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Citation
Nagasaki University (長崎大学) 博士 医学 (2013-08-07)

Issue Date
2013-08-07

URL
http://hdl.handle.net/10069/35228

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FK506 reduces abnormal prion protein through the activation of autolysosomal degradation and prolongs survival in prion-infected mice

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Keywords: FK506, prion, autophagy, therapy, microglia, degradation, tacrolimus

Introduction

The transmissible spongiform encephalopathies (TSE), prion diseases, are fatal neurodegenerative disorders and include Creutzfeldt-Jakob disease (CJD) in humans. Histologically, TSEs are characterized by neuronal loss, maculation and activation of astrocytes and microglia, and the conformational conversion of normal prion protein (PRNP) to the abnormal form (PRNPSc) is central to the pathogenesis.1,2 For this reason, drug discovery for the prion diseases has focused primarily on compounds capable of inhibiting the conversion of PRNP. A number of drugs, including pentosan polysulfate (PPS),1 quinacrine,4 anti-PRNP antibodies5–6 and others,7–8 have been proposed as potential anti-prion agents. Most act by binding to PRNPSc and inhibiting the interaction between PRNPSc and PRNP, resulting in a reduction of the conversion of PRNPSc into PRNPSc. PPS has been shown to prolong the survival of infected animals, but only when the drug is administered into the brain directly because it cannot cross the blood brain barrier.9 Anti-PRNP antibody treatments present the same problem. Quinacrine is strongly inhibitory in vitro but has little effect on patient survival.10 Other small compounds that show anti-prion effect in vitro have been reported to possess prophylactic effect in vivo but failed to stop pathogenesis of the disease after onset. Put simply, an effective treatment for patients with prion disease might be needed.7,8

Prion diseases are generally thought to be caused by the accumulation of misfolded, aggregate-prone proteins such as α-synuclein in Parkinson disease or dementia with Lewy bodies, and...
polyglutamine-containing proteins in Huntington disease or spinocerebellar ataxia, and PRNP in prion diseases. The clearance of these aggregated proteins depends mainly upon autophagy, which is the major molecular mechanism for degrading cytoplasmic materials in the lysosome, and over the past decade, many researchers have focused on the autophagy-lysosomal system as a quality control regulator of protein in neurodegenerative diseases. Of note, the chemical-induced enhancement of autophagy has been reported to decrease aggregates in the CNS and retard the progression of neurological symptoms in several animal and cell culture models of neurodegenerative diseases, including TSE. FKBP12-FK506 complex is known to interact with and inhibit calcineurin, resulting in the suppression of both T-lymphocyte signal transduction and IL2 transcription. Recent studies reveal neuroprotective effects in cerebral ischemia and several neurodegenerative disorders, including TSE. On the other hand, FKBP39 has recently been reported to suppress autophagy in drosophila. This result raises the possibility that FK506 could also influence the autophagy-lysosomal system in mammalian cells. In this study, we examined the effect of FK506 treatment on prion infection, autophagic pathways, and the relationship between them, using prion-infected cell cultures and animal models.

Results

FK506 decreases PRNPSc in cell culture models. To investigate the effect of FK506 on the amount of PRNPSc, we treated neuroblastoma cells overexpressing PRNP (N2a58 cells) and infected them with the mouse-adapted Gerstmann-Sträussler-Schenker syndrome (GSS) Fukuoka-1 strain (N2a58/Fukuoka-1), together with different concentrations of FK506 over 48 h, and found that the amount of PRNPSc decreased in correlation with the concentration of FK506 (Fig. 1A and B). The effective concentration for 50% reduction of PRNPSc (IC50) over 48 h was 11.9 μM. We next tested whether FK506 reduces PRNPSc in a microglial cell line overexpressing PRNP (MG20 cells). The inhibitory effect on PRNPSc accumulation was also observed for MG20 cells infected with Fukuoka-1 (Fig. S1A). Furthermore, we determined the effect of FK506 on PRNPSc levels in uninfected cells. FK506-treated N2a58 (Fig. 1C) and MG20 (Fig. S1B) cells exhibited a mild reduction in PRNPSc levels, suggesting that a decrease in PRNPSc may also contribute to a reduction in PRNPSc accumulation.

FK506, also named tacrolimus, is well known as an immunosuppressive drug that is mainly used post-transplantation to decrease the activity of the recipient’s immunity. It has been reported that FK506 binds to the immunophilin FKBP12 (FK506 binding protein), and creates a new complex. This complex is known to interact with and inhibit calcineurin, resulting in the suppression of both T-lymphocyte signal transduction and IL2 transcription. Recent studies reveal neuroprotective effects in cerebral ischemia and several neurodegenerative disorders, including TSE. On the other hand, FKBP39 has recently been reported to suppress autophagy in drosophila. This result raises the possibility that FK506 could also influence the autophagy-lysosomal system in mammalian cells. In this study, we examined the effect of FK506 treatment on prion infection, autophagic pathways, and the relationship between them, using prion-infected cell cultures and animal models.

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To examine whether FK506 directly binds to PRNPSc, we examined the affinity of FK506 to recombinant PRNP (recPRNP) using the surface plasmon resonance (SPR) method. The positive control, GN8, that is known to bind to PRNPSc and inhibit the conversion, showed the typical binding signals in a dose-dependent manner, while the affinity of FK506 to recPRNP was very low (Fig. S3). Although most of the compounds reported to have therapeutic action in prion diseases bind to PRNPSc, SPR revealed that FK506 did not bind to recPRNP, indicating that the anti-prion effect of FK506 is indirect.
FK506 increases the levels of autophagy-related molecules and the formation of autolysosomes in prion-infected cell culture models. To investigate the effect of FK506 on the activation of autophagy, we first analyzed the levels in N2a58/Fukuoka-1 cells of several autophagy-related molecules: LC3-II, ATG7, ATG12–ATG5 complex and BECN1/Beclin 1.30 An increase in all the molecules tested was observed following treatment with FK506 (Fig. 1A). Autolysosomes were evaluated by staining with an auto-fluorescent drug, monodansylcadaverine (MDC), which detects acidic compartments including autolysosomes.30 Because autophagy is strongly induced by starvation, increased fluorescence was seen in the cells treated with Hank’s Balanced Salt Solutions (HBSS), as the positive control (Fig. 2A). A similar increase in MDC signals was seen in the FK506-treated N2a58/Fukuoka-1 cells, which had twice as many vacuoles as the untreated cells (Fig. 2A). The increased signals were also observed for uninfected N2a58 cells treated with FK506 (Fig. S2).

To determine whether the degradation of PRNPSc was due to autolysosomal activity, we treated the cells with NH4Cl,31 which is an effective inhibitor of lysosomal hydrolases that acts by increasing the pH inside lysosomes. The levels of LC3-II were observed in the following order: FK506 + NH4Cl > NH4Cl > FK506 > no treatment (Fig. 2B). These observations can be explained by the fact that LC3-II itself is also degraded by lysosomal hydrolases. The decreased levels of PRNPSc by FK506 were recovered when the cells were added with NH4Cl (Fig. 2C, lane 4). These results indicate that PRNPSc is actively degraded by autolysosomes when cells are treated with FK506.

FK506 prolongs survival of prion-infected mice. To examine the effect of FK506 on the survival periods of prion-infected mice, CD-1 mice were inoculated intracerebrally with Fukuoka-1 strain. FK506 was intraperitoneally administered (1.0 or 0.1 mg/kg/day) from 20 or 60 d post-inoculation (d.p.i.). In the untreated controls, symptoms appeared at 110 d.p.i. and the mice died around 120 d.p.i. (Fig. 3A; Table 1). While there was no significant difference in symptom onset or survival between the mice treated from 60 d.p.i. and the control, those mice treated from either 20 d.p.i. survived until about 140 d.p.i (Fig. 3A; Table 1). Next, transgenic mice overexpressing Syrian hamster Prnp32 [Tg(Sha Prnp)] were inoculated intracerebrally with hamster scrapie-prion strain 263K. FK506 (1.0 mg/kg/day, orally) was administered either from 14 d.p.i. or 28 d.p.i. The mice treated from 14 d.p.i. survived about 14 d longer than the mice receiving vehicle only (p < 0.05) (Fig. S4). These results indicate that FK506 treatment led to prolonged survival in prion-infected animal models, although the effect was influenced by the timing of administration.

FK506 upregulates autophagy and decreases PRNPSc in the brains of mice. Some of the mice treated from 20 d.p.i. were sacrificed at 110 d.p.i. and their brains examined to determine the degree of accumulation of PRNPSc and levels of autophagy-related molecules. The amount of PRNPSc in the treated mice was considerably less than that in the control at 110 d.p.i. (Fig. 3D and E). In contrast, there was no significant difference between the brains of treated mice and those of the control by the terminal stage. Levels of LC3-II, ATG7 and ATG12–ATG5 complex were significantly increased in the brains of mice receiving FK506 (Fig. 4), supporting the view that FK506 decreased the accumulation of PRNPSc via activation of autolysosomes in animal brains as well as in cells.

FK506 partially suppresses pathological changes associated with prion diseases in Fukuoka-1-infected mouse brains

Next, we analyzed the degree of vacuolation, gliosis and PRNPSc in the brains of Fukuoka-1-infected mice. We evaluated the degree of vacuolation by calculating the percentage of
Figure 3. FK506 prolongs survival in Fukuoka-1-infected mice. (A) Survival curves in the Fukuoka-1-infected mice administered FK506 intraperitoneally. The control mice (n = 6, circle) and FK506-treated mice (group 1: from 20 d.p.i., n = 10, square; group 2: from 60 d.p.i., n = 5, triangle) were compared. Mice in group 1 survived significantly longer than those of the control group (p < 0.01, Logrank test). (B) Comparison of spongiform change at 110 d.p.i. between control animals (vehicle only) and group 1 mice. The brain sections of cortex (Cx), hippocampus (Hip), thalamus (Tha), striatum (St) and cerebellum (Ce) stained with hematoxylin and eosin are shown. Scale bars: 100 μm. (C) In 3 to 5 randomly selected areas of each tissue sample, individual vacuoles were measured and the total vacuolated area was expressed as a percentage of the entire surface of the specimen. Statistical significance was determined using the two-tailed Student’s t-test. *p < 0.05 compared with the control. Error bars indicate SD (n = 3). (D) Accumulation of PRNPSc in brain tissues at 110 d.p.i. (upper panel) and terminal stage (lower panel) from controls (vehicle only) or group 1 mice was analyzed by western blotting. (E) Immunohistochemical staining of PRNPSc in thalamus samples of mice sacrificed at 110 d.p.i. and those sacrificed at the terminal stage, using SAF32 antibody. Scale bars: 100 μm. Data are representative of at least three mice.
Table 1. Survival periods of prion-infected mice administered FK506

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>FK506 (mg/kg/day)</th>
<th>Start point (d.p.i)</th>
<th>Number</th>
<th>Mean ± SD (days)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-1 Fukuoka-1</td>
<td>0</td>
<td>6</td>
<td>125.5 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>60</td>
<td>5</td>
<td>128 ± 2.1</td>
<td>n.s</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>60</td>
<td>5</td>
<td>123 ± 2.1</td>
<td>n.s</td>
</tr>
</tbody>
</table>

n.s, no significance

Discussion

In our experiments, accumulation of PRNPSc was suppressed by FK506 treatment in both prion-infected cells and mice. Furthermore, increased amounts of autophagy-related molecules such as LC3-II, ATG12–ATG5 complex and ATG7 were detected in FK506-treated prion-infected cells. These results support the notion that FK506 enhances the degradation of PRNPSc via upregulation of autophagy, although the molecular basis remains to be elucidated.

There are two major pathways involved in the degradation of abnormally folded proteins in cells: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system. The UPS is involved in the targeted degradation of most short-lived proteins or proteins that fold improperly within the endoplasmic reticulum. Although inhibition of the UPS causes cytosolic PRNPSc accumulation, it is unlikely that the degradation of PRNPSc occurs mainly in the UPS, as PRNPSc aggregates are unable to enter the narrow channel into the proteasome. On the other hand, the UPS has been reported to be impaired in TSEs, and it has been demonstrated that both PRNPSc and aggregated β-sheet-rich recPRNP inhibit the activity of proteasomes. Thus, it is reasonable to postulate that autophagy is one of the major degradation pathways of PRNPSc.

Mukherjee et al. have recently reported that FK506 suppressed the function of calcineurin leading to the reduction of prion-induced neurodegeneration, and concluded that FK506 treatment does not alter the extent of accumulation of PRNPSc, astrocytosis or microglial activation at the terminal stage of the disease. These findings are consistent with our observation that no significant difference was observed between the brains of FK506-treated mice and those of control animals at the terminal stage. However, in our study, both accumulation of PRNPSc and microgliosis in the mice treated from 20 d.p.i. were decreased at 110 d.p.i., when symptoms were already in evidence in the untreated controls. These observations indicate that both the rate of accumulation of PRNPSc and microgliosis were decreased in the FK506-treated mice, and it is most likely that the delayed accumulation of PRNPSc was primarily due to the increased degradation of PRNPSc via activation of autophagy. Furthermore, our SPR experiments revealed that there was no direct interaction between recPRNP and FK506, providing further evidence that the principle anti-prion effect of FK506 is not due to inhibition of the conversion into PRNPSc.

The importance of autophagy in the clearance of PRNPSc is also supported by the fact that certain chemicals such as trehalose, lithium and rapamycin decrease the amount of PRNPSc in prion-infected cultured cells or mice by inducing the upregulation of autophagy. In contrast, treatment with trehalose or lithium did not significantly prolong the survival of prion-infected mice, although these findings may be partially explained by the fact that the effective concentration of both drugs is extremely high (mM range), and therefore difficult to achieve in vivo. Rapamycin did extend survival to a moderate extent only under certain experimental conditions. In our study, the early administration at 20 d.p.i of FK506 prolonged the survival of Fukuoka-1-infected mice. In contrast, the late administration (60 d.p.i) had little effect on survival duration. The difference in the protective effect afforded by FK506 as a consequence of administration time may be explained by the assumption that activation of autophagy can completely disrupt small aggregates of PRNPSc, but not large PRNPSc aggregates. It is reasonable to postulate that the size of PRNPSc aggregates has increased by the late stage. Moreover, autophagy may sometimes cause fragmentation of large aggregates of PRNPSc, resulting in enhancement of PRNPSc formation. The above possibilities also explain why there was no difference in accumulated PRNPSc at the terminal stage. However, further studies are required to investigate the molecular basis of these results and the optimum time for administration of FK506.

Increasing evidence suggests that inflammation processes such as gliosis are involved in the pathogenesis of prion diseases.
Suppression of microglial activation by FK506 has also been reported in cerebral ischemia and a tauopathy mouse model. Microglia are thought to exert two opposing effects in prion diseases. Following exposure to PRNPSc, microglia produce neurotoxins that exacerbate neurodegeneration. In contrast, it is reported elsewhere that activated microglia may afford protection to neurons through phagocytosis and digestion of aggregated PRNPSc. It is possible that the balance between the two contrary effects of microglial activation may proceed in a time-dependent manner. When treatment was initiated at an early stage (20 d.p.i.) microglia appeared to play a deteriorative role, while at a late stage (60 d.p.i.) they offered a protective role. Nevertheless, further studies are needed to determine the exact role of microgliosis in the pathogenesis of prion diseases, and to establish the contribution by FK506 to the suppression of microglial activation, with that of stimulating autophagy. FK506 immunosuppression is clearly mediated by the inhibition of calcineurin in T-cells, but it remains to be determined whether suppression of microglial activation and activating autophagy depend on the inhibition of calcineurin. Thus, it would be worthwhile examining the effect of non-calcineurin inhibiting derivatives such as V-10,367 and GPI-1046.44 Inhibition of the peptidyl-prolyl isomerase activity of FKBPs may constitute another mechanism for the anti-prion effect of FK506, as a number of FKBPs are suspected to accelerate the aggregation of abnormal proteins.42,43 Thus, it would be worthwhile examining the effect of non-calcineurin inhibiting derivatives such as V-10,367 and GPI-1046.44

FK506 has the advantage of having been widely used for many years in the clinical setting, in contrast to most of the other substances that have been proposed as therapeutic agents for TSEs. Of particular interest is its effect on Crohn disease, since carriers of the ATG16L1 gene and certain other mutations which are thought to impair autophagy, have increased susceptibility to the disease.45,46 Furthermore, oral administration of FK506 has clinical benefit because of its ease of administration and its low cost. Taken together, these characteristics raise the possibility that FK506 may become a valuable therapeutic agent for prion diseases. It would be of great value to additionally examine whether combination therapy using FK506 together with other drugs possessing different areas of action would be an even more beneficial strategy for the effective management of TSEs.

In conclusion, we have shown that FK506 treatment decreases the levels of PRNPSc in prion-infected cell culture models and prolongs the survival of prion-infected mice, both of which are accompanied by upregulation of autophagy-related molecules. Our findings provide evidence that FK506, in addition to attenuation of microglial activation and neuroprotection, induces the activation of the autophagy-lysosomal system and facilitates the elimination of accumulated PRNPSc via this mechanism.

Materials and Methods

Ethics statement. All animal experiments were permitted by the committee of the Nagasaki University in accordance with the Guidelines for Animal Experimentation of Nagasaki University and conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Reagents. M-20 antibody (Santa Cruz Biotechnology, sc7694) is a goat polyclonal antibody recognizing the C-terminus of mouse PRNP. SAF32 antibody (SPI-BIO, A03202) is a mouse monoclonal antibody recognizing the octapeptide repeats in mouse PRNP. The anti-AIF1/IBA1 antibody to detect microglia (Wako Pure Chemical Industries, 016–20001 for western blotting, 019-19741 for immunohistochemistry) and the anti-GFAP antibody to detect astrocytes (DAKO, Z033429) are rabbit polyclonal antibodies. Antibodies to detect autophagy-related molecules were rabbit polyclonal antibodies: anti-ATG5 (Cell Signaling Technology, 2630), -ATG12 (2011), -BECN1 (3738), -LC3 (Medical and Biological Laboratories, PM036) and

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-ATG7 (Sigma Aldrich, A2856). FK506 (20% powder dissolved in water) for use in the animal studies was a gift from Astellas Pharma Inc. FK506 (Sigma-Aldrich, F4679) for use in the cell culture model assay was dissolved in dimethyl sulfoxide (DMSO; Sigma, D4540). Monodansylcadaverine (MDC; Sigma, 30432) was used for labeling autolysosomes and dissolved in ethanol (Nacalai, 14713-95).

**Animal models.** Four-week-old transgenic mice overexpressing hamster PRNP [Tg(Sha Prnp)], and CD-1 male mice (Charles River Laboratories International), were inoculated intracerebrally with 20 μl of brain homogenate from 263K-infected hamster and Fukuoka-1-infected mice, respectively. Mice were monitored weekly until the terminal stage of disease or until sacrificed. Clinical onset was defined as the presence of 3 or more of the following signs: greasy and/or yellowish hair, hunchback, weight loss, yellow pubes, ataxic gait and nonparallel hind limbs. Brains were removed, and the right hemispheres frozen and homogenized at 20% (w/v) in phosphate-buffered saline (PBS; Nacalai Tesque, 14249). Total proteins were extracted by mixing with the same amount of 2 × lysis buffer [1% Triton X-100 (Wako, 168-11085), 1% Deoxycholic acid (Wako, 046-18811), 300 mM NaCl (Nacalai, 31320-05), 50 mM Tris (Nakalai, 35434-2)–HCl (Wako, 080-01066), pH 7.5].

**In vivo administration of FK506.** In Fukuoka 1-infected CD-1 mice, FK506 (1.0 or 0.1 mg/kg/day) was intraperitoneally administered from 20 or 60 d post-inoculation (d.p.i.). In 263K-infected Tg(Sha Prnp) mice, treatment with FK506 (1.0 mg/kg/day, orally) was started either from 14 d.p.i. or 28 d.p.i.

**Cell culture.** N2a58 and MG20 cells were prepared as described previously.47,48 Fukuoka-1-infected N2a58 (N2a58/Fukuoka-1) and MG20 cells (MG20/Fukuoka-1) were produced by inoculation with brain homogenates harvested from Fukuoka-1-infected, terminally ill ddY mice. All cell media was supplemented with 10% fetal bovine serum and penicillin-streptomycin (Nacalai, 09367-8500) and Opti-MEM (Gibco-Invitrogen, 31985), respectively. MG20 and MG20/Fukuoka-1 cells were cultured in low-glucose DMEM (Wako, 041-29775) supplemented with 10 μM 2-mercaptoethanol (Sigma, M3148) and 10 μg/ml insulin (Sigma, I3536). Both N2a58/Fukuoka-1 and MG20/Fukuoka-1 cells stably produced PRNPSc for over 30 passages.

**FK506 treatment in cell cultures.** Cells (3.5 × 105 cells/well) were grown in 6-well plates for 24 h prior to the addition of different concentrations of FK506 diluted in the same volume of DMSO. As a negative control, DMSO alone was used. After treatment for 48 h, the proteins were collected in lysis buffer (0.5% Triton X-100, 0.5% Deoxycholic acid, 150 mM NaCl and 50 mM TRIS-HCl, PH 7.5) and analyzed by western blotting. To inhibit lysosomal activity, cells were initially treated with 10 mM of NH4Cl (Wako, 017-02995) for 24 h, after which 30 μM of FK506 was added and the cells were cultured for a further 24 h.

**MDC assay.** Cells were treated with 10 μM of FK506 for 24 h or HBSS for 30 min, and then incubated with 0.1 mM of MDC in PBS for 30 min at 37°C. The cells were then washed with PBS twice and observed using an Axio Observer Z1 (Carl Zeiss, 431007-9901). The granules of MDC were counted using an INCell Analyzer 1000 (GE Healthcare, 25-8010-26).

**Western blotting.** Total protein concentrations were measured using a BCA protein assay kit (Pierce, 23227). To detect PRNPSc, the samples were digested with PK (40 μg/ml protein) for 30 min at 37°C. Loading buffer [50 mM TRIS-HCl (pH 6.8), containing 5% glycerol (Kanto Chemical, 17029-00), 1.6% SDS (Nakalai, 31606-75) and 100 mM dithiothreitol (Nakalai, 14128-62)] was added to the proteins, and the mixtures incubated at 95°C for 10 min. SDS-PAGE was performed using 15% acrylamide gels. The proteins were transferred onto an Immobilon-P membrane (Millipore, IPVH10100) in a transfer buffer containing 20% methanol, and the membrane was blocked with 5% nonfat dry milk in TBST [10 mM TRIS-HCl (pH 7.8), 100 mM NaCl, 0.1% Tween 20 (Wako, 591-09825)] for 60 min at room temperature and reacted with primary antibody overnight at 4°C. Immunoreactive bands were visualized using the enhanced ECL plus chemiluminescence system (GE Healthcare, RPN2132).

**Histochemistry.** The brain tissues were fixed in 10% neutral buffered formalin (Wako, 066-03847). The fixed hemispheres were embedded in paraffin and sectioned into 3 μm slices. To evaluate the spongeiform change, the tissue sections were stained with hematoxylin (Wako, 131-09665) and eosin (Wako, 056-06722). For AIF1 and GFAP staining, after deparaffinization and rehydration, the sections were treated with 0.3% hydrogen peroxidase (Wako, 086-07445) in methanol (Hayashi Pure Chemical, 130-02069) for 30 min to inactivate endogenous peroxidase and then incubated with 3% nonfat dry milk (Megmilk Snow Brand, FA-08) in TBST for 60 min at room temperature. The blocked sections were reacted with primary antibody overnight at room temperature, then reacted with enzyme polymer horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G antibodies (Dako, K4002) for 60 min at room temperature. Immunostaining was visualized using 3, 3-diaminobenzidine (DAB; Dojindo Lab, D06722). The hydrolytic autoclaving and formic acid method for PRNPSc staining has been described previously.39

**Statistical analysis.** The unpaired t-test or Welch’s correction was used for comparison between the two groups. For multiple comparison the one-way ANOVA followed by the Tukey-Kramer test was used. The log rank test was used for analyzing the survival time of prion-infected mice. All statistical analysis was performed using GraphPad Prism software.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Acknowledgments**
We thank Dr. Hitoki Yamanaka for helpful discussions, and Mari Kudo, Atsuko Matsuo and Ayumi Yamakawa for technical assistance. This work was supported by the Global Centers of Excellence Program (F12); a grant-in-aid for scientific research (B;


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