1. Introduction

Conduction of impulses, either individually or in trains, can produce long-lasting effects on nerve excitability. In 1935, Gasser found that when axons conducted trains of impulses, they underwent hyperpolarizaion (Gasser, 1935). Previous studies using high-frequency electrical stimulation for rat axons have shown that the phenomenon presumably occurs due to activity of the electrogenic Na^+–K^+ pump (Bostock and Grafe, 1985; Gordon et al., 1990). In a human motor axon, the extent and duration of hyperpolarization depend on both discharge rate and train length (Bostock and Bergman, 1994). Vagg et al. (1998) found that axonal hyperpolarization can be produced in human motor axons by the natural activity associated with a maximal voluntary contraction. It has recently been shown that voluntary contraction causes the change of other indices of axonal excitability (Kuwabara et al., 2001) and that the extent and pattern of axonal hyperpolarization could be measured by using threshold tracking technique (Kuwabara et al., 2002). Kiernan et al. (2004) clarified the axonal hyperpolarization in human median nerve by 8 Hz steady stimulation with surface electrodes. The reason why they used 8 Hz stimulation is because motor axons can maintain a tonic discharge at this frequency or even higher in voluntary contractions, and >10-Hz electric stimulation to the nerve trunk is painful and therefore not tolerable.

The safety margin for impulse conduction is normally high and the activity-dependent hyperpolarization induced by voluntary contraction is insufficient to jeopardize nerve conduction in healthy axons, but in demyelinated axons which have critical impairment of the safety margin, the conduction block can be induced by tetanic stimulation (Bostock and Grafe, 1985). The activity-dependent conduction block is potentially important in patients with demyelinating neuropathy such as chronic inflammatory demyelinating polyneuropathy and multifocal motor neuropathy. It has been reported that voluntary contraction produced (or accentuate) conduction block in patients with such demyelinating neuropathy (Cappelen-Smith et al., 2000; Kaji et al., 2000). In addition, Inglis et al. have shown by using microneurography that natural activity can produce conduction block in acutely injured single human axons (Inglis et al., 1998).

The single fiber electromyography (SFEMG) technique was developed to study the microphysiology of the motor unit, such as the propagation of muscle fibers (Stålberg, 1966) and neuromuscular jitter (Sanders and Stålberg, 1996). SFEMG recordings can be performed during intramuscular electrical stimulation. This stimulated SFEMG (s-SFEMG) technique can be used in patients who cannot cooperate (unconscious patients, children, and patients with very weak muscles) (Stålberg et al., 1992). The technique enables motor axons to fire at a constant frequency and provides the continuous data of latency from the time of the axonal stimulation to the onset of muscle-fiber action potential (MAP) including axonal conduction time. The continuous high frequency axonal stimulation during about 30 s may detect activity-dependent conduction block in patients with prominently reduced axonal safety factor because continuous high frequency stimulation could be equivalent to maximum voluntary muscle contractions. Moreover, when there are conduction blocks in the proximal nerves (e.g., the nerve roots), the impulse load at the more distal tested site at the nerve trunk should be less than expected, and it is impossible to estimate the extent of impulse load. In contrast by using the method of intramuscular axonal stimulation, it is possible to use a constant and steady impulse load. The present study has been undertaken to develop a method to assess activity-dependent hyperpolarization and block in single motor axons of human subjects by using s-SFEMG.

2. Methods

2.1. Subjects

Ten healthy subjects (5 males, 5 females; median age, 30 years; range, 20–50 years) were examined. None of them had a peripheral nerve disorder, and systematic disease or medication affecting peripheral nerve function. Informed consent was provided by each subject and all experiments were conducted in accordance with the *Declaration of Helsinki* and with the approval by the Ethics Committee of Chiba University School of Medicine for Human Research Studies.

2.2. Single fiber electromyography

Axonal s-SFEMG was performed in the right extensor digitorum communis muscle (EDC) using a Nicolet Viking 4 EMG machine (Nicolet Biomedical Japan, Tokyo, Japan) and conventional procedures as described in a previous report by Trontelj et al. (1986). The recordings were made intra-muscularly with a concentric needle electrode (30 G; TECA elite US53153). The high pass filter was set to 2 kHz, and the low pass filter was 10 kHz. Intra-muscular axonal stimulation was performed with a monopolar needle electrode (28 G; TECA U0809P02) and a reference surface electrode placed 2 cm laterally. The stimulus duration was 0.1 ms. With stimulating at 3 Hz with stimulus intensity around 1 mA, contraction of EDC was confirmed, and a recording electrode was inserted into the site of muscle bundle contraction. The distance between the stimulating and recording electrodes was 2 cm. Both needles were fixed by metallic strut to minimize needle moving during stimulation.

The stimulus intensity was determined as 20% above the activation threshold of the target MAP. The fingers of subjects were fixed with a strap to reduce motion artifacts. Although the muscle fibers can be stimulated either directly or indirectly via its axon in this s-SFEMG method, we excluded direct stimulation of muscle fibers by the very low jitter value (mean consecutive difference <5 μs) (Trontelj et al., 1986). By contrast, increased jitter with intermittent blocking indicates subthreshold stimulation. When it was observed, we performed a new recording 1 min later with increased stimulus intensity by further 20% of the previous intensity.

A total of 500 stimuli were delivered at the frequencies of 5, 10, and 20 Hz. Latencies of the initial rising phase of MAP were measured. A special program for latency measurements (Analysis of SFEMG software) was developed by Medical Try System Co. Ltd. (Tokyo, Japan). If a prominent latency change was observed after 500 times stimulation at 20 Hz, short time stimulation at the same frequency was repeated 1 min later to observe whether the latency prolongation was recovered or not.

2.3. Data analyses and statistics

Latency change (%) at 100th, 200th, 300th, 400th and 500th stimulus with each of the three stimulus rate was measured. Latency change was defined as "latency at 500th stimulation (or at 25 s after starting the stimulation)/latency at baseline × 100. We also calculated latency change at 25 s after the start of the stimulation with each stimulus rate (e.g., at 125th stimulus with 5 Hz stimulation, at 250th with 10 Hz and at 500th with 20 Hz) to compare the latency change at a constant time length.

The data of latency change were checked for normality using the Shapiro–Wilk test (Shapiro and Wilk, 1965) and Q–Q plot before using parametric tests. A repeated measurement analysis (Fitzmaurice et al., 2004) was conducted to evaluate the main effects of stimulation with the covariance among repeated measures

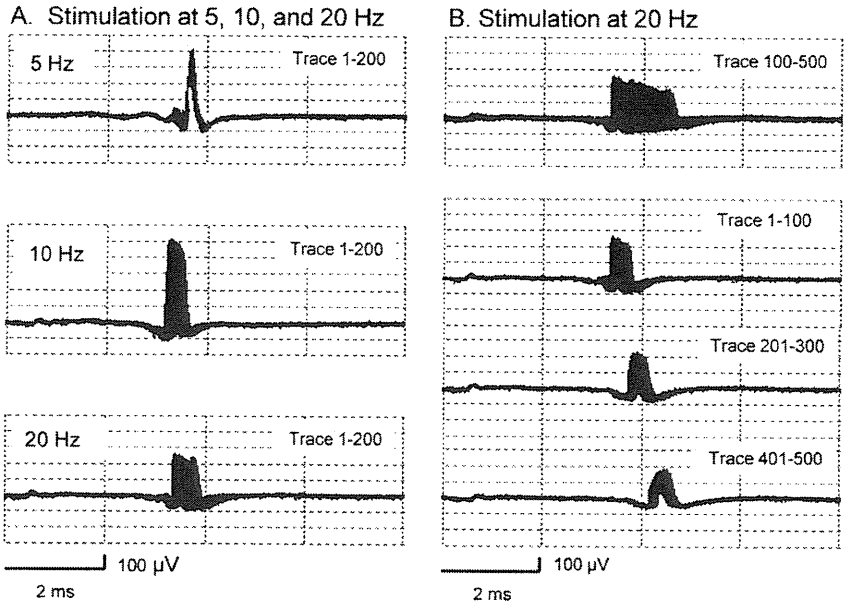

Fig. 1. Examples of superimposed single muscle action potentials during prolonged tetanic intramuscular microstimulation in the extensor digitorum communis muscle of a normal subject. (A) Characteristic responses during the first 200 stimuli delivered at 5, 10, and 20 Hz. Each superimposed response is recorded from a different site. (B) An example of superimposed responses recorded during 20 Hz stimulation. Traces 100–500, 1–100, 201–300, and 401–500 are separately shown. Note a progressive increase in latencies.

modeled as compound symmetry (CS). Tukey–Kramer's method (Westfall and Young, 1993) was applied for adjustment of multiple comparisons between any two groups. All comparisons were two-sided, and p-value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed by the SAS software program, version 9.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

A total of 585 MAPs were examined at 5 Hz ($n = 190$), 10 Hz ($n = 210$), and 20 Hz ($n = 185$) steady stimulation. The number of MAPs per subject were 12–27 at 5 Hz, 15–37 at 10 Hz and 12–27 at 20 Hz. The data of latency change (%) were analyzed for reducing the influence of latency at baseline determined by the distance between the stimulating electrode and the recording electrode. The tetanic electric stimulation was well-tolerated in all 10 subjects, whereas three of them complained of slight pain during 20 Hz stimulation.

There was a progressive linear prolongation of latencies, as the stimulus rate increased. Representative recordings from a single subject are shown in Fig. 1. The latency prolongation during stimulation was not observed at 5 Hz, but occurred slightly at 10 Hz, and prominently at 20 Hz. A gradual progressive latency prolongation was clearly visible at 20 Hz (Fig. 1B and Supplementary Video S1).

Fig. 2 shows changes in latency during 500 stimuli in two subjects. The baseline latencies varied because all three recordings in each subject were from different site to present characteristic examples. There were no obvious changes in latencies at 5 Hz, a slight increase in latency was seen at 10 Hz, and a clear latency prolongation at 20 Hz stimulation. The latency prolonged linearly and the slope of the regression line was steepest for 20 Hz stimulation. The slope of the formula of the regression line indicates the latency elongation (μs) per stimulus. In all recordings, the mean slope of the regression line was 0.07 μs/stimulus at 5 Hz, 0.26 μs/stimulus at 10 Hz and 0.53 μs/stimulus at 20 Hz.

Fig. 3 shows box plots of the latency change during 500 stimulations at 5, 10, and 20 Hz in the three different rate groups. The data of each stimulus rate group were normally distributed. There was a significant increase in latency, as the stimulus rate was increased. The least square means (SEM) of latency change by applying a mixed effect model were 100.7 (0.28)% at 5 Hz, 102.3 (0.27)% at 10 Hz and 105.3 (0.28)% at 20 Hz. The difference was more prominent between 10 and 20 Hz than between 5 and 10 Hz stimulation.

Fig. 4 shows comparison of latency change at constant time length, 25 s (125 stimuli at 5 Hz, 250 at 10 Hz, and 500 at 20 Hz). The data of three stimulus groups were also normally distributed. The least square means (SEM) of latency change were 100.2 (0.28)% at 5 Hz, 101.3 (0.28)% at 10 Hz and 105.3 (0.29)% at 20 Hz. Again there were statistically significant differences among three stimulus rate groups. During 500 stimuli, there was a linear chronological change in latency at 5, 10, and 20 Hz stimulation, and the latency increase was most prominent at 20 Hz (Fig. 5).

In most of recordings at 20 Hz stimulation in which we observed prominent latency prolongation, the same stimulation was repeated 1 min after the first trial. We confirmed the recovery from latency prolongation.

4. Discussion

The present study has demonstrated in healthy human motor axons, activity-dependent nerve conduction slowing by a constant frequency of prolonged electric stimulation, which presumably reflects activity-dependent hyperpolarization. The extent of impulse conduction slowing varied with stimulus rates. The method using axonal stimulation SFEMG appears to be able to assess the extent of activity-dependent hyperpolarization due to the activation of Na^+/K^+ pump in single motor axons. However, our method had some limitations. We could not demonstrate direct evidence that axonal membrane was hyperpolarized when the latency was prolonged (i.e., a threshold increase). Secondly, our study was on a small scale. To investigate the reliability of this method, further

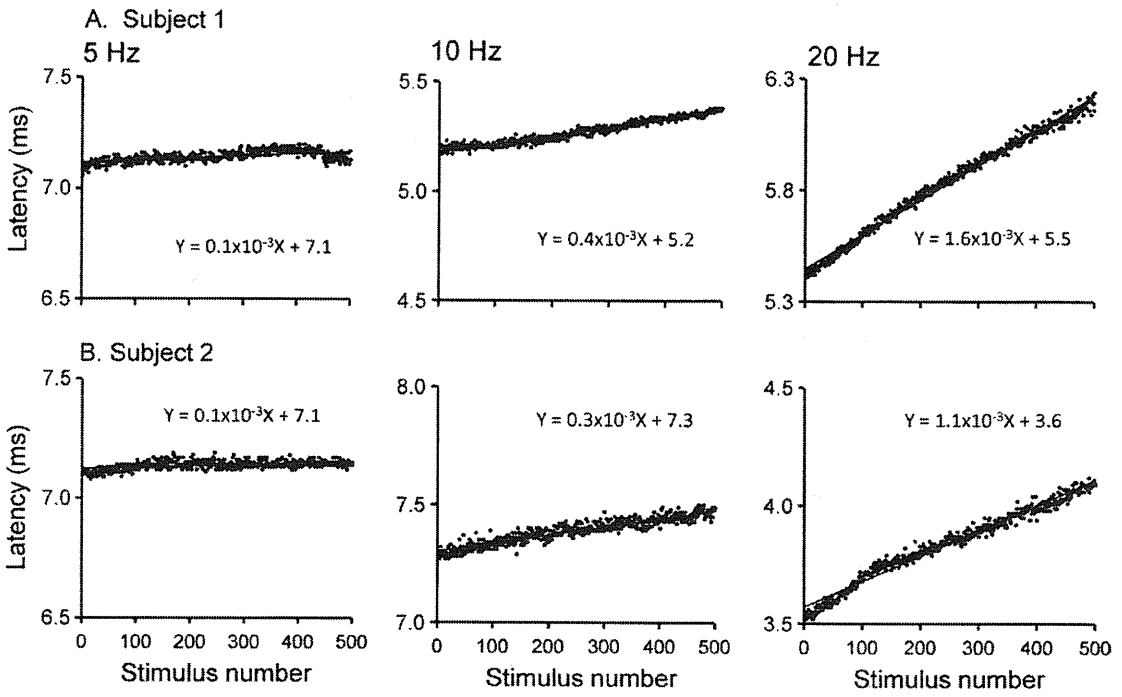

Fig. 2. Examples of the scatter plot of the latencies of single muscle action potentials at each stimulus rate recorded from different sites of two subjects. The equation of the linear function in each plot indicates the formula of the regression line. The slope of the formula represents the latency prolongation per stimulus. Note a progressive increase in latencies as the stimulus frequency increases.

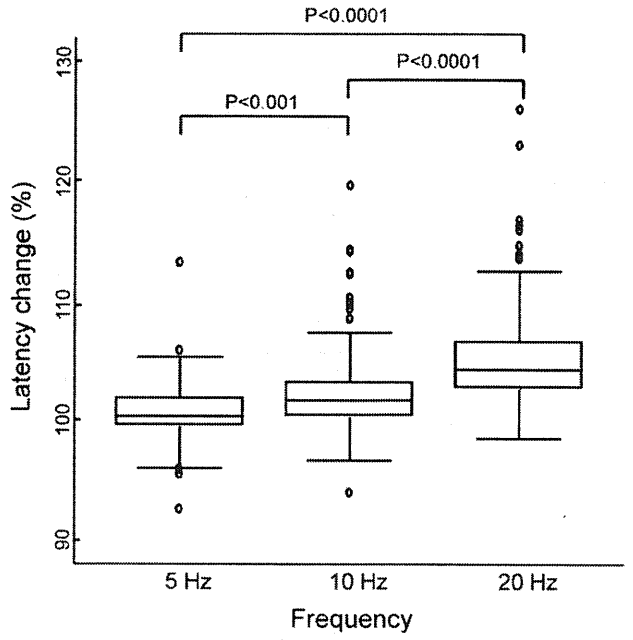

Fig. 3. Box plots of the latency change (%) at 500 times stimulation at 5 Hz (n = 190), 10 Hz (n = 210), and 20 Hz (n = 185). 5 Hz: median 100.6%; range 92.5–113.1% ; 10 Hz: median 101.7%; range 93.9–119.5%; 20 Hz: median 104.2%; range 98.5–125.9%. The data of latency change (%) are analyzed by means of repeated measurement analysis (F = 95.6, p < 0.001) and multiple comparisons are calculated by means of Tukey–Kramer's method. There are statistically significant differences among latency changes among each frequency (p-values are given in the figure).

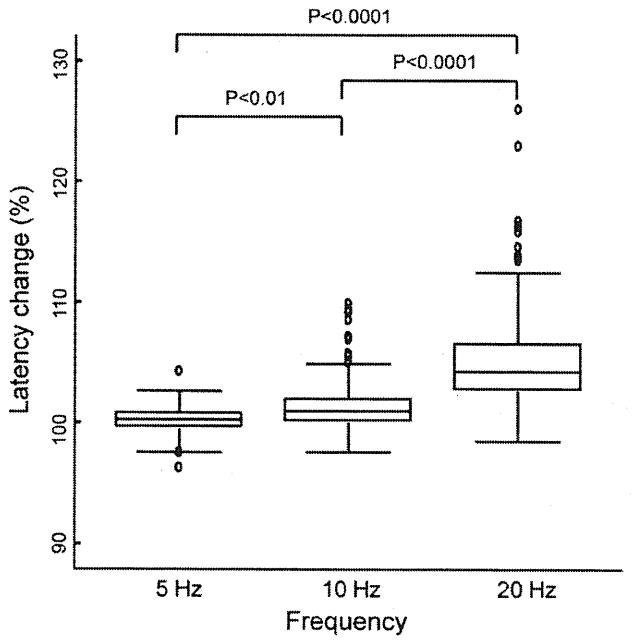

Fig. 4. Box plots of the latency change (%) during a constant stimulus length (25 s). 5 Hz: median 100.3%; range 96.2–104.2% ; 10 Hz: median 101.0%; range 97.6–109.8%; 20 Hz: median 104.2%; range 98.5–125.9%. The data of latency change (%) are analyzed by means of repeated measurement analysis (F = 196.8, p < 0.001) and multiple comparisons are calculated by means of Tukey–Kramer's method. There are statistically significant differences between frequencies (p-values are given in the figure).

studies involving a larger number of subjects or other population will be required.

Particularly, prolonged repetitive stimulation at 20 Hz results in obvious latency prolongation of MAPs. In most part of recordings with obvious latency prolongation, we observed slightly decreasing amplitude and increasing duration of the action potential as the latency prolonged (Supplementary Video S1). The change of

the amplitude and duration of action potential might be due to slowing of the propagation velocity of the muscle fiber action potential by continuous activation.

4.1. Mechanisms of the activity-dependent latency prolongation

Along with previous studies, the latency increases are supposed to be induced by activation of the electrogenic Na^+/K^+ pump and resulting axonal hyperpolarization (Vagg et al., 1998; Kuwabara et al., 2001, 2002). Two major mechanisms could be responsible for the hyperpolarization that follows activity; activation of slow potassium channels and activation of the electrogenic Na^+/K^+ pump.

It has been reported that with trains of 10–20 impulses, slow potassium channels can be a main mechanism for axonal hyperpolarization, and more prolonged impulse trains cause activation of the electrogenic Na^+/K^+ pump and thereby axonal hyperpolarization (Bergman, 1970; Lin et al., 2000). It is therefore likely that trains of 500 impulses used in this study result in activation of the pump. The latency reflects axonal conduction time, neuromuscular junction transmission time and muscle fiber conduction time. The neuromuscular transmission is established by presynaptic acetylcholine release, and the intervals of the release are random, not steady. Scatter plots of all recordings in this study could apply to linear models, and the mean slope of the regression line was steeper, as stimulus frequency increased.

The linear prolongation of the latency is likely to reflect prolongation of axonal conduction time due to membrane hyperpolarization, although the possibility that changes in muscle membrane properties may partly contribute to the observed latency changes during tetanic stimulation, could not be excluded. This is a limitation of our method, and further studies are required to clarify the precise mechanisms for the activity-dependent changes.

4.2. The advantage of the s-SFEMG method

Our s-SFEMG method could provide quantitative assessment in single motor axons because axons can be activated at a fixed frequency. In a previous study by Kiernan et al. (2004), axonal stimulation has been demonstrated by 8 Hz surface electrode stimulation at the wrist (Kiernan et al., 2004). It has been noted that higher frequency stimulation over 8 Hz was more painful and this made it more difficult for naïve volunteers to relax in their study. However, high frequency stimulation over 10 Hz is available by the s-SFEMG method without pain. In steady voluntary contractions, motor axons discharge at 6–20 Hz (Burke and Jankelowitz, 2009). Therefore 20 Hz stimulation is more equivalent to physiological maximum voluntary contraction than 8 Hz stimulation.

4.3. Clinical implications

The present study shows that the s-SFEMG method could be used to assess the degree of membrane hyperpolarization in normal human motor axons. The procedure is easy and safe, and therefore can be applied to patients with neuromuscular disorder, particularly who complained of fatigability. Activity-dependent conduction block could be responsible for fatigue in patients with demyelinating neuropathy, such as chronic inflammatory demyelinating polyneuropathy (Burke and Jankelowitz, 2009).

In normal axons, the activity-dependent hyperpolarization causes nerve conduction slowing, but does not cause conduction block because of the sufficiently high safety margin for impulse transmission at each node. By contrast, previous studies have demonstrated that activity-dependent conduction block occurred in demyelinated axons in rat and patients with demyelinating neuropathy (Bostock and Grafe, 1985; Kaji et al., 2000; Cappelen-Smith et al., 2000). We speculate that patients with a number of diseases in which the safety factor for impulse transmission is critically lowered by not only demyelination but also increased axonal branching due to collateral sprouting suffer from fatigability due to activity-dependent conduction block. Our technique by using 20 Hz stimulation may detect activity-dependent conduction block if the safety margin of impulse transmission is reduced, and would provide the mechanism for fatigue in disease.

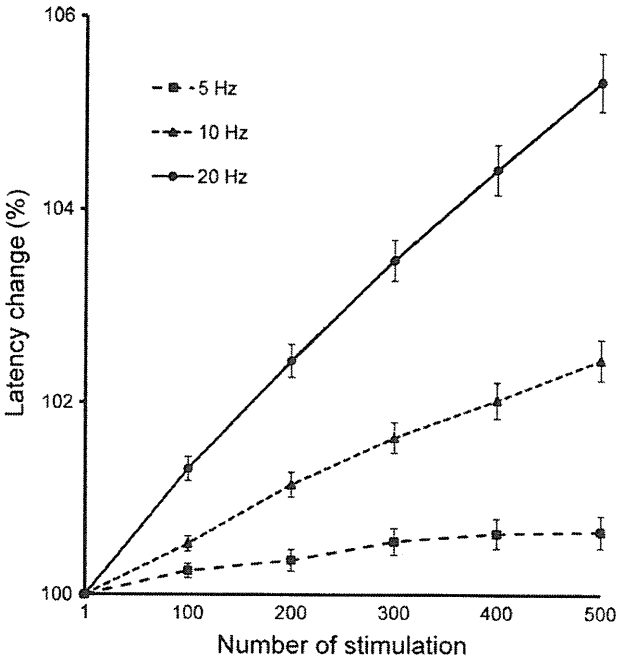

Fig. 5. Change in the mean latency prolongation at 5, 10, and 20 Hz stimulation. There was a linear trend in the chronological changes in the rate of the latency prolongation at each stimulus rate, and the slopes become steeper as the stimulus frequency is increased.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.clinph.2011.05.005.

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ORIGINAL COMMUNICATION

Nationwide survey of Alexander disease in Japan and proposed new guidelines for diagnosis

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Abstract Alexander disease (AxD) is a rare neurodegenerative disorder characterized by white matter degeneration and formation of cytoplasmic inclusions. *Glial fibrillary acidic protein* (*GFAP*) mutations have been reported in various forms of AxD since 2001. However, a definitive diagnosis remains difficult because of uncertain prevalence, and different clinical features seen in infantile AxD and adult AxD may lead to confusion and misdiagnosis. Here we report an epidemiological study conducted in Japan. Two nationwide questionnaire-based surveys were conducted using tentative diagnostic criteria. We gathered information regarding prevalence, neurological findings, magnetic resonance imaging (MRI) findings, electrophysiological findings, genetic information, and the results of therapeutic interventions and home care. Prevalence of various forms of AxD was determined as 27.3% (infantile), 24.2% (juvenile), and 48.5% (adult). Prevalence of AxD in Japan was estimated to be approximately 1 case per 2.7 million individuals. The main characteristics of infantile and juvenile AxD include delayed psychomotor development or mental retardation, convulsions, macrocephaly, and predominant cerebral white matter abnormalities in the frontal lobe on brain MRI. The main characteristics of adult AxD include bulbar signs, muscle weakness with hyperreflexia, and signal abnormalities and/or atrophy of medulla oblongata and cervical spinal cord on MRI. To ensure correct diagnosis of AxD, the physician should understand the importance of the process of *GFAP* genetic testing, which provides definitive diagnosis. Therefore, we propose new clinical guidelines for diagnosing AxD based on simplified classifications: cerebral AxD (type 1), bulbospinal AxD (type 2), and intermediate form (type 3).

Keywords Glial fibrillary acidic protein · Alexander disease · Genetics · Magnetic resonance imaging · Prevalence

Abbreviations

AxD	Alexander disease
GFAP	Glial fibrillary acidic protein
EEG	Electroencephalogram
ABR	Auditory brainstem response
TRH	Thyroid-releasing hormone

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Introduction

Alexander disease (AxD) is a rare neurodegenerative disorder characterized by white matter degeneration and formation of cytoplasmic inclusions known as Rosenthal fibers, which accumulate primarily in the astrocyte end-feet of subpial and perivascular zones and consist of glial fibrillary acidic protein (GFAP), heat shock protein 27, and αB-crystallin [1–3]. *Glial fibrillary acidic protein* mutations have been reported in various forms of AxD since 2001 [4]. AxD in adults was identified following research on various clinical forms of AxD and their associated findings detected by magnetic resonance imaging (MRI) [5]. Although adult AxD demonstrates a *GFAP* mutation, the clinical symptoms and MRI findings are different from those found in infantile AxD. Clinical features and MRI findings characteristic of AxD were recently identified by performing case studies and a systematic review [6–10]. However, a definitive diagnosis remains difficult because of uncertain prevalence; furthermore, different clinical features seen in infantile AxD and adult AxD may lead to confusion and misdiagnosis.

This study reports the results of a nationwide survey conducted in Japan to gather information on the prevalence, neurological findings, MRI findings, electrophysiological findings, genetic information, and the results of therapeutic interventions and home care.

Materials and methods

Inclusion criteria specified for the diagnosis of AxD are summarized in Table 1. These criteria were decided on the basis of a literature review on AxD, and were decided for the three common age-dependent clinical subtypes, namely infantile, juvenile, and adult. MRI findings were based on previously reported proposed criteria [11].

First survey

To determine the proportion of AxD patients in Japan, a survey questionnaire was sent to the members of educational facilities listed by the Japanese Societies of Neurology, Pediatrics, and Child Neurology, requesting information on patients who fulfilled the clinical inclusion criteria. Cases reported between 2004 and 2009 were examined in this survey.

Second survey

A second set of survey questionnaires was mailed in November 2009 to facilities that were reported as having AxD patients in the first survey. Information was requested on patients' neurological, electrophysiological, and pathological findings, results of genetic analyses, treatment provided, and their clinical outcomes. We confirmed that these cases did not overlap with other reported cases by documenting age and place of birth.

Receipt of responses to the second survey was closed in February 2010, and the collected data were then analyzed.

The survey aimed at collecting information without many details, to obtain the maximum possible answers. Therefore, results were superficial, e.g., normal/abnormal, presence/absence of muscle weakness.

Table 1 Requirements for the proposed diagnosis of Alexander disease for the first survey

Definite Alexander disease: existence of numerous Rosenthal fibers in addition to gliosis and loss of myelin in pathological study or *GFAP* gene mutation, and satisfaction of the following neurological and MRI findings 1, 2, or 3

1. Infantile Alexander disease: onset age is under 2 years with one or more items of (a) and one or more items of (b)
   (a) Neurological findings: psychomotor developmental delay/mental retardation, convulsion, macrocephaly spastic paralysis, bulbar or pseudobulbar signs, cerebellar ataxia
   (b) MRI findings: cerebral white matter abnormalities with frontal lobe predominance, signal abnormalities with swelling or atrophy of basal ganglia and thalami, periventricular rim, brainstem lesions, contrast enhancement
2. Juvenile Alexander disease: onset age is between 2 and 12 years with one or more items of (a) and one or more items of (b) (i) or (b) (ii)
   (a) Neurological findings: mental retardation or dementia, convulsion, macrocephaly spastic paralysis, bulbar or pseudobulbar signs, cerebellar ataxia, autonomic dysfunction, nystagmus, palatal myoclonus
   (b) MRI findings
      (i) Cerebral white matter abnormalities with frontal lobe predominance, signal abnormalities with swelling or atrophy of basal ganglia and thalami, periventricular rim, brainstem lesions, contrast enhancement
      (ii) Signal abnormalities or atrophy of medulla, oblongata, and/or cervical cord
3. Adult Alexander disease: onset age is over 12 years with one or more items of (a) and one or more items of (b)
   (a) Neurological findings: paralysis, bulbar or pseudobulbar signs, cerebellar ataxia, autonomic dysfunction, nystagmus, palatal myoclonus, dementia
   (b) MRI findings: signal abnormalities or atrophy of medulla oblongata and/or cervical cord

Results

The results of the first and second surveys are summarized in Fig. 1.

Thirty-five patients with definite AxD were identified at the end of the surveys and were further analyzed. Prevalence of various forms of AxD was determined as infantile, 9/33 (27.3%); juvenile, 8/33 (24.2%); and adult, 16/33 (48.5%). Cases of twins with infantile AxD, and those with familial adult AxD were considered as one case; this was reflected in the calculations of the proportion of patients in each category.

Patient profiles are summarized in Table 2. Infantile AxD predominantly affected males (8 boys, 2 girls), while no gender predominance was observed in juvenile or adult AxD. Sixty-five percent of adult AxD patients had family members with AxD-like symptoms. Only one pair of identical twins presenting with similar clinical findings were diagnosed with infantile AxD, because brain biopsy of one of the twins showed characteristic Rosenthal fibers. In both cases, a *GFAP* mutation (R79C) was identified later.

Neurological findings are summarized in Table 3. Incidence of delayed psychomotor development or mental retardation, convulsions, macrocephaly, hyperreflexia, dysarthria, and dysphagia was high, whereas incidence of palatal tremor was zero in infantile AxD patients. Incidence of muscle weakness, hyperreflexia, dysarthria, dysphonia, dysphagia, and sphincter abnormalities was high in adult AxD patients. Three cases described as having "unilateral or asymmetric muscle weakness" were included. In addition, three cases that showed asymmetry in the initial stage of the disease, but not at the time of reporting, were also included. Asymmetry was identified in at least 35.0% of the adult AxD cases. Dementia and muscle rigidity were observed in 25.0% and 29.4% of adult AxD

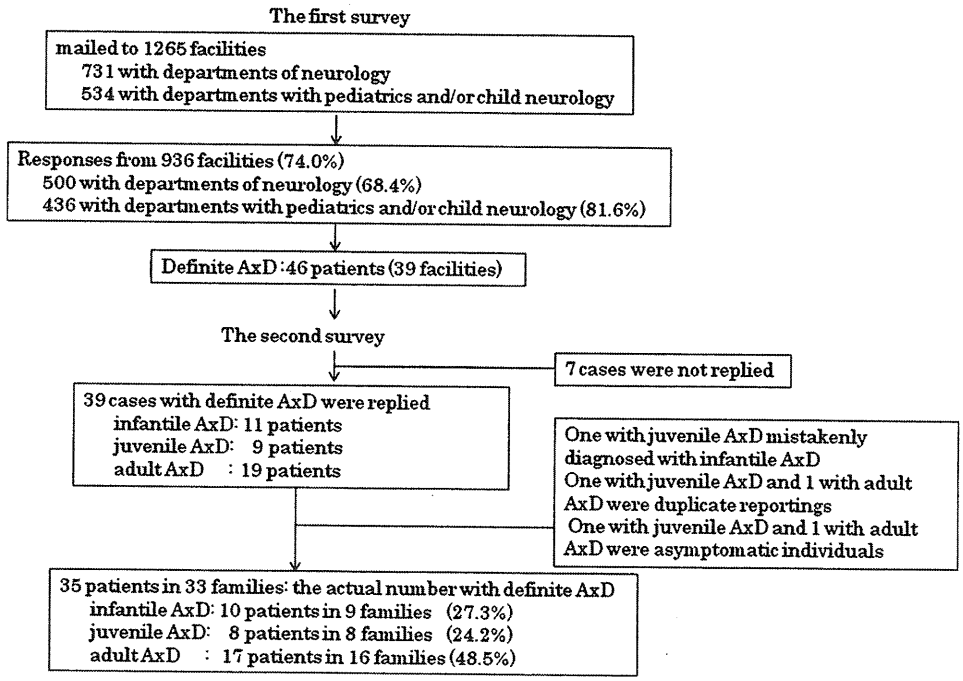

Fig. 1 Flow of the survey

Table 2 Patient profiles in the second survey

	Infantile form	Juvenile form	Adult form
Cases	10	8	17
M:F	8:2	4:4	9:8
Age at onset (m: months, y: years)	10.7 ± 6.7 m (3 to 24 m)	4 0 ± 2.3 y (2 y 10 m to 9 y)	44.1 ± 12.9 y (26 to 61 y)
Family history	2 (twins)	0	10 (9 families)
Period from onset to diagnosis	60 ± 4 4 m (0 to 14 m)	6.3 ± 3.1 y (<1 y 2 m to 10 y)	6.9 ± 6.3 y (0 to 22 y)
GFAP gene analysis[a]	10	8	17
Pathological examination	1 (brain biopsy)	0	2 (autopsy)

[a] *Glial fibrillary acidic protein* gene analysis was also preformed in two asymptomatic individuals

Table 3 Summary of neurological signs of Alexander disease in the second survey

	Infantile form	Juvenile form	Adult form
Muscle weakness		42.9% (3/7)	82.4% (15/17)
Tender reflex abnormality	85.7% (6/7)	71.4% (5/7)	94.1% (16/17)
Hyperreflexia	85.7% (6/7)	71.4% (5/7)	94.1% (16/17)
Hyporeflexia or areflexia			12.5% (2/16)
Babinski sign		57.1% (4/7)	82.4% (14/17)
Parkinsonism		0.0% (0/7)	29.4% (5/17)
Sensory disturbance		0.0% (0/7)	17.6% (3/17)
Dysarthria	100.0% (6/6)	100.0% (8/8)	88.2% (15/17)
Dysphonia	66.7% (6/9)	37.5% (3/8)	70.6% (12/17)
Dysphagia	77.8% (7/9)	25.0% (2/8)	88.2% (15/17)
Nystagmus	0.0% (0/6)	0.0% (0/8)	64.7% (11/17)
Limb ataxia	20.0% (1/5)	37.5% (3/8)	30.8% (4/13)
Truncal ataxia	20.0% (1/5)	50.0% (4/8)	50.0% (6/12)
Palatal myoclonus	0.0% (0/6)	0.0% (0/7)	37.5% (6/16)
Orthostatic hypotension		20.0% (1/5)	7.7% (1/13)
Sphincter abnormalities	33.3% (3/9)	12.5% (1/8)	57.1% (8/14)
Sleep disorder		25.0% (1/4)	30.8% (4/13)
Convulsions	100.0% (9/9)	87.5% (7/8)	6.3% (1/16)
Mental retardation/psychomotor developmental delay	100.0% (8/8)	87.5% (7/8)	6.3% (1/16)
Dementia			25.0% (4/16)
Macrocephaly	75.0% (6/8)	50.0% (4/5)	
Scoliosis	44.4% (4/9)	50.0% (4/8)	13.3% (2/15)

Table 4 Summary of MRI findings of Alexander disease in the second survey

	Infantile form	Juvenile form	Adult form
White matter lesion	100% (10/10)	100% (8/8)	37.5% (6/16)
Abnormalities of basal ganglia, thalamus	100% (9/9)	50.0% (4/8)	50.0% (8/16)
Abnormalities of brainstem			
Medulla oblongata	14.3% (1/7)	62.5% (5/8)	100% (16/16)
Pons	14.3% (1/7)	62.5% (5/8)	75.0% (12/16)
Midbrain	28.6% (2/7)	57.1% (4/7)	75.0% (12/16)
Abnormalities of cervical cord	25.0% (1/4)	20.0% (1/5)	100% (16/16)
Abnormalities of cerebellum	37.5% (3/8)	50.0% (4/8)	62.5% (10/16)
Periventricular rim	100% (7/7)	62.5% (5/8)	31.3% (5/16)
Enhancement	75.0% (3/4)	50.0% (1/2)	8.3% (1/12)

patients, respectively. Three cases with clinical findings similar to frontotemporal dementia were mentioned in the free comments section of the questionnaire. Sleep disorders, such as sleep apnea syndrome or rapid eye movement (REM) sleep behavior disorder, and palatal tremor were observed in 30.8% and 37.5% of the adult AxD patients, respectively. Incidence of dysarthria, convulsions, mental retardation, and hyperreflexia was high in juvenile AxD patients.

MRI findings are summarized in Table 4. Cerebral white matter abnormalities in the frontal lobe, signal abnormalities indicating swelling or atrophy of the basal ganglia and thalami, and periventricular rim of low signal intensity on T2-weighted images and high signal intensity on T1-weighted images were predominantly observed in all infantile AxD patients, whereas brainstem lesions were present in only a small number of these patients. Medulla oblongata and cervical spinal cord abnormalities were observed in all adult AxD patients, while abnormalities of other structures related to the brainstem and cerebellum were observed in 60–70% of adult AxD patients. Furthermore, abnormal signals of nucleus dentatus and middle cerebellar peduncle were observed in a few cases reported in the free comments section of the second survey form.

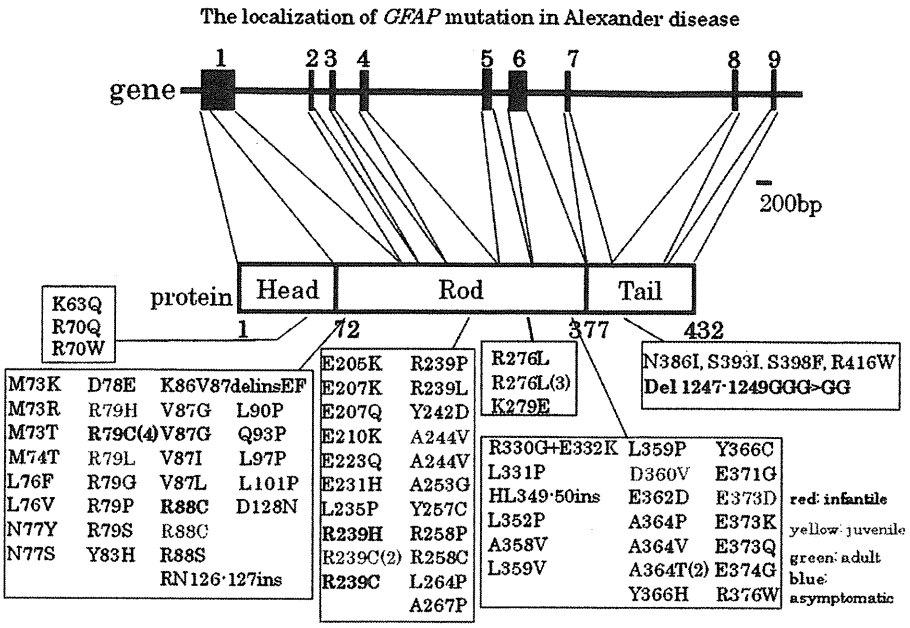

Fig. 2 Schematic demonstrating the *GFAP*, corresponding proteins, and localization of *GFAP* mutations in AxD. Colors indicate mutations reported in this survey: *red* infantile AxD, *yellow* juvenile AxD, *green* adult AxD, *blue* asymptomatic individuals. Mutations shown in *black* indicate published *GFAP* mutations that were excluded from this survey

Although white matter abnormalities were observed in all juvenile AxD patients, abnormalities of the basal ganglia and thalami, periventricular rim, and abnormalities of the brainstem were only observed in approximately 50% of these patients.

Gene analysis is summarized in Fig. 2. *Glial fibrillary acidic protein* analysis was performed for all 35 AxD patients. The mutation sites were unknown for five cases despite the existence of genetic abnormalities, because each patient underwent gene analysis at different facilities; hence a total of 30 patients were available for further analysis. All mutations were identified as *GFAP* point mutations except for one infantile AxD patient, who showed one base deletion in exon 7. The residues affected by point mutation in 70.0% of infantile AxD patients included R79, R88, and R239. Amino acid mutations in juvenile AxD included mutations at R79, R88, R239, A244, D360, and E373. Mutations detected in adult AxD were spread throughout *GFAP* and included M74T, V87G, L101P, A244V, L264P, R258C, R276L, A364T, R376W, S393I, and S398F. The A244V mutation was detected in juvenile and adult AxD.

Electrophysiological findings, treatments, and prognosis are summarized in Table 5. Electroencephalogram (EEG) recordings revealed abnormalities in 8 of 9 (88.9%) infantile AxD patients, 6 of 7 (85.7%) juvenile AxD patients, and 6 of 13 (46.2%) adult AxD patients. Auditory brainstem response (ABR) findings revealed abnormalities in four of five (80.0%) infantile AxD patients, five of seven (71.4%) juvenile AxD patients, and four of five (80.0%) adult AxD patients. Nerve conduction studies were conducted in 13 adult AxD patients, which revealed abnormalities in motor and sensory nerve conduction in 4 (30.8%) and 2 (15.4%) of these patients, respectively. However, data for these studies contained information only about whether it was normal or abnormal. There was no information about the sites of examination or details of the abnormalities.

The survey results did not specify any therapy for AxD. However, some symptomatic treatments were described and evaluated. Antiepileptic drugs seemed to be effective for treating convulsions in infantile or juvenile AxD patients. The efficacy of thyroid-releasing hormone (TRH) therapy was evaluated in three patients, being effective for treating ataxia and some brainstem abnormalities in one juvenile AxD patient. In this patient, envelope-area improvement was observed by balance testing using a stabilometer. Two adult AxD patients were treated with L-dopa, an antiparkinsonism drug, and one adult AxD patient was treated with tizanidine, an antispasticity drug.

The disease duration of the surviving cases ranged from 7 months to 18 years for infantile AxD, from 4 years 6 months to 21 years for juvenile AxD, and from 1 to 16 years for adult AxD patients. Nine of the ten infantile AxD patients were still alive and receiving home care when the first survey was conducted. All juvenile AxD patients were also alive; seven were receiving home care, and one was undergoing treatment in a hospital. Three adult AxD patients failed to survive, and 13 were alive. There was no information on one patient. One nonsurvival case was a patient with V87G who died at the age of 67 years with disease duration of 13 years. In the second case, a patient with R276L died at the age of 51 years with disease duration of 18 years. In the third case, the patient with R258C died at the age of 77 years with disease duration of 9 years.

Table 5 Summary of abnormalities of electrophysiological examinations, therapy, and prognosis in the second survey

	Infantile form	Juvenile form	Adult form
Abnormalities of electrophysiological examinations			
Motor nerve conduction study	0% (0/5)	0% (0/4)	30.8% (4/13)
Sensory nerve conduction study	0% (0/3)	0% (0/2)	15.4% (2/13)
Electroencephalogram	88.9% (8/9)	85.7% (6/7)	46.2% (6/13)
Sensory evoked potential	100% (2/2)	50.0% (2/4)	42.9% (3/7)
Motor evoked potential	100% (1/1)		100% (3/3)
Auditory brainstem response	80.0% (4/5	71.4% (6/7)	80.0% (4/5)
Visual evoked potential	100% (2/2)	50.0% (12)	50.0% (12)
Therapy			
Antiepileptic drugs	8 cases (7 were effective)	3 cases (all were effective)	2 cases (1 was effective)
TRH	1 case	1 case (effective)	1 case
Other			L-Dopa antispasticity dugs
Prognosis	9 cases: alive	8 cases: alive	13 cases: alive
	1 case unknown		3 cases: dead
			1 case: unknown

Discussion

A definitive diagnosis of Alexander disease requires genetic analysis or pathological diagnosis, and diagnoses are almost exclusively given by neurologists or neuropediatricians. Therefore, the estimated number of patients was reported as (patients in the second survey)/(sampling rate × response rate in the first survey) = (reported number of patients)/(response rate in first survey) = 35/0.74 = 47 patients, which is a minimal prevalence assuming that it is unlikely that other units have diagnosed AxD. Considering that the population in Japan is approximately 128 million, we estimate that the disease prevalence is approximately 1 in 2.7 million people, which indicates the frequency of AxD for a 5-year period. The number of actual patients indicates only the number reported in this study, and does not include affected family members who were not reported. Also, the time parameter for prevalence was 5 years, which was also the duration of investigation for this study. Although genetic testing for AxD is mainly conducted in the facility where the present authors work, we cannot rule out the existence of uncertain cases because of the small number of Japanese facilities that conduct genetic testing for AxD. Therefore, the actual prevalence may possibly be higher than that indicated by this study.

Regarding the frequency of the various forms of the disease, adult AxD accounted for 48.5% of the total cases evaluated in this study, and was the most frequent form reported in the survey. However, it was previously reported that infantile AxD accounts for 51% of the total number of AxD cases and is the most frequent form, while adult AxD is the least frequent form, accounting for only 27.2% of total cases [12]. Nevertheless, we consider the results of the present study to be reliable, based on the high response rate to both the first and the second survey. Adult AxD is more difficult to diagnose than infantile AxD, because the MRI criteria and typical clinical symptoms in infantile AxD patients show more regions of brain involvement [11]. Therefore, the frequency of occurrence of adult AxD is not related to that of diagnosis of infantile AxD patients.

We based this study on the presently conducted surveys. However, one case of infantile AxD (1247-1249GGG>GG [13]), two cases of juvenile AxD (D360V [14], R79H [15]), and nine cases of adult AxD (S398F [16], L264P [17], V87G [18], R276L [19, 20], R376W [21], L101P [22], R258C [23], and M74T [24]) have been previously reported. Two cases included in this study, A364T (juvenile AxD) and E373D (adult AxD), are new variations that have not been previously reported.

Cerebral symptoms, such as delayed psychomotor development or mental retardation, convulsions, and macrocephaly, are the most significant neurological findings considered for the diagnosis of infantile AxD. The general characteristics of AxD on brain MRI include predominant cerebral white matter abnormalities in the frontal lobe, signal abnormalities indicating swelling or atrophy of the basal ganglia and thalami, and periventricular rim. Furthermore, progressive bulbospinal symptoms, such as bulbar signs, motor signs, and autonomic dysfunction, may aid

diagnosis of adult AxD. Characteristic findings on MRI in adult AxD patients included signal abnormalities and/or atrophy of medulla oblongata and cervical spinal cord, and abnormalities of other structures related to the brainstem and cerebellum. These results support the results of previous MRI studies [7–9, 23].

Glial fibrillary acidic protein mutations at R79, R88, and R239 accounted for 75.0% of mutations identified in infantile and juvenile AxD patients; however, these mutations were not detected in any of the adult AxD cases. The abnormalities at R79, R88, and R239, which are reported worldwide, have a tendency to be associated with infantile or juvenile AxD. R79 mutations are reported in adult AxD patients, although this is rare [23]. Approximately 60% of the *GFAP* mutations identified in adult AxD cases in this survey were only detected in the Japanese population. Sixty-five percent of adult AxD patients had family members with AxD. In contrast, infantile or juvenile AxD patients had no family histories of AxD, except for one pair of twins. The R276L mutation was observed in three independent families from the same region of Japan. This mutation was not a mutational site with many known cases seen across different races, such as R79 and R239, but was observed in cases found in the same area. Although we cannot rule out that it was a de novo mutation, this mutation is suggestive of the founder effect. Two unrelated patients from the same prefecture in the northern part of Japan were identified with the A244V mutation. However, this mutation was also observed in a patient of other race [6]. Therefore, the A244V mutation may not be the result of a founder effect in Japan.

All infantile or juvenile AxD patients suffered seizures and hence showed EEG abnormalities. However, approximately 50% of adult AxD patients also showed EEG abnormalities, suggesting subclinical cerebral dysfunction resulting from pathological abnormalities in the cerebral matter. ABR abnormalities were independent of AxD type, indicating that radiological and pathological abnormalities of medulla oblongata were observed in all AxD types.

Unfortunately, this survey did not specify any therapies for AxD. However, antiepileptic drugs were reported to be effective in controlling seizures, although seizures that present in infantile or juvenile AxD are considered intractable. The efficacy of TRH therapy was evaluated in three patients. Thyroid-releasing hormone is a neuromodulator of the cerebellum and brainstem and is expected to improve cerebellar ataxia and symptoms of brainstem dysfunction [14]. However, the effectiveness of TRH needs to be confirmed by further large-scale studies. Ceftriaxone, which was not reported in this survey, was recently reported as a potential therapy for halting the progression of some neurological symptoms of AxD [25, 26].

The prognosis of AxD was reported to be relatively good in this survey, which may be due to various reasons. First neonatal AxD, which results in severe disability or death within 2 years [12], was not reported. Second, improvements in general care, nutritional requirements, and respiratory care have contributed to extending the lifespan of AxD patients. However, most patients with AxD receive care in their own homes; therefore, better care is required for such patients.

We inferred the prevalence of AxD in Japan from the high response rate achieved in both our studies. However, we cannot rule out that there may be more than a few undiagnosed cases, one reason being the lack of enough facilities that perform genetic testing for definitive diagnosis of the disease. Another reason is that diagnosis of juvenile AxD may be difficult, particularly in Japan. This is supported by the fact that, in our first survey, no cases of onset during the teens (there are no reports of this originating in Japan) were found, which may be due to several reasons. First, extremely varied cases were found such as cases that presented clinical features of infantile AxD, of adult AxD, of both, and even cases that presented changes in medulla oblongata and cervical spinal cord but that were seen as tuberous in imaging findings [27–29]. These variations may lead to confusion among physicians and misdiagnosis. Second, in Japan, patients in their teens are examined by pediatricians, and MRI diagnostic standards established by van der Knaap et al. [11] are very well known, hence symptoms that are similar to adult AxD, which falls outside those guidelines, may be overlooked. We also inferred this because no such cases of juvenile AxD were reported. Furthermore, although neurologists usually diagnose cases in older teens, we speculate that some cases of AxD are confused for other diseases (such as multiple sclerosis or cancer). This confusion could be similar to cases of nodular lesions that do not present with atrophy, but rather are believed to be observed in juvenile AxD, when patients present atrophy of medulla oblongata or cervical spinal cord, or in cases where signal abnormality that accompanies atrophy indicates possible adult AxD [8, 9, 23]. Therefore, to ensure correct diagnosis of AxD, the physician should understand the importance of the process of *GFAP* genetic testing, which provides definitive diagnosis, but before that the neuropediatrician and neurologist should understand the importance of suspecting AxD from the patient's clinical condition and MRI findings, and place high importance on identifying juvenile AxD, which presents the most complex clinical features. For this reason, we believe that a new classification that helps physicians to suspect AxD based only on neurological and neuroradiological findings, instead of the age at which symptoms present, would be beneficial.

Table 6 Guideline for diagnosing Alexander disease

1. Cerebral Alexander disease (type 1)
   I. Neurological findings
      (a) Core features
         Psychomotor developmental delay/mental retardation, convulsions, macrocephaly
      (b) Supportive features
         Dysarthria, dysphagia, dysphonia, hyperreflexia, cerebellar ataxia, sphincter abnormalities, scoliosis
   II. MRI findings
      (a) Core feature
         Cerebral white matter abnormalities with frontal lobe predominance
      (b) Supportive features
         Signal abnormalities with swelling or atrophy of basal ganglia and thalami, periventricular rim, brainstem lesions, contrast enhancement
2. Bulbospinal Alexander disease (type 2)
   I. Neurological findings
      (a) Core features
         Muscle weakness, hyperreflexia (sometimes hypo- or areflexia), positive Babinski sign, dysarthria, dysphagia, dysphonia
      (b) Not frequent but specific features
         Palatal myoclonus
      (c) Supportive features
         Cerebellar ataxia nystagmus, scoliosis, sleep disorder (i.e., sleep apnea syndrome, REM behavior disorder), parkinsonism, dementia, psychosis, sphincter abnormalities
   II. MRI findings
      (a) Core feature
         Signal abnormalities or atrophy of medulla oblongata and/or cervical cord
      (b) Supportive features
         Signal abnormalities and/or atrophy of cerebellum, white matter lesion, signal abnormalities of basal ganglia and thalami, contrast enhancement
3. Intermediate form (type 3)
   I. Neurological findings
      At least one of the core features in type 1 and at least one of the core features in type 2
   II. MRI findings
      Core feature of type 1 and core feature of type 2

For a case satisfying any of the above-mentioned types, the following definite diagnosis is recommended

Definite diagnosis
   I. Pathological findings
      Existence of numerous Rosenthal fibers in addition to gliosis and loss of myelin
   II. Gene analysis
      GFAP mutation

Approximately 10% of Alexander disease cases seem to show negative *GFAP* mutation in spite of showing typical clinical features of Alexander disease and pathological findings. Therefore, cases satisfying the above clinical features but with negative *GFAP* mutation may be Alexander disease ("possible" Alexander disease)

Based on the above findings, we propose a new guideline where the clinical forms of AxD are classified into the following three types based on neurological and MRI findings: (1) cerebral (type 1), (2) bulbospinal AxD (type 2), and (3) intermediate form (type 3) (Table 6). The primary objective of our guidelines is to increase diagnostic yield by suspecting AxD based on neurological symptoms and MRI findings, which will lead to genetic or pathological testing. Hence, we decided to increase the sensitivity instead of increasing the positive predictive value. On the basis of these new guidelines, we have corrected and updated the neurological and MRI findings for the cases included in our study (Table 7, 8) to 12 cases of type 1 (previously, 9 cases were classified as infantile AxD and 3 as juvenile AxD), 16 cases of type 2 (all cases were previously classified as adult AxD), and 7 cases of type 3 (previously, 1 case was classified as infantile AxD, 5 cases as juvenile AxD, and 1 case as adult AxD).

The phenotypes may seem to vary greatly between type 1 and type 2, but from changes in the images of

Table 7 Summary of neurological signs of Alexander disease classified by the proposed guideline

	Type 1 ($n = 12$)	Type 2 ($n = 16$)	Type 3 ($n = 7$)
Age at onset	3 m to 5 y	26 to 61 y	9 m to 30 y
Muscle weakness	33.0% (1/3)	87.5% (14/16)	60.0% (3/5)
Tendon reflex abnormality	77.8% (7/9)	93.8% (15/16)	83.3% (5/6)
Hyperreflexia	77.8% (6/9)	93.8% (15/1)	83.3% (5/6)
Hyporeflexia or areflexia		12.5% (2/16)	
Babinski sign	33.0% (1/3)	81.3% (13/16)	80.0% (4/5)
Parkinsonism	0.0% (0/3)	25.0% (4 16)	20.0% (1/5)
Sensory disturbance	0.0% (0/3)	18.8% (3/16)	0.0% (0/5)
Dysarthria	100.0% (8/8)	87.5% (14/16)	100.0% (7/7)
Dysphonia	63.8% (7/11)	68.8% (11/16)	42.9% (3/7)
Dysphagia	54.5% (6/11)	87.5% (14/16)	57.1% (4/7)
Nystagmus	0.0% (0/8)	68.8% (11/16)	0.0% (0/7)
Limb ataxia	14.3% (1/7)	33.3% (4/12)	42.9% (3/7)
Truncal ataxia	0.0% (0/7)	50.0% (6/12)	83.3% (5/6)
Palatal myoclonus	0.0% (0/8)	40.0% (6/15)	0.0% (0/6)
Orthostatic hypotension	0.0% (0/3)	7.7% (1/13)	50.0% (1/2)
Sphincter abnormalities	27.3% (3/11)	53.8% (7/13)	28.6% (2/7)
Sleep disorder	0.0% (0/2)	30.8% (4/13)	50.0% (l/2)
Convulsions	90.9% (10/11)	0.0% (0/15)	100.0% (7/7)
Mental retardation/psychomotor developmental delay	90.0% (9/10)	0.0% (0/15)	100.0% (7/7)
Dementia		26.7% (4/15)	0.0% (0/1)
Macrocephaly	80.0% (8/10)		50.0% (3/6)
Scoliosis	45.5% (5/11)	13.3% (2/15)	50.0% (3/6)

Table 8 Summary of MRI findings of Alexander disease classified by the proposed guideline

	Type 1 ($n = 12$)	Type 2 ($n = 16$)	Type 3 ($n = 7$)
White matter lesion	100.0% (12/12)	33.3% (5/15)	100.0% (7/7)
Abnormalities of basal ganglia, thalamus	81.8% (9/11)	46.7% (7/15)	71.4% (5/7)
Abnormalities of brainstem			
Medulla oblongata	0.0% (0/9)	100.0% (15/15)	100.0% (7/7)
Pons	0.0% (0/9)	73.3% (11/15)	100.0% (7/7)
Midbrain	11.1% (1/9)	73.3% (11/15)	100.0% (6/6)
Abnormalities of cervical cord	0.0% (0/6)	100.0% (15/15)	75.0% (3/4)
Abnormalities of cerebellum	20.0% (2/10)	60.0% (9/15)	94.3% (6/7)
Periventricular rim	77.8% (7/9)	26.7% (4/15)	94.3% (6/7)
Enhancement	66.7% (4/6)	8.3% (1/12)	

recently reported long-term survival infantile cases [30], and from the progression of medulla oblongata and cervical spinal cord atrophy seen in an adult cases [31], we believe that certain factors related to the developmental stage determine the severity of cerebral white matter pathological change.

In conclusion, we report a large number of AxD patients in Japan, and provide an estimate of the overall prevalence of the disease with relative frequencies of the three forms. In addition, we propose new clinical guidelines for diagnosing AxD based on simplified classifications. We hope that this report and the guidelines we propose will lead to higher diagnostic yield in the future.

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Conflicts of interest None.

Appendix

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Research Report

Zonisamide-induced long-lasting recovery of dopaminergic neurons from MPTP-toxicity

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ABSTRACT

Zonisamide is an antiepileptic drug that also improves the cardinal symptoms of Parkinson's disease. This study investigated the effects of zonisamide on dopaminergic neuronal degeneration in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. Six groups of mice were treated as follows: 1) normal saline; 2) MPTP, 15 mg/kg×4 every 2 h; 3) MPTP and zonisamide, 40 mg/kg×1, 1 h after the last MPTP dose; 4) MPTP and zonisamide, 1 day after the last dose of MPTP; 5) MPTP and zonisamide, 1 h before the first MPTP dose; and 6) zonisamide, 40 mg/kg. MPTP-treatment decreased the contents of dopamine as well as the number and area of tyrosine hydroxylase (TH)-positive neurons. Concurrent treatment of mice with zonisamide and MPTP did not show any inhibition of the toxic effect of MPTP towards dopamine contents at 1 week after treatment but it increased the number and area of TH-positive neurons compared to the MPTP-treated group. Surviving TH-positive neurons had recovery of dopamine production after several weeks. Moreover, zonisamide increased the number of S100β-positive and glial fibrillary acidic protein (GFAP)-positive astrocytes and dopamine turnover. These results suggest that zonisamide acts as a neuro-protectant against MPTP-induced dopaminergic neuronal degeneration as shown by an increase of TH-positive neurons and this may be mediated by increased S100β secretion.

1. Introduction

Zonisamide (1,2-benzisoxazole-3-methanesulfonamide) was synthesized in 1972 in Japan and has been used as an anti-epileptic drug (Sackellares et al., 1985). The anti-parkinsonian effect of zonisamide was discovered during clinical experience where it reduced tremor or akinesia in patients with Parkinson's disease (Murata et al., 2007). An open-label trial of zonisamide (50–200 mg/day) treatment showed lessening of symptoms, especially wearing-off of Parkinson's disease, when it was used in combination with anti-parkinsonian drugs (Murata et al., 2001) and more than 30% improvement of

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total Unified Parkinson's Disease Rating Score for up to 3 years (Murata, 2004). The activation of dopamine synthesis and a moderate degree of monoamine oxidase type B (MAO-B) inhibition have been proposed as the mechanisms for the effects of zonisamide in Parkinson's disease (Murata, 2004). Zonisamide attenuates 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity by reversible MAO-B inhibition (Sonsalla et al., 2010). Zonisamide is effective in alleviating the neurotoxicity of dopamine quinones which cause dopaminergic neuron-specific oxidative stress (Asanuma et al., 2008) and it has also shown selective neuroprotection against complex I mitochondrial dysfunction (Costa et al., 2010). Zonisamide treatment attenuates MPTP neurotoxicity by an increase of tyrosine hydroxylase (TH) protein in the dopaminergic system (Yano et al., 2009; Sonsalla et al., 2010) and increases striatal dopamine turnover (Yabe et al., 2009). Moreover, the nigral dopaminergic neurotoxic effects of MPTP were attenuated by zonisamide in marmosets (Choudhury et al., 2010). A recent study has shown that zonisamide shows neuroprotective effects by enhancing astroglial cyst(e)ine transport and/or astroglial S100β production or secretion (Asanuma et al., 2010). The present investigation was conducted to examine the effects of zonisamide on dopaminergic neuronal degeneration and astrocyte cell density in MPTP-treated mice.

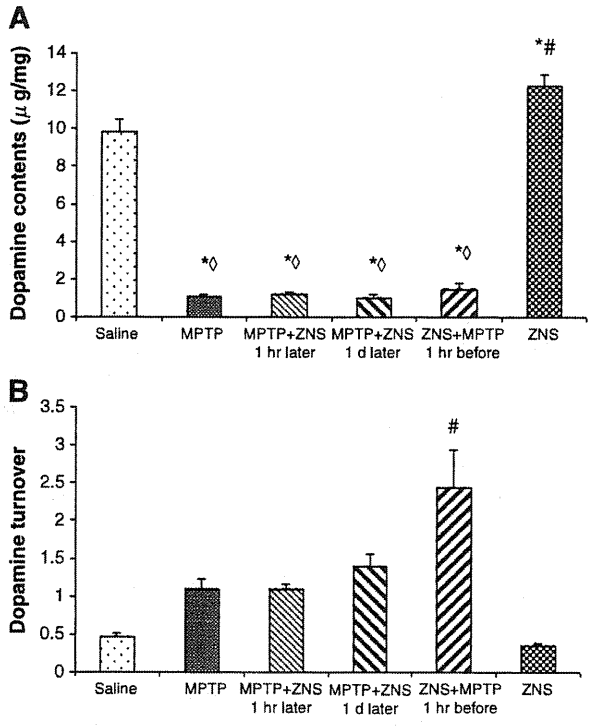

Fig. 1 – Effects of zonisamide administered 1 week after MPTP on (A) dopamine contents and (B) dopamine turnover [(DOPAC+HVA)/DA]. Saline: normal control; MPTP: MPTP, 15 mg/kg×4 every 2 h; MPTP+ZNS (1 h later): MPTP treatment followed by zonisamide 40 mg/kg 1 h later; MPTP+ZNS (1 d later): MPTP treatment followed by zonisamide 1 day later; ZNS+MPTP (1 h before): zonisamide 1 h before MPTP treatment; and ZNS: zonisamide, 40 mg/kg. Values are presented as the mean±SEM (n=7–9 per group). The levels of significance were analyzed by ANOVA, with *post hoc* Tukey test: *$P<0.05$ vs. saline; #$P<0.05$ vs. MPTP; ◊$P<0.05$ vs. ZNS.

2. Results

2.1. *Effects of zonisamide on dopamine contents and its metabolites 1 week after MPTP administration*

The contents of dopamine and dopamine turnover in the striatum at 1 week after MPTP-treatment are shown in Fig. 1A and B, respectively. MPTP treatment reduced the contents of dopamine to 11% of the normal control group ($P<0.001$ vs. normal control). Post-treatment with zonisamide 1 h after MPTP administration reduced dopamine contents to 12% of the normal control group ($P<0.001$ vs. normal control) and post-treatment with zonisamide 1 day after MPTP administration also reduced dopamine contents to 10% of the normal control group ($P<0.001$ vs. normal control). Pre-treatment with zonisamide prior to MPTP reduced the dopamine contents to 15% of the normal control group ($P<0.001$ vs. normal control). The group that was pre-treated with zonisamide prior to MPTP significantly increased dopamine turnover ($P<0.01$ vs. MPTP) compared with MPTP.

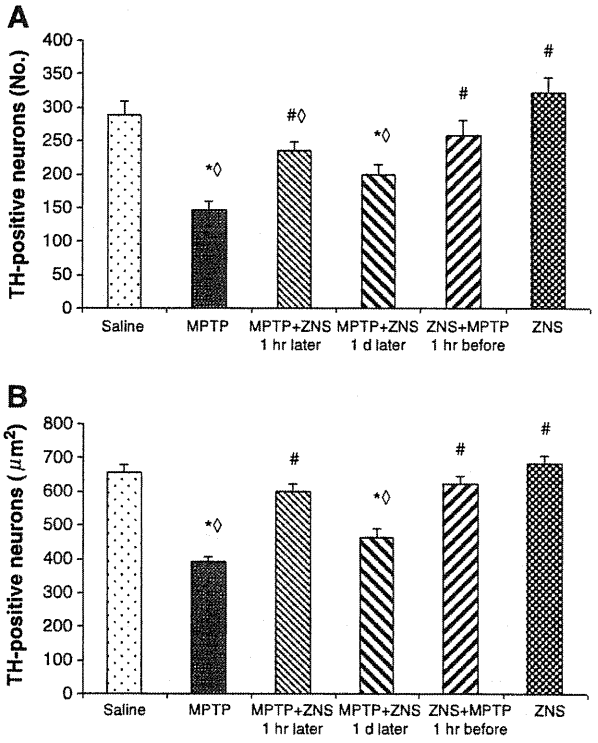

Fig. 2 – Effects of zonisamide administered 1 week after MPTP on (A) the number and (B) the area of TH-positive neurons in substantia nigra. Values are presented as the mean±SEM (n=6 per group). The levels of significance were analyzed by ANOVA, with *post hoc* Tukey test: sequence of groups and symbols for statistical analysis are same with those in Fig. 1.

2.2. *Effects of zonisamide on TH-positive neurons 1 week after MPTP administration*

2.2.1. *Number*

MPTP treatment produced a remarkable reduction of TH-positive neurons to 51% of the normal control group ($P<0.001$ vs. normal control) and pre-treatment with zonisamide prior to MPTP increased the number of TH-positive neurons by 75% over the MPTP group ($P<0.01$ vs. MPTP) (Figs. 2A and 3). Post-treatment with zonisamide 1 h after MPTP increased the number of TH-positive neurons by 60% over the MPTP group ($P<0.05$ vs. MPTP) (Figs. 2A and 3).

2.2.2. *Area*

In the normal control group, the average area of TH-positive neurons was 654.87 μm^2 (Figs. 2B and 3A) and MPTP reduced the area of TH-positive neurons to 60% of the control group ($P<0.001$ vs. normal control) (Figs. 2B and 3B). Pre-treatment with zonisamide prior to MPTP increased the area of TH-positive neurons by 59% over the MPTP group ($P<0.001$ vs. MPTP) (Figs. 2B and 3E). Post-treatment with zonisamide 1 h after MPTP increased the area of TH-positive neurons by 54% over the MPTP group ($P<0.001$ vs. MPTP) (Figs. 2B and 3C) and post-treatment with zonisamide 1 day after MPTP also increased the area of TH-positive dopaminergic neurons by 18% over the MPTP group (Figs. 2B and 3D).

2.3. *Effects of zonisamide on S100β-positive astrocytes 1 week after MPTP administration*

MPTP treatment increased the number of S100β-positive astrocytes by 67% over the normal control group ($P<0.001$ vs. normal control) and pre-treatment with zonisamide prior to MPTP increased the number of S100β-positive astrocytes by 82% over the MPTP group ($P<0.001$ vs. MPTP) (Figs. 4A and 5). Post-treatment with zonisamide 1 h after MPTP increased the number of S100β-positive astrocytes by 27% over the MPTP group ($P<0.001$ vs. MPTP) and post-treatment with zonisamide 1 day after MPTP

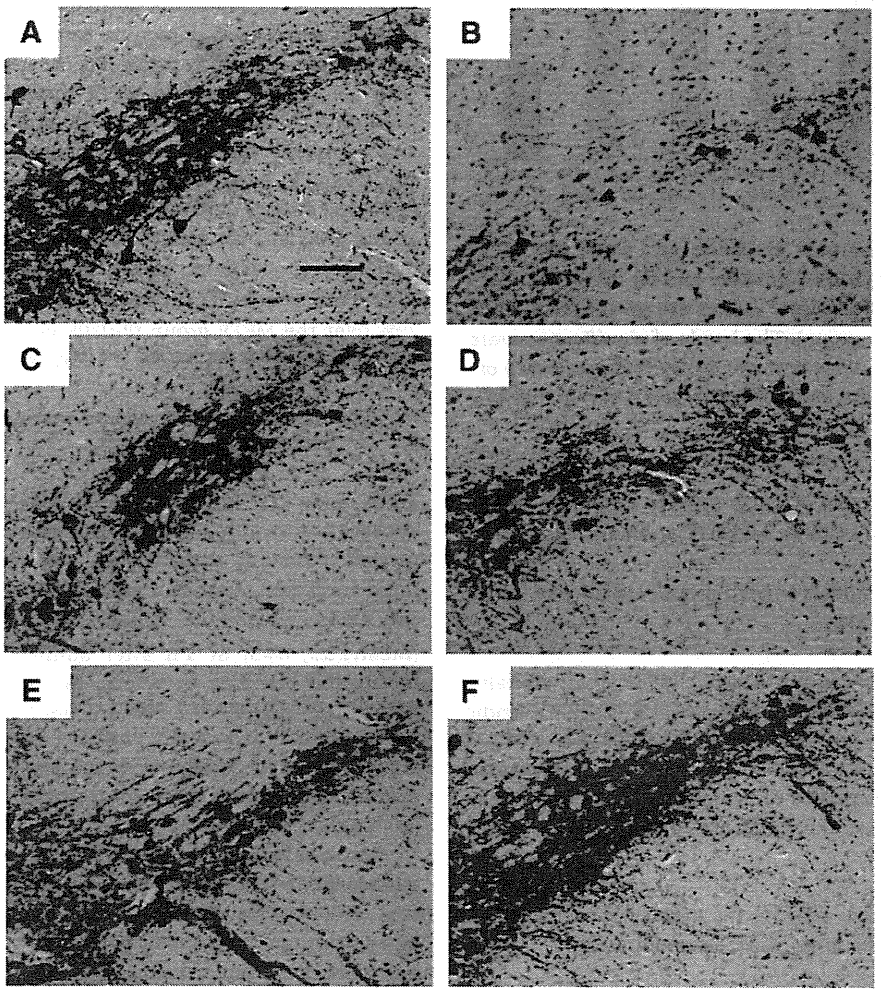

Fig. 3 – Images of TH-positive neurons with DAB immunostaining in the substantia nigra counterstained with hematoxylin (1 week after MPTP-treatment): (A) normal control; (B) MPTP 15 mg/kg × 4 every 2 h; (C) MPTP treatment followed by zonisamide 40 mg/kg 1 h later; (D) MPTP treatment followed by zonisamide 1 day later; (E) zonisamide 1 h before MPTP treatment; and (F) zonisamide 40 mg/kg. Bar scale, 100 μm.

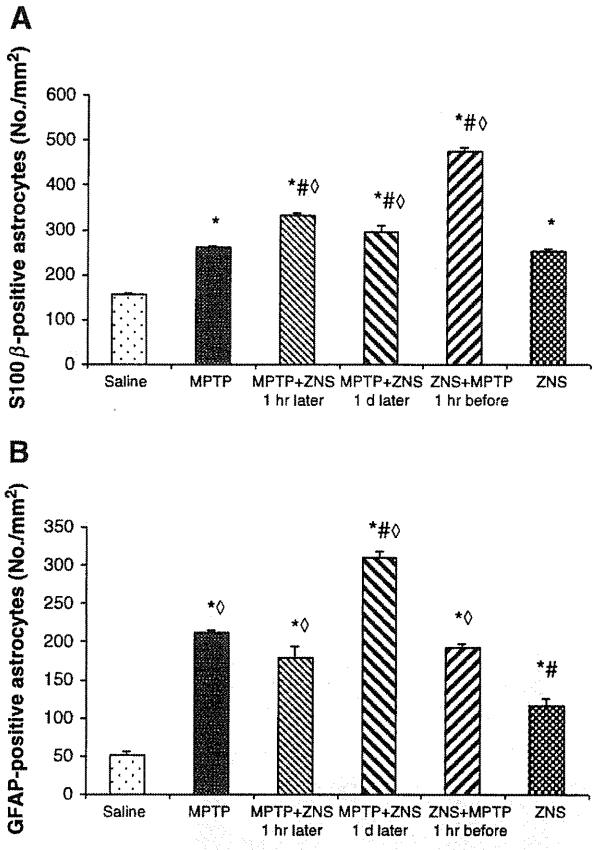

Fig. 4 – Effects of zonisamide administered 1 week after MPTP on the number of (A) S100β-positive and (B) GFAP-positive astrocytes in substantia nigra. Values are presented as the mean ± SEM ($n=6$ per group). The levels of significance were analyzed by ANOVA, with post hoc Tukey test: sequence of groups and symbols for statistical analysis are same with those in Fig. 1.

also increased the number of S100β-positive astrocytes by 13% over the MPTP group ($P<0.05$ vs. MPTP) (Figs. 4A and 5).

2.4. *Effects of zonisamide on GFAP-positive astrocytes 1 week after MPTP administration*

Compared to the normal control group, MPTP treatment also significantly increased the number of glial fibrillary acidic protein (GFAP)-positive astrocytes ($P<0.001$ vs. normal control) (Figs. 4B and 6). Post-treatment with zonisamide 1 day after MPTP increased the number of GFAP-positive astrocytes by 46% over the MPTP group ($P<0.001$ vs. MPTP) (Figs. 4B and 6).

2.5. *Effects of zonisamide on the dopamine contents and its metabolites 3 weeks after MPTP administration*

The striatal dopamine contents and its turnover at 3 weeks after MPTP treatment are presented in Fig. 7. Dopamine contents were reduced to 16% of the normal control group at 3 weeks after MPTP treatment ($P<0.001$ vs. normal control) and dopamine turnover was also increased ($P<0.001$ vs. normal control). Post-treatment with zonisamide 1 h after MPTP administration reduced dopamine contents to 33% of the normal control group ($P<0.001$ vs. normal control) and dopamine turnover was increased ($P<0.01$ vs. normal control). Post-treatment with zonisamide 1 day after MPTP administration also reduced dopamine contents to 22% of the normal control group ($P<0.001$ vs. normal control) and dopamine turnover was also increased ($P<0.001$ vs. normal control). Pre-treatment with zonisamide prior to MPTP reduced dopamine contents to 31% of the normal control group ($P<0.001$ vs. normal control) and dopamine turnover was increased ($P<0.001$ vs. normal control). However, compared to MPTP treatment, the group that was pre-treated with zonisamide 1 h prior to MPTP and the group that was post-treated with zonisamide 1 h after MPTP significantly decreased dopamine turnover ($P<0.01$ vs. MPTP and $P<0.05$ vs. MPTP, respectively).

2.6. *Effects of zonisamide on the dopamine contents and its metabolites 9 weeks after MPTP administration*

Nine weeks after MPTP-treatment, striatal dopamine contents were reduced to 24% of the normal control group ($P<0.001$ vs. normal control) and dopamine turnover was also increased ($P<0.001$ vs. normal control) (Fig. 8A and B). Pre-treatment with zonisamide prior to MPTP increased the contents of dopamine by 168% over the MPTP group ($P<0.001$ vs. MPTP) and dopamine turnover was decreased ($P<0.001$ vs. MPTP) (Fig. 8A and B). Post-treatment with zonisamide 1 h after MPTP increased striatal dopamine contents by 175% over the MPTP group ($P<0.001$ vs. MPTP) while dopamine turnover was decreased ($P<0.001$ vs. MPTP). Post-treatment with zonisamide 1 day after MPTP increased the contents of dopamine by 79% over the MPTP group ($P<0.05$ vs. MPTP) and dopamine turnover was also decreased ($P<0.01$ vs. MPTP) (Fig. 8A and B).

3. Discussion

Zonisamide is a drug used for seizure therapy and is also approved as an adjunctive treatment for Parkinson's disease in Japan. For our experiments, zonisamide was administered subcutaneously at a dose of 40 mg/kg. With this dose, the blood zonisamide concentration was 21.3 ± 1.2 μg/ml (mean ± SD, $n=5$) at 1 h after administration in common marmosets (Yabe et al., 2009). This is well correlated with both the recommended blood concentration of zonisamide (10–30 mg/ml) in human patients with epilepsy (Murata, 2004) and to a previous report showing the maximum plasma concentration of zonisamide in rhesus monkeys (Matsumoto et al., 1983).

Zonisamide attenuated the depletion of dopamine contents in MPTP-treated mice by an increase of TH-protein (Yano et al., 2009) and also by MAO-B inhibition (Sonsalla et al., 2010). By contrast, post-treatment with zonisamide after MPTP did not prevent the reduction of dopamine contents (Yokoyama et al., 2010). In this study, concurrent treatment of mice with zonisamide and MPTP did not show any significant effect on MPTP-treated dopamine depletion after one week of administration (Fig. 1A). Nonetheless, pre-treatment with zonisamide or post-treatment with zonisamide prevented a decrease