Human superoxide dismutase 1 overexpression in motor neurons of
*Caenorhabditis elegans* causes axon guidance defect and neurodegeneration

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**ABSTRACT**

Strong evidence indicates that mutant Cu, Zn-superoxide dismutase 1 (SOD1) exerts toxic effect on motor neurons in amyotrophic lateral sclerosis (ALS). However, the nature of mutant SOD1-mediated motor neuron degeneration is poorly understood. To provide new insight into the mechanism by which mutant SOD1 induces motor neuron injury, we developed novel *Caenorhabditis elegans* models of ALS. Expression of human wild type or G93A SOD1 specifically in motor neurons of *C. elegans* caused progressive locomotion defect and paralytic phenotype, which recapitulate some characteristic features of ALS including age-dependent motor dysfunction and degeneration of motor neurons associated with SOD1 aggregation. In addition, the motor neuron loss is independent of cell death protein 3 (CED-3)/cell death protein 4 (CED-4) caspase pathway. We also found that before motor neurons began to die in adulthood, axon guidance defect of motor neuron appeared during the development stages. When green fluorescent protein (GFP)-tagged proteins related to axon guidance were examined in motor neurons, a significantly decreased density and number of GFP-tagged puncta were observed in the transgenic worms. Our models mimic axon developmental defect and the adult-onset degeneration of motor neurons in ALS. Using this model, we uncovered the cell-autonomous damage caused by human SOD1 to motor neurons in vivo, and provided a new insight into the developmental defect mechanism that may contribute to motor neuron degeneration in ALS.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset disorder characterized by progressive degeneration of motor neurons. Most ALS cases occur sporadically (SALS), and approximately 10% of the ALS cases are familial (FALS) (Cleveland and Rothstein, 2001; Rowland and Schneider, 2001). Mutations in the Cu, Zn-superoxide dismutase 1 (SOD1) are the most prevalent form of FALS, accounting for approximately 20% of all the FALS cases (Chen et al., 2013). Since the first SOD1 missense mutation was discovered in 1993 (Rosen et al., 1993), more than 160 different mutations have been identified in the 153-amino acids protein in SOD1-associated FALS cases (Chen et al., 2013; Wroe et al., 2008). Mutations in SOD1 usually confer a dominant gain of function, rather than a loss of dismutase activity (Valentine et al., 2005). Loss of SOD1 function in mice does not cause motor neuron degeneration (Chen et al., 2013; Reaume et al., 1996). However, mice harboring overexpressed mutant SOD1 develop a progressive motor neuron degeneration, which is similar to that observed in ALS patients (Gurney et al., 1994; Turner and Talbot, 2008; Wong et al., 1995). The mutant SOD1-linked FALS and SALS have some clinical similarities (Bosco et al., 2010; Gruzman et al., 2007). Thus, revealing how abnormal SOD1 leads to the dysfunction of motor neurons might shed light on the cause and pathogenesis of FALS and SALS. Although it has been widely accepted that motor neuron injury is associated with SOD1 misfolding and aggregation (Pasinelli and Brown, 2006), the exact molecular event and pathway attributing to the dysfunction of motor neurons remain elusive.

To address this question, we used *Caenorhabditis elegans* as a model organism. *C. elegans* has been used in modeling various neurodegenerative diseases, including polyglutamine expansion diseases, α-synuclein-linked Parkinson disease, and amyloid beta-associated Alzheimer’s disease (Cohen et al., 2006; Kuwahara et al., 2008; Satyal et al., 2000; Silva et al., 2011; Tauffenberger et al., 2013). Although the pan-neuronal expression of human mutant SOD1 has been used for ALS research (Wang et al., 2009), a motor neuron-specific expression of the mutant SOD1 model has not been established in *C. elegans*. 

0197-4580/$ – see front matter © 2014 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.neurobiolaging.2013.09.003
Here, we engineered *C. elegans* by overexpressing human wild type or mutant G93A SOD1 specifically in the D-type motor neurons, which have been used to study motor neuron degeneration in *C. elegans* (Liachko et al., 2010; Vaccaro et al., 2012a, 2012b). We found that during adulthood, the wild type and G93A SOD1 transgenic worms showed locomotor defects and became paralyzed. The worms had SOD1 aggregation and motor neuron degeneration, which were independent of the Ced-3/CED-4 programmed cell death pathway. We also observed axon guidance defect in motor neurons during the development of the transgenic worms. Furthermore, the density and number of green fluorescent protein (GFP)-tagged axon guidance proteins were significantly decreased, suggesting that the axon guidance pathway is impaired by human SOD1 during motor neuron development.

2. Methods

2.1. Molecular biology

The 1819-base pair unc-25 promoter was amplified using KOD-Plus Neo DNA polymerase (Toyobo) from N2 worm genomic DNA. Then, the unc-25 promoter was inserted into the *HindIII* and BarnHI sites of pPD95.77 to generate pPD95.77-unc-25::GFP. The 465-base pair human wild type SOD1 with or without a TAATT stop codon was inserted into the downstream site of NotI to generate pPD95.77-unc-25::wild type SOD1 or pPD95.77-unc-25::wild type SOD1::GFP. The plasmids of pPD95.77-unc-25::G93A SOD1 or pPD95.77-unc-25::G93A SOD1::GFP were made using the same method. The plasmids of pPD95.77-unc-25::UNC-51::GFP, pPD95.77-unc-25::UNC-51::GFP, and pPD95.77-unc-25::UNC-40::GFP were kindly provided by Dr Ogura (Ogura and Goshima, 2006).

2.2. Transgenic strains

Extrachromosomal arrays were attained using injection into the germline and standard techniques (*Mello et al., 1991*). For the wild type or G93A SOD1 expressions, each sample of 100 ng/µL DNA and myo-2::mcherry of 5 ng/µL was injected into the adult gonad. For the wild type or G93A SOD1-GFP expression, each sample of 100 ng/µL DNA was injected. GFP control worms were generated using injection of 100 ng/µL unc-25::GFP plasmid. The plasmids of unc-25::UNC-5::GFP, unc-25::UNC-40::GFP, and unc-25::UNC-51::GFP were injected at 50 ng/µL with 5 ng/µL myo-2::mcherry. Multiple extrachromosomal lines for each transgene were generated based on the fluorescent markers. The SOD1 transgenes were integrated into the genome to generate stable transgenic lines by exposing worms to 4,5-dimethylaminolevulinate (4,5-DALA) combined with UV light. At least 3 independent stable lines were produced for each variant, and the lines were outcrossed to N2 worms 6 times. The main transgenic strains used in this study include: ngl819(unc-25p::GFP), ngl823(unc-25p::wild type SOD1), ngl827(unc-25p::G93A SOD1), ngl836(unc-25p::G93A SOD1::GFP), ngl837(unc-25p::wild type SOD1::GFP), ngl401(unc-25p::UNC-5::GFP), nglEx62(unc-25p::UNC-40::GFP), and nglEx64(unc-25p::UNC-51::GFP).

2.3. *C. elegans* strains

Bristol strain N2 worms were used as the standard nontransgenic wild type worms. Experiments were performed at 20 °C using standard *C. elegans* techniques (Brenner, 1974). The following mutant strains were used in this study: unc-40(n324) I, unc-5(e152) IV, unc-51(e1189) V, ced-3(n717) IV, and ced-4(n162) III.

2.4. Quantitative real-time polymerase chain reaction and primers

We performed real-time polymerase chain reaction (PCR) to quantify gene expression levels. PCR amplification was performed using an Applied Biosystems 7500 Real-Time PCR System in a mixture of SYBR Green real-time PCR mix, SYBR Premix Ex Taq II (Takara) and 0.4 µM/µL of each primer in a final volume of 20 µL. Expression levels relative to the wild type N2 worms were normalized to 2 endogenous reference genes (act-1 and ama-1), and were calculated using the Applied Biosystems 7500 software V2.0.5.

Real-time PCR primers were: ama-1 (forward primer: 5'-CAGTCA-GAAAGGCTATCGAG-3'; reverse primer: 5'-CCAACTCTCTGAGACCTGTG-3'), act-1 (forward primer: 5'-GCCTGACGTGATCTTTCTGATTCC-3'; reverse primer: 5'-GTAGCAGACCTCTCTGTCGATC-3'), and human sod-1 (forward primer: 5'-GCTGGGCGCAA-AAGATGAAAGAG-3'; reverse primer: 5'-CCACAGGCACACAGACTCC-3').

2.5. Locomotion analysis

A video-based assay was used to assess the locomotion speed of *C. elegans* as reported (Wang et al., 2009). The experiment was carried out immediately after the worm was transferred to a fresh nematode growth media (NGM) plate and then measured using video in 30 seconds. The ratio was measured as net distance traveled during 30 seconds, divided by the body length, and then normalized to the nontransgenic N2 the fourth larval stage (L4) worms (Wang et al., 2009). n = 20 for each time point. The ratio of the movement distance to the body length, measured by ImageJ software, was used as the movement index in Fig. 1C–E.

2.6. Paralysis analysis

Worms were scored as paralyzed if they moved their noses but failed to move their bodies when their noses were tapped with a platinum worm picker. Experiments were performed with more than 20 worms per plate in triplicate.

2.7. Fluorescence microscopy

Worms were immobilized in 5 mM sodium azide in M9 buffer on 2% agar pads slides. Images were collected using a Leica TCS SP5 laser confocal scanning microscope. For fluorescence recovery after photobleaching (FRAP), the Leica TCS SP5 laser confocal microscope was used. To analyze the axon guidance defects of motor neurons in *C. elegans*, the number of D-type dorsal/ventral axons that reached the dorsal nerve cord in L4 larvae was counted. At least 20 worms were examined for each strain. Human SOD1 (hSOD1) accumulation was analyzed in each strain by observing at least 20 worms and 19 motor neurons per worm, and the inclusion was categorized according to uniform standards (no inclusion, small, medium, or large inclusion) (Wang et al., 2009; Watson et al., 2008).

2.8. Protein analysis

L4 to young adult worms were washed 4 times with M9 buffer and the packed worms were lysed using sonication in extraction buffer (10 mM Tris-HCl, pH 8.0, with 1 mM Ethylene diamine tetraacetic acid (EDTA)), 100 mM NaCl, and 0.5% NP-40 supplemented with protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF), and 50 mM iodoacetamide) on ice. The lysates were then centrifuged at 130,000 g for 5 minutes using a Beckman-Coulter Optima L-100 XP ultracentrifuge. The supernatant was kept as the soluble sample. The pellet was sonicated in extraction buffer and was centrifuged again at 130,000 g for 5 minutes at 4 °C. Then
the remaining pellet was resolved in resuspension buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 0.5% deoxycholic acid, and 2% sodium dodecyl sulfate [SDS]) and sonicated until the pellet was resuspended in the solution, and this preparation was kept as the insoluble sample (Zhang et al., 2011). For the denaturing and nonreducing SDS-polyacrylamide gel electrophoresis, the soluble and insoluble samples were mixed in Laemmli sample buffer without β-mercaptoethanol. The samples were boiled and were run on SDS-polyacrylamide gel electrophoresis gels. Anti-human SOD1 antibody (SOD-100; Stressgen) and anti-actin antibody (C4; MP Biomedicals) were used for the Western blot analysis.

2.9. Life span analysis

L4 stage worms (n = 60) were placed on NGM plates containing 2′-deoxy-5-fluorouridine (FUDR) (25 μg/mL) and were counted every 2 days from adult Day 1 until death. Worms were scored as dead if they did not respond to external stimuli.

3. Results

3.1. Specific expression of human SOD1 in the D-type motor neurons of C. elegans causes progressive locomotor defects

To examine the effect of ALS-associated human mutant SOD1 in motor neurons, we generated transgenic C. elegans expressing human wild type or G93A SOD1 in the 19 D-type dorsal and ventral motor neurons under the unc-25 promoter. G93A is a gain-of-function mutation and the G93A SOD1 transgenic mice develop the similar disease phenotypes as human ALS (Rosen et al., 1993). To observe the expressed SOD1 proteins directly, 2 additional transgenic worms that had GFP-fused wild type or G93A SOD1 expression in motor neurons were also used. Multiple transgenic C. elegans lines stably integrated with GFP-fused or unfused wild type and G93A SOD1 were obtained. The messenger ribonucleic acid (mRNA) levels of SOD1 were determined for each line using quantitative real-time polymerase chain reaction, and transgenic lines with similar levels of expression (1.0–1.7-fold of WT-SOD1) were chosen for subsequent experiments (Fig. 1A).

C. elegans body wall muscle cells receive excitatory and inhibitory inputs to coordinate muscle contraction and relaxation to facilitate movement (McIntire et al., 1993). The GABAergic D-type motor neurons act as cross-inhibitors for body wall muscle contractions, and they are required for relaxation of the body wall muscles. When these neurons are killed using a laser, the worm only has excitatory input into its muscles. After this procedure, the worm can still move to a sinusoidal posture, but the wave amplitudes decrease (Baran et al., 2010; Jorgensen, 2005). Wild type nontransgenic N2 worms and transgenic worms that express GFP in motor neurons moved in a wavelike pattern (Fig. 1B–D), and the hSOD1 transgenic worms were uncoordinated and the amplitude of the wave pattern was severely reduced compared with the GFP worms. (E) Rates of forward movement of the GFP transgenic worms and hSOD1-GFP transgenic worms. Error bars indicate SD. Abbreviations: 2D, the second day after the fourth larva stage; 4D, the fourth day after the fourth larva stage; 6D, the sixth day after the fourth larva stage; 8D, the eighth day after the fourth larva stage; GFP, green fluorescent protein; hSOD1, human SOD1; L4, the fourth larva stage; mRNA, messenger ribonucleic acid; NGM, nematode growth media; PCR, polymerase chain reaction; SOD1, superoxide dismutase 1; WT, wild type.

Fig. 1. hSOD1 causes motor defects in C. elegans. (A) The levels of mRNA in each transgenic strain. The mRNA levels of all stains used in the present study were measured using real-time PCR, and ranged between 1.0- and 1.7-fold of mRNA expression in wild type SOD1 worms. Error bars indicate SD. (B) L4 worms were placed on OP50 NGM plates. Worms expressing wild type or G93A SOD1 were uncoordinated. N2 worms moved in a wavelike pattern; hSOD1 transgenic worms were uncoordinated and the amplitude of the wave pattern was severely reduced. (C) Rates of forward movement of the nontransgenic N2 worms and hSOD1 transgenic worms. Error bars indicate SD. (D) Worms expressing wild type or G93A SOD1-GFP were uncoordinated, and the amplitude of the wave pattern was severely reduced compared with the GFP worms. (E) Rates of forward movement of the GFP transgenic worms and hSOD1-GFP transgenic worms. Error bars indicate SD. Abbreviations: 2D, the second day after the fourth larva stage; 4D, the fourth day after the fourth larva stage; 6D, the sixth day after the fourth larva stage; 8D, the eighth day after the fourth larva stage; GFP, green fluorescent protein; hSOD1, human SOD1; L4, the fourth larva stage; mRNA, messenger ribonucleic acid; NGM, nematode growth media; PCR, polymerase chain reaction; SOD1, superoxide dismutase 1; WT, wild type.
neurons, although G93A SOD1 leads to a more severe defect in *C. elegans*. The locomotion speed of the GFP-fused SOD1 transgenic worms was also examined. The GFP-fused wild type SOD1 transgenic worms exhibited more severe locomotor defect compared with the nonfused wild type SOD1 worms (Fig. 1C–E), suggesting that the addition of the GFP moiety might aggravate the effect of wild type SOD1 in motor neurons of *C. elegans*. On the contrary, GFP-fused G93A SOD1 worms were somewhat faster than the G93A SOD1 worms. These observations are consistent with the previous report that the addition of the yellow fluorescent protein (YFP) has opposite effects on the wild type and mutant SOD1 in *C. elegans* (Wang et al., 2009).

### 3.2. The hSOD1 transgenic worms display age-dependent paralysis

Because the hallmark of ALS is the paralysis of voluntary muscles, we next investigated whether the uncoordinated motility phenotype of the hSOD1 transgenic worms could progress to paralysis. Although the life spans of the hSOD1 worms were not obviously different from the control worms (wild type non-transgenic N2 worms and GFP transgenic worms), the hSOD1 worms became paralyzed during adulthood (Fig. 2A–C). Paralysis was age-dependent and occurred at a higher rate for hSOD1 worms compared with the control worms. After 12 days of adulthood on plates, nearly 90% of the G93A SOD1 worms became paralyzed and more than 60% of the wild type SOD1 worms were paralyzed, and only 10% of the control worms were affected. Therefore, these results indicate that the wild type SOD1 and G93A SOD1 exert toxic effects on motor neurons of *C. elegans*. In addition, the GFP-fused wild type SOD1 transgenic worms and GFP-fused G93A SOD1 also showed age-dependent paralysis during adulthood although the lifespan was as normal as the control worms. Typically, after 12 days of adulthood, approximately 80% of the GFP-fused hSOD1 transgenic worms became paralyzed (Fig. 2B). The observations indicate that SOD1 or GFP-fused SOD1 causes age-dependent paralysis during adulthood when expressed in motor neurons of *C. elegans*.

### 3.3. Wild type SOD1 and G93A SOD1 aggregate in cytoplasm and axons of motor neurons

Toxic protein-induced neuron dysfunction is often associated with protein aggregation. Abnormal accumulation of SOD1-positive inclusions in the spinal motor neurons is a pathological hallmark in SOD1-related FALS (Jonsson et al., 2008). Fluorescence microscopy observation of GFP fused wild type SOD1 and G93A SOD1 transgenic worms during development and adulthood strikingly revealed that wild type SOD1 and G93A SOD1 were accumulated within motor neurons (Fig. 3A and B). Inclusions appeared as early as the first larva stage (L1) stage and increased with age in number and size (Fig. 3E and F). The inclusions were also visible within the axon of motor neurons (Fig. 3C and D). Although wild type and G93A SOD1 aggregated in motor neurons of *C. elegans*, the G93A SOD1 tended to aggregate at a higher frequency and formed larger inclusions than the wild type SOD1 (Fig. 3C and D; \( \chi^2 \ p < 0.001 \)). Next, we performed biochemical analysis to detect the insoluble SOD1 and soluble SOD1 in the hSOD1 transgenic worms. Homogenized protein extracts from the transgenic worms were separated into soluble supernatant and insoluble pellets using ultracentrifugation at 130,000g for 5 minutes, and the supernatant and pellet fractions were solubilized in nonreducing conditions and analyzed using immunoblotting with anti-SOD1 antibody. As shown in Fig. 3G, G93A SOD1-GFP worms exhibited a higher portion of SOD1 protein in the insoluble fraction and all of the SOD1 protein in the wild type SOD1-GFP worms was soluble. This observation suggests that although wild type and G93A SOD1 form inclusions in motor neurons of *C. elegans*, their biochemical properties are different. The G93A mutation is the replacement of a hydrophilic glycine to hydrophobic alanine, and it causes a SOD1 protein constitutive conformation change and leads to SOD1 aggregation. Although the wild type SOD1 was not found in the insoluble fractions, it could also take on abnormal conformations and become toxic in the context of *C. elegans* motor neurons, probably because of the posttranslational processes, such as oxidation, Cu\(^{2+}\), and Zn\(^{2+}\).

![Graphs showing paralysis and survival](https://example.com/graphs)

**Fig. 2.** hSOD1 causes adult-onset, age-dependent paralysis in *C. elegans*. (A) Wild type SOD1 and G93A SOD1 transgenic worms showed age-dependent paralysis. (B) Wild type SOD1-GFP worms and G93A SOD1-GFP worms showed age-dependent paralysis. Error bars indicate SD. (C) Survival curves of wild type and G93A SOD1 transgenic worms. (D) Survival curves of wild type and G93A SOD1-GFP worms. Abbreviations: GFP, green fluorescent protein; hSOD1, human SOD1; SOD1, superoxide dismutase 1; WT, wild type.
binding. This suggests that when the cell environment is changed, wild type SOD1 could acquire some aberrant conformations and toxic properties of FALS-linked SOD1 mutants.

3.4. hSOD1 causes degeneration of motor neurons independent of the CED-3/CED-4 programmed cell death pathway in C. elegans

In ALS patients, the motor defects and paralysis are because of motor neuron degeneration. We therefore investigated whether there was a gross loss of motor neurons in the wild type SOD1 and G93A SOD1 transgenic worms. To visualize the motor neurons in the living hSOD1 transgenic worms, we crossed the unc-25p:GFP reporter transgene which drives expression of GFP in the 19 D-type motor neurons (Fig. 4A) with the hSOD1 transgenic worms. We observed that motor neuron degeneration occurred in the wild type and G93A SOD1 transgenic worms (Fig. 4B and C). The G93A SOD1 caused a higher rate of neuron degeneration compared with the wild type SOD1 (Fig. 4D). Before the L4 stage, no obvious neuron loss was observed in the hSOD1 transgenic worms. However, after adulthood, the motor neurons died rapidly, indicating that the degeneration is adult-onset and age-dependent (Fig. 4D). Motor neuron degeneration was also observed in the hSOD1-GFP transgenic worms (Fig. 4E). Therefore, our hSOD1 transgenic worms mimic the adult-onset, gradual motor neuron degeneration seen in ALS. Caspase activation has been implicated in the death of motor neurons by mutant SOD1 in the ALS mice model (Pasinelli et al., 2000). To examine whether the caspase pathway is involved in SOD1-induced cell death in our models, we tested CED-3, a major programmed cell death caspase expressed in the neuron of the C. elegans, and CED-4, a CED-3 activating protein (Seshagiri and Miller, 1997). We crossed the hSOD1 transgenic worms with the ced-4 and ced-3 mutants and found that the mutants could not block hSOD1-induced motor neuron death (Fig. 4F). These results from ced-4 and ced-3 mutants document that the motor neuron degeneration induced by hSOD1 is independent of the CED-3/CED-4 programmed cell death pathway.

3.5. hSOD1 transgenic worms show axon guidance defect of motor neurons during development

Neuronal dysfunction always occurs before degeneration in many neurodegenerative diseases (Saxena and Caroni, 2011). Our previous data showed that worms expressing hSOD1 exhibited uncoordinated movement as early as the L4 stage, when the motor neurons did not begin to degenerate (Figs. 1B and 5D). Therefore, we next examined the motor neurons of the hSOD1 worms at the L4...
stage. In *C. elegans*, the D type motor neurons are located along the ventral nerve cord. During development, motor neurons first project their axons anteriorly, then their axons branch to the dorsal nerve cord where they establish synapse connections to the dorsal and ventral body muscles (Ogura and Goshima, 2006) (Fig. 4A). In wild type and G93A SOD1 worms, we found that the axons frequently failed to reach the dorsal destination (Fig. 5A and B). The same results were also observed in the hSOD1-GFP transgenic worms (Fig. 5C and D). We speculated that hSOD1 might destroy the dorsal axon guidance pathway. Therefore, we analyzed some axon guidance mutants and found that the axon guidance defect in hSOD1 transgenic worms was similar to those of Netrin/UNC-40/
UNC-5 signaling mutants unc-5, unc-40, and unc-51 (Fig. 4F–H). UNC-40 and UNC-5 are receptors that are expressed in neurons and both of UNC-40 and UNC-5 are required for dorsally-extending axons to be repulsed by the ventral ligand Netrin (Hedgecock et al., 1990). UNC-51 is a serine/threonine kinase that is expressed in neurons and it is known for its role in vesicle trafficking of UNC-5 in motor neurons of C. elegans (Ogura and Goshima, 2006). Therefore, we speculated that UNC-5, UNC-40, and/or UNC-51 might be affected in motor neurons by hSOD1 during development.

To clarify the problem, we generated transgenic lines that were carried out with GFP-tagged UNC-5, UNC-40, or UNC-51 in motor neurons. Wild type and G93A SOD1 transgenic worms were crossed with the 3 lines separately and then the double transgenic lines were examined for the distribution of the fluorescence proteins in motor neurons.

In the control worms (which do not express hSOD1 in motor neurons), the functional UNC-5::GFP, which can rescue the guidance defects of unc-5 (Killeen et al., 2002; Ogura and Goshima, 2006), was associated with small vesicles that are evenly distributed in the cell bodies and axons of the motor neurons (Fig. 6A). When wild type or G93A SOD1 was introduced, the UNC-5::GFP puncta along the axons, its brightness, and the UNC-5::GFP-positive neurons were greatly reduced (Fig. 6A and B). A similar decrease in number and brightness of the fluorescence proteins was observed in the hSOD1 worms as well (Fig. 6C).

Fig. 6. hSOD1 worms show decreased brightness of puncta of GFP-tagged axon guidance proteins. (A) The distribution of UNC-5::GFP, UNC-40::GFP, and UNC-51::GFP were examined in motor neurons of L4 stage N2 control worms and L4 stage hSOD1 worms. Scale bar, 10 μm. (B) Left panel: quantification the fluorescence of UNC-5::GFP puncta along a 50-μm distance of the axon. Five worms of each genotype at the L4 stage were examined and then normalized to N2 worms. In each case, the fluorescence intensity at a 50-μm distance of the axon was measured using ImageJ software. Right panel: quantification of the number of UNC-5::GFP-positive neurons in the indicated strains. Five worms of each strain at the L4 stage were examined. The fluorescence of UNC-5::GFP puncta and the number of UNC-5::GFP-positive neurons were significantly reduced in hSOD1 transgenic worms; p < 0.0001. Error bars indicate SD. (C) hSOD1 worms exhibited diminished fluorescence recovery of UNC-5::GFP after photo bleach. Left: example of photobleach of UNC-5::GFP on a ventral axon. Right: graphical representation of fluorescence recovery after photo bleaching in the left panels. White circles indicate the areas of photobleach. Relative fluorescence intensity (RFI) value on the y-axis represents percentage of fluorescence corrected for background bleaching; the x-axis represents the time of recovery; n = 5 for each strain. Error bars indicate SD. (D) Axon degeneration of the hSOD1 transgenic worms during adulthood. n = 20 for each strain. Error bars indicate SD. Abbreviations: GFP, green fluorescent protein; hSOD1, human SOD1; L4, the fourth larval stage; SOD1, superoxide dismutase 1; WT, wild type.
also observed when the GFP-tagged UNC-40 or UNC-51 was similarly crossed into the hSOD1 worms (Fig. 6A). These results suggest that wild type and G93A SOD1 might affect the axon guidance proteins in motor neurons during development. Thus, the hSOD1-affected motor neurons cannot innervate the dorsal and ventral body muscles and the transgenic worms became uncoordinated. To verify whether the axon guidance protein changes precede the axon guidance defects, we made the double transgenic worms, unc-25::UNC-5/UNC-40/UNC-51::GFP; unc-25::WT/G93A SOD1::GFP. We found that the axon guidance proteins could partially rescue the axon guidance defect in the SOD1 transgenic worms, suggesting that the axon guidance protein changes might precede the axon guidance defect (Supplementary Fig. 1). The partial rescue result indicates that SOD1 might damage other axon development proteins in addition to the 3 proteins mentioned in our model.

Furthermore, a FRAP experiment was carried out on the UNC-5::GFP worms, which showed a much slower recovery of fluorescence in the axons of motor neurons in wild type and G93A SOD1 worms compared with the control worms (Fig. 6C). This reflects the immobile state and abnormal dynamic state of the UNC-5 receptor influenced by hSOD1 in motor neurons of C. elegans. During adulthood, the remaining axons that reached the dorsal nerve cord in the hSOD1 transgenic worms also degenerated (Fig. 6D), which corresponded to the loss of motor neurons caused by hSOD1 (Fig. 4).

4. Discussion

In this report, we presented the C. elegans model of ALS by expressing hSOD1 in motor neurons. Our findings support a role for cell-autonomous damage to motor neurons by hSOD1. It suggests that hSOD1 has an intrinsic toxicity to the motor neurons in C. elegans and the damage within motor neurons alone suffices to produce motor defects.

Initial reports assumed that damage solely within motor neurons and ALS was cell-autonomous. However, studies with mice showed no toxicity with neuron-restricted expression of mutant hSOD1 (Lino et al., 2002; Pramatarova et al., 2001). It was reported that motor neurons, which did not express the mutant protein, can display ALS-like pathology when surrounded by other types of cells that expressed the mutant protein (Clement et al., 2003). In other studies, it was shown that astrocytes expressing mutant SOD1 can release factors toxic to motor neurons (Marchetto et al., 2008; Nagai et al., 2007). Although such studies in mice suggested that motor neurons might not be the primary site of damage in ALS models, reducing mutant hSOD1 expression in motor neurons delayed disease onset and extended the lifespan in an ALS model (Boillee et al., 2006; Ralph et al., 2005). In a later study it was reported that neuronal expression of mutant hSOD1 was sufficient to cause motor neuron degeneration and paralysis in transgenic mice when the neuron-specific Thy1.2 promoter was used, which drove much higher expression of mutant SOD1 in neurons (Jaarsma et al., 2008). These findings indicate that SOD1 indeed plays a critical role in damaging motor neurons. The establishment of the novel hSOD1 C. elegans model provides an useful tool to define toxic properties of SOD1 specifically in motor neurons.

In our experiments, wild type SOD1 was originally used as the control for G93A SOD1 because it was reported that wild type SOD1 driven by a pan-neuronal snb-1 promoter caused no toxic effect in C. elegans (Wang et al., 2009). However, in our models, overexpressed wild type SOD1 also exerted toxicity on motor neurons, although it was moderately less than that of the G93A SOD1. Wild type SOD1 was also found to aggregate and formed inclusions in motor neurons, which was thought to be a unique feature expected of a mutant but not wild type SOD1. The different observations of toxicity in wild type SOD1 might be because of the following reasons. First, our transgenic worms were generated using injection of concentrations (100 ng/µL) of SOD1 plasmids greater than the pan-neuronal SOD1 models (20 ng/µL). Thus, our models might represent a greater expression of hSOD1 in motor neurons. Second, the unc-25 promoter might drive more hSOD1 expression in motor neurons than does the pan-neuronal snb-1 promoter. This similar observation was reported in mice; the midbrain dopamine neuron-specific promoter drove more expression of A53T α-synuclein than did the pan-neuronal promoter in the midbrain neurons (Lin et al., 2012). Third, our unpublished data showed that motor neurons were more susceptible to the toxicity of hSOD1 than other types of neurons, such as the dopamine neurons posterior deirid (PDE) neuron, cephalic sensilla (CPE) neurons, and anterior deirid (ADE) neurons in C. elegans. Similar results were also observed in a Drosophila model of ALS: wild type SOD1 caused motor neuron damage and induced progressive motor dysfunction (Watson et al., 2008). It was proposed that when posttranslational processes were altered or nonheritable modifications (such as oxidation, Cu2+ and Zn2+ binding) were introduced, wild type SOD1 acquired aberrant conformation and the toxic property of FALS-linked SOD1 mutants (Bosco et al., 2010; Estevez et al., 1999; Ezzi et al., 2007; Furukawa, 2012; Silva et al., 2011). Study in beta-amyloid cytotoxicity suggests that intracellular soluble peptides might be the culprit for neuronal degeneration, and it is possible that soluble SOD1 might be responsible for the motor neuron injury. Therefore, at least in the context of C. elegans D-type motor neurons, the overexpressed wild type SOD1 conferred abnormal conformation change, aggregated, and became toxic. The effects from wild type SOD1 are slightly less than that of G93A SOD1. Consistent with the observation that wild type SOD1 inclusions found in motor neurons of C. elegans, SOD1-positive inclusions can be detected not only in FALS, but also in some cases of SALS (Jonsson et al., 2008; Matsumoto et al., 1996; Shibata et al., 1996).

Motor defects of the hSOD1 transgenic worms are probably the results of the damage and death of motor neurons caused by hSOD1. Loss of the cell bodies and axons of the motor neurons were observed during the adulthood of C. elegans. Furthermore, we found that hSOD1-induced motor neuron death is independent of the CED-4/CED-3 apoptotic pathway. It was reported that in the G93A SOD1 mice model, caspase-3 activation was not crucial for degeneration of motor neurons; lack of obvious Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the degenerating motor neurons also suggests that the motor neuron degeneration might not occur through the caspase pathway (Kang et al., 2003). It is possible that other mechanisms of cell death pathway such as necrosis or autophagy may contribute to hSOD1-induced motor neuron degeneration. Future work should include whole genome RNA interference to precisely identify the genes and pathways that participate in motor neuron degeneration in our models.

Pathological changes in the axons of motor neurons appear to precede clinical symptoms and motor neuron degeneration in ALS mice models (Pun et al., 2006), which are generally in line with our findings in the transgenic worms that severe dorsal axon guidance defect occurs prior to obviously motor neuron loss during the development of the worm when hSOD1 was expressed in motor neurons. This indicates that hSOD1 impairs dorsal axon guidance signaling. After analyzing the GFP-tagged proteins that are critical for dorsal axon guidance in motor neurons, we found that the levels of UNC-5::GFP, UNC-40::GFP, and UNC-51::GFP were much reduced in the hSOD1 transgenic worms. The cell bodies and axons showed diminished distribution of the GFP-tagged proteins. FRAP assay showed that UNC-5::GFP were immobile in axons when wild type or G93A SOD1
was introduced, reflecting the stalling of vesicle transport along the axon. In the mouse model of ALS, the axonal transport defect was also observed in wild type SOD1 mice and G93A SOD1 mice (Marinkovic et al., 2012). Axon guidance proteins are crucial in regulating motor axon guidance and motor neuron connectivity during development, and the defects in the expression or function of axon guidance proteins might underlie the pathological changes of motor axons that are associated with ALS (Schmidt et al., 2009). Future work should include Ethyl methanesulfonate (EMS)-forward genetic screening to identify key genes that might potentially participate in the hSOD1-induced axon guidance defect. Furthermore, questions concerning how the GFp-tagged axon guidance proteins become diminished by hSOD1 remain to be resolved.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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This work was supported by grants from the National Natural Science Foundation of China (81171201 and 81370470), and the National Basic Research Program of China from the Science and Technology Commission (2010CB945200 and 2011CB510003).

The authors thank Ken-ichi Ogura (Yokohama City University Graduate School of Medicine) for some vectors, Dr Zhang Hong (NIBS, Beijing) for the help of microinjection, and Dr Cai Shiqing (ION, SIBS, Shanghai) for the help of UV integration. Strains used in this report were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.09.003.

References

Boller, S., Yamanao, K., Lobsiger, C.S., Copeland, N.G., Jenkins, N.A., Kassiotis, G., Baran, R., Castelblanco, L., Tang, G., Shapiro, I., Goncharov, A., Jin, Y., 2010. Motor axonal transport defect. Furthermore, questions associated with ALS (Schmidt et al., 2009). Future work should include Ethyl methanesulfonate (EMS)-forward genetic screening to identify key genes that might potentially participate in the hSOD1-induced axon guidance defect. Furthermore, questions concerning how the GFp-tagged axon guidance proteins become diminished by hSOD1 remain to be resolved.

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