PATHOPHYSIOLOGICAL STUDY OF AXONAL DEGENERATION IN THE GRACILE AXONAL DYSTROPHY (GAD) MUTANT MOUSE

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ABSTRACT

The gracile axonal dystrophy (gad) mouse, which shows hereditary sensory ataxia and motor paresis, has been morphologically characterized by the dying back type of axonal degeneration in the nerve terminals of dorsal root ganglion cells and motor neurons. In the present study, developmental changes in the functional properties of peripheral nerves were investigated in the gad mouse using electrophysiological and immunohistochemical methods. In the gastrocnemius muscle of the gad mouse, the incidence of fibrillation increased with age. Although the conduction velocity of the tibial nerve stayed within the normal range, the amplitude of the M wave decreased significantly with age in this mutant. Ligation study of the sciatic nerve showed that both antero- and retrograde axonal transport was functional in the gad mouse even when the disease had fully progressed. The morphometry of the sciatic nerve showed no differences between gad and control mice. Nicotinic acetylcholine receptors started to disperse over the surface of skeletal muscles in the gad mouse after 11 weeks of age when motor paresis was clinically manifested. Our results show that the function of peripheral nerve of the gad mouse decreases with age through an axonal degeneration process. The possibility raised by previous morphological studies that the dysfunction of axonal transport causes the pathogenesis of the gad mouse is unlikely.

The gracile axonal dystrophy (gad) mouse is a neurological mutant mouse that exhibits sensory ataxia at the age of 4 weeks, followed by tremor and dragging of the hindlimbs at 12 weeks of age (15, 16). The mutation is transmitted by an autosomal recessive gene on chromosome 5. Previous morphological studies of this mutant mouse showed focal axonal swellings and subsequent loss of central terminals of the nerve processes of dorsal root ganglia cells (DRG) in the regions of the gracile nucleus of the medulla oblongata and the gracile fasciculus of the spinal cord (7, 10). Recent reports showed that the gad mouse exhibits dying back

type axonal degeneration also in the peripheral nerve terminals of DRG followed by the same type of the degeneration of motor neurons (9, 11). However, despite these extensive morphological studies, little is known about the functional properties of the neurons which degenerate in the gad mouse. For instance, dysfunction of axonal transport has previously been suggested to play a crucial role in the pathogenesis of the gad mouse. In the present study, to address these questions and on the way to identify the *gad* gene, we further analyzed the axonal degeneration of the gad mouse at various ages using pathophysiological methods. Electromyography was carried out to study developmental changes in physiological properties of the gastrocnemius muscle and the tibial nerve. Both

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antero- and retrograde axonal transport in the sciatic nerve was studied by a ligation experiment to evaluate the possibility of dysfunction raised by previous studies. In addition, the effects of degeneration of the sciatic nerve on the gastrocnemius muscle were analyzed by labeling nicotinic acetylcholine receptors (nAChRs).

MATERIALS AND METHODS

Animals

Homozygous *gad/gad* mice and normal littermates (+/+ or *gad/+*) of the *gad* strain were used throughout the present study. The animals were bred under specific pathogen-free conditions in the Laboratory Animal Research Center, National Institute of Neuroscience, fed on a commercial diet with water *ad libitum* and kept under controlled conditions: Temperature $23 \pm 3^\circ\text{C}$, humidity $65 \pm 5\%$, with a 12 h light/dark cycle.

Electrophysiology

Mice were divided into 3 groups according to age: group I, aged 6 weeks ± 2 days; group II, 9 weeks ± 2 days; group III, 12 weeks ± 2 days. Each group was composed of 5 *gad* mice and 5 normal littermates, and was subjected to electromyography and measurement of the conduction velocity in the tibial nerve. Animals were sedated by intraperitoneal administration of 0.13 ml/10 g body weight of ethanol (20% v/v), and the limbs and tail were fixed lightly with thin surgical tape. A concentric needle electrode mounted on a micromanipulator was introduced into the right gastrocnemius muscle, and spontaneous electrical activity such as fibrillation was measured using a 4-channel electromyography (EMG; Dantec). The conduction velocity in the right tibial nerve was measured by stimulation at the ankle and mid-thigh, and by recording the evoked potential (M wave) from the plantar muscle.

Morphometry of the Sciatic and Tibial Nerve

Homozygous *gad* mice aged 16 weeks and age-matched normal littermates were used for the morphometric study of the sciatic and tibial nerves. Procedures for the preparation of peripheral nerve tissues were according to a previously described method (7). Transverse sections were stained with toluidine blue, and total fiber diameters (axon plus myelin sheath) of at least 1,000 fibers were meas-

ured on enlarged photomicrographs.

Ligation of the Sciatic Nerve

The right sciatic nerve of homozygous *gad* mice aged 10, 12 and 16 weeks and of age-matched normal littermates were ligated very tightly under anesthesia as described previously (4). After 24 h, small pieces of nerves both proximal and distal to the ligated portion were removed and stained longitudinally with anti-kinesin and anti-dynein antibodies, respectively.

Preparation of Anti-Cytoplasmic Dynein Antibodies

Anti-dynein antibodies were raised in rabbits against a cytoplasmic dynein previously purified from rat brain (3). The antibodies were further affinity-purified by a modification of the previously described method (13) using an Immobilon-P membrane (Millipore) on which purified rat brain dynein 57 kD light chain was blotted. The specificity of the antibodies was studied by Western blot analysis.

Immunofluorescence Microscopy

The sections were air-dried on slides coated with 0.5% gelatin, and fixed with acetone. After preincubation with 5% skimmed milk in phosphate-buffered saline (PBS, pH 7.4) to eliminate any non-specific reaction, the sections were incubated for 60 min at room temperature with monoclonal anti-kinesin antibodies (Sigma) and affinity-purified rabbit anti-rat dynein antibodies diluted 1:40 and 1:20 with PBS, respectively. Following three 10-min washes in PBS containing 0.05% Tween-20, the sections for kinesin staining were incubated with fluorescence-conjugated goat anti-mouse immunoglobulin (Dako) diluted 1:100 in PBS. For dynein staining, fluorescence-conjugated goat anti-rabbit IgG (Cappel) diluted 1:200 was used as the second antibody. The sections were mounted with 50% glycerol in PBS, and examined by fluorescent microscopy.

nAChRs Labeling

The right sciatic nerves of homozygous *gad* mice aged 9, 10, 11, 12, 14 and 16 weeks and of age-matched normal littermates were dissected as described previously (8). After 10 days, the dener-

Table 1 *Incidence of Fibrillation, Amplitude of M Wave and Conduction Velocity of Gad Mice and Normal Littermates*

Group		Incidence of fibrillation	Amplitude of M wave (mV)	Conduction velocity (m/s)
I (6 weeks)	Control	0/5	5.9 $\pm$ 0.2	25.3 $\pm$ 0.7
	Gad	0/5	5.7 $\pm$ 0.3	24.8 $\pm$ 0.8
II (9 weeks)	Control	0/5	6.4 $\pm$ 0.6	27.2 $\pm$ 0.6
	Gad	3/5	5.3 $\pm$ 0.5*	25.5 $\pm$ 0.8
III (12 weeks)	Control	0/5	6.8 $\pm$ 0.6	26.8 $\pm$ 0.4
	Gad	5/5	4.4 $\pm$ 0.7*	26.4 $\pm$ 0.5

*Significantly different compared with respective controls ($P < 0.05$). Values are mean $\pm$ SEM (n=5).

Table 2 *The Mean Diameters of Myelinated Sciatic and Tibial Nerve Fibers in the 16-Week-Old Gad Mice and Age-Matched Normal Littermates*

Genotype	Number of mice examined	Sciatic nerve* (μ m)	Tibial nerve* (μ m)
<i>gad/gad</i>	6	5.50 $\pm$ 0.09	4.35 $\pm$ 0.06
<i>gad/+</i> , <i>+/+</i>	6	5.44 $\pm$ 0.08	4.46 $\pm$ 0.05

*No significant difference between both genotypes. Values are mean $\pm$ SEM.

vated and the contralateral control gastrocnemius muscles were sectioned (10 μ m) on a cryostat and stained with rhodamine-labeled α -bungarotoxin (Molecular Probes, 1:50 dilution with PBS) as described previously (12).

RESULTS

Electrophysiology

The control mice showed no spontaneous electrical activity of the gastrocnemius muscle in any of the three groups. In gad mice, the incidence of muscle fibrillation increased with age (Table 1). The mean conduction velocity of the tibial nerve of gad mice was not significantly different from that of the controls at any stage. However, the amplitude of the M wave decreased significantly with age in the gad mouse (Table 1).

Morphometry of Peripheral Nerve Fibers

As shown in Table 2, the mean diameters of neither myelinated sciatic nor tibial nerve fibers in the gad mouse aged 16 weeks were significantly different from those of their normal littermates.

Characterization of Anti-Cytoplasmic Dynein Antibodies

The specificity of the anti-dynein antibodies was confirmed by Western blot analysis using a purified rat brain cytoplasmic dynein and homogenates of whole mouse brain (Fig. 1).

Axonal Transport in the Peripheral Nerve

Proximal and distal portions of the ligated sciatic nerve were stained with monoclonal anti-kinesin and affinity-purified rabbit anti-rat dynein antibodies, respectively. As shown in Fig. 2, A and B, immunofluorescence microscopic examination of the ligated sciatic nerves of 12-week-old gad mice showed strong staining with the anti-kinesin antibody in the region of the nerve proximal to the site of ligation. The pattern of staining was gradational; brighter staining was observed at the regions closer to the ligated portion. The anti-dynein antibody stained the portions both proximal and distal to the ligated site in the gad mouse (Fig. 2, C and D). Similar results were obtained in the normal littermates. The pattern and intensity of the staining with

Fig. 1 Immunoblotting with anti-cytoplasmic dynein antibodies. Lanes 1 and 2; purified rat brain cytoplasmic dynein. Lanes 3 and 4; whole mouse brain homogenate. Lanes 1 and 3 were incubated with blot-purified anti-cytoplasmic dynein 57 kD light chain antibodies, followed by HRP-anti rabbit second antibodies. Detection was performed using an ECL Western blotting detection kit (Amersham, Tokyo). In lanes 2 and 4, the first antibodies were replaced by nonspecific IgG as controls. Double arrowhead shows the 57 kD band and single arrowhead the 55 kD band. *Denotes single 57 kD band in the whole mouse brain homogenate.

these antibodies showed no changes with age in either gad or control mice (data not shown). Contralateral non-ligated nerves showed much lower levels of staining with both antibodies (data not shown).

Distribution of nAChR Clusters

Rhodamine-labeled α -bungarotoxin was used in the present study to detect nAChR clusters on the gastrocnemius muscles. In all sections examined, nAChRs were clustered at the neuromuscular junction in the homozygous gad mice by 11 weeks

Fig. 2 Immunofluorescence photomicrographs of ligated 12-week-old gad mouse sciatic nerves stained with anti-kinesin (A, B) and anti-dynein antibodies (C, D) as described in Materials and Methods. Left and right columns are portions proximal and distal to the ligated site, respectively. Scale bar, 50 μ m

old. Fig. 3 shows the labeling of nAChRs on the gastrocnemius muscle of a 10-week-old gad mouse. However, at 12 weeks of age, some fibers of the muscle expressed nAChRs over the entire surface (Fig. 4). This disorganization of the expression of nAChRs on the gastrocnemius muscle of the gad mouse increased with age (data not shown). The sciatic nerve was dissected out from gad mice and normal littermates at 10 weeks of age, and nAChRs were distributed over the entire surface of all fibers of the gastrocnemius muscle from both mutants and controls (Fig. 3, B and D).

DISCUSSION

Pathophysiology of the dying back type of axonal degeneration was studied in the gad mutant mouse which shows hereditary sensory and motor neuropathies. The electromyograph of the gastrocnemius muscle showed that the incidence of muscle fibrillation increased with age in this mutant. Since fibrillation results from the discharge of denervated muscle fibers (6), this observation suggests that the denervation process which progresses with age is

Fig. 3 Photomicrographs of the rhodamine-labeled α -bungarotoxin-stained gastrocnemius muscles from gad mice aged 10 weeks (A, B) and from age-matched normal littermates (C, D). Right column, denervated muscle; left column, contralateral control muscle. Scale bar, 50 μ m

neurogenic. While the conduction velocity in the tibial nerve of the gad mouse was not significantly different from that of age-matched controls, the amplitude of the M wave decreased significantly with age in the mutant. These observations confirm our previous morphological findings that the neuropathy in the gad mouse is an axonal type and not a demyelinating type. It is widely accepted that the conduction velocity in motor axons is slowed in demyelinating neuropathies but is normal in axonal neuropathies, which have reductions

in the amplitude of the evoked muscle action potential or sensory nerve action potential reflecting the degree of fiber loss (1). In the present study, both fibrillation and the decrease in amplitude of the M wave in gad mice began to be exhibited around the age of 9 weeks, prior to the onset of clinical motor paresis. These findings suggest that the gad mouse is a useful model for studying the function of subclinically degenerating axons, where degeneration progresses with time leading to the manifestation of neurological symptoms.

Previous morphological studies raised the possibility that the axonal transport of DRG and motor neurons was impaired and involved in the pathogenesis of the gad mouse (9–11). To address this question, we studied the axonal transport of the sciatic nerve of the gad mouse. Recent studies have identified two motor proteins for antero- and retrograde axonal transport; kinesin (2) and cytoplasmic dynein (14). When a nerve is ligated, anterogradely and retrogradely moving organelles are known to accumulate on the proximal and distal edges, respectively, of the ligated portion. Kinesin was shown to accumulate proximally to a much greater extent than distally to the ligation (4). Since brain dynein is localized not only on retrogradely transported membranous organelles but also on those transported anterogradely, this molecule accumulates on both proximal and distal sides (5). In the gad mouse at ages of 10, 12 and 16 weeks, immunofluorescent staining patterns using anti-kinesin and anti-dynein antibodies on the ligated sciatic nerve were similar to those of normal littermates in the present study. These results suggest that normal axonal transport remained in the sciatic nerve of the gad mouse even when the disease had fully

Fig. 4 Photomicrographs of 12-week-old gad mouse gastrocnemius muscles. Hematoxylin-eosin staining (A), rhodamine-labeled α -bungarotoxin immunostaining (B). Scale bar, 50 μ m

progressed. The present morphometric study of the peripheral nerves of the gad mouse supports this normal axonal transport in the gad mouse, since the diameters of the sciatic and the tibial nerve fibers of the gad mouse with severe motor paresis were not significantly different from those of their normal littermates.

It is interesting that dispersion of nAChRs was observed *in vivo* after the age of 12 weeks in the gad mutant. At this age, motor paresis begins to be manifested clinically (9), although previous histological studies showed that endplates of the hind-limb muscles become denervated at an earlier stage. Our present findings suggest that the disorganization of the expression of nAChRs in the gad mouse may play a crucial role in the progression of clinical symptoms.

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