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Satoru, Takahashi ; Ashok B. Kulkarni

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Satoru Takahashi and Ashok B. Kulkarni

Functional Genomics Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892, USA

Abbreviations used: ALS, amyotrophic lateral sclerosis; Cdk5, cyclin-dependent kinase 5; ChAT, choline acetyltransferase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SOD1, superoxide dismutase 1

To whom correspondence should be addressed:
Ashok B. Kulkarni, Ph.D.
Functional Genomics Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health,
Bldg. 30, Room 527, 30 Convent Drive, Bethesda, MD 20892, USA
Tel.: 301-435-2887; Fax: 301-435-2888; E-mail: ak40m@nih.gov

Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by selective loss of motor neurons in the brain and spinal cord. Neurotoxicity mediated by glutamate is thought to play a role in the neuronal death through intracellular calcium-dependent signaling cascades. Cyclin-dependent kinase 5 (Cdk5) has been proposed as one of the calcium-dependent mediators that may cause neuronal death observed in this disease. Cdk5 is activated in neurons by the association with its activators, p35 or p39. The calcium-activated protease calpain cleaves p35 to its truncated product, p25, which eventually causes the cellular mislocalization and prolonged activation of Cdk5. This deregulated Cdk5 induces cytoskeletal disruption and apoptosis. To examine whether inhibition of the calpain-mediated conversion of p35 to p25 can delay the disease progression of ALS, we generated double transgenic mice in which ALS-linked copper/zinc superoxide dismutase 1 mutant ($SOD1^{G93A}$) was expressed in a p35-null background. The absence of p35 neither affected the onset and progression of motor neuron disease in the mutant SOD1 mice nor ameliorated the pathological lesions in these mice. Our results provide direct evidence that the pathogenesis of motor neuron disease in the mutant SOD1 mice is independent of the Cdk5 activation by p35 or p25.

Key words: ALS, SOD1, Cdk5, p35, Alzheimer's disease, neurodegeneration

Running title: Cdk5 and neurodegeneration in a mouse model of ALS

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset, neurodegenerative disease characterized by loss of motor neurons in the brain and spinal cord, leading to paralysis and ultimately to death (Cleveland 1999). Mutations in the copper/zinc superoxide dismutase 1 (SOD1) gene have been known to cause the inherited form of ALS through an undefined toxic property of the mutant SOD1 protein (Rosen *et al*. 1993; Gurney *et al*. 1994). However, recent studies have demonstrated that neuron-specific expression of the mutant SOD1 did not cause motor neuron disease (Pramatarova *et al*. 2001; Lino *et al*. 2002), suggesting that its toxic effects on motor neurons are indirect. Furthermore, the mutant SOD1 has been shown to inactivate the glial glutamate transporter that plays an important role in maintaining low extracellular glutamate levels (Rothstein *et al*. 1995; Trotti *et al*. 1999). There is substantial evidence to support the notion that an excess of extracellular glutamate may be a critical factor in causing motor neuron disease (Ludolph *et al*. 2000). Overstimulation by glutamate results in elevated intracellular calcium levels, which in turn activates intracellular calcium-dependent signaling cascades, eventually leading to neuronal death (Sattler and Tymianski 2000). The main pathological feature of ALS is characterized by the selective vulnerability of motor neurons. ALS-vulnerable motor neurons are relatively deficient in the calcium-binding proteins, calbindin D-28K and/or parvalbumin, while extraocular motor neurons with abundant levels of these calcium-binding proteins are less likely to degenerate with the disease progression (Alexianu *et al*. 1994; Siklos *et al*. 1998; Nimchinsky *et al*. 2000). Thus, the inability to accommodate a large calcium load may be a prerequisite for the

selective vulnerability of the motor neurons. Deposits containing calcium have been observed in the spinal motor neurons of ALS patients (Siklos *et al*. 1996) and also of the animal model (Siklos *et al*. 1998), suggesting that the pathogenesis of ALS may be associated with impaired calcium homeostasis in motor neurons. A study showing that overexpression of the calcium binding protein, parvalbumin, delayed the disease onset in a transgenic mouse model of ALS further supports this hypothesis (Beers *et al*. 2001). Thus, the search for molecular targets involved in impaired calcium homeostasis will help in developing effective therapies aimed at protecting the vulnerable motor neurons in ALS patients.

Cyclin-dependent kinase 5 (Cdk5) is an active enzyme in postmitotic neurons; the activation of Cdk5 requires its association with the neuronal activators, p35 or p39 (Lew *et al*. 1994; Tsai *et al*. 1994; Tang *et al*. 1995). We have earlier identified the important role of Cdk5 in neuronal migration as well as in brain development (Ohshima *et al*. 1996, 1999, 2002; Tanaka *et al*. 2001). Apart from the role of Cdk5 in developing brain, it has also been proposed as a possible target for the downstream molecules causing the neuronal death induced by elevated intracellular calcium. Disturbances in cellular calcium homeostasis induce the cleavage of p35 to its truncated product, p25, through the activation of the calcium-dependent cysteine protease, calpain, which eventually causes the mislocalization of Cdk5 in the cellular compartments and its prolonged activation (Kusakawa *et al*. 2000; Lee *et al*. 2000). This deregulated Cdk5 activity is implicated in neuronal death through both cytoplasmic and nuclear pathways. In the cytoplasm, Cdk5/p25 induces hyperphosphorylation of tau and subsequent cytoskeletal disruptions

leading to neuronal death (Patrick *et al*. 1999; Lee *et al*. 2000). In the nucleus, Cdk5/p25 phosphorylates a prosurvival transcription factor, MEF2, and inhibits its anti-apoptotic functions (Gong *et al*. 2003). The involvement of p25 in neurodegeneration is further supported by a study showing that transgenic mice expressing p25 specifically in neurons developed a motor neuron disease and paralysis reminiscent of ALS (Bian *et al*. 2002). Moreover, elevated levels of p25 and higher Cdk5 activity have been found in the spinal cord of $SOD1^{G37R}$ transgenic mice (Nguyen *et al*. 2001). These observations suggest that the deregulation of Cdk5 induced by conversion of p35 to p25 may be involved in the pathogenic mechanism underlying neurodegeneration caused by mutant SOD1. Thus, it is possible that inhibition of the calpain-mediated conversion of p35 to p25 may provide a potential therapeutic approach to slow down the disease progression of ALS.

To test this hypothesis, we generated double transgenic mice in which human SOD1 with a G93A mutation ($SOD1^{G93A}$) was expressed in a p35-null background. We show here that the absence of p35, and hence the lack of p25 generation, did not affect the onset, progression and pathology of motor neuron disease in $SOD1^{G93A}$ mice, a mouse model of ALS.

Materials and Methods

Materials

Polyclonal antibodies to p35 (C-19) and Cdk5 (C-8) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to human superoxide dismutase 1 (hSOD1) and choline acetyltransferase (ChAT) were obtained from Chemicon International, Inc. (Temecula, CA). Polyclonal antibody to ubiquitin was purchased from Novocastra Laboratories Ltd. (Newcastle, UK). Monoclonal antibodies to phosphorylated neurofilaments (SMI 34) and α-tubulin were obtained from Sternberger Monoclonals, Inc. (Lutherville, MD) and Sigma (St. Louis, MO), respectively. Monoclonal antibodies to tau were AT-8 (Endogen, Woburn, MA), Tau-1 (Boehringer Mannheim, Indianapolis, IN) and Tau-5 (BIOSOURCE International, Inc., Camarillo, CA).

Generation of mutant SOD1 transgenic mice lacking p35

The transgenic mice expressing the human SOD1 gene with a G93A mutation ($SOD1^{G93A}$) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as hemizygotes by mating transgenic males with B6/SJL F1 females. This line was originally generated and characterized by Gurney *et al*. (1994). p35 mutants were maintained in a C57BL/6 x 129/SvJ hybrid background (Ohshima *et al*. 2001). A two-step breeding strategy was employed to obtain the mutant SOD1 mice lacking p35. Initially, the $SOD1^{G93A}$ mice were crossed with p35+/- mice to generate the mutant SOD1

mice with a single functional p35 allele. These mice were then crossed with p35+/- mice to generate mutant SOD1 mice, either with or without p35. Thus, these lines were maintained as hemizygotes of the $SOD1^{G93A}$ transgene. The genotypes of these mice were determined by polymerase chain reaction using DNA extracted from the tail (Gurney *et al*. 1994; Ohshima *et al*. 2001). Mice were housed under a 12 h light/ 12 h dark cycle. All care was given in compliance with National Institutes of Health guidelines on the use of laboratory and experimental animals.

Preparation of the spinal cord extracts

Lumbar spinal cord lysates were prepared in a lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin. To examine the phosphorylation state of tau, phosphatase inhibitors (phosphatase inhibitor cocktail I and II, SIGMA, St. Louis, MO) were further added in the lysis buffer. Following a 30-min incubation on ice, insoluble material was removed by centrifugation at 4°C, and the protein concentration of the supernatant was determined using the BCA protein assay (Pierce, Rockford, IL). Fractionation of spinal cord lysates into a cytosolic fraction and a membrane fraction was performed as previously reported (Kusakawa et al. 2000). Briefly, the lumbar spinal cords were homogenated in 10 volumes of HEPES buffer (20 mM HEPES, pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA) at 4°C and then centrifuged at 10,000 X *g* for 15 min. The resulting supernatant was further centrifuged at 100,000 X *g* for 60 min. The supernatants were collected as a cytosolic

fraction. The remaining pellets were resuspended in HEPES buffer containing 150 mM NaCl and 1% Triton X-100 and used as a membrane fraction.

Western blot analysis

Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred onto a nitrocellulose membrane. The membranes were blocked in 1× PBS containing 5% skim milk and 0.05% Tween 20, and incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: anti-p35 (1:1,000), anti-Cdk5 (1:1,000), anti-hSOD1 (1:4,000), AT-8 (diluted to 5 μg/ml), Tau-1 (1:5,000), Tau-5 (1:6,000) and anti-α-tubulin (1:4,000) antibodies. Incubation with peroxidase-conjugated anti-mouse or rabbit IgG (1:10,000) was performed at room temperature for 60 min. A signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL) and relative optical densities of the bands were quantified using an image analysis system with NIH Image software, version 1.62. To determine the p35 and p25 protein levels, the data obtained with the p35 antibody (C-19) were normalized to α-tubulin levels on the stripped and reprobed membranes. For reuse, the membranes were stripped for 30 min at 50°C in 63 mM Tris-HCl (pH 6.8) containing 100 mM 2-mercaptoethanol and 2% SDS.

Cdk5 kinase activity assay

Lumbar spinal cord lysates were prepared with the same lysis buffer except with a NaCl concentration of 50 mM, to allow Cdk5 association with p35 and p39 as previously

described (Takahashi *et al*. 2003). The lysates were immunoprecipitated with anti-Cdk5 (C-8) antibody. The Cdk5 immunoprecipitates were prepared by incubation of 300 μl of the lysate (corresponding to 300 μg of protein) with anti-Cdk5 antibody (3 μg) overnight at 4°C followed by further incubation with 25 μl of Protein A-Agarose beads (50% slurry in lysis buffer; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 3 hours at 4°C. The immunoprecipitates were washed twice with the lysis buffer and twice with a kinase buffer consisting of 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM dithiothreitol, and resuspended in 60 μl of the kinase buffer. Kinase activity assays were performed using either histone H1 or bacterially-expressed human tau as a substrate as described previously (Takahashi *et al*. 2003). Briefly, a total volume of 50 μl of kinase assay mixture was used, containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM microcystin LR, 10 μl of the immunoprecipitate and either 10 μg of histone H1 or 5 μg of tau. The phosphorylation reaction was initiated by the addition of 0.1 mM [γ-^{32}P] ATP and incubated at 30°C for 60 min. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiled immediately for 5 min. Samples were separated by SDS-PAGE on a 15% polyacrylamide gel, and autoradiography was performed to detect the phosphorylation of histone H1 or tau.

Determination of disease onset and progression

A Rotarod (Rota-Rod for Mice 7650; Ugo Basile, Comerio, Italy) was used to evaluate motor function. All mice were initially trained for a total of ~20 min on the rotating rod

at four different speeds (5, 10, 15 and 20 rpm; 5 min per each speed) everyday for 3 days. Performance was then determined at intervals of 3-4 days before the disease onset and thereafter at intervals of 10 days throughout the disease progression, and the time each mouse remained on the rotating rod at the different speeds was registered. The onset of disease was defined as the age at which the mouse could not remain on the rotarod for 7 min at a speed of 20 rpm, as described previously (Li *et al*. 2000). If the mouse remained on the rod for >7 min, the test was completed and scored as 7 min. Terminal illness was defined by either the inability of a mouse to right itself within 10 sec when left on its back or the inability to reach for food or water as described previously (Subramaniam *et al*. 2002).

Immunohistochemistry

The $SOD1^{G93A}$ mice, either with or without p35, were studied at two different disease stages, at the ages of 80 days (late pre-symptomatic stage) and 120 days (end-stage of disease). The littermate controls included wild-type and p35-/- mice. Three animals from each genotype were subjected to the immunohistochemical analysis at the indicated ages. Mice were anesthetized with avertin (250 mg/kg, i.p., Fluka, Milwaukee, WI) and perfused transcardially with 0.1 M sodium phosphate buffer (pH 7.4), followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The lumbar spinal cord from L1-L5, which included the lumbar enlargement, was dissected out and postfixed in the same fixative overnight at 4°C. The spinal cords were then treated in 30% sucrose overnight at 4°C until saturated. The frozen sections (14 μm) were serially cut from the

lumbar enlargement and subjected to immunohistochemical analysis. Antibodies specific for ChAT (1:1,000 dilution), phosphorylated neurofilaments (SMI 34, 1:1,000 dilution) and ubiquitin (1:2,500 dilution) were applied to the sections overnight at 4°C after blocking nonspecific binding with 10% normal goat serum in PBS containing 0.01% Triton X-100 (PBS-T) for 60 min at room temperature. After washing in PBS-T, biotin-labeled secondary antibodies were applied for 60 min at room temperature. The sections were subsequently washed with PBS-T and incubated with ABC solution (Vector Laboratories Inc., Burlingame, CA). The immunoreaction was developed using 3, 3'-diaminobenzidine as a chromogen. Finally, the sections were counterstained with Methyl Green (Zymed, San Francisco, CA) and mounted with Micromount (Surgipath Medical Industries, Inc., Richmond, IL). The staining specificity of these antibodies was assessed by omission of the primary antibodies.

To compare the number of motor neurons in the lumbar spinal cord of $SOD1^{G93A}$ mice, either with or without p35, we counted neurons on the ChAT-immunostained sections spanning the lumbar enlargement in each group, as previously described (Lewis *et al*. 2000). For each mouse, at least 20 sections, collected at every seventh serial section (14 μm), were subjected to counting. The results were expressed as the number of motor neurons per anterior horn.

Results

Generation and characterization of SOD1^{G93A} transgenic mice lacking p35

To examine the possible relevance of Cdk5 to the pathogenesis of ALS, we generated SOD1^{G93A} transgenic mice lacking the neuronal Cdk5 activator, p35, by crossing the mutant SOD1 transgenic mice with p35+/- mice. Our breeding strategy maintained the SOD1^{G93A} transgene in a hemizygous state and allowed us to examine the SOD1^{G93A} mice, in the presence or absence of p35, as well as their littermate controls (wild-type and p35-/- mice). We first examined the protein levels of mutant SOD1 and Cdk5 in the lumbar spinal cord of these mice at the age of 80 days, by which time none of them had developed any motor impairment. The amount of mutant SOD1 in the lumbar spinal cord of SOD1^{G93A} mice was not altered by a lack of p35 (Fig. 1a). Moreover, there was no difference in the amount of Cdk5 protein in these mice. So, we examined the effects of the presence or absence of p35 on the Cdk5 kinase activity, using Cdk5 immunoprecipitates from the lumbar spinal cord extracts. The SOD1^{G93A}; p35-/- mice showed a significant reduction of Cdk5 activity (less than 20%) as compared to SOD1^{G93A}; p35+/+ mice (Fig.1b). In contrast, there was no difference in Cdk5 kinase activity for histone H1 between p35-/- and SOD1^{G93A}; p35-/- mice (Fig. 1b), suggesting that Cdk5/p39 activity was unaffected by the mutant SOD1. We did not detect an increased conversion of p35 to p25 as well as higher Cdk5 activity in the SOD1^{G93A} mice at the age of 80 days as compared to the age-matched wild-type littermates (Fig. 1a and b), in contrast to a previous report that demonstrated these changes in SOD1^{G37R} mice at

the end-stage of disease (Nguyen *et al*. 2001). Because our results were obtained from the 80-day-old mice, we wondered whether the use of the pre-symptomatic mice failed to detect the deregulation of Cdk5. Therefore, we examined the conversion of p35 to p25 and Cdk5 activity with the disease progression, using the $SOD1^{G93A}$ mice at the different stages of disease and their age-matched wild-type littermates. Most of the mutant SOD1 mice developed the motor impairment by 100 days of age and died by 130 days of age (Table 1). Thus, we selected three different disease stages based on rotarod performance tests: day 80 as the pre-symptomatic stage, day 100 as the symptomatic stage and day 120 as the end-stage of disease. The levels of p35 protein gradually decreased in the $SOD1^{G93A}$ mice with the disease progression, while the p25 levels decreased to a lesser extent (Fig. 2a, b and c). Consequently, the ratio of p25 to p35 seemed to increase in the $SOD1^{G93A}$ mice at 120 days of age (Fig. 2d), while this change was not statistically significant ($p = 0.23$). Cdk5 kinase activity significantly decreased in the $SOD1^{G93A}$ mice with the disease progression, which resulted in a 60% reduction in the activity at the end-stage of disease (120 days of age) as compared to the age-matched wild-type littermates (Fig. 2e and f). This change of Cdk5 activity in $SOD1^{G93A}$ mice seemed to occur in parallel with the progressive decrease in the amount of p35, and thus p25 protein.

The subcellular localization of p35 has been known to be different from that of p25: p35 is associated with membranes, while p25 is present in a cytosolic fraction (Patrick *et al.* 1999; Kusakawa *et al.* 2000). Therefore, we have further analyzed the amount of p35 and p25 proteins in the fractionated spinal cord lysates from $SOD1^{G93A}$ mice at 120 days of age and their age-matched wild-type littermates. As previously reported, p35 was

more abundant in the membrane fraction, while p25 remained in the supernatant as a soluble form. The decreased levels of p35 and p25 proteins in the SOD1^{G93A} mice was pronounced in the membrane fraction and in the cytosolic fraction, respectively, further confirming that the levels of p35 and p25 proteins decreased in spinal cord of SOD1^{G93A} mice (Fig. 2g). Cdk5 kinase activity in the fractionated lysates was measured using either histone H1 or tau as a substrate, and was found to decrease in the SOD1^{G93A} mice as compared to that in the wild-type littermates (Fig. 2h). It was noteworthy that Cdk5 in the cytosolic fraction from SOD1^{G93A} mice had a decreased ability to phosphorylate tau, because the deregulation of Cdk5 by p25 has been known to promote the hyperphosphorylation of tau (Patrick *et al*. 1999). These results indicated that neurodegeneration caused by the SOD1^{G93A} mutant protein resulted in a significant reduction of Cdk5 activity and in a progressive decrease of p35 and p25 proteins.

p35-independent development of motor deficits in SOD1^{G93A} transgenic mice

To examine the effects of Cdk5/p35 or Cdk5/p25 on the phenotype of motor neuron disease in mutant SOD1 mice, we determined the age of disease onset and the life span of SOD1^{G93A} mice in the presence or absence of p35. Because the mutant SOD1 mice lacking p35 were generated with a mixed genetic background by crossing the mutant SOD1 mice (B6/SJL background) with p35 mutants (C57BL/6 x 129/SvJ hybrid background), analysis of the motor functions was carried out using a large number of littermate mice (SOD1^{G93A}; p35+/+ mice, n=17 and SOD1^{G93A}; p35-/- mice, n=15). The onset of disease (Table 1) was not significantly different in the SOD1^{G93A}; p35-/- mice as

compared to the SOD1^{G93A}; p35+/+ mice (Fig. 3a). In addition, the mean life span of the SOD1^{G93A}; p35-/- mice (Table 1) was similar to that of the SOD1^{G93A}; p35+/+ mice (Fig. 3b). However, it appeared that between 120 and 130 days of age, the probability of survival dropped 60% in SOD1^{G93A}; p35-/- mice, but only 24% in SOD1^{G93A}; p35+/+ mice (Fig. 3b). This observation suggested that the loss of Cdk5/p35 activity might affect the survival of SOD1^{G93A} mice at the end-stage of disease (120 days of age) when the Cdk5 activity in SOD1^{G93A} mice had dropped to approximately 60% of that in wild-type mice. To further determine how the presence or absence of p35 affects motor deterioration in the mutant SOD1 mice, we tested the performance of these mice on a rotarod every 10 days beginning at 70 days of age (pre-symptomatic stage) and continued the tests until most of the mice could no longer remain on the rod at 120 days of age. These tests revealed that the absence of p35 did not affect the disease progression in the mutant SOD1 mice (Fig. 4). Furthermore, we did not find any motor impairment in rotarod performance of their littermate controls, wild-type (n=14) and p35-/- mice (n=12) (data not shown). Taken together, these results suggest that Cdk5/p35 or Cdk5/p25 has no impact on the onset and progression of motor neuron disease in SOD1^{G93A} mice, while the life span of SOD1^{G93A} mice may be affected to some extent by p35 deficiency.

Pathological lesions in SOD1^{G93A} mice remain unaltered in the absence of p35.

To further examine the features of motor neuron disease in SOD1^{G93A} mice, either with or without p35, we carried out neuropathological analysis of these mice as well as their wild-type and p35-/- littermates. At the ages of 80 days (pre-symptomatic stage) and 120

days (end-stage of disease), three animals from each genotype were sacrificed and the lumbar spinal cords were histopathologically evaluated. In order to label the motor neurons in the spinal cord specifically, the sections were immunostained for choline acetyltransferase (ChAT) (Fig. 5). Labeling was restricted to large neurons in the anterior horn of the spinal cord, and the pattern of ChAT-immunoreactivity was similar to that observed by retrograde labeling of motor neurons through the peripheral nerve (Takahashi *et al*. 1999). There was no difference in cell number of the ChAT-immunoreactive neurons from wild-type and p35-/- mice at the ages of 80 and 120 days (Fig. 5). However, a significant reduction in the number of the ChAT-immunoreactive neurons was observed in the mutant SOD1 mice, irrespective of the presence or absence of p35, as the disease progressed from the pre-symptomatic stage (80 days of age) to the end-stage of disease (120 days of age) (Fig. 5).

Abnormal cytoplasmic immunoreactivities for phosphorylated neurofilaments and ubiquitin have been known to be histopathological hallmarks of ALS. Therefore, we examined these abnormalities in 120-day-old mice by immunostaining the sections with anti-phosphorylated neurofilament and anti-ubiquitin antibodies (Fig. 6). In wild-type and p35-/- mice, immunoreactivity for phosphorylated neurofilaments was mainly present within the neuropils of the spinal cord (Fig. 6a and c), while the numerous motor neurons in the mutant SOD1 mice exhibited a strong immunoreactivity for phosphorylated neurofilaments in the cell bodies and in the axon hillocks, irrespective of the presence or absence of p35 (Fig. 6b and d). Weak immunoreactivity for ubiquitin was observed in the cytoplasm of motor neurons from wild-type and p35-/- mice (Fig. 6e and g). In the

mutant SOD1 mice, either with or without p35, strong immunoreactivity for ubiquitin was observed in the cytoplasm of motor neurons and in the neurites (Fig. 6f and h). Thus, the aggregation of phosphorylated neurofilaments and ubiquitinated deposits observed in spinal motor neurons from the mutant SOD1 mice were not altered by the absence of p35. These results indicated that the histopathological lesions in $SOD1^{G93A}$ mice were independent of the presence or absence of p35 and p25 proteins.

We further examined the phosphorylation state of tau in the spinal cord lysates of 120-day-old $SOD1^{G93A}$ mice, either with or without p35, as well as their wild-type and p35-/- littermates, because hyperphosphorylation of tau has been found in another ALS model animal, $SOD1^{G37R}$ mice (Nguyen *et al*. 2001). We used an AT-8 antibody that recognizes tau only when Ser-202 and Thr-205 are phosphorylated (Biernat *et al*. 1992), and a Tau-1 antibody that recognizes a dephosphorylated epitope of tau at amino acid 191-224 (Iqbal and Grunke-Iqbal 1995). To determine the total tau levels, the phosphorylation-independent monoclonal antibody, Tau-5, was used (LoPresti et al. 1995). No AT-8 immunoreactivity was found in the samples from wild-type and $SOD1^{G93A}$ mice as well as from p35-/- and $SOD1^{G93A}$; p35-/- mice, while the strong AT-8 immunoreactivity was detected in samples from E18.5 wild-type embryos which served as a positive control (Fig. 7). The signal intensity for Tau-1 immunoreactivity decreased in samples from $SOD1^{G93A}$ mice, either with or without p35, as compared to those from the wild-type and p35-/- mice (Fig. 7). However, the decreased levels of Tau-1 immunoreactivity in samples from $SOD1^{G93A}$; p35+/+ and $SOD1^{G93A}$; p35-/- mice were accompanied by the reduction of total tau levels (Fig. 7), which may reflect the loss of

spinal motor neurons observed in these animals at the end-stage of disease. In the light of a previous observation that tau is hyperphosphorylated in spinal cord of $SOD1^{G37R}$ mice (Nguyen *et al*. 2001), our results suggest that the phosphorylation state of tau in mutant SOD1 mice may be differentially affected by the specific mutations of the SOD1 gene.

Discussion

Transgenic mice expressing mutant SOD1 develop a dominantly inherited adult-onset paralytic disorder that replicates many of the clinical and pathological features of familial ALS (Gurney *et al*. 1994). Existence of this animal model with a time lag between the disease onset and death allowed us to test the hypotheses that the deregulation of Cdk5 induced by conversion of p35 to p25 may be involved in the pathogenesis of mutant SOD1-linked ALS, and that Cdk5/p35 may be a possible therapeutic target to slow down the disease progression. We generated the $SOD1^{G93A}$ mice lacking p35 to determine the effects of the absence of p35, and hence the subsequent inhibition of p25 generation, on the clinical and pathological features of the mutant SOD1 mice. We found that the p35 deficiency had no effects on the onset and progression of the motor neuron disease, while the absence of p35 resulted in a significant reduction of Cdk5 activity in the spinal cord of the mutant SOD1 mice. Furthermore, the pathological analysis of the mutant SOD1 mice, in the presence or absence of p35, indicated no differences in the progression of motor neuron loss and in the altered immunoreactivities to ubiquitin and phosphorylated neurofilaments. These results provide a compelling evidence that Cdk5/p35 and Cdk5/p25 do not participate in the pathogenesis of motor neuron degeneration caused by the mutant SOD1 ($SOD1^{G93A}$). Thus, inhibition of the calpain-mediated conversion of p35 to p25 is unlikely to confer protective effects on motor neuron disease in mutant SOD1-linked ALS.

We also found that the levels of p35 protein in the spinal cord of $SOD1^{G93A}$ mice gradually decreased with the disease progression, while the p25 levels decreased to a

lesser extent. These changes seemed to occur in parallel with a progressive loss of spinal motor neurons and resulted in a significant reduction of Cdk5 activity. This is in contrast to a previous report that demonstrated the increased levels of p25 and higher Cdk5 activity in $SOD1^{G37R}$ mice (Nguyen *et al*. 2001). The reasons for this discrepancy are not apparent because both studies used the mutant SOD1 mice at the end stage of disease. It is unlikely that the methodological differences may contribute to this discrepancy because we used the same procedures to those used by Nguyen *et al*. (2001). One factor that might be responsible for this discrepancy is the choice of SOD1 mutant strains ($SOD1^{G93A}$ vs. $SOD1^{G37R}$ mice). The clinical and pathological features of motor neuron disease in mutant SOD1 mice are likely to depend upon the expression levels of the mutant SOD1 as well as the specific mutation in the SOD1 gene. A higher level of Cdk5 activity has been found in a $SOD1^{G37R}$ mouse line expressing a higher level of the mutant SOD1, which showed a shorter life span (average 24 weeks) relative to another line expressing a lower level of the mutant SOD1 (average life span, 52 weeks). In contrast, Cdk5 kinase activity in the $SOD1^{G93A}$ mice (average life span, 18 weeks) used in this study significantly decreased with the disease progression, resulting in a 60% reduction in the activity at the end-stage of disease as compared to the non-transgenic littermates. In addition to the differences in clinical features between the $SOD1^{G93A}$ and $SOD1^{G37R}$ mice, the pathological features are also not similar between these strains: astrocytosis in presymptomatic mice was observed in $SOD1^{G37R}$ mice, but not in $SOD1^{G93A}$ mice (Tu *et al*. 1996; Wong *et al*. 1995), and the aggregation of cytoplasmic proteins observed in $SOD1^{G93A}$ mice was very rare in $SOD1^{G37R}$ mice (Watanabe *et al*. 2001). Furthermore, we

found no immunoreactivity for phosphorylated tau at serine-199 and threonine-205 in the spinal cord extracts of $SOD1^{G93A}$ mice, while hyperphosphorylation of tau at these sites has been found in the spinal motor neurons of $SOD1^{G37R}$ mice (Nguyen *et al*. 2001). Another difference was the propensity of neurons in these mouse lines to convert p35 to p25. The p25 protein level was increased in spinal cord extracts of $SOD1^{G37R}$ mice, while it was decreased in those of $SOD1^{G93A}$ mice, resulting in the reduced ability of Cdk5 to phosphorylate tau. Thus, the overexpression of $SOD1^{G93A}$ or $SOD1^{G37R}$ may have different effects on the Cdk5 pathway. However, our findings indicating that the absence of p35, and hence the subsequent inhibition of p25 generation, had no effect on the clinical and pathological features of motor neuron disease linked to mutant SOD1, suggest that Cdk5 deregulation is unlikely to contribute to ALS-like disease progression in mutant SOD1 transgenic mice.

The generation of p25 from p35 through the activation of calpain, is induced by various neurotoxic insults such as ischemia, oxidative stress or amyloid β-peptide (Kusakawa *et al*. 2000; Lee *et al*. 2000). Amyloid β-peptide is a primary constituent of the amyloid plaques in Alzheimer's disease brain that have been suggested to trigger the cascade of molecular events leading to neurodegeneration (Harkany *et al*. 2000; Masliah and Rockenstein 2000). Interestingly, inhibition of either Cdk5 or calpain activities has been reported to prevent amyloid β-peptide induced neuronal death (Alvarez *et al*. 1999; Lee *et al*. 2000). Furthermore, upregulation of the p35 conversion to p25, has been found in brains of Alzheimer's disease patients (Patrick *et al*. 1999). These observations suggested that alteration in the regulatory pattern of Cdk5 might play a key role in the

sequence of molecular events leading to neurodegeneration in Alzheimer's disease. However, postmortem cleavage of p35 to p25 has been known to proceed within a relatively short time (Taniguchi *et al*. 2001). Therefore, it still remains to be determined whether the increased conversion of p35 to p25 is a postmortem event or an *in vivo* condition in Alzheimer's disease brains. Transgenic mouse models that overexpress mutant amyloid precursor protein under the regulatory control of neuronal promoters have been reported to exhibit Alzheimer's disease-like neuropathology (Masliah and Rockenstein 2000). Analysis of these transgenic mice bred in a p35-null background, as we did here in an animal model of ALS, will provide important insights regarding the involvement of Cdk5 deregulation in the pathogenesis of Alzheimer's disease. Generation of genetically altered animal models for neurodegenerative diseases and their careful analysis will be a helpful strategy not only for determining the precise pathogenic mechanism underlying these diseases, but also for identifying the effective therapeutic approaches to treat the patients with these debilitating diseases.

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Figure legends

Figure 1. Generation of $SOD1^{G93A}$; p35-/- double transgenic mice.

$SOD1^{G93A}$ transgenic mice were crossed with p35+/- mice to generate the mutant SOD1 mice lacking p35 as described in Materials and Methods. Lumbar spinal cord extracts were prepared from the mutant SOD1 mice, either with or without p35, as well as from their non-transgenic littermates (wild-type and p35-/- mice) at the age of 80 days. (a) The spinal cord extracts were analyzed by SDS-PAGE and Western blotting with anti-p35, anti-human specific SOD1 (hSOD1) and anti-Cdk5 antibodies. Equal amount of protein (20 μg) loaded on the gels was examined by reprobing the same membrane with anti-α-tubulin antibody. (b) Cdk5 kinase assay was carried out using histone H1 as a substrate. Autoradiography was used to detect the phosphorylation of histone H1. The amounts of substrate and immunoprecipitated Cdk5 used were examined by staining the gels with Coomassie Blue and by Western blotting (WB) with an anti-Cdk5 antibody, respectively. Quantitative results indicate the relative Cdk5 kinase activity, expressed as a percentage of the value for wild-type mice and shown as the mean ± S.E. from three independent experiments. These data were analyzed using the unpaired, two-tailed Student's *t* test and considered to be significantly different when $p < 0.05$ (*).

Figure 2. Progressive decrease in p35 protein level and Cdk5 kinase activity in $SOD1^{G93A}$ transgenic mice at the advanced stages of disease. Lumbar spinal cord extracts were prepared from G93A SOD1 transgenic mice ($SOD1^{G93A}$) and their non-transgenic littermates (WT) at the ages of 80 days (pre-symptomatic stage), 100 days (symptomatic stage) and 120 days (end-stage of disease). (a) The levels of p35, p25 and mutant SOD1 were examined by SDS-PAGE and Western blotting with anti-p35 and anti-human specific SOD1 (hSOD1) antibodies. Equal amount of protein (20 μg) loaded on the gels was examined by reprobing the same membrane with anti-α-tubulin antibody. (b and c) To quantitatively determine the levels of p35 and p25 protein, the data obtained with the anti-p35 antibody were normalized to the α-tubulin levels. The results indicate the relative amount of p35 (b) or p25 (c) expressed as a relative value to that of wild-type mice at the age of 80 days (n=3). (d) The ratio of p25 to p35 was calculated by measuring the optical densities of the p25 and p35 bands (n=3). (e) Cdk5 kinase assay was carried out using histone H1 as a substrate. Autoradiography was used to detect the phosphorylation of histone H1. The amounts of substrate and immunoprecipitated Cdk5 used were examined by staining the gels with Coomassie Blue and by Western blotting (WB) with an anti-Cdk5 antibody, respectively. (f) Relative Cdk5 kinase activities were expressed as a percentage of the value for wild-type mice at the age of 80 days and shown as the mean ± S.E. from three independent experiments. These data were analyzed using the unpaired, two-tailed Student's *t* test and considered to be significantly different when $p < 0.05$ (*). (g) Spinal cord lysates from $SOD1^{G93A}$ mice and their non-transgenic littermates at the age of 120 days were fractionated into the supernatants as a

cytoplasmic fraction and the pellets as a membrane fraction by ultracentrifugation at 100,000 X *g* for 60 min, as described in Materials and Methods. The fractionated samples were examined by Western blotting with anti-p35 and anti-α-tubulin antibodies. (h) Cdk5 kinase activities in the fractionated samples were examined using either histone H1 or tau as a substrate.

Figure 3. Onset of motor neuron disease and life span in $SOD1^{G93A}$ mice, either with or without p35. (a) Disease onset of the $SOD1^{G93A}$ mice was not altered by the absence of p35. The onset of disease was determined by a motor function deficit seen in rotarod performance tests, as described in Materials and Methods. (b) Kaplan-Meier survival curves of the $SOD1^{G93A}$ mice in the presence or absence of p35. The life span of the mutant SOD1 mice was not altered by the absence of p35. $SOD1^{G93A}$; p35+/+ mice (n=17) and $SOD1^{G93A}$; p35-/- mice (n=15).

Figure 4. p35-independent deterioration of the motor function in $SOD1^{G93A}$ mice. Disease progression in motor function was tested on the rotarod at four different speeds of 5 rpm (a), 10 rpm (b), 15 rpm (c) and 20 rpm (d) for 7 minutes each. Testing was terminated either when the mouse fell from the rod or at 7 min if the mouse remained on the rod. Data are shown as the mean ± S.E. from $SOD1^{G93A}$; p35+/+ (n=17) and $SOD1^{G93A}$; p35-/- (n=15) mice.

Figure 5. p35 deficiency does not confer protective effects on progressive motor neuron loss caused by the mutant SOD1 ($SOD1^{G93A}$). Spinal motor neurons were defined by choline acetyltransferase (ChAT) immunoreactivity in the lumbar spinal cord of wild-type (a and e), $SOD1^{G93A}$; p35+/+ (b and f), p35-/- (c and g) and $SOD1^{G93A}$; p35-/- (d and h) mice at the ages of 80 (a, b, c and d) and 120 (e, f, g and h) days. (i and j) Three animals from each genotype were used at the indicated ages, and the number of ChAT-immunoreactive neurons was counted on at least 20 sections from each animal as described in Materials and Methods. The data were shown as the average numbers of neurons per anterior horn and analyzed using the unpaired, two-tailed Student's *t* test (* $p < 0.05$). The directional arrows point toward the ventral (V) and lateral (L) aspects of the spinal cord. Scale bars = 50 μm.

Figure 6. p35 deficiency does not ameliorate abnormal immunoreactivities for phosphorylated neurofilaments and ubiquitin in the lumbar spinal cord of $SOD1^{G93A}$ mice. Immunoreactivities for phosphorylated neurofilaments (a, b, c, and d) and ubiquitin (e, f, g and h) were examined in the lumbar spinal cord of wild-type (a and e), $SOD1^{G93A}$; p35+/+ (b and f), p35-/- (c and g) and $SOD1^{G93A}$; p35-/- (d and h) mice at the age of 120 days as described in Materials and Methods. In wild-type and p35-/- mice, immunoreactivities for phosphorylated neurofilaments and ubiquitin were present in the neuropil and in the somata of the spinal cord, respectively. In contrast, motor neurons in the mutant SOD1 mice harbored aggregates of phosphorylated neurofilaments and

ubiquitinated deposits in the cell bodies as well as in the neurites, irrespective of the presence or absence of p35. Scale bars = 50 μm.

Figure 7. Mutant SOD1^{G93A} affects the protein levels of tau in spinal cord of the transgenic mice, but not the phosphorylation state, irrespective of the presence or absence of p35. Spinal cord lysates were prepared from wild-type, SOD1^{G93A}; p35+/+, p35-/-, and SOD1^{G93A}; p35-/- mice at the age of 120 days as well as from wild-type embryos (E18.5). The samples were examined by SDS-PAGE and Western blotting using the phosphorylation-dependent tau antibodies, AT-8 (recognizes a phosphorylated epitope of tau at Ser-202 and Thr-205) and Tau-1 (recognizes a dephosphorylated epitope of tau at amino acids 191-224), and the phosphorylation-independent tau antibody, Tau-5. The amount of protein loaded on the gels was examined by reprobing the same membranes with an anti-α-tubulin antibody. No AT-8 immunoreactivity was detected in samples from wild-type, SOD1^{G93A}; p35+/+, p35-/- and SOD1^{G93A}; p35-/- mice, while the strong AT-8 immunoreactivity was detected in samples from wild-type embryos. Note that the reduced Tau-1 immunoreactivity in samples from SOD1^{G93A} mice, either with or without p35, was accompanied by the decreased levels of total tau.

Table 1 (Takahashi et al.)

Table 1. Onset of motor deficit and life span of $SOD1^{G93A}$ mice in the presence or absence of p35.

	$SOD1^{G93A}$; p35+/+ (n=17)	$SOD1^{G93A}$; p35-/- (n=15)	*P* value
Onset (days)	98.8 ± 1.8	102.8 ± 2.1	0.155
Life span (days)	126.1 ± 2.5	122.9 ± 1.6	0.346

Note: Data were analyzed using the unpaired, two-tailed Student's *t* test and expressed as the mean ± S.E.

Figure 1 (Takahashi et al.)

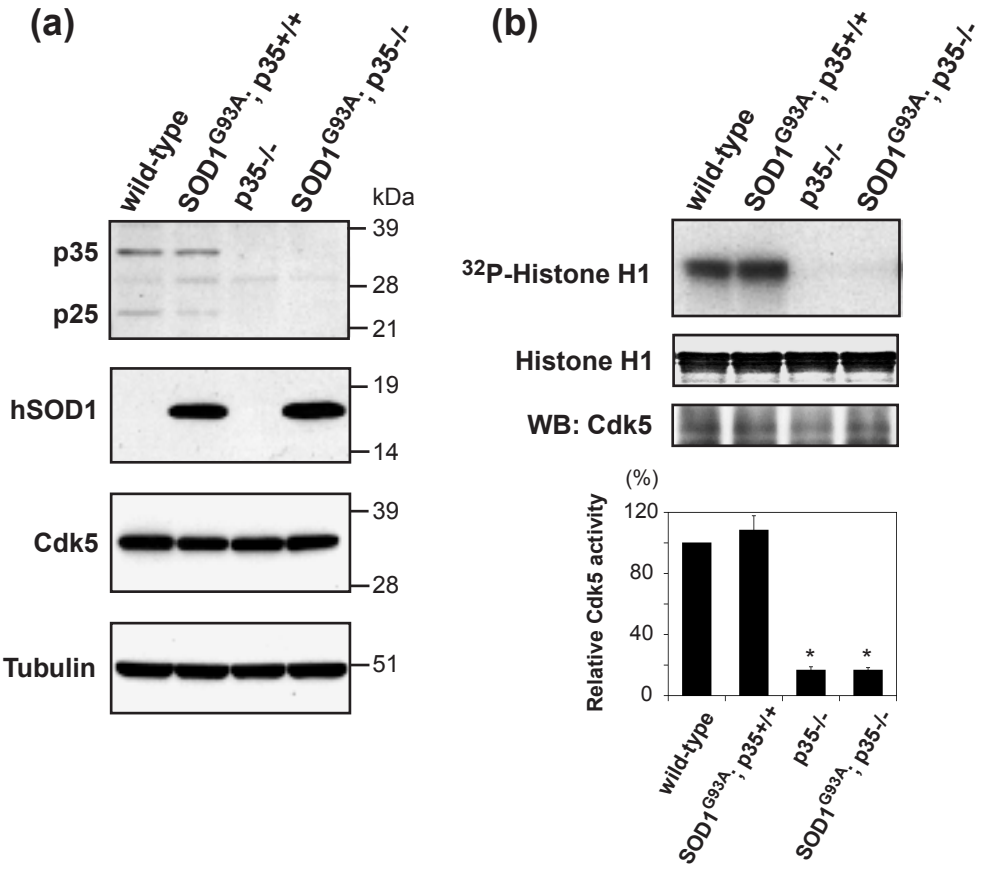
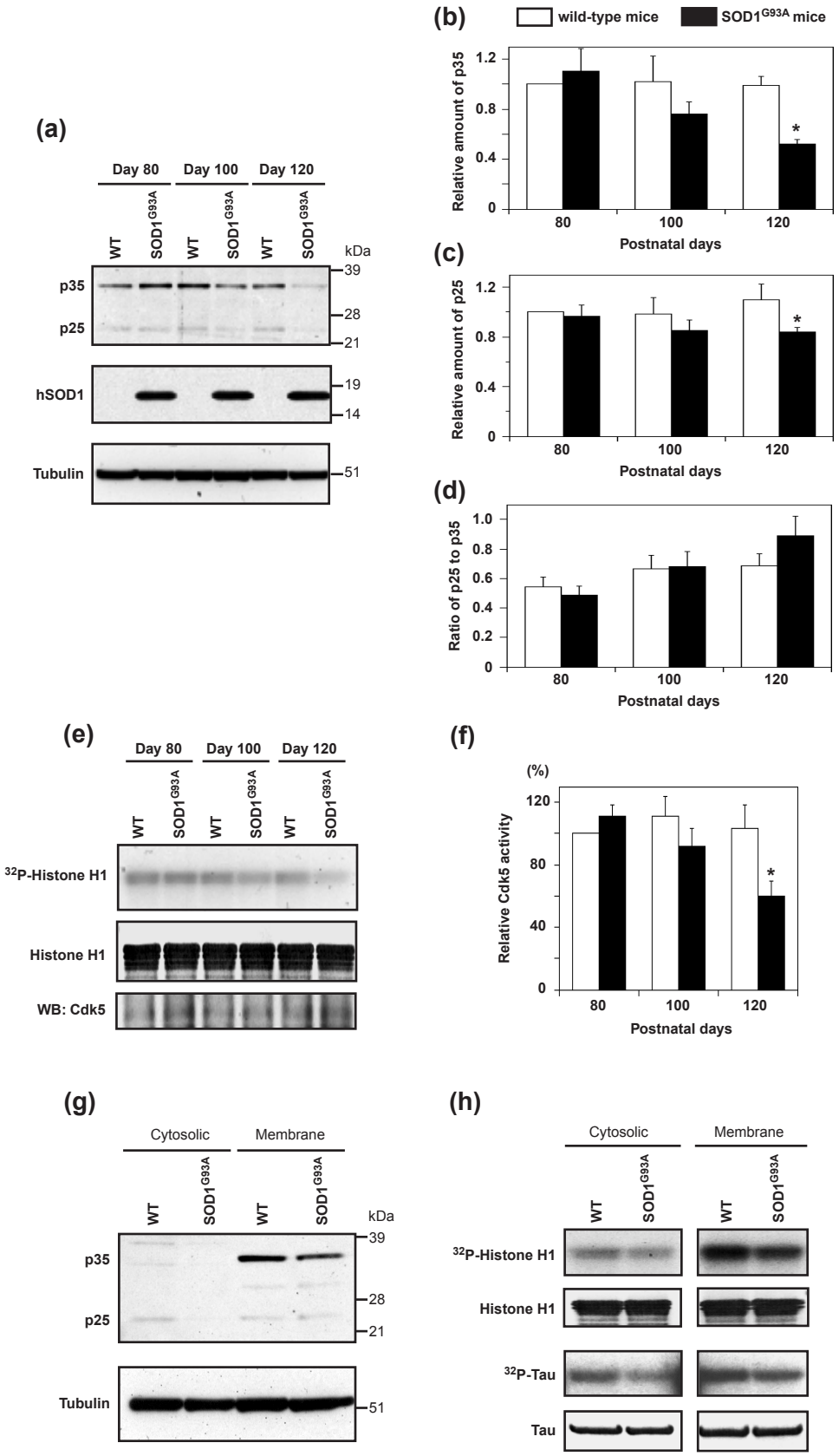


Figure 2 (Takahashi et al.)



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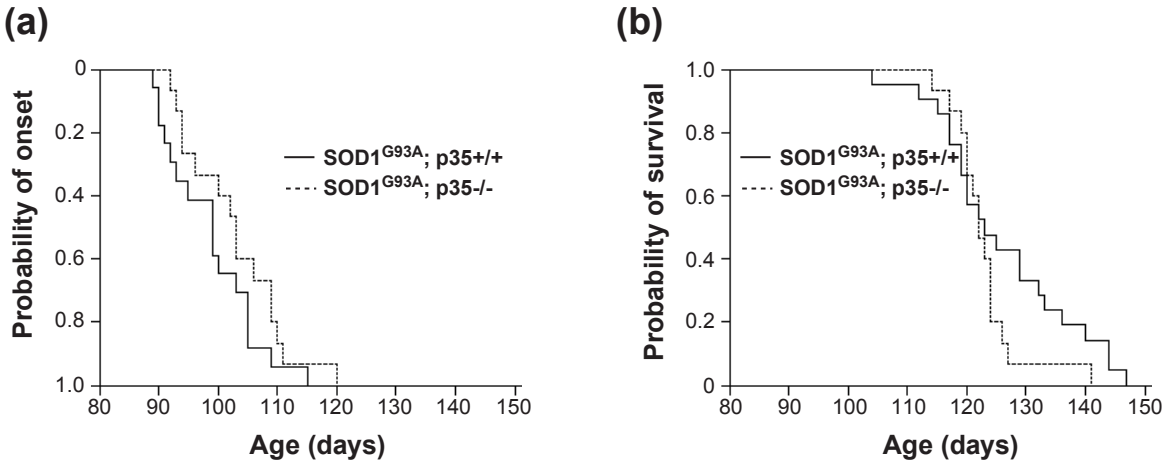

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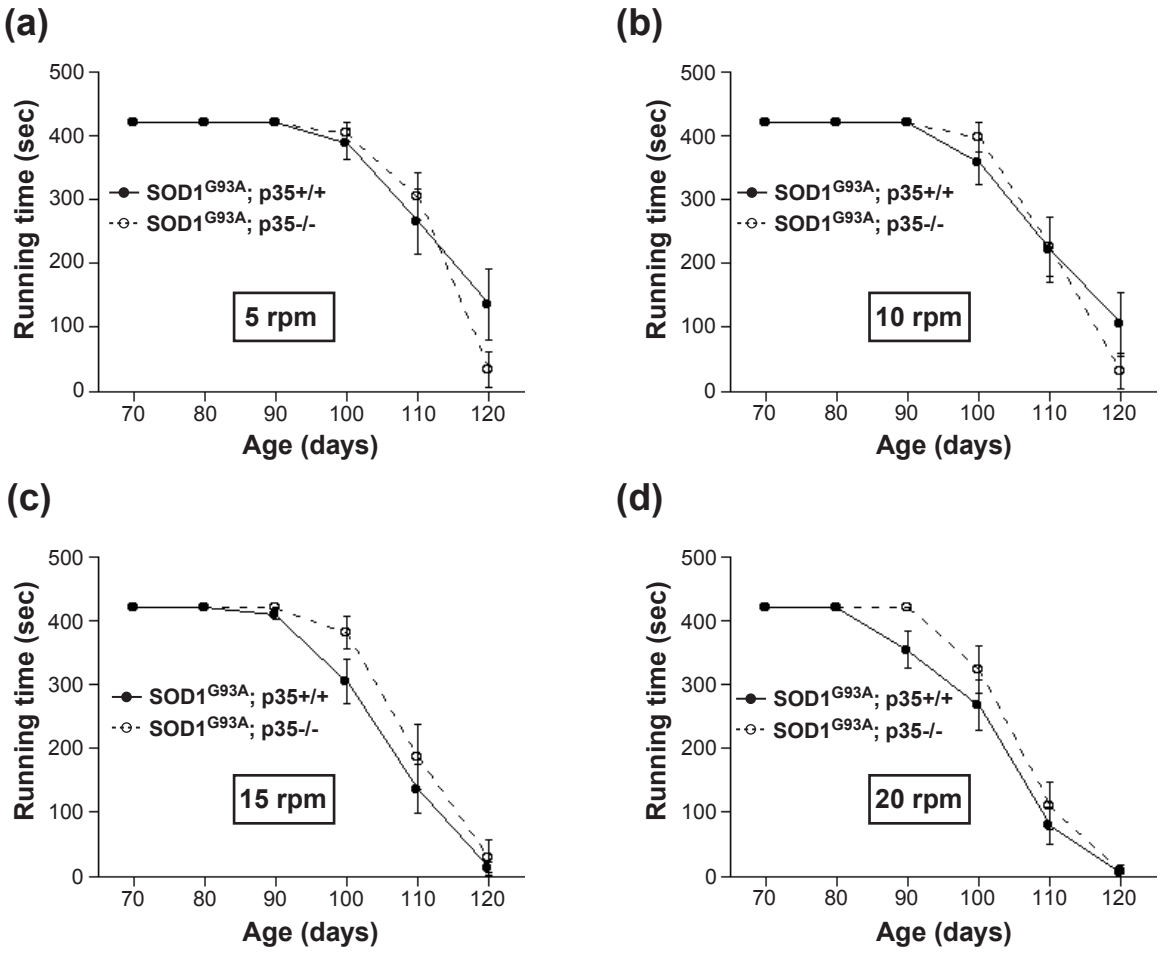

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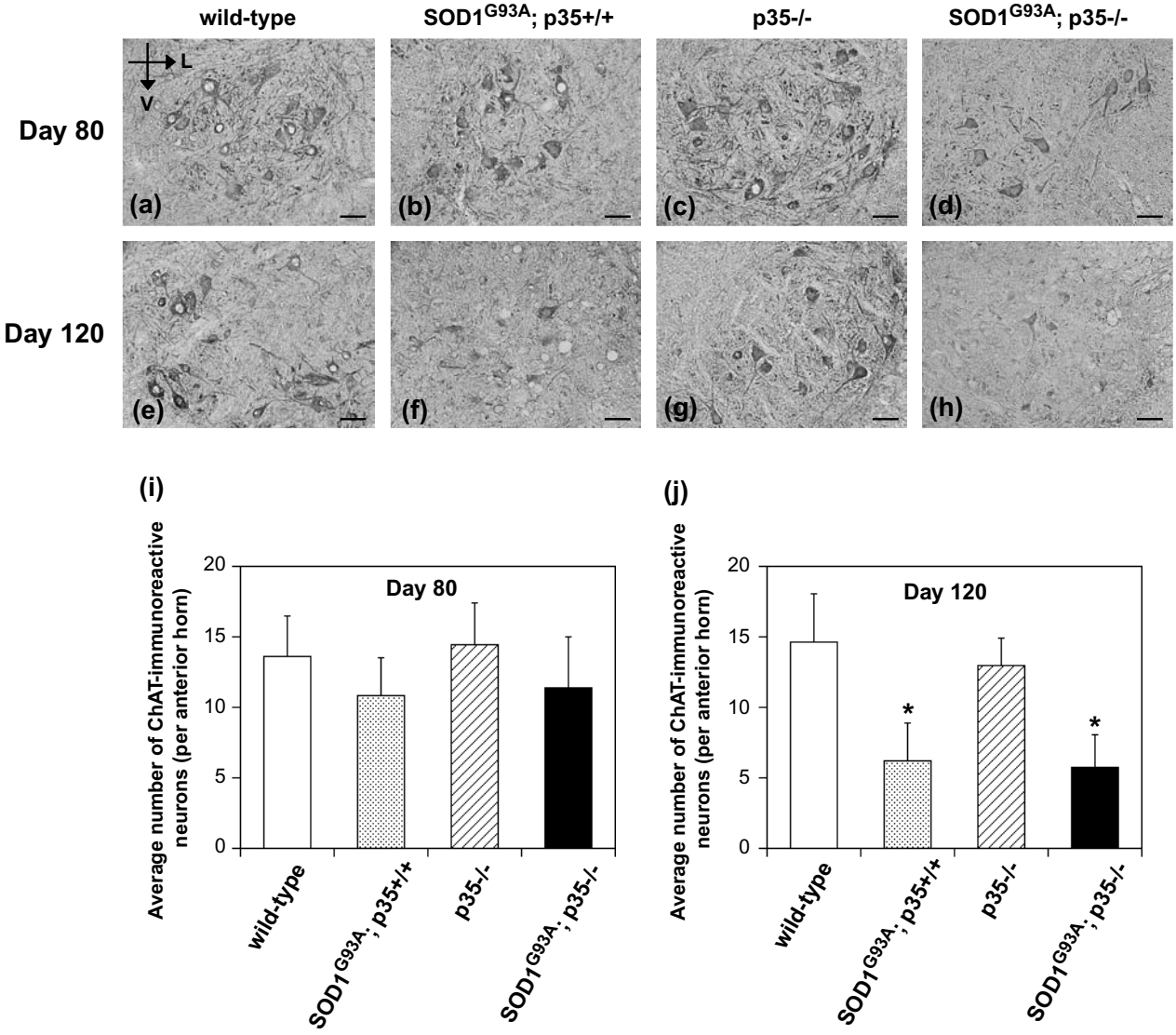

Figure 6 (Takahashi et al.)

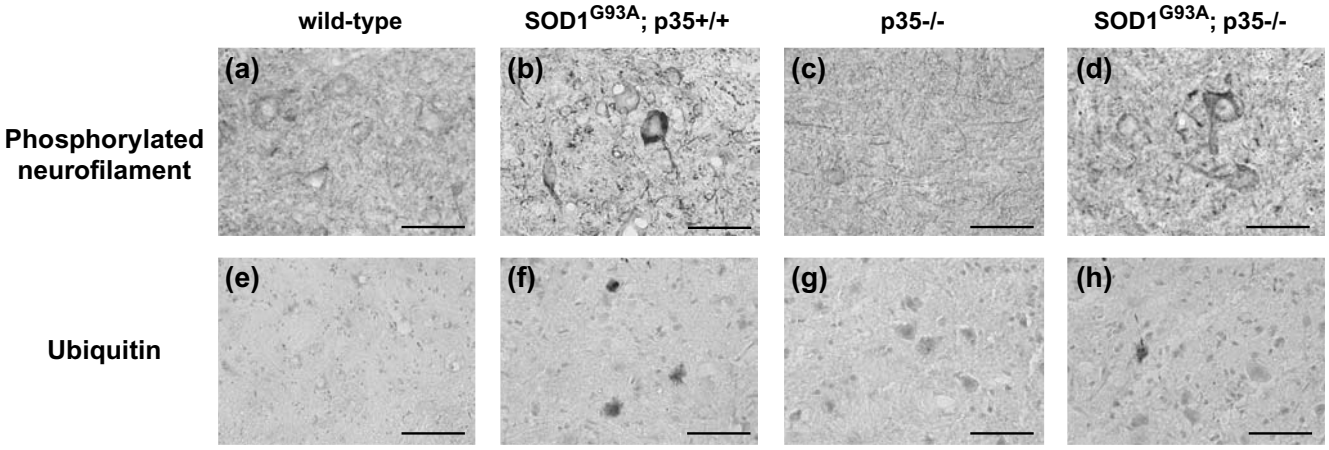

Figure 7 (Takahashi et al.)