings at this time. At 80 postnatal days, sensory nerve endings frequently showed dystrophic changes characterized by axonal dilatation with accumulations of neurofilaments, tubulovesicular structures, mitochondria and myelin-like figures. These findings suggest that axonal transport in the sensory nerve endings is impaired in this mutant mouse. Motor nerve endings were usually well preserved and normal structures even at 80 postnatal days. Intrafusal fibrosis, decrease in number of sensory nerve endings and atrophy of intrafusal muscle fibres were clearly recognized by 100 days of age.

A. Takagi · H. Kajihara (✉)
Department of Pathology, Institute of Health Sciences,
Hiroshima University School of Medicine,
Kasumi 1-2-3, Minami-ku, Hiroshima, 734 Japan
Tel.: (81) 82-257-5405, Fax: (81) 82-257-5409

A. Takagi
Department of Physical Therapy, Institute of Health Sciences,
Hiroshima University School of Medicine, Hiroshima, 734 Japan

K. Oda
Department of Neurology, Kyoto Red Cross Hospital,
Kyoto, 606 Japan

T. Kikuchi
Department of Animal Models for Human Disease,
National Institute of Neuroscience, NCNP, Kodaira, Tokyo, 187 Japan

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Introduction

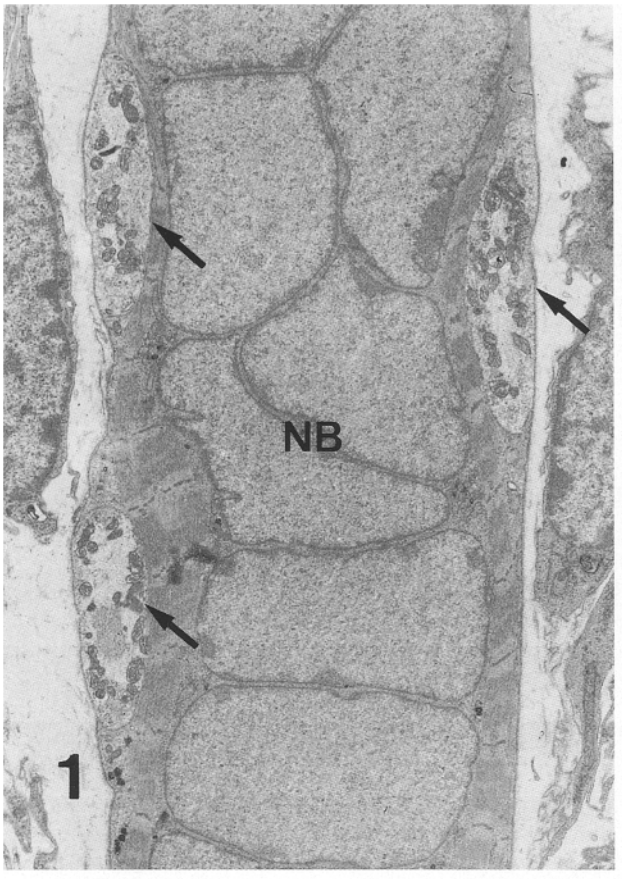
The gracile axonal dystrophy (GAD) mutant mouse was found in a randomly bred strain of mice [28]. This disorder is transmitted by an autosomal recessive gene, *gad*, and characterized by progressive sensory ataxia first detectable at 30 days after birth and by paresis of the hindlimbs that is evident 80 days after birth [27]. In neuropathological examinations, neuroaxonal degeneration and spheroid formation (axonal dystrophy) were observed in the gracile tract, dorsal spinocerebellar tract and spinal trigeminal nucleus, and the pattern of these changes was quite similar to that in human spinocerebellar ataxia [13, 30]. Electron microscopic findings showed an accumulation of neurofilaments, tubulovesicular structures and mitochondria in the dystrophic axons of the central nervous system [8, 13].

Peripheral nerves including muscle spindles were studied morphologically by a method combining acetylcholinesterase and silver impregnation in this mutant mouse and dying back type axonal degeneration of sensory nerve endings in the muscle spindles was demonstrated [8, 14]. In the present study, muscle spindles in the extensor digitorum longus (EDL) of this mutant mouse were studied by electron microscopy.

Materials and methods

Homozygous *gad/gad* and normal littermates (+/+ or *gad/*+) aged from 20 to 120 days after birth were used. The animals were bred under specific pathogen-free conditions in the Laboratory Animal Research Center of the National Institute of Neuroscience, fed on a commercial diet with water ad libitum and kept under controlled conditions.

The animals were killed by exsanguination under deep ether anaesthesia. Both EDL muscles were quickly removed and the right sided muscle was cut into small pieces and fixed overnight in cold 2.5% phosphate buffered glutaraldehyde solution at pH 7.4.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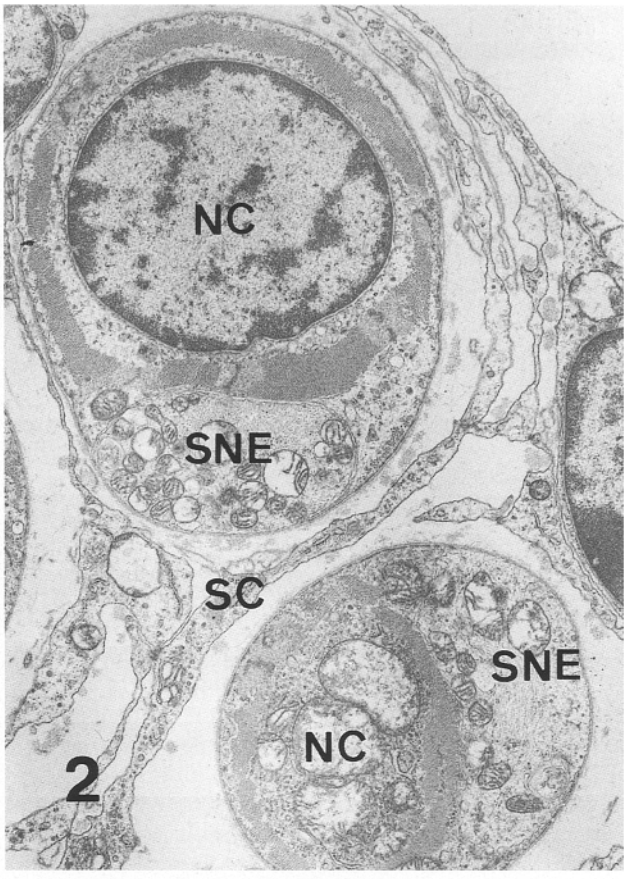
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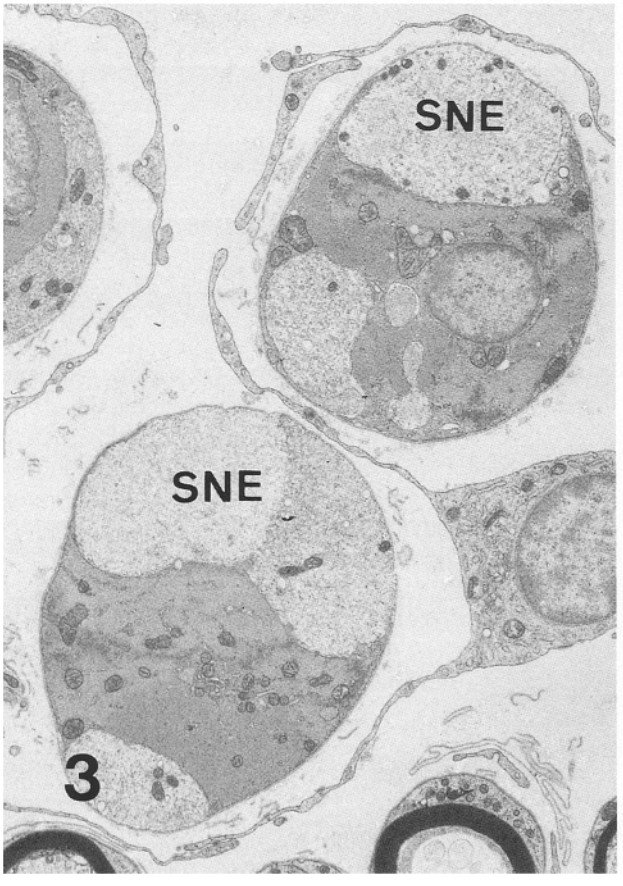
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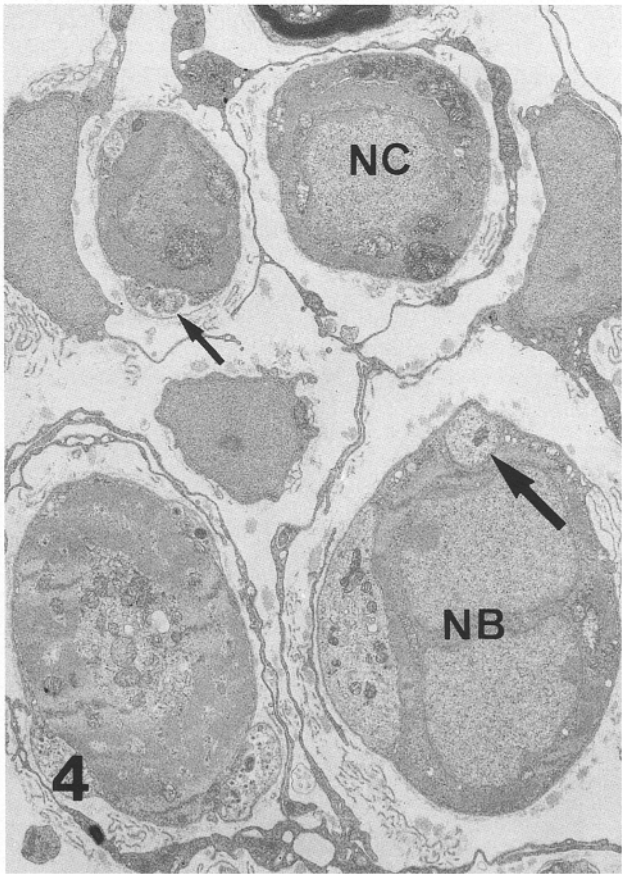
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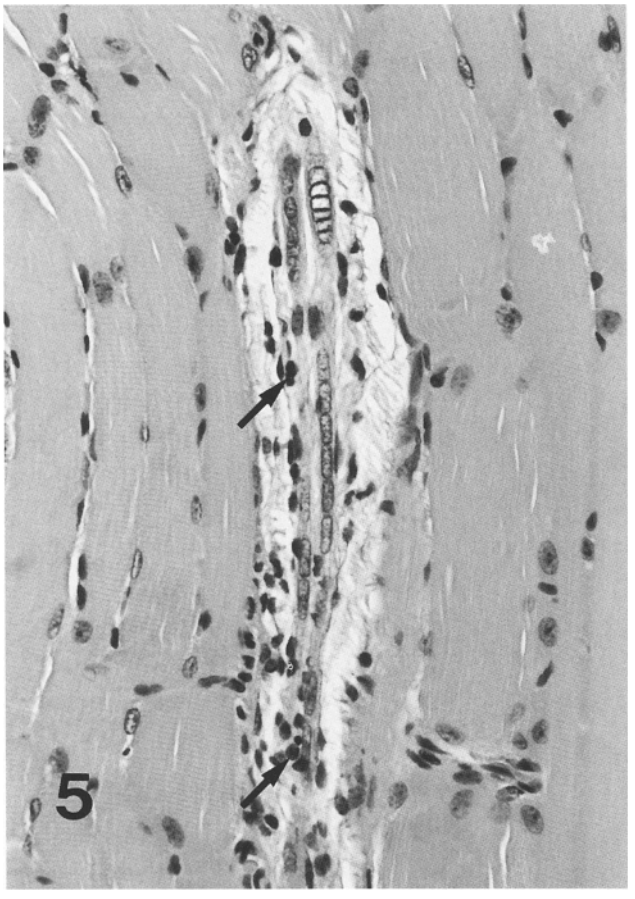


They were then post-fixed for 1 h in 1% phosphate buffered osmium tetroxide solution, dehydrated through graded alcohols, and embedded in epoxy resin. Semithin sections were cut with a Porter-Blum ultramicrotome, equipped with glass knives, and stained with methylene blue (Richardson's solution). Thin sections were cut with the same ultramicrotome, equipped with a diamond knife, stained with uranyl acetate and lead citrate and examined with a Hitachi H-7000 transmission electron microscope at 75 kV.

For light microscopic observations, the left EDL was fixed with 10% phosphate buffered formaline solution and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin, periodic acid-Schiff and Klüver-Barrela for myelin.

Fig. 1 Fine structure of nuclear-bag fibre (*NB*) in control animal at 30 days after birth. In longitudinal section, the muscle fibre contains closely packed large nuclei in the central region. Sensory nerve endings (*arrows*) appear as periodic series of transversely cut sections deeply indenting borders of the muscle fibre. ×5300

Fig. 2 Cross section of nuclear-chain fibres (*NC*) in the same animal of **Fig. 1**. Sensory nerve endings (*SNE*) are semilunar in shape, and contain a considerable number of neurofilaments, and a few mitochondria, neurotubules and small vesicles. *SC* Septal cell, ×8600

Fig. 3 Cross section of intrafusal muscle fibres of the gracile axonal dystrophy (GAD) mouse at 20 days after birth. *SNE* are markedly swollen and contain a decreased number of cell organelles. ×6700

Fig. 4 Cross section of intrafusal muscle fibres of the GAD mouse at 50 days after birth. Some SNE (*small arrow*) show degenerative and atrophic changes. In addition, regenerative changes of SNE (*large arrow*) are frequently recognizable at this stage. ×5200

Fig. 5 Light microscopic changes of muscle spindle of the GAD mouse at 80 days after birth. Both NB and NC are slightly atrophied with increase in number of mesenchymal cells (*arrows*). H & E, ×400

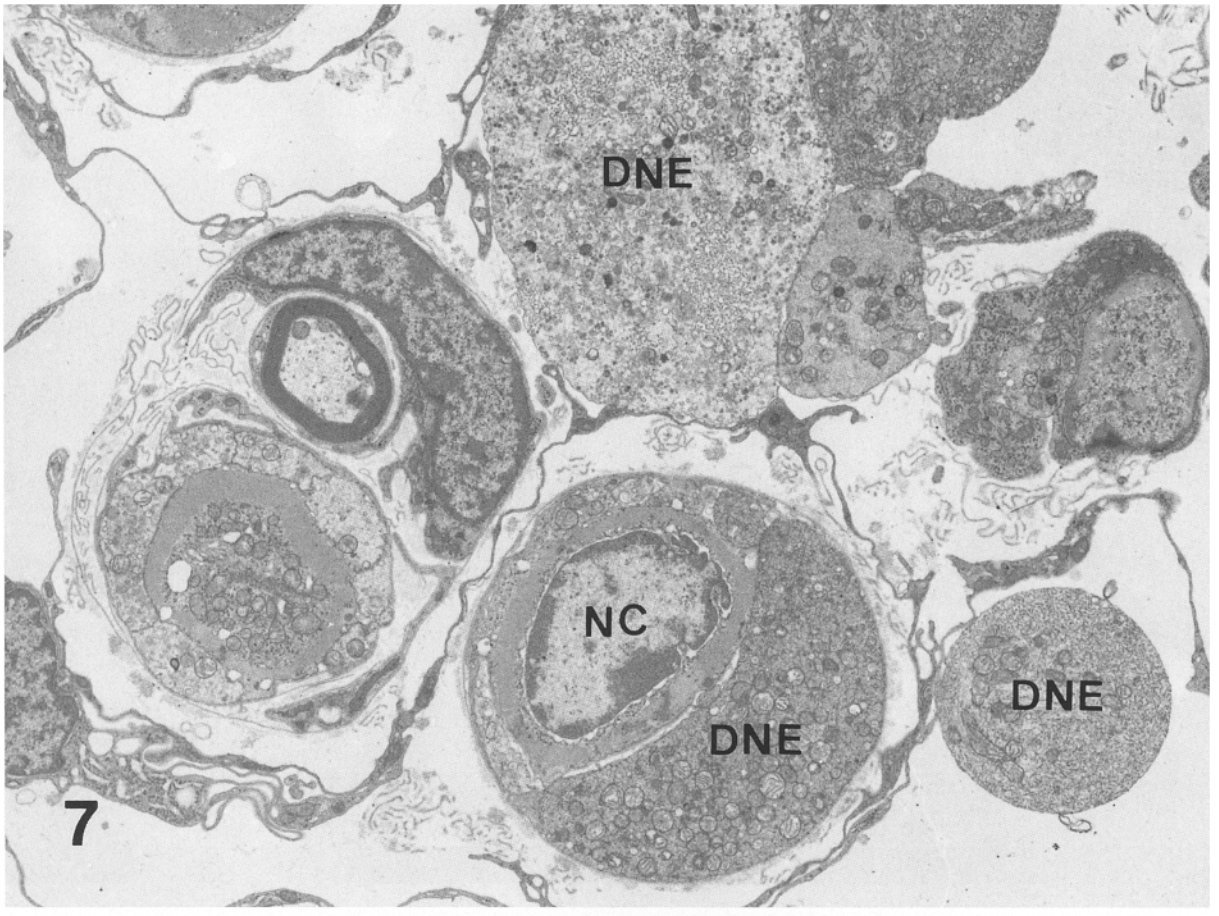
Fig. 6 Light microscopic changes of muscle spindle of the GAD mouse at 120 days after birth. Intrafusal muscle fibres (*arrows*) are markedly atrophied with slight fibrosis. Mesenchymal cells are also increased in number. Epon section with methylen blue stain. ×400

Results

Muscle spindles in EDL muscles of control animals

As described elsewhere [1, 3, 10, 11, 15, 16], four intrafusal muscle fibres, two nuclear-bag and two nuclear-chain fibres were usually found in the muscle spindles of the rodents.

The nuclear-bag fibre was larger than the nuclear-chain fibre and contained closely packed large nuclei in the central region of the muscle fibre (Fig. 1). The nuclear-chain fibre, in contrast, was smaller and contained a single row of slightly elongated nuclei located in the central region of the muscle fibre. The sensory nerve endings in mature muscle spindles formed a typical annulo-spiral feature around intrafusal muscle fibres. In longitudinal section, the intrafusal muscle fibres appeared as a periodic series of transversely cut sections of sensory nerve endings deeply indenting the borders of the intra-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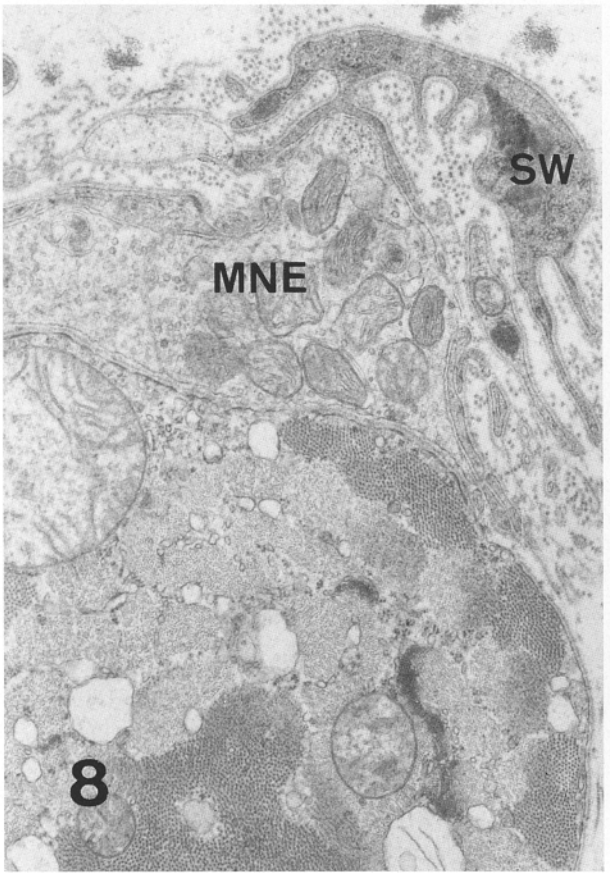
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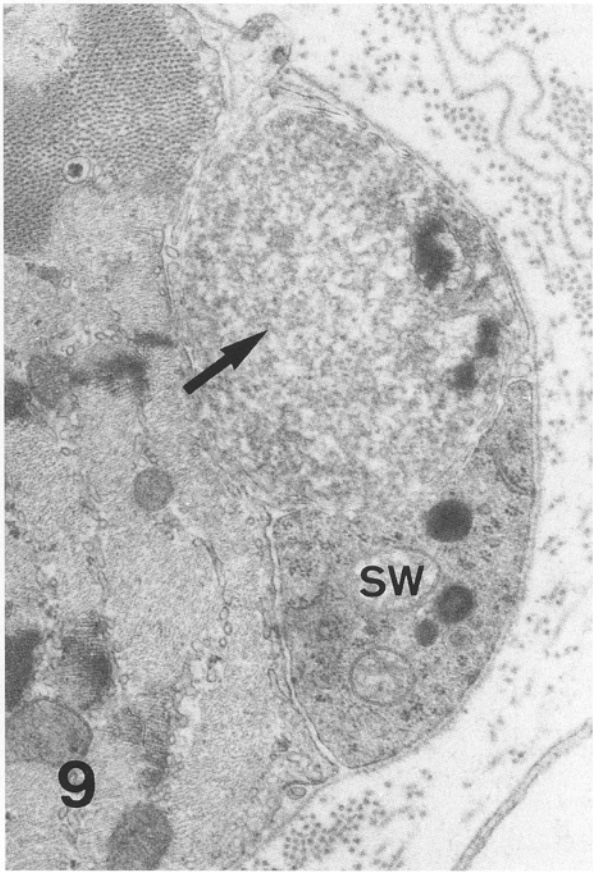
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fusal muscle fibres (Fig. 1). In cross section, they usually showed a semilunar or horseshoe shape around the intrafusal muscle fibres (Fig. 2). The nerve endings were devoid of Schwann cells. The outer surfaces of the nerve endings were covered with basement membrane continuous with that of the extrafusal muscle fibres. The nerve endings contained a variable number of mitochondria, a few small vesicles 200–700 Å in diameter, a small number of neurotubules and neurofilaments, and a few elements of the smooth surfaced endoplasmic reticulum in the relatively electron-lucent cytoplasm.

Intrafusal muscle fibres were usually separated from one another by thin cytoplasmic processes of inner capsular cells (Fig. 2). Many myelinated and non-myelinated nerve fibres were recognizable in the intracapsular spaces. The capsules of the muscle spindles consisted of two to more than ten layers of the thin cytoplasm of the capsular cells, collagen fibres and elastic fibres.

Muscle spindles in EDL muscles of the GAD mouse

At 20–50 days after birth muscle spindles of EDL showed no structural abnormalities by light microscopy. In electron microscopic observations, however, the sensory nerve endings distributed around the surface of the intrafusal muscle fibres, both nuclear-bag and nuclear-chain fibres showed degenerative changes by 20 days after birth (Fig. 3). These degenerated nerve endings were variable in size, some swollen and some atrophied, and they usually had electron-lucent cytoplasm, while an accumulation of neurofilaments was found in some of the degenerated nerve endings. The fine structure of the intrafusal muscle fibres was usually well preserved. Satellite cells, septal cells and capsular cells showed no structural abnormalities.

By 40–50 days after birth, degenerative changes in the sensory nerve endings had become more conspicuous. Amorphous and heterogeneous material such as cell debris were frequently observed in the spaces between the basement membrane and indented cytoplasmic membrane of intrafusal muscle fibres (Fig. 4). In addition, many fragments of basement membrane appeared around the satellite cells, Schwann cells and septal cells. Collagen fibres were also increased in numbers in the intrafusal spaces (Fig. 4).

Fig. 7 Fine structure of muscle spindle in the GAD mouse at 80 days after birth. Many dystrophic nerve endings (*DNE*) frequently appear in this stage. Numerous mitochondria, neurofilaments, neurotubules and small vesicles accumulate in the dystrophic nerve endings. ×6100

Fig. 8 The motor nerve ending (*MNE*) is usually well preserved even after 80 postnatal days and contains numerous synaptic vesicles and a few mitochondria. Basement membrane can be seen between cytoplasmic membranes of an MNE and of a muscle fibre. *SW* Schwann cell, × 10000

Fig. 9 Degenerative MNE (*arrow*) containing electron-dense materials is occasionally seen after 80 postnatal days. ×13000

Degenerative nerve endings were frequently detached from the surface of the muscle fibres. In addition, regenerative changes of the sensory nerve endings were also recognizable at this stage. Small sprouts or regenerative branches of the sensory nerve endings were found in the narrow spaces between the cytoplasmic membrane of intrafusal muscle fibre and basement membrane (Fig. 4). These small sprouts contained a considerable number of neurofilaments, neurotubules and mitochondria, and appeared the same as normal sensory nerve endings. Motor nerve endings were usually well preserved at this stage.

At 80–120 days after birth the intrafusal muscle fibres in the muscle spindles of the EDL were more or less atrophied (Fig. 5). An increasing number of mesenchymal cells and mild fibrosis were usually accompanied by morphological changes in the intrafusal muscle fibres. At 120 days after birth, mesenchymal cell proliferation and fibrosis became more distinct, and intrafusal muscle fibres were decreased in number and size (Fig. 6).

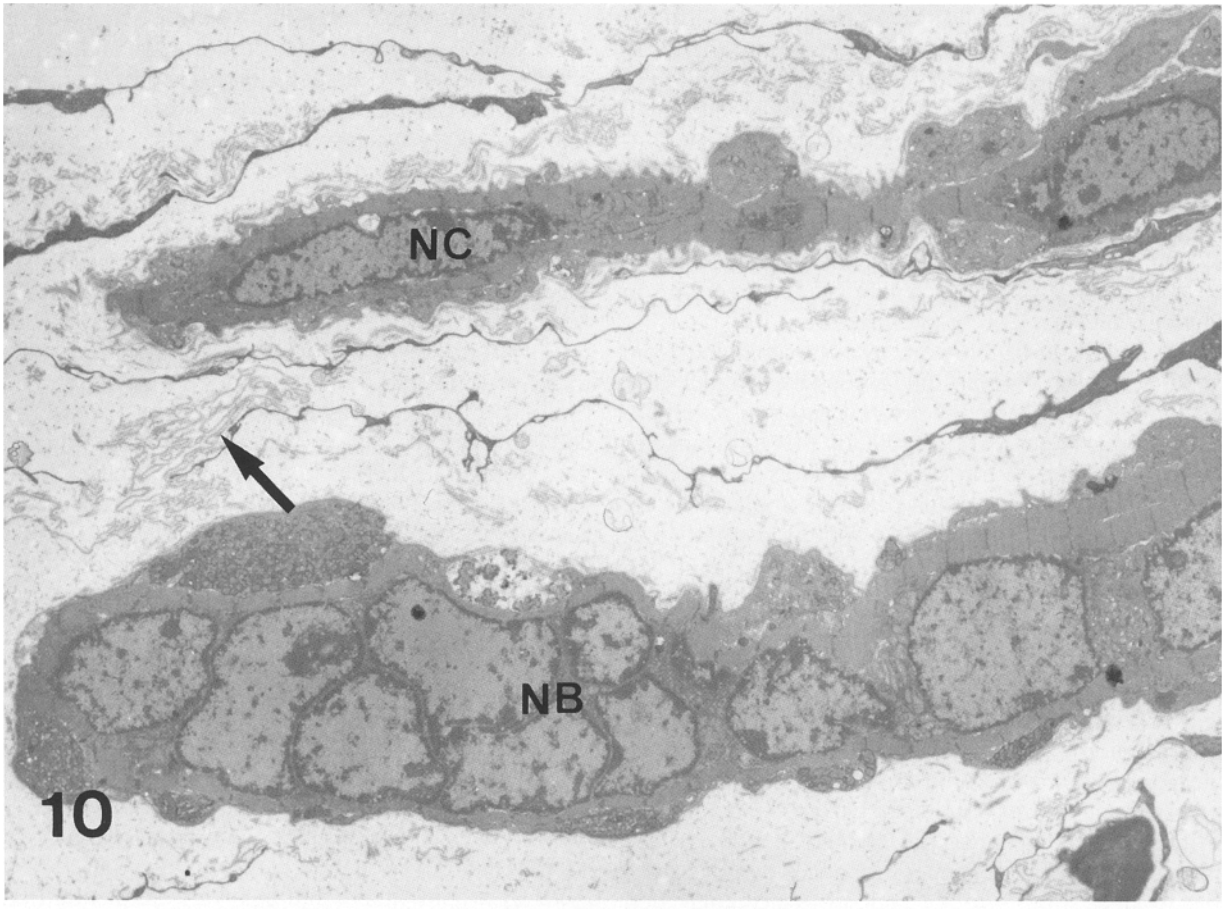
By electron microscopy, the sensory nerve endings usually showed hypertrophic or dystrophic changes at 80 days after birth. A large number of mitochondria, neurofilaments, microtubules and vesicular structures accumulated in the axoplasm of these dystrophic nerve terminals (Fig. 7). The mitochondria were variable in size and shape, and many of them showed degenerative changes containing myelin-like figures. Accumulated neurofilaments were usually tangled. The microtubules were irregularly crooked and tended to aggregate. Some of these dystrophic nerve endings were frequently detached from the surface of the intrafusal muscle fibres (Fig. 7). The regenerative changes showing small axonal sprouts were also found in some sensory nerve endings.

Motor nerve endings were usually well preserved in this period (Fig. 8) but some nerve endings were severely damaged with loss of the cytoplasmic membrane and amorphous and electron-dense areas in the cytoplasm (Fig. 9). Intrafusal muscle fibres were atrophied and frequently contained hydropic vacuoles. Satellite cells were increased in number.

At 100–120 postnatal days, the sensory nerve endings were markedly decreased in number. The surviving nerve endings were dystrophic and variable in size and shape (Fig. 10). The cytoplasm of these dystrophic nerve endings was filled with numerous cell organelles, such as mitochondria, neurofilaments, microtubules and vesicular structures. Most of these organelles were degenerate and aggregated (Fig. 11). Neurofilaments were densely accumulated and tended to be located in peripheral regions, aggregated neurotubules were irregularly arranged, mitochondria were irregular in size and shape and many contained myelin-like figures. In addition, numerous dense granules appeared in the cytoplasm of these dystrophic nerve endings (Fig. 11).

Some motor nerve endings were free of intrafusal muscle fibres and contained numerous vesicular and vacuolar structures without dense cores.

Intrafusal muscle fibres were usually atrophic and contained a few lipofuscin granules at the nuclear poles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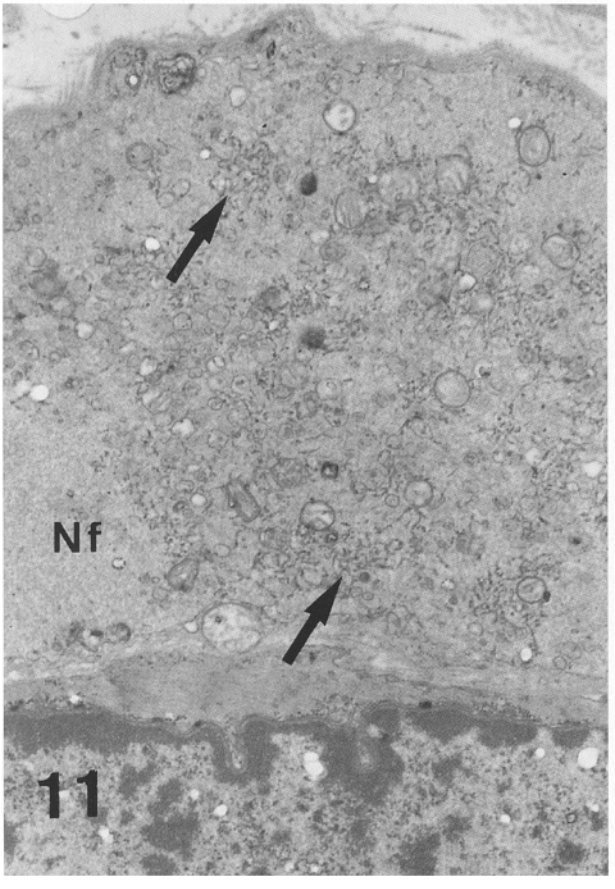
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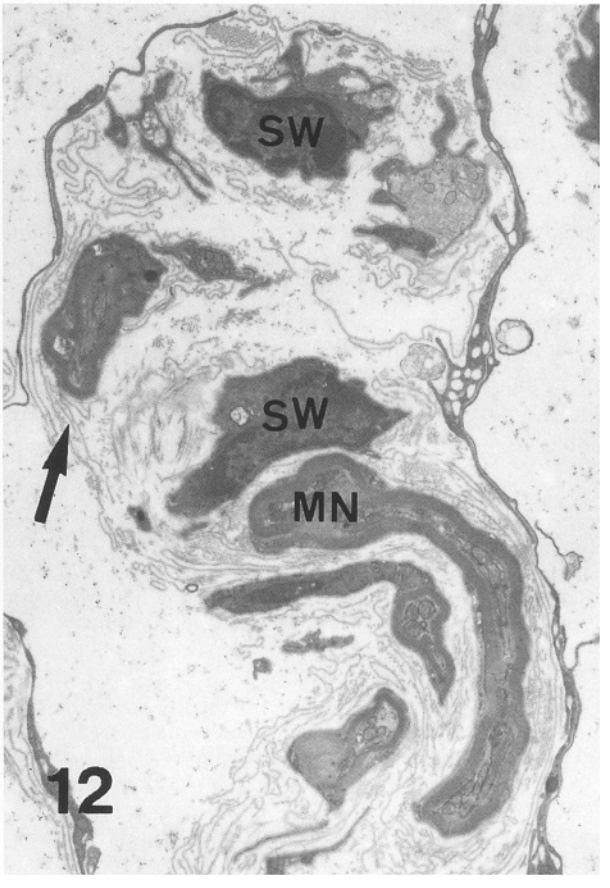
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(Fig. 10). Satellite cells and other intrafusal mesenchymal cells were increased in number with synthesis of collagen fibres. In addition to increase in fibrous elements, numerous lamellar and fragmented basement membranes accumulated around the intrafusal muscle fibres, septal cells, nerve fibres, Schwann cells and other mesenchymal cells (Fig. 12).

Discussion

On electron microscopy, degenerative sensory nerve endings were noted in muscle spindles of the GAD mouse on the 20th day after birth. These nerve endings were swollen and contained electron-lucent cytoplasm. In addition regenerative nerve endings were frequently observed in muscle spindles at 50 postnatal days and identified as finger-like axonal sprouts in close contact with muscle fibres. Schröder [21] has previously reported similar regenerative nerve terminals in muscle spindles of the rat at 1 and 2 months after sciatic nerve crush. They retained normal structures even 14 and 24 months after the nerve crush. In the present study, dystrophic changes of the sensory nerve endings were frequently observed after the 80th postnatal day. The dystrophic nerve endings usually contained numerous mitochondria, neurofilaments, microtubules and small vesicular structures.

Concerning the accumulation of axoplasmic mitochondria, Webster [26] has previously reported that numerous mitochondria moved close to Ranvier's nodes of myelinated fibres and to non-myelinated nerves during the early stage of Wallerian degeneration in crushed sciatic nerve. Similar finding were also observed in the axoplasm of constricted sciatic nerve segments excised and oxygenated for up to 8 h [20]. An increase in succinic dehydrogenase activity and an accumulation of axonal mitochondria were noted in both proximal and distal regions of the constriction. In addition to the mitochondrial accumulation, other axoplasmic organelles, such as tubulovesicular structures and filamentous structures, were also increased in the axoplasm adjacent to the ligation. From these results, the focal accumulation of axoplasmic mitochondria has generally been considered to be due to the energy demand for regenerative or reparative processes at the site of injury.

Some investigators who studied the chemically induced "dying back" type neuropathies found that the accumulation of tubulovesicular structures appeared in the axoplasm of peripheral nerve fibres with saccular or fusiform dilatation [2, 7, 17, 19]. Marked accumulations of neurofilaments in the axoplasm of peripheral nerve fibres were also found in acrylamide induced neuropathy [18, 24]. A striking accumulation of both tubulovesicular structures and neurofilaments were observed together with proliferations of mitochondria in the dilated axoplasm of the peripheral nerves of animals with vitamin E deficiency [23, 25]. Southam and his co-workers [23] indicated that both fast anterograde and retrograde axonal transport were impaired in the animals with vitamin E deficiency, and that the disturbance of axonal transport might be a secondary phenomenon following damage to the function of mitochondria and other intra-axonal membranous structures with lack of the antioxidant effect of vitamin E. A defect of cytoplasmic turnaround in the distal axons and plugging of the distal axons with accumulated axoplasmic organelles might induce the dying back type neuropathies [23].

After 100–120 postnatal days, in our present study, sensory nerve endings were markedly decreased in number and the nerve endings remaining were also dystrophic. Axoplasmic organelles that accumulated in the nerve endings were severely damaged in this stage. These results support the idea of Southam and his coworkers [23] that axonal transport is impaired in the dying back type neuropathies.

Since the GAD mouse has no dietary related deficiency (for example, with vitamin E [29]), dysfunction of the cytoplasmic and intra-axonal membrane system in the sensory nerve endings can reasonably be assumed to originate from metabolic abnormalities associated with a deficient enzyme. The accumulation of axoplasmic organelles might be due to disturbance of axoplasmic streaming. However, it is not known whether the degeneration of axoplasmic organelles reflects primary or secondary changes.

Recently, Ernfors and his co-workers [4] reported neurotrophin-3-deficient mice generated by gene targeting. The homozygotes of these mutant mice showed complete absence of muscle spindles and Golgi tendon organs as well as of spinal proprioceptive afferents with severe movement disturbance of the limbs. The neurotrophin family, including neurotrophin-3, nerve growth factor, brain-derived neurotrophic factor and neurotrophin-4/5, plays an important role in the development of sensory neurons [5, 6, 9]. Our findings might be considered to be due to absence of gene-related neutrophic substances although no data are avaliable to confirm this.

The fine structural changes of motor nerve terminals on extrafusal muscle fibres have been shown to appear by the 60th postnatal day [12]. In this study these degenerative changes were first recognized on the 80th postnatal day, suggesting that the intrafusal nerve endings

Fig. 10 Fine structure of muscle spindles in the GAD mouse after 120 postnatal days. Intrafusal muscle fibres (*NB* and *NC*) are atrophied. Dystrophic nerve endings are decreased in number and irregular in size and shape. Fragments of basement membrane (*arrow*) are scattered in the interstitial space. ×3600

Fig. 11 High magnification of DNE after 100 postnatal days. Accumulated cell organelles are usually degenerative. Neurofilaments (*Nf*) are densely packed and usually located in peripheral areas. Microtubules (*arrows*) are aggregated and run in irregular directions. Mitochondria are variable in size and shape. ×16000

Fig. 12 Degenerated *SW* with a distinct basement membrane are seen in the interstitial space. Numerous fragments of basement membrane (*arrow*) and bundles of collagen fibres appear around the myelinated nerve fibres (*MN*), *SW* and other cell elements. ×6000

might be more resistant than those of extrafusal muscle fibres.

Atrophy of intrafusal muscle fibres was distinct after 100–120 postnatal days. In a morphometric study of intrafusal muscle fibres of rats after denervation and reinnervation, Schröder and his co-worker [22] previously reported that atrophy of intrafusal muscle fibres was present in both denervated and reinnervated rats with an increase in number of intrafusal muscle fibres. In our GAD mutant mice, however, an increase in number of intrafusal muscle fibres was not found at 100–120 postnatal days.

Muscle spindles of lower lumbrical muscles are known to form lamellar accumulation of basement membranes after crushing of the sciatic nerve, and degeneration and regeneration of intrafusal muscle fibres take place repeatedly in these diseased muscle spindles [21]. The GAD mouse, showing dying back type degeneration and dystrophic changes in the primary and secondary sensory neurons resembles some human hereditary neuropathies in their pathomorphological and clinical features. This mutant mouse might thus be useful for studying the pathogenesis of human hereditary neuropathies.

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