Overexpressed wild-type SOD1 exhibits ALS-related misfolded conformation in iPSC-derived spinal motor neurons

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Short title: Wild-type SOD1 adopts “toxic conformation” by overexpression

Key words: ALS, misfolded SOD1, motor neuron, iPSC

Conflict of interest: none declared

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a late-onset, fatal disorder in which motor neurons selectively degenerate. *Superoxide dismutase 1 (SOD1)* was found as a causative gene of familial ALS, and mutant *SOD1* transgenic mice recapitulated ALS phenotypes. Analysis of these mice revealed accumulation of misfolded SOD1 protein in motor neurons. Misfolded SOD1 accumulation was found in spinal motor neurons of both familial ALS patients with *SOD1* mutation and sporadic ALS patients. However, it is unclear what condition causes wild-type SOD1 misfolding in patients without *SOD1* mutation. Here, we generated induced pluripotent stem cells from mutant *SOD1* transgenic mice, wild-type *SOD1* transgenic mice, and control mice, and differentiated them into spinal motor neurons to analyze misfolded SOD1 accumulation. We found that misfolded SOD1 protein was accumulated in spinal motor neurons of both mutant and wild-type *SOD1* transgenic mice as detected by sensitive antibody against misfolded conformation of SOD1. These results suggest that an increased expression level of wild-type *SOD1* may accelerate the ALS pathology, and our *in vitro* model would be a useful tool for misfolded SOD1 research.

Keywords: iPSCs, amyotrophic lateral sclerosis (ALS), superoxide dismutase 1 (SOD1),

misfolded protein, motor neurons

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating disorder in which progressive motor neuronal degeneration leads to muscle weakness, respiratory failure and death within as short as several years [1,2]. The precise pathomechanism of ALS is still unknown, and effective treatment has not been established. Genetic analysis of ALS patients identified *SOD1* as a causative gene of familial ALS in 1993 [3], and mutant *SOD1* transgenic mice were established as a well recapitulated ALS model [4]. Analysis of these *SOD1* transgenic mice developed understandings of ALS, and revealed that accumulated SOD1 protein was misfolded in spinal motor neurons.

SOD1 protein is considered to gain a toxic function via its misfolding [5], and misfolded SOD1 is highly observed in mutant *SOD1* transgenic mouse spinal cord [6,7]. Many previous studies have presented molecular mechanisms of SOD1 toxicity in ALS pathology including excitotoxicity, oxidative stress, mitochondrial dysfunction, axonal transport, and non-cell autonomous toxicity [8,9]. On the other hand, misfolded SOD1 protein accumulation was reported in not only familial ALS patients with *SOD1* mutation but also in sporadic ALS

patients by histopathological analysis of postmortem tissues [10]. However, it is unclear why misfolded SOD1 accumulations are exhibited in spinal motor neurons without *SOD1* mutation. Here, we analyzed misfolded SOD1 protein accumulation with sensitivity-different antibodies using induced pluripotent stem cell (iPSC)-derived spinal motor neurons, and revealed that overexpression of wild-type *SOD1* causes its misfolding.

MATERIAL AND METHODS

Transgenic animals

This animal study was approved by our institutional review board. Transgenic mice overexpressing $SOD1^{WT}$ (B6.Cg-Tg(SOD1)2Gur/J) or $SOD1^{G93A}$ mutation (B6SJL-Tg(SOD1*G93A)1Guj/J) were obtained from the Jackson Laboratory (Bar Harbor, ME).

iPSC generation

iPSCs were generated from mouse embryonic fibroblasts (MEFs) of wild-type human *SOD1* transgenic mice, mutant human $SOD1^{G93A}$ transgenic mice, and non-transgenic mice. Four reprogramming factors (Oct3/4, Sox2, Klf4 and c-Myc) were introduced into the MEFs using retroviral vectors as reported previously [11]. iPSCs were cultured on SNL feeder cells.

RT-PCR

RNA was isolated with Trizol (Thermo Fisher Scientific, Waltham, MA), and first-strand cDNA was synthesized with PrimeScript RT reagent Kit (TaKaRa BIO, Kusatsu, Japan). RT-PCR was performed using ExTaq (Takara BIO). Primer sets are listed in Table S1.

In vitro differentiation to three germ layers

The iPSCs were trypsinized into single cells and plated on Petri dishes (Kord-Valmark, Brampton, Canada) to form embryoid bodies (EBs) as previously reported [13]. After three days, they were transferred to gelatin-coated dishes and cultured for another three days. Cells were fixed and stained with anti-β-III tubulin (1:500, Millipore, Billerica, MA), anti-α-smooth muscle actin (1:100, Dako, Glostrup, Denmark) or anti-α-fetoprotein (1:500, R&D Systems, Minneapolis, MN) antibodies.

Differentiation to spinal motor neurons

iPSCs were differentiated into motor neurons according to methods previously described [14,15]. iPSCs were trypsinized into single cells, then resuspended in DM1 medium (DMEM/F12 (Thermo Fisher Scientific), 10% knockout serum (Thermo Fisher Scientific), penicillin, streptomycin, glutamine (Thermo Fisher Scientific) and 2-mercaptoethanol (Thermo Fisher Scientific), and plated at a concentration of 200,000 cells per ml in Petri dishes to form EBs. 48 hours later, EBs were divided from one dish into four Petri dishes containing DM1

medium supplemented with 1 μM retinoic acid (Sigma-Aldrich, St. Louis, MO) and 1 μM Smoothened agonist (Enzo Life Sciences, Farmingdale, NY). On Day 7, the EBs were dissociated into single cells using papain and DNase, resuspended into MN medium containing DMEM/F12, 5% horse serum (Thermo Fisher Scientific), B-27 supplement (Thermo Fisher Scientific), N2 supplement (Thermo Fisher Scientific), neurotrophic factors (GDNF, CNTF, NT3 and BDNF: 10 ng/ml (Peprotech, Princeton, NJ), and penicillin/streptomycin (Thermo Fisher Scientific), and then replated at a concentration of $1.5x10^5$ per cm^2 onto poly-D-lysine/laminin coated plates. Two-thirds of the medium was changed every three days.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (pH 7.4) for 30 min at room temperature and rinsed with phosphate-buffered saline (PBS). The cells were permeabilized in PBS containing 0.2% Triton X-100 for 10 min at room temperature, followed by rinsing with PBS. Non-specific binding was blocked with PBS containing 10% donkey serum for 60 min at room temperature. Cells were incubated with primary antibodies overnight at 4°C and then labeled with appropriate fluorescently tagged secondary antibodies. DAPI (4′,6-diamidino-2-phenylindole) (Nacalai Tesque, Kyoto, Japan) was used to label nuclei. Fluorescence images

were obtained using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) or an ArrayScan VTI HCS reader (Thermo Fisher Scientific). The following primary antibodies were used: β-III tubulin (1:500, Millipore, or 1:2000, CST), α-smooth muscle actin (1:100, Dako), α-fetoprotein (1:500, R&D Systems,), Nestin (1:500, Millipore), HB9 (1:200, Developmental Studies Hybridoma Bank, Iowa, IA), Islet-1 (1:200, Developmental Studies Hybridoma Bank), choline acetyltransferase (ChAT) (1:100, Millipore), misfolded human SOD1 (A5C3) [6] (1:100, MediMabs, Montreal, QC, Canada), misfolded human SOD1 (B8H10) [16] (1:100, MediMabs). The number of misfolded SOD1-positive cells and βIII-tubulin-positive cells was quantified by IN Cell Analyzer 6000 (GE Healthcare, Chicago, IL) and IN Cell Developer toolbox software 1.9 (GE Healthcare). The ratio of misfolded SOD1 and βIII-tubulin-positive cells to total βIII-tubulin-positive cells was shown as a percent of misfolded SOD1-positive neurons.

Statistical analysis

All data were shown as mean ± SEM. Results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine statistical significances of the data. A p-value < 0.05 was considered statistically significant. Analyses were performed with IBM SPSS statistics software (IBM, Armonk, NY).

RESULTS

Generation of iPSCs and spinal motor neuron differentiation

iPSCs were generated from mouse embryonic fibroblasts (MEFs) of mutant human *SOD1*^{G93A} transgenic mice (SOD1 G93A), wild-type human *SOD1* transgenic mice (SOD1 WT), and non-transgenic mice (control) (Figure 1A). These generated iPSCs expressed embryonic stem cell (ESC) markers (Figure 1B). Pluripotency of these iPSCs was confirmed by *in vitro* three-germ layer assay (Figure 1C), and generated iPSCs showed normal karyotypes (Figure 1D).

iPSCs were differentiated into spinal motor neurons (Figure 2A). The generated spinal motor neurons were immunostained by motor neuron markers HB9, Isl1, and pan-neuron marker β-III tubulin on Day 11 (Figure 2B).

Evaluation of misfolded SOD1 protein in spinal motor neurons

We evaluated the accumulation of misfolded SOD1 in spinal motor neurons using two misfolded SOD1-specific antibodies with different epitopes; A5C3 recognizes 80-118 amino acids of SOD1, and B8H10 recognizes 57-78 amino acids of SOD1 [8]. SOD1 G93A motor neurons were clearly immunostained by one of the misfolded SOD1-specific antibodies

(A5C3), whereas SOD1 WT motor neurons and control motor neurons were not immunostained by that antibody. The other misfolded SOD1 antibody (B8H10) clearly stained both SOD1 WT motor neurons and SOD1 G93A motor neurons, but did not stain control motor neurons (Figure 3A,B). Our results suggested that misfolded SOD1 antibodies with different epitopes exhibited different sensitivity to misfolded SOD1 protein, and overexpression of wild-type *SOD1* presented protein misfolding in spinal motor neurons as well as mutant *SOD1* overexpression.

DISCUSSION

We generated iPSCs from mutant human SOD1 transgenic mice, wild-type human SOD1 transgenic mice, and non-transgenic mice, and evaluated the accumulation of misfolded SOD1 protein using iPSC-derived spinal motor neurons. We found that overexpression of wild-type SOD1 caused protein misfolding as well as mutant SOD1, and antibody sensitivity to misfolded SOD1 protein differed depending on the epitopes.

It has been suggested that TDP-43, another important molecule in ALS, might more generally be involved in ALS pathology, and ALS caused by SOD1 mutation has been regarded as a separate entity from general ALS [17]. However, accumulation of misfolded

SOD1 protein was reported in sporadic ALS patients without SOD1 mutation [10]. This suggested the contribution of misfolding of wild-type SOD1 to the pathogenesis of sporadic ALS.

Previous studies have presented the toxic effects of wild-type SOD1 *in vivo*. Old mice expressing heterozygous wild-type human *SOD1* exhibited a loss of motor neurons in the spinal cord although life span was not affected [4]. Further, homozygous wild-type *SOD1* transgenic mice developed ALS-like symptoms and exhibited a short life span[18]. Wild-type *SOD1* mice also showed exacerbated disease caused by mutant *SOD1*[19-21]. These reports indicated that increased expression levels of wild-type *SOD1* may accelerate the ALS pathology. Although it has not yet been revealed how structural loosening is caused as a post-translational modification by the overexpression of *SOD1*, our *in vitro* model would be a useful tool for analyzing the pathogenesis of misfolded SOD1-mediated ALS.

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CONTRIBUTIONS

H.I. conceived the project; K.K., K.I. and H.I. designed the experiments; K.K. and K.I. performed the experiments; K.K., K.I., H.Y., R.T. and H.I. analyzed the data; R.T. and H.I. contributed reagents, materials and analysis tools; K.K., K.I. and H.I. wrote the paper.

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2010; 19 (24):4774-4789.

FIGURE LEGENDS

Figure 1. Generation of iPSCs

A. iPSCs were morphologically identical to embryonic stem cells. Scale bar = 100 μm.

B. iPSCs expressed the ESC markers Nanog, Esg1, Rex1, Oct3/4 and Sox2 in RT-PCR.

C. Immunostaining confirmed *in vitro* differentiation ability to all three germ layers. Scale bar = 50 μm.

D. Each iPSC line maintained a normal karyotype.

Figure 2. Motor neuron differentiation

A. Protocol for motor neuron differentiation.

B. Neural stem cell marker nestin was stained on Day 9, and motor neuron markers HB9 and Isl1 were stained on Day 11. Scale bar = 100 μm.

Figure 3. Detection of misfolded SOD1 in iPSC-derived motor neurons

A. Immunostaining of motor neurons on Day 21. SOD1 G93A motor neurons distinguished by ChAT staining were clearly immunolabeled by a misfolded-specific anti-SOD1 monoclonal antibody (A5C3), while SOD1 WT motor neurons and control motor neurons

had less immunoreactivity (upper panels). The other misfolded-specific anti-SOD1 monoclonal antibody (B8H10) showed immunoreactivity to SOD1 G93A motor neurons and SOD1 WT motor neurons (lower panels).. Scale bar = 100 μm.

B. Quantification of misfolded SOD1-positive neurons. The misfolded SOD1-positivity in SOD1 G93A motor neurons was higher than in SOD1 WT motor neurons or control motor neurons by evaluation with misfolded SOD1 antibody (A5C3). Each group represents mean ± SEM, n = 3 wells with each 9 fields images; *P < 0.05, one-way ANOVA followed by Tukey's multiple comparison *post hoc* test. The misfolded SOD1-positivity in SOD1 G93A motor neurons or SOD1 WT motor neurons was higher than in control motor neurons by evaluation with misfolded SOD1 antibody (B8H10). Each group represents mean ± SEM, n = 3 wells, with each providing 9 image fields; *P < 0.05, one-way ANOVA followed by Tukey's multiple comparison *post hoc* test.

TABLE S1. Primer list

Primer	Sequence (5' to 3')
ERas_F	actgcccctcatcagactgctact
ERas_R	caaccactggtttttctgccaccg
Esg1_F	gaagtctggttccttggcaggatg
Esg1_R	actcgatacactggcctagc
Rex1_F	acgagtggcagtttcttcttggga
Rex1_R	tatgactcacttccaggggcact
Oct3/4_F	tctttccaccaggcccccggctc
Oct3/4_R	tgcgggcggacatggggagatcc
Sox2_F	tagagctagactccgggcgatga
Sox2_R	ttgccttaaacaagaccacgaaa
Gapdh_F	accacagtccatgccatcac
Gapdh_R	tccaccaccctgttgctgta

Figure 1

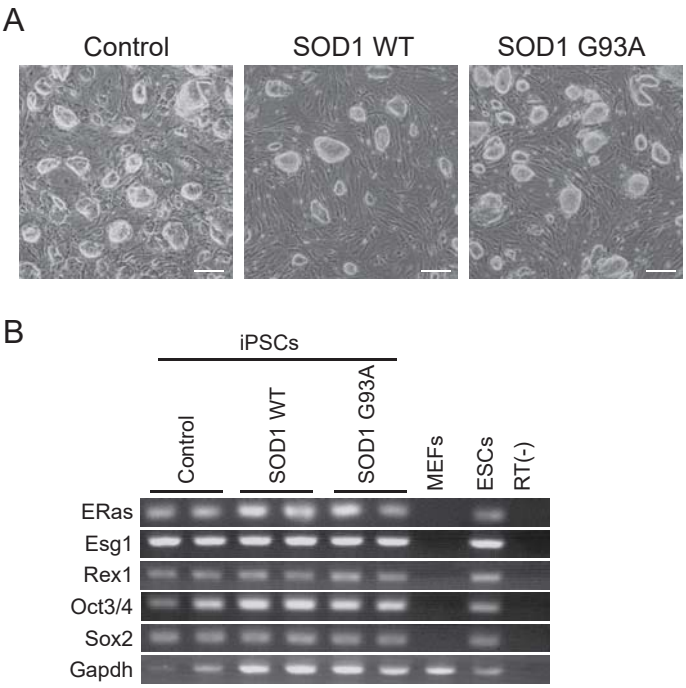
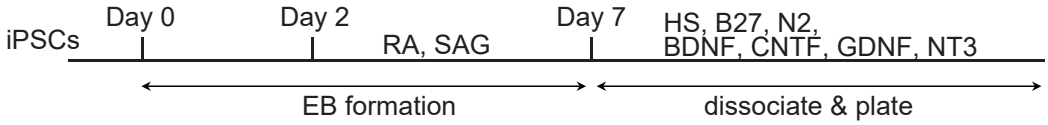

A
Control
SOD1 WT
SOD1 G93A
B
iPSCs
Control
SOD1 WT
SOD1 G93A
MEFs
ESCs
RT(-)
ERas
Esg1
Rex1
Oct3/4
Sox2
Gapdh
C
Control
SOD1 WT
SOD1 G93A
α-fetoprotein/DAPI
α-SMA/DAPI
β-III tubulin/DAPI
D
Control
SOD1WT
SOD1G93A

Figure 2

A


iPSCs
Day 0
Day 2
RA, SAG
Day 7
HS, B27, N2,
BDNF, CNTF, GDNF, NT3
EB formation
dissociate & plate

B

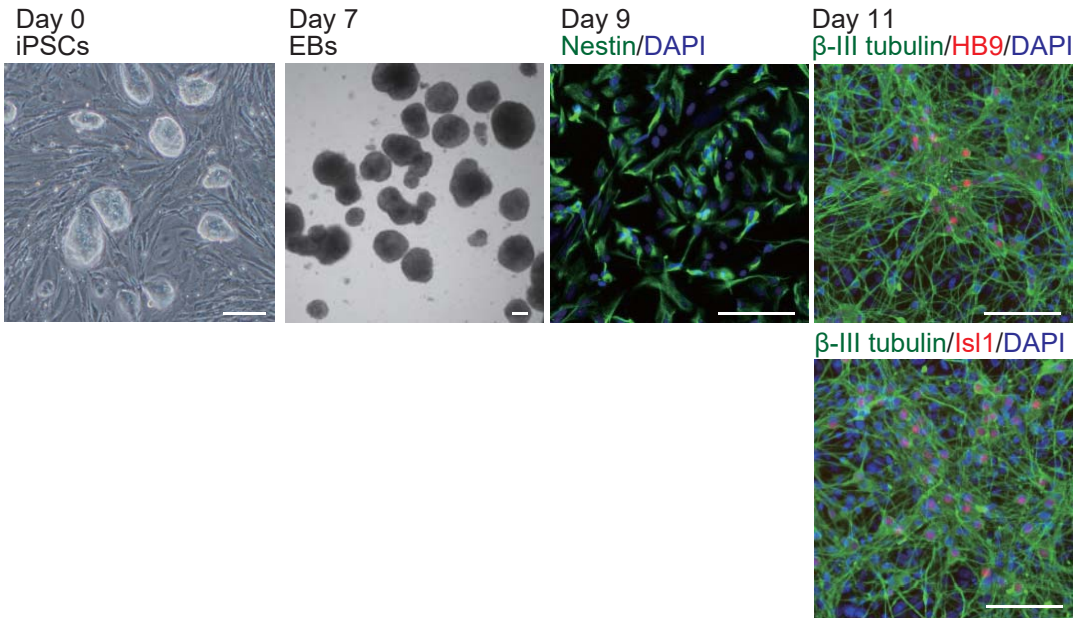
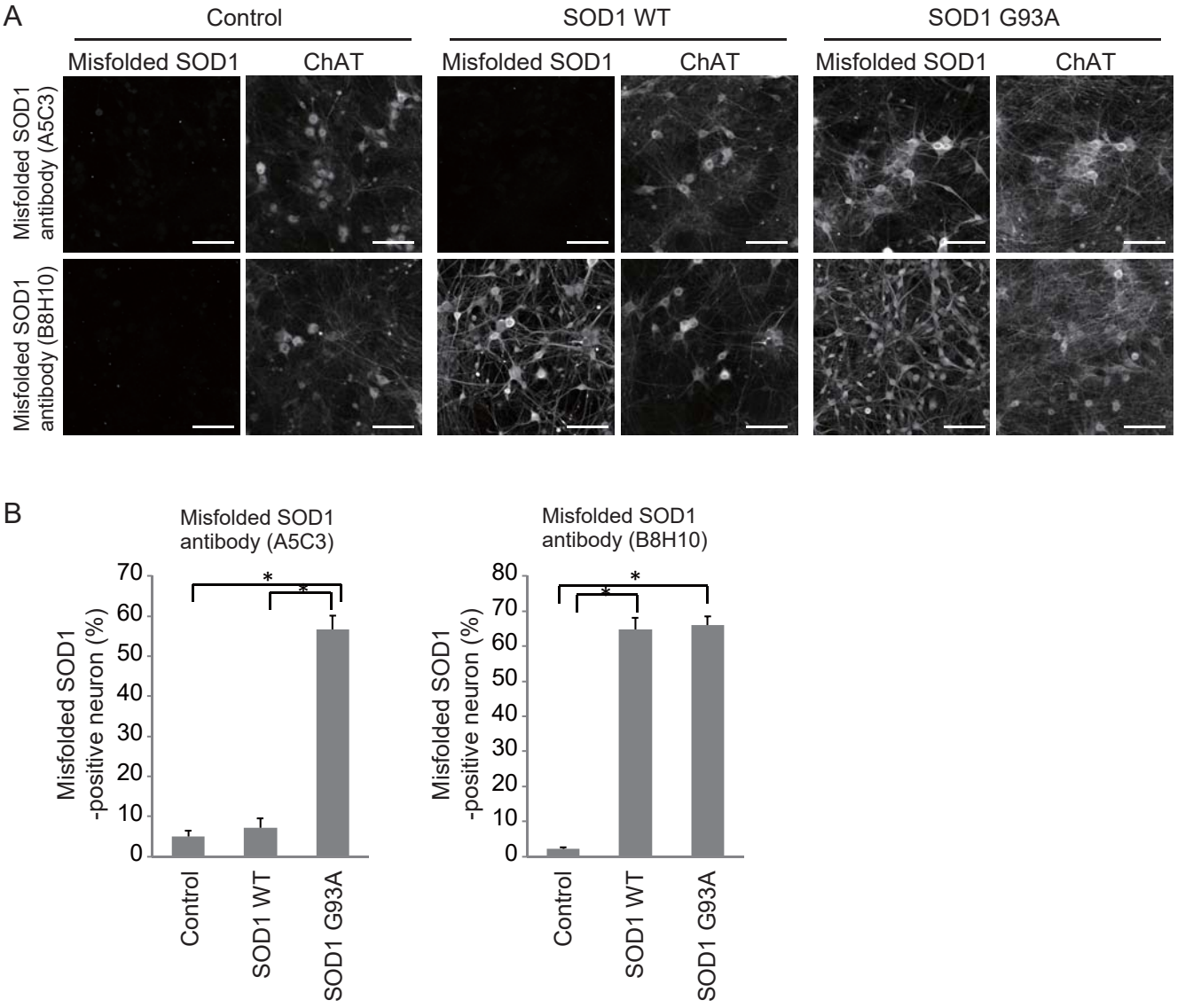

Day 0
iPSCs
Day 7
EBs
Day 9
Nestin/DAPI
Day 11
β-III tubulin/HB9/DAPI
β-III tubulin/Isl1/DAPI

Figure 3


A
Control
SOD1 WT
SOD1 G93A
Misfolded SOD1
ChAT
Misfolded SOD1
ChAT
Misfolded SOD1
ChAT
Misfolded SOD1 antibody (A5C3)
Misfolded SOD1 antibody (B8H10)
B
Misfolded SOD1 antibody (A5C3)
Misfolded SOD1 -positive neuron (%)
70
60
50
40
30
20
10
0
*
*
Control
SOD1 WT
SOD1 G93A
Misfolded SOD1 antibody (B8H10)
Misfolded SOD1 -positive neuron (%)
80
70
60
50
40
30
20
10
0
*
*
Control
SOD1 WT
SOD1 G93A