protein deposition remains unknown. This study strongly supports a seed-dependent mechanism for the formation of the intracellular protein aggregates. In the context of our propagation hypothesis, it will be crucial to inhibit not only the production of intracellular amyloid seeds but also their spread into the extracellular space. Vaccination against the intracellular amyloid proteins, such as α-syn (41) or Tau may be an effective approach, together with inhibition of intracellular amyloid filament formation by small molecular inhibitors, for the therapy of these diseases.

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REFERENCES

1. Chiti, F., and Dobson, C. M. (2006) *Annu. Rev. Biochem.* **75,** 333–366
2. Goedert, M., Spillantini, M. G., and Davies, S. W. (1998) *Curr. Opin Neurobiol.* **8,** 619–632
3. Prusiner, S. B. (2001) *N. Engl. J. Med.* **344,** 1516–1526
4. Soto, C., Estrada, L., and Castilla, J. (2006) *Trends Biochem. Sci.* **31,** 150–155
5. Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M. S., Shen, J., Takio, K., and Iwatsubo, T. (2002) *Nat. Cell Biol.* **4,** 160–164
6. Kirschner, D. A., Inouye, H., Duffy, L. K., Sinclair, A., Lind, M., and Selkoe, D. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84,** 6953–6957
7. Eanes, E. D., and Glenner, G. G. (1968) *J. Histochem. Cytochem.* **16,** 673–677
8. Nguyen, J. T., Inouye, H., Baldwin, M. A., Fletterick, R. J., Cohen, F. E., Prusiner, S. B., and Kirschner, D. A. (1995) *J. Mol. Biol.* **252,** 412–422
9. Serpell, L. C., Berriman, J., Jakes, R., Goedert, M., and Crowther, R. A. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97,** 4897–4902
10. Berriman, J., Serpell, L. C., Oberg, K. A., Fink, A. L., Goedert, M., and Crowther, R. A. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 9034–9038
11. Perutz, M. F. (1999) *Trends Biochem. Sci.* **24,** 58–63
12. Harper, J. D., and Lansbury, P. T., Jr. (1997) *Annu. Rev. Biochem.* **66,** 385–407
13. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) *Cell* **73,** 1055–1058
14. Jakes, R., Spillantini, M. G., and Goedert, M. (1994) *FEBS Lett.* **345,** 27–32
15. Nonaka, T., Iwatsubo, T., and Hasegawa, M. (2005) *Biochemistry* **44,** 361–368
16. Aoyagi, H., Hasegawa, M., and Tamaoka, A. (2007) *J. Biol. Chem.* **282,** 20309–20318
17. Taniguchi, S., Suzuki, N., Masuda, M., Hisanaga, S., Iwatsubo, T., Goedert, M., and Hasegawa, M. (2005) *J. Biol. Chem.* **280,** 7614–7623
18. Nonaka, T., Kametani, F., Arai, T., Akiyama, H., and Hasegawa, M. (2009) *Hum. Mol. Genet.* **18,** 3353–3364
19. Nonaka, T., and Hasegawa, M. (2009) *Biochemistry* **48,** 8014–8022
20. Sung, J. Y., Kim, J., Paik, S. R., Park, J. H., Ahn, Y. S., and Chung, K. C. (2001) *J. Biol. Chem.* **276,** 27441–27448
21. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6469–6473
22. Conway, K. A., Rochet, J. C., Bieganski, R. M., and Lansbury, P. T., Jr. (2001) *Science* **294,** 1346–1349
23. Masuda, M., Suzuki, N., Taniguchi, S., Oikawa, T., Nonaka, T., Iwatsubo, T., Hisanaga, S., Goedert, M., and Hasegawa, M. (2006) *Biochemistry* **45,** 6085–6094
24. Giasson, B. I., Murray, I. V., Trojanowski, J. Q., and Lee, V. M. (2001) *J. Biol. Chem.* **276,** 2380–2386
25. Desplats, P., Lee, H. J., Bae, E. J., Patrick, C., Rockenstein, E., Crews, L., Spencer, B., Masliah, E., and Lee, S. J. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 13010–13015
26. Hasegawa, M., Fujiwara, H., Nonaka, T., Wakabayashi, K., Takahashi, H., Lee, V. M., Trojanowski, J. Q., Mann, D., and Iwatsubo, T. (2002) *J. Biol. Chem.* **277,** 49071–49076
27. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292,** 1552–1555
28. Gilon, T., Chomsky, O., and Kulka, R. G. (1998) *EMBO J.* **17,** 2759–2766
29. Friedhoff, P., von Bergen, M., Mandelkow, E. M., Davies, P., and Mandelkow, E. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 15712–15717
30. Wood, S. J., Wypych, J., Steavenson, S., Louis, J. C., Citron, M., and Biere, A. L. (1999) *J. Biol. Chem.* **274,** 19509–19512
31. Tanaka, M., Chien, P., Yonekura, K., and Weissman, J. S. (2005) *Cell* **121,** 49–62
32. Luk, K. C., Song, C., O'Brien, P., Stieber, A., Branch, J. R., Brunden, K. R., Trojanowski, J. Q., and Lee, V. M. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 20051–20056
33. Clavaguera, F., Bolmont, T., Crowther, R. A., Abramowski, D., Frank, S., Probst, A., Fraser, G., Stalder, A. K., Beibel, M., Staufenbiel, M., Jucker, M., Goedert, M., and Tolnay, M. (2009) *Nat. Cell Biol.* **11,** 909–913
34. Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S., and Roses, A. D. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90,** 1977–1981
35. Strittmatter, W. J., Saunders, A. M., Goedert, M., Weisgraber, K. H., Dong, L. M., Jakes, R., Huang, D. Y., Pericak-Vance, M., Schmechel, D., and Roses, A. D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11183–11186
36. Olesen, O. F., Mikkelsen, J. D., Gerdes, C., and Jensen, P. H. (1997) *Brain Res. Mol. Brain Res.* **44,** 105–112
37. Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E., and Ikeda, K. (1991) *Brain Res.* **541,** 163–166
38. Zerbinatti, C. V., Wahrle, S. E., Kim, H., Cam, J. A., Bales, K., Paul, S. M., Holtzman, D. M., and Bu, G. (2006) *J. Biol. Chem.* **281,** 36180–36186
39. Cataldo, A. M., Peterhoff, C. M., Troncoso, J. C., Gomez-Isla, T., Hyman, B. T., and Nixon, R. A. (2000) *Am. J. Pathol.* **157,** 277–286
40. Braak, H., and Braak, E. (1991) *Acta Neuropathol.* **82,** 239–259
41. Masliah, E., Rockenstein, E., Adame, A., Alford, M., Crews, L., Hashimoto, M., Seubert, P., Lee, M., Goldstein, J., Chilcote, T., Games, D., and Schenk, D. (2005) *Neuron* **46,** 857–868
42. Yonetani, M., Nonaka, T., Masuda, M., Inukai, Y., Oikawa, T., Hisanaga, S. I., and Hasegawa, M. (2009) *J. Biol. Chem.* **284,** 7940–7950

Mutant SOD1 in cell types other than motor neurons and oligodendrocytes accelerates onset of disease in ALS mice

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Dominant mutations in ubiquitously expressed superoxide dismutase (SOD1) cause familial ALS by provoking premature death of adult motor neurons. To test whether mutant damage to cell types beyond motor neurons is required for the onset of motor neuron disease, we generated chimeric mice in which all motor neurons and oligodendrocytes expressed mutant SOD1 at a level sufficient to cause fatal, early-onset motor neuron disease when expressed ubiquitously, but did so in a cellular environment containing variable numbers of non-mutant, non-motor neurons. Despite high-level mutant expression within 100% of motor neurons and oligodendrocytes, in most of these chimeras, the presence of WT non-motor neurons substantially delayed onset of motor neuron degeneration, increasing disease-free life by 50%. Disease onset is therefore non-cell autonomous, and mutant SOD1 damage within cell types other than motor neurons and oligodendrocytes is a central contributor to initiation of motor neuron degeneration.

cell autonomous | motor neuron disease

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the selective loss of motor neurons, which results in progressive and ultimately fatal paralysis of skeletal muscles leading to death usually within 2 to 5 years after disease onset. Ten percent of cases of ALS are inherited, and the most frequent causes of familial ALS are dominant mutations in the gene for Cu/Zn superoxide dismutase (SOD1). Whereas deletion of SOD1 from mice does not cause motor neuron disease, ubiquitous expression of mutant SOD1 in rodents leads to progressive, selective motor neuron degeneration, arising from an acquired toxicity of mutant SOD1. Although several hypotheses have been proposed to explain the mechanism of mutant SOD1-mediated toxicity (including formation of protein aggregates because of reduced conformational stability, mitochondrial dysfunction, excitotoxicity, abnormal axonal transport, mutant-derived oxidative damage, lack of growth factors, and inflammation), the exact mechanisms responsible for the onset, and then the progression, of motor neuron degeneration remain unknown (1–3).

A central unsolved question is how the surrounding environment affects mutant SOD1-expressing motor neurons. Whereas initial efforts to selectively express mutant SOD1 in neurons (4, 5) failed to cause motor neuron degeneration or disease, pan-neuronal mutant expression (from the Thy1 promoter) did yield very late onset disease in one mouse line, but even that disease progressed very slowly (6). Construction and analysis of chimeric mice initially implicated cells neighboring motor neurons as important players in determining survival (7). Subsequently, we and others demonstrated that selective reduction in mutant SOD1 expression within microglia (8, 9) and astrocytes (10) significantly slows the progression of disease, but, surprisingly, does not affect the timing of disease onset. Among several possibilities, acceleration of progression by mutant astrocytes could depend on glutamate-dependent excitotoxicity due to loss of the EAAT2 glutamate transporter from affected regions (11), an enhanced inflammatory response from microglia mediated by mutant-expressing astrocytes (10), inability to regulate glutamate receptor subunit 2 (GluR2) expression in motor neurons by mutant astrocytes (12), or release by astrocytes of one or more unknown toxic factors, as suggested by accelerated death *in vitro* of embryonic stem cell-derived motor neurons when cocultured with mutant expressing glial cells or conditioned media from those cells (13, 14). Together, these data raised the possibility that accelerated disease progression does not alter the timing of disease onset.

Evidence that disease onset might have different genetic and cellular determinants than disease progression comes from the recent demonstration that selective down-regulation of mutant SOD1 solely within the postnatal motor neurons (for example, using vesicular acetylcholine transporter promoter-driven Cre mice (15) and a deletable "floxed" mutant SOD1 transgene) significantly slowed onset without affecting disease progression (10). Recent work also demonstrated that pan-neuronal expression of mutant SOD1 in mice (using the Thy-1 promoter) resulted in very late onset motor neuron disease (6). However, unresolved is whether onset of disease from this neuron-specific mutant synthesis is delayed when compared with mice expressing mutant SOD1 at comparable levels within motor neurons and also expressing the mutant ubiquitously.

It therefore remains untested whether the onset of mutant SOD1-mediated ALS is determined solely by cell-autonomous mechanisms. Whereas mutant microglia and astrocytes strongly accelerate disease progression (8–10), and mutant synthesis selectively within astrocytes can yield astrocytic activation, but not motor neuron degeneration (16), the influence of mutant expression within cell types beyond motor neurons on the onset of degeneration is unknown. To resolve these issues, we created aggregation chimeras derived from embryonic cells of $SOD1^{G37R}$ mice (17) and embryonic cells from mice lacking the Olig1 and Olig2 transcription factors ($Olig^{-/-}$) (18) to analyze disease in mice in which all motor neurons (and oligodendrocytes) express high levels of mutant SOD1.

Results

$Olig^{-/-}$::$SOD1^{G37R}$ Chimeric Mice. Our goal was to generate mice in which all motor neurons were derived solely from an $SOD1^{G37R}$ mutant mouse line that develops early-onset disease (17) but in

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The authors declare no conflict of interest.

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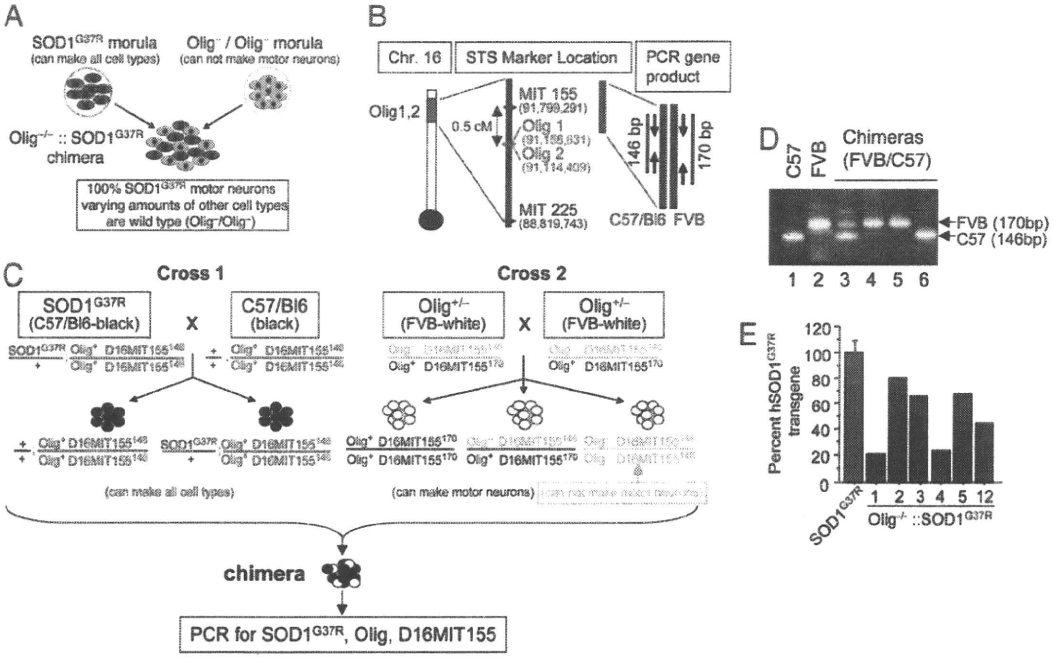

Fig. 1. Strategy for generating Olig$^{-/-}$::SOD1^{G37R} chimeric mice. (*A*) Experimental design for creation of chimeric mice that have 100% SOD1^{G37R} motor neurons. (*B*) Location of the Olig1/2 locus and D16MIT155 STS marker on mouse chromosome 16. (*C*) Mating and genotyping strategy for generating Olig$^{-/-}$::SOD1^{G37R} chimeric mice. Olig$^{+/-}$ mice used for Cross 2 were generated by the mating of Olig$^{+/-}$ mice (C57BL/6 genetic background) with WT FVB mice. The SOD1^{G37R} mice were of a C57BL/6 genetic background (the WT Olig^{+} allele from SOD1^{G37R} mice is highlighted in red). Expected genotypes of each embryo are described. Olig$^{-/-}$ embryos were generated by intercrossing Olig$^{+/-}$ mice (Cross 2). Chimeras derived from Olig$^{-/-}$ embryos were distinguished from Olig$^{+/-}$ by using the D16MIT155 STS marker, which is closely located near the Olig1/2 locus. The Olig^{-} allele derived from the C57BL/6 background carries MIT155^{146} (highlighted in green). (*D*) PCR products of the D16MIT155 STS marker. A PCR product of 146 bases is produced from the C57BL/6 background or Olig$^{-/-}$::SOD1^{G37R} or Olig$^{-/-}$::WT chimeras (lanes 1 and 6), whereas chromosome 16 derived from FVB mice produces a PCR product of 170 base (D16MIT155^{170}, lanes, 2, 4, and 5). (*E*) Degree of chimerism of Olig$^{-/-}$::SOD1^{G37R} mice assessed by Q-PCR for the SOD1^{G37R} transgene. Duplex Q-PCR (three independent runs) on genomic DNA from tail extracts was used to analyze the levels of SOD1 mutant transgene. Tail DNA extracts from the original SOD1^{G37R} line 42 ($n = 2$) were used as positive controls and assigned a 100% transgene content (red bar).

which other cell types would have varying proportions of mutant and normal cells. Such chimeric mice were produced by aggregation of cells from morula stage SOD1^{G37R} embryos (which can generate all cell types) and embryos deficient in the Olig1 and Olig2 (Olig$^{-/-}$) transcription factors; these homozygous mutants are unable to generate motor neurons and oligodendrocytes and ordinarily die before birth (Fig. 1*A*).

To distinguish chimeric mice derived from Olig$^{+/-}$ and Olig$^{-/-}$ mice, we used differential marking of the Olig^{+} allele. Thus, Olig$^{-/-}$ embryos were generated by intercrossing of Olig$^{+/-}$ mice in which the Olig^{+} allele was marked with a sequence length polymorphism found in the FVB mouse strain (a 24-base insertion) at the D16MIT155 locus (Gene ID: 62773, UniSTS:127788) that lies 0.5 cM from the Olig gene (Fig. 1*B*). The tight linkage between D16MIT155 and the Olig genes was confirmed by the observed lack of recombination among 113 offspring obtained from the breeding of Olig$^{+/-}$ mice (D16MIT155$^{170/146}$). Thus, all chimeras derived from either WT or heterozygous Olig embryos would generate a 170-bp fragment (D16MIT155^{170}) after PCR of tail genomic DNA (Fig. 1 *B* and D and supporting information (SI) Fig. S1). However, the absence of the expanded D16MIT155 allele in similar PCRs of genomic DNA from each chimera would be diagnostic for chimeras derived from Olig$^{-/-}$ embryos (Fig. 1 *B* and D, lane 6). Subsequently immunocytochemistry was used to confirm that all motor axons in these Olig$^{-/-}$ chimeras were in fact mutant (see Fig. 2*A*).

Although no Olig1/2-deficient or Olig2-deficient mice are viable postnatally because of the absence of motor neurons (18–20), 25 of 100 viable chimeras (as judged by mottled coat color) contained Olig$^{-/-}$ cells (Fig. 1*C* and Fig. S1). Approximately half of these Olig$^{-/-}$ chimeras showed abnormal limb posture, which was evident early in life (Table 1). In most cases, one or two limbs (either forelimbs or hindlimbs) were contracted (Movies S1 and S2). Because these abnormalities of limb posture and ambulatory activity were not progressive and were of comparable severity and frequency in Olig$^{-/-}$ chimeras fused with either WT or SOD1^{G37R} mutant embryos, they cannot result from SOD1 mutant derived damage. Instead, they must reflect congenital abnormalities probably resulting from one or more developmental deficits associated with lack of proper Olig function, i.e., Olig$^{-/-}$.

Seven chimeras derived from an SOD1^{G37R}-positive embryo and an Olig$^{-/-}$ embryo were identified by the presence of the SOD1^{G37R} transgene and homozygosity at the marked Olig$^{-/-}$ locus (D16MIT155$^{146/146}$) (Fig. S1). These Olig$^{-/-}$::SOD1^{G37R} chimeras were found at approximately the expected frequency and with varying degrees of chimerism, as measured either by coat color or by proportion of SOD1^{G37R} transgenes within genomic tail DNA (Fig. 1*E*).

All Motor Neurons in Olig$^{-/-}$::SOD1^{G37R} Chimeras Are Mutant SOD1-Expressing. To test whether all motor neurons of Olig$^{-/-}$::SOD1^{G37R} chimeras expressed mutant SOD1 as expected from the prior genetic efforts, the fifth lumbar (L5) ventral and dorsal roots were immunostained with human-specific SOD1 antibody. Transverse sections of L5 ventral roots showed that all motor axons of Olig$^{-/-}$::SOD1^{G37R} chimeras expressed human SOD1^{G37R} (Fig. 2 *A* and *B*), yielding images (and fluorescence intensities) comparable with the analysis of germ-line SOD1^{G37R} roots (Fig. 2*D*). Lumbar spinal cord sections from different levels were stained with human SOD1 antibody and SMI-32, a marker for neurons. All motor neurons examined from different levels of lumbar spinal cord expressed human SOD1 (Fig. 2 *F–K*), confirming that

Table 1. Characteristics of Olig$^{-/-}$::SOD1^{G37R} and Olig$^{-/-}$::WT chimeric mice

ID	SOD1^{G37R}	Survival, days	Disease	Abnormal limb posture at birth	L4 motor axon (R/L)	L5 motor axon (R/L)
1	+	239	No	Yes	271/326	454/407
2	+	234	No	No	403/549	595/680
3	+	224	No	Yes	341/488	410/ND
4	+	211	No	Yes	405/284	543/314
5	+	192	Yes	Yes	544/ND	757/746
6	−	395	No	No	477/541	511/886
7	−	368	No	No	463/473	839/743
8	−	366	No	Yes	ND/241	323/456
9	−	269	No	Yes	584/457	ND/517
10	−	269	No	No	612/399	664/606
11	−	267	No	No	845/760	873/908
12	+	65	No	No	573/486	492/397
13	−	65	No	Yes	438/301	418/435
Mean Olig$^{-/-}$::SOD1^{G37R}					424 ± 109 (n = 11)	526 ± 150 (n = 11)
Mean Olig$^{-/-}$::WT					507 ± 166 (n = 13)	629 ± 202 (n = 13)

Survival ages of SOD1^{G37R} positive chimeras (ID nos. 1–5 and 12) and control chimeras (nos. 6–11 and 13). All chimeras but chimera no.5 were killed for the histological analysis before showing hindlimb paralysis or other disease. ND, not determined.

Olig$^{-/-}$::SOD1^{G37R} chimeras had 100% mutant SOD1-expressing motor neurons. In contrast, the dorsal roots of Olig$^{-/-}$::SOD1^{G37R} chimeras showed only a proportion of human SOD1-positive axons, indicating that sensory axons were chimeric for WT and mutant SOD1-expressing axons (Fig. 2*C*).

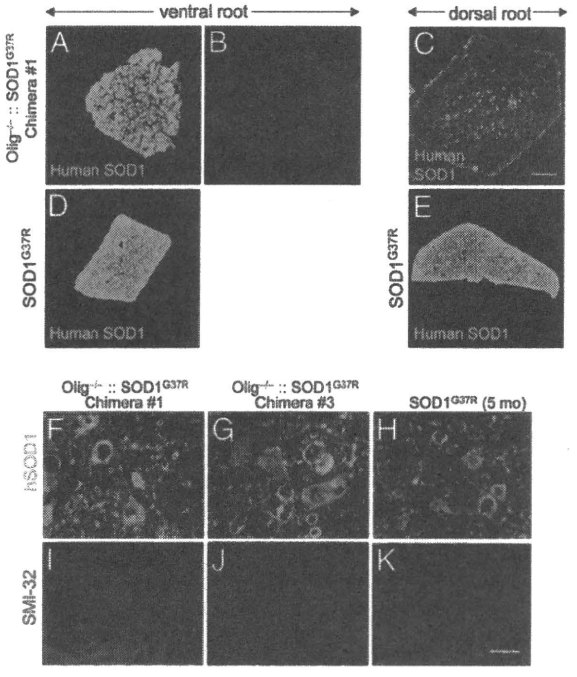

Fig. 2. Olig$^{-/-}$::SOD1^{G37R} chimeric mice express mutant SOD1 in all motor neurons but only in a subset of sensory axons. (*A–E*) L5 ventral (motor), (*A*, *B*, and *D*) and dorsal (sensory) (*C* and *E*) roots from 8-month-old Olig$^{-/-}$::SOD1^{G37R} mice (*A–C*) or 2-month-old germ-line SOD1^{G37R} mice (*D* and *E*) were stained with human specific SOD1 antibody (*A* and *C–E*), or myelin basic protein antibody (*B*). (*F–K*) Lumbar spinal cord sections from two different Olig$^{-/-}$::SOD1^{G37R} and 5-month-old SOD1^{G37R} mice immunostained with the anti-human SOD1 antibody (*F–H*) and the motor neuron marker SMI-32 (*I–K*). Note that all of the motor neurons are SMI-32-positive (red) and also expressed human SOD1 (green). [Scale bars: 50 μm (*A–E*) and 40 μm (*F–K*).]

Variable, Olig$^{-/-}$-Mediated Reduction in Motor Neuron Number in WT or SOD1^{G37R} Chimeras. Examination of transverse sections (Fig. 3 *A–G* and Fig. S2) of motor roots (stained with toluidine-blue) revealed that the number of both L5 and L4 motor axons of the Olig$^{-/-}$ chimeras had unexpected variability relative to those found in germ-line normal or SOD1^{G37R} mice. The reduced number of motor axons (frequently also highly asymmetric in comparing right and left roots; Table 1) was not, however, the result of mutant SOD1-dependent axonal loss. Similarly reduced numbers and variability of motor axons were seen in Olig$^{-/-}$::WT chimeras and Olig$^{-/-}$::SOD1^{G37R} chimeras. The reduced axonal number was in some cases extraordinary: instead of the typical ≈1,000 axons in the L5 root, some WT and SOD1^{G37R} chimeras had roots with as few as 323 and 314 motor axons in Olig$^{-/-}$::WT and Olig$^{-/-}$::SOD1^{G37R} chimeras, respectively. There was no statistically significant difference in number of motor axons from Olig$^{-/-}$::SOD1^{G37R} and Olig$^{-/-}$::WT (mean axon number ± standard error for L4, SOD1^{G37R}, 424 ± 109; WT, 507 ± 106, $P = 0.17$; for L5, SOD1^{G37R}, 526 ± 150; WT, 629 ± 202, $P = 0.18$, unpaired t test) (Table 1). Because reduced axon numbers were found early in life and did not seem to increase with age (Table 1; Fig. 3*E*), and because there was an absence of signs of significant active axonal degeneration (Fig. 3*F* and Fig. S2), the variable number of motor axons almost certainly reflects loss of motor neurons early in development that normally would have been contributed by Olig$^{-/-}$ progenitor cells.

Motor neuron disease from expression of mutant SOD1 has previously been demonstrated to arise from loss of the pool of large caliber motor axons (21), including for end-stage mutant SOD1^{G37R} (22) (see also Fig. 3 *A* and *E*). With the exception of one Olig$^{-/-}$::SOD1^{G37R} chimera (no. 5, Table 1, Fig. 3*D*) that developed ALS-like hindlimb weakness, the Olig$^{-/-}$::SOD1^{G37R} chimeras (nos. 1–4, 7–8 months old), however, did not have a reduced proportion of the large (>3 μm in diameter) axons when compared with WT mice (Fig. 3 *A*, C, and E), reflecting preservation of large caliber motor axons of Olig$^{-/-}$::SOD1^{G37R} chimeras (Fig. 3*F*). Further, examination of transverse sections of spinal cords from

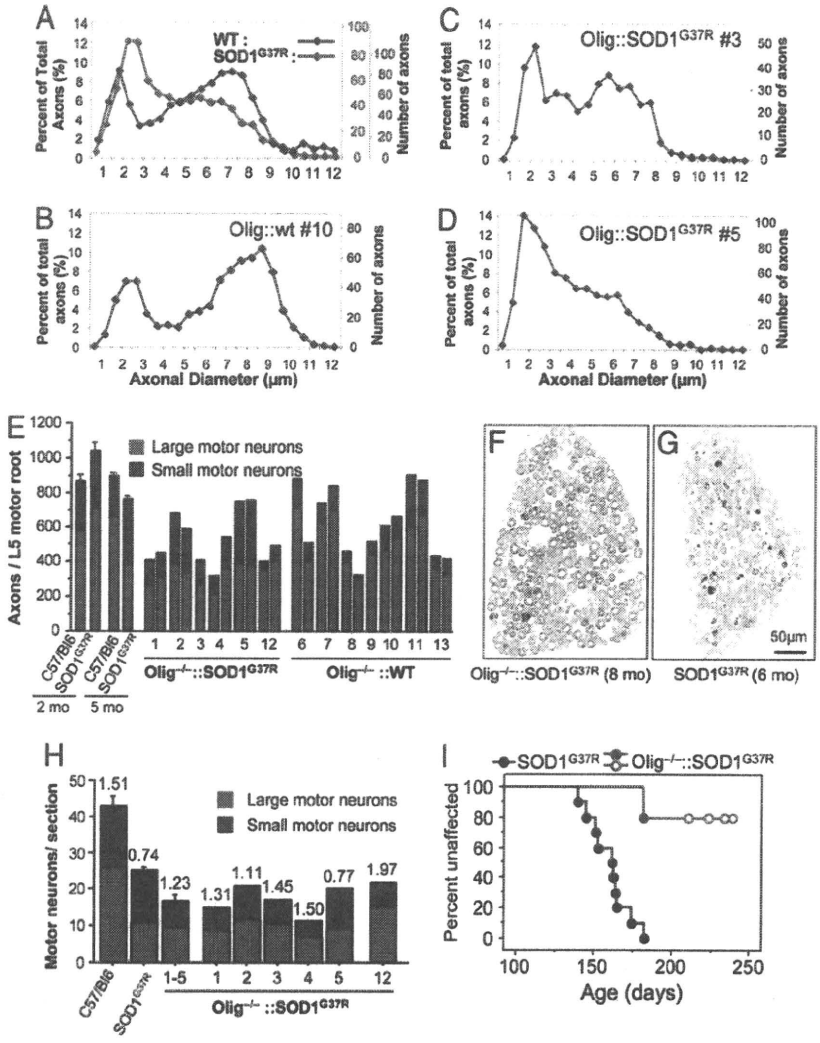

Fig. 3. Mutant SOD1-expressing motor neurons of Olig$^{-/-}$::SOD1^{G37R} escaped degeneration. (*A–D*) Representative L5 motor axonal caliber of WT (*A*, blue), end-stage SOD1^{G37R} (*A*, red), Olig$^{-/-}$::WT chimera (*B*), and Olig$^{-/-}$::SOD1^{G37R} chimeras (*C* and *D*). (*B–D*) Averaged axon caliber counts of both side of L5 motor roots (an average of three to four animals per genotype in *A*). (*D*) Note only no. 5 chimera developed ALS-like disease. (*E*) The number and proportions of large (>3-μm diameter, red bars) and small caliber motor axons (blue bars) in the L5 motor roots from various chimeras (numbers corresponding to the ID in Table 1), germ-line SOD1^{G37R}, and C57BL/6 mice. For the number of axons in SOD1^{G37R} and C57BL/6 mice, an average from three to four animals per genotype was shown. (*F* and *G*) Representative toluidine-blue-stained L5 motor axon images of 8-month-old Olig$^{-/-}$::SOD1^{G37R} mice (*F*), and end-stage SOD1^{G37R} mice (6 months old, *G*). (*H*) Quantification of large motor neurons (red bars) and small motor neurons (blue bars); sample images are in Fig. S3). Bars represent the mean number of motor neurons per section averaged from 10–12 sections cut throughout the lumbar spinal cords. The numbers on top of the bars represent the ratio of the number of large motor neurons to the number of small motor neurons. [Scale bar: 50 μm (*F* and *G*).] (*I*) Olig$^{-/-}$::SOD1^{G37R} chimeric mice are viable and escaped from motor neuron disease. Shown are onset data of germ-line SOD1^{G37R} mice (blue) and Olig$^{-/-}$::SOD1^{G37R} chimeric mice (red). Open circles indicate four chimeric mice that never developed motor neuron disease but that were killed at 211–239 days of age.

Olig$^{-/-}$::SOD1^{G37R} chimeras confirmed that large motor neurons, which were mainly lost in the end-stage SOD1^{G37R} mice (Fig. S3*B*), were preserved in the Olig$^{-/-}$::SOD1^{G37R} chimeras (Fig. S3 *C* and *D*). The ratio of large to small motor neurons in most Olig$^{-/-}$::SOD1^{G37R} chimeras (nos. 1–4) was comparable with that in normal mice, but not to the reduced ratio measured in end-stage SOD1^{G37R} mice (Fig. 3*H*). Combined with the absence of active degeneration within the motor roots, we conclude that, although all motor neurons were mutant and some chimeras (e.g., nos. 1 and 3) had the substantial disadvantage of as few as one-third the normal number of motor neurons surviving during development, there was little, if any, mutant-dependent motor neuron loss in the Olig$^{-/-}$::SOD1^{G37R} chimeras at ages 28 (chimera no. 4) to 56 (chimera no. 1) days past the 183 days of maximal age of disease onset of the germ-line SOD1^{G37R} mice. Finally, examination of spinal cord sections of these disease-free chimeric mice for hallmark cellular signs of disease revealed an absence of ubiquitinated protein accumulation in SOD1 mutant motor neurons, and a comparatively modest level of astrocytic and microglial activation relative to end-stage germ-line SOD1^{G37R} mice, and relative to chimeric mouse no. 5, which developed disease (Figs. S4 and S5).

Most Olig$^{-/-}$::SOD1^{G37R} Chimeras Escape Early-Onset Motor Neuron Disease. Of seven Olig$^{-/-}$::SOD1^{G37R} chimeras that were carefully monitored during aging, one died suddenly from an unknown cause at 151 days of age, but without prior motor neuron symptoms. One (chimera no. 12) was killed at 65 days of age for histological experiments. For the remaining five, one chimera (no. 5, Table 1) was killed at 192 days of age, after it developed partial hindlimb paralysis, a symptom indicating initiation of motor neuron disease. The remaining four chimeras, killed at ages between 211 and 239 days (Fig. 3*I*, open circles), all continued to move without difficulty and could reach food and water without any assistance despite some limb abnormalities present as early as weaning age. None, however, developed any phenotypic signs of age-dependent motor neuron disease (Movies S3 and S4) by an age of 51–79 days beyond the mean onset of germ-line SOD1^{G37R} mice (160 days of age, Fig. 3*I*), indicating as much as a 1.5-fold increase of disease-free life achieved in those chimeras. These disease-free mice also did not exhibit the weight loss that typically accompanies phenotypic disease onset. Thus, these animals exhibited no signs of the onset of ALS despite having all of their motor neurons mutant for SOD1.

Mutant Upper Motor Neurons Escape Age-Dependent Degeneration in Olig$^{-/-}$::SOD1^{G37R} Chimeras. In symptomatic mutant SOD1 mice, spinal lateral axons, which contain axons of the upper motor neurons, are severely degenerated (23). To examine whether the WT non-motor neuronal cells affect degeneration of these upper motor axons, morphology of spinal lateral axons in the lumbar spinal cords was examined in the Olig$^{-/-}$::SOD1^{G37R} chimeras.

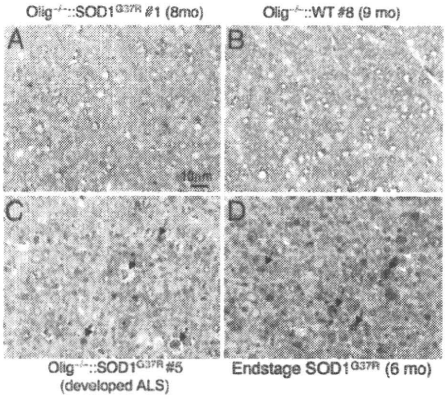

Fig. 4. Upper motor axons in the lateral spinal cord are preserved in Olig$^{-/-}$::SOD1^{G37R} mice. Transverse sections of upper lumbar spinal cord of Olig$^{-/-}$::SOD1^{G37R} chimera no. 1 (*A*), Olig$^{-/-}$::WT (*B*), Olig$^{-/-}$::SOD1^{G37R} chimera no. 5 (*C*, developed ALS), or end-stage SOD1^{G37R} mice (*D*) were stained with toluidine blue. Arrows, degenerating axons. (Scale bar: 10 μm.)

Parallel analysis of end-stage SOD1^{G37R} mice (6 months old) showed severe degeneration in the spinal lateral axons as expected (Fig. 4*D*), as well as in the one Olig$^{-/-}$::SOD1^{G37R} chimera (no. 5, Fig. 4*C*) that developed motor neuron disease at 192 days of age. In contrast, despite all motor neurons and their ensheathing oligodendrocytes expressing mutant SOD1, almost all lateral axons of the remaining Olig$^{-/-}$::SOD1^{G37R} chimeras including the one killed at the oldest age (8 months) (Fig. 4*A*) were preserved (just as they were in Olig$^{-/-}$::WT chimeras (Fig. 4*B*)).

Discussion

Despite the fact that all motor neurons of the Olig$^{-/-}$::SOD1^{G37R} mice expressed the SOD1^{G37R} transgene at a level comparable with that in germ-line SOD1 mutant mice that develop early-onset motor neuron disease when expressed ubiquitously, four of five Olig$^{-/-}$::SOD1^{G37R} chimeras escaped any sign of onset of motor neuron disease without showing overt motor neuron degeneration for as much as 53 days beyond the mean life span of germ-line SOD1^{G37R} mice. Evidence that the expression level of mutant SOD1 in motor neurons of Olig$^{-/-}$::SOD1^{G37R} mice was comparable with that in germ-line SOD1^{G37R} mice comes from the one chimera (no. 5) that developed motor neuron disease, probably because of a large contribution of mutant-expressing cells other than motor neurons. In fact, and to our surprise, a highly significant, 1.5-fold, increase in disease-free life was documented in these surviving animals before killing for experimental analysis. Preservation of upper and lower motor neurons in these chimeras clearly indicated that expression of mutant SOD1 in all motor neurons was not sufficient to cause onset and hence progression of early-onset disease or motor neuron degeneration. Thus, although the number of Olig$^{-/-}$::SOD1^{G37R} chimeric mice produced for this study was relatively small because of the complexity of the experimental design, our data establish that onset of motor neuron degeneration is non-cell autonomous and that non-autonomous cell contributors to onset may be distinguishable from those that drive progression because accelerating disease progression [from reduced mutant synthesis in astrocytes (10) or microglia (9)] was not previously observed to affect the timing of disease onset.

We note that Olig$^{-/-}$ chimeras had fewer motor neurons as early as 65 days of age independent of the expression of the SOD1^{G37R} transgene. Although there was variation in the number of L4 and L5 motor axons (ranging from 241 to 845 axons in L4, 314–908 axons in L5), the average number of motor axons of Olig$^{-/-}$ chimeras was approximately half that of WT mice. This finding is also striking because these chimeric mice escaped phenotypic signs of motor neuron disease onset despite a comparative disadvantage beginning early in life, i.e., having substantially fewer motor neurons than normal, which might have been expected to sensitize these animals to motor neuron damage. Whereas prior lesioning of motor axons using peripheral axotomy has been reported to increase the proportion of small caliber axons and reduce motor neuron vulnerability to mutant SOD1 (24), our Olig$^{-/-}$::SOD1^{G37R} mice retain a high proportion of the large caliber motor axons that are vulnerable to motor neuron disease. Nevertheless, these large-caliber mutant-containing motor axons escaped degeneration.

Selective gene inactivation within motor neurons has proven that the timing of disease onset can be delayed by reducing mutant SOD1 solely within postnatal motor neurons (10). Whereas no work to date had provided any insight into whether damage to cell types beyond motor neurons was a determinant of disease onset, our chimeras now establish that mutant SOD1 action within one or more additional cell types affects timing of disease onset. Indeed, in addition to escape from disease onset in most Olig$^{-/-}$::SOD1^{G37R} mice, only a small subset of mutant-expressing motor axons exhibited any degenerative changes. Even then, the degree of degeneration never reached the level that was required to support motor neuron disease.

As to the identity of the cell types beyond motor neurons that contribute to disease onset, mutant synthesis within muscle has previously been shown not to affect either onset or progression (25). Mutant synthesis within microglia (8, 9) or astrocytes (10) strongly accelerates disease progression, but expression in neither cell type seems to affect onset. The inability to generate oligodendrocytes in Olig1/2$^{-/-}$ mice (18) predicts that Olig$^{-/-}$::SOD1^{G37R} mice express mutant SOD1 in all oligodendrocytes as well as motor neurons (technical limitations precluded documenting that all oligodendrocytes were mutant). If so, then mutant SOD1 expression by oligodendrocytes is also not a significant contributor to degeneration of upper motor neurons and disease onset. Although it remains untested whether mutant toxicity solely within motor neurons is sufficient to provoke motor neuron disease, pan-neuronal expression of mutant SOD1 in mice was recently shown to result in very late onset motor neuron disease (6), suggesting that neurons other than motor neurons are contributors to onset of disease. Potential contributors to disease onset remain untested, but include mutant SOD1 action within interneurons, the myelinating Schwann cells of the periphery, and the endothelial cells of the vasculature. Consistent with damage directly developed within the last of these cell types, SOD1 mutants of different biochemical characteristics disrupt the blood–spinal cord barrier by reducing the levels of the tight junction proteins ZO-1, occludin and claudin-5, resulting in microhemorrhages with release of neurotoxic hemoglobin-derived products, reductions in microcirculation, and hypoperfusion. SOD1-mutant mediated endothelial damage accumulates before motor neuron loss and a neurovascular inflammatory response (26), supporting a central contribution to disease initiation.

In any event, the cumulative evidence supports the hypothesis that it is ALS-linked mutant SOD1 within multiple cell types that, in collaboration with damage directly within motor neurons, drives non-cell-autonomous onset of motor neurodegeneration.

Materials and Methods

Generation of Chimeric Mice. Olig$^{-/-}$::SOD1^{G37R} chimeric mice were generated by aggregation of embryos (morulae at eight-cell stage) derived from Olig$^{+/-}$ × Olig$^{+/-}$ and from SOD1^{G37R} $^{+/-}$ (line 42) × C57BL/6 breedings (17). Olig$^{+/-}$ mice were generated by the breeding of FVB mice and Olig1/2 mutant heterozygous mice (a gift from D Anderson, California Institute of Technology) (18).

Mice were genotyped by PCR for the presence of the mutant SOD1 transgene as described in ref. 27, and for the Olig mutant locus using the following primers: sense, GAA CAG CAG CAG CAG CAG CTG; antisense, CAG CTC CTG CG CGA GCT ACC.

To distinguish chimeras derived from Olig$^{-/-}$ embryos from those from Olig$^{+/-}$ embryos, a Sequence-Tagged-Site (STS) marker D16MIT155 located 0.7 Mb telomeric from the Olig1/2 locus was used. We exploited the situation that in our

matings the Olig WT locus we used was derived from FVB mice, whereas the Olig mutant locus was derived from a C57BL/6 allele. This situation yielded a 170-bp PCR product (D16MIT155^{170}) for the STS locus in any animal with one unmutated Olig allele (see Fig. 1 *B* and *D*), but only a 146-bp product (D16MIT155^{146}) for chimeras generated from mutant SOD1 embryos and embryos homozygously deleted for Olig. The primers used for D16MIT155 were the following: primer 1, AAA CAC AAG GTT ATC TTA GTG GTG G; primer 2, TGT GTT TGT ATA TGT GAA TGT ATG TCA.

For survival experiments, the time of onset was defined at the time when denervation-induced muscle atrophy had produced a 10% loss of maximal body weight, and end-stage was determined by paralysis sufficiently severe that the animal could not right itself within 20 seconds when placed on its side, an endpoint frequently used for SOD1 mutant-expressing mice (e.g., refs. 9 and 28) and one that was consistent with the requirements of the Animal Care and Use Committee of the University of California. Animals were observed every 24 h.

Quantification of SOD1 Transgene Level by Real-Time PCR. To estimate the overall degree of chimerism, genomic DNA extracted from tails was analyzed by quantitative PCR of the human SOD1 gene as described in ref. 9. In brief, DNA (33 ng) was amplified with iQ supermix (Bio-Rad) and 100 nM of each primer and probe in a Bio-Rad iCycler real time PCR machine. The specific primers and probe used for the human SOD1 gene were the following: hSOD1-forward, CAATGTGACTGCTGACAAAG; hSOD1-reverse, GTGCGGC-CAATGATGCAAT; and hSOD1 probe, fam-CCGATGTGTCTATTGAAGATTCTG-BHQ. Primers and probe for the apolipoprotein B (apoB) as a normalizer were as follows: apoB-forward, CACGTGGGCTCCAGCATT; apoB-reverse, TCAC-CAGTCATTTCTGCCTTTG; and apoB probe, Texas Red-CCAATGGTCGGG-CACTGCTCAA-BHQ2.

Tissue Preparation and Immunofluorescence. Mice were perfused transcardially with PBS followed by 4% paraformaldehyde in phosphate buffer. Before freezing, tissues were cryoprotected in 30% (wt/vol) sucrose in PBS. Frozen tissues were sectioned with a cryostat (Leica, Bannockburn, IL) in 30-μm or 20-μm sections for spinal cord or roots, respectively, and processed for immunofluorescence.

Floating sections of spinal cords were incubated in blocking buffer [1% (wt/vol) BSA, 0.2% (wt/vol) fat-free milk, 5% (vol/vol) normal goat serum, 0.3% (vol/vol) Triton X-100 in PBS, pH 7.4], followed by overnight incubation with a set of two primary antibodies; an antibody recognizing unphosphorylated neurofilaments, SMI32 (1:1000, Sternberger Monoclonals, Lutherville MA) and hSOD1 (1:100) (7, 29) diluted in PBS/0.3% Triton X-100 (PBST). Subsequently, the sections were incubated with species-specific secondary antibodies that were conjugated with either FITC or Cy3 (Jackson ImmunoResearch, West Grove, PA).

For the staining of roots, sections were directly mounted on slides and incubated in blocking buffer [1% (wt/vol) BSA, 1% (vol/vol) goat serum, 0.3% (vol/vol) Triton X-100 in PBS, pH 7.4], followed by incubation with hSOD1 antibody (diluted at 1:300) and myelin basic protein (MBP) antibody (1:100, Chemicon) in PBST overnight and subsequently with anti-rabbit and anti-rat IgG antibodies that were conjugated with FITC and Texas Red (Jackson ImmunoResearch, West Grove, PA), respectively. Sections were mounted in ProLong Antifade reagent (Invitrogen, Carlsbad, CA) and analyzed on an Olympus confocal microscope (FV1000).

Immunohistochemistry and Evaluation of Microgliosis and Astrogliosis. Floating sections of spinal cords were incubated with primary antibodies followed by biotinylated species-specific secondary antibodies. Details are provided in *SI Materials and Methods*.

Motor Neuron Counting. Motor neuron numbers were determined from 30-μm-serial sections across the lumbar spinal cords (a total of 10–12 sections per animal). Motor axons were counted in every 24th section after staining with anti-ChAT antibody.

Morphological Analysis of Axons. Spinal cords and lumbar roots, postfixed in 4% paraformaldehyde/PBS, transversely sectioned into 5-mm blocks were treated with 2% Osmium tetroxide in 0.05 M cacodylate buffer, washed, dehydrated, and embedded with Epon (Electron Microscopy Sciences). One-micrometer cross-sections were stained with 1% toluidine blue. Axonal diameters of L5 or L4 roots were measured by Bioquant software. Entire roots were imaged, imaging thresholds were selected individually, and the cross-sectional area of each axon was calculated and reported as a diameter of a circle of equivalent area. Axon diameters were grouped into 0.5-μm bins.

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1. Bruijn LI, Miller TM, Cleveland DW (2004) Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu Rev Neurosci* 27:723–749.
2. Boillee S, Vande Velde C, Cleveland DW (2006) ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron* 52:39–59.
3. Pasinelli P, Brown RH (2006) Molecular biology of amyotrophic lateral sclerosis: Insights from genetics. *Nat Rev Neurosci* 7:710–723.
4. Pramatarova A, *et al.* (2001) Neuron-specific expression of mutant superoxide dismutase 1 in transgenic mice does not lead to motor impairment. *J Neurosci* 21:3369–3374.
5. Lino MM, Schneider C, Caroni P (2002) Accumulation of SOD1 mutants in postnatal motoneurons does not cause motoneuron pathology or motoneuron disease. *J Neurosci* 22:4825–4832.
6. Jaarsma D, *et al.* (2008) Neuron-specific expression of mutant superoxide dismutase is sufficient to induce amyotrophic lateral sclerosis in transgenic mice. *J Neurosci* 28:2075–2088.
7. Clement AM, *et al.* (2003) Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302:113–117.
8. Beers DR, *et al.* (2006) Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 103:16021–16026.
9. Boillee S, *et al.* (2006) Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312:1389–1392.
10. Yamanaka K, *et al.* (2008) Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* 11:251–253.
11. Howland DS, *et al.* (2002) Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci USA* 99:1604–1609.
12. Van Damme P, *et al.* (2007) Astrocytes regulate GluR2 expression in motor neurons and their vulnerability to excitotoxicity. *Proc Natl Acad Sci USA* 104:14825–14830.
13. Nagai M, *et al.* (2007) Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 10:615–622.
14. Di Giorgio FP, *et al.* (2007) Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* 10:608–614.
15. Misawa H, *et al.* (2003) VAChT-Cre.Fast and VAChT-Cre.Slow: Postnatal expression of Cre recombinase in somatomotor neurons with different onset. *Genesis* 37:44–50.
16. Gong YH, *et al.* (2000) Restricted expression of G86R Cu/Zn superoxide dismutase in astrocytes results in astrocytosis but does not cause motoneuron degeneration. *J Neurosci* 20:660–665.
17. Wong PC, *et al.* (1995) An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14:1105–1116.
18. Zhou Q, Anderson DJ (2002) The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* 109:61–73.
19. Lu QR, *et al.* (2002) Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* 109:75–86.
20. Takebayashi H, *et al.* (2002) The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. *Curr Biol* 12:1157–1163.
21. Bruijn LI, *et al.* (1997) ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18:327–338.
22. Lobsiger CS, Garcia ML, Ward CM, Cleveland DW (2005) Altered axonal architecture by removal of the heavily phosphorylated neurofilament tail domains strongly slows superoxide dismutase 1 mutant-mediated ALS. *Proc Natl Acad Sci USA* 102:10351–10356.
23. Yamanaka K, *et al.* (2006) Progressive spinal axonal degeneration and slowness in ALS2-deficient mice. *Ann Neurol* 60:95–104.
24. Kong J, Xu Z (1999) Peripheral axotomy slows motoneuron degeneration in a transgenic mouse line expressing mutant SOD1 G93A. *J Comp Neurol* 412:373–380.
25. Miller TM, *et al.* (2006) Gene transfer demonstrates that muscle is not a primary target for non-cell-autonomous toxicity in familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 103:19546–19551.
26. Zhong, Z. *et al.* (2008) ALS-causing SOD1 mutants generate blood-spinal cord barrier breakdown and reductions in microcirculation prior to motor neuron degeneration. *Nat Neurosci* 11:420–422.
27. Williamson TL, Cleveland DW (1999) Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nat Neurosci* 2:50–56.
28. Kaspar BK, *et al.* (2003) Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* 301:839–842.
29. Pardo CA, *et al.* (1995) Superoxide dismutase is an abundant component in cell bodies, dendrites, and axons of motor neurons and in a subset of other neurons. *Proc Natl Acad Sci USA* 92:954–958.

Schwann cells expressing dismutase active mutant SOD1 unexpectedly slow disease progression in ALS mice

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Neurodegeneration in an inherited form of ALS is non-cell-autonomous, with ALS-causing mutant SOD1 damage developed within multiple cell types. Selective inactivation within motor neurons of an ubiquitously expressed mutant *SOD1* gene has demonstrated that mutant damage within motor neurons is a determinant of disease initiation, whereas mutant synthesis within neighboring astrocytes or microglia accelerates disease progression. We now report the surprising finding that diminished synthesis (by 70%) within Schwann cells of a fully dismutase active ALS-linked mutant ($SOD1^{G37R}$) significantly accelerates disease progression, accompanied by reduction of insulin-like growth factor 1 (IGF-1) in nerves. Coupled with shorter disease duration in mouse models caused by dismutase inactive versus dismutase active SOD1 mutants, our findings implicate an oxidative cascade during disease progression that is triggered within axon ensheathing Schwann cells and that can be ameliorated by elevated dismutase activity. Thus, therapeutic down-regulation of dismutase active mutant SOD1 in familial forms of ALS should be targeted away from Schwann cells.

neurodegeneration | non-cell-autonomous | superoxide dismutase | amyotrophic lateral sclerosis | motor neurons

Amyotrophic lateral sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease that causes premature death of brain and spinal cord motor neurons (1). Most cases of ALS are of unknown etiology, but some familial forms are linked to dominant missense mutations in the ubiquitously expressed *SOD1* (superoxide dismutase 1) gene (1). SOD1 is an abundant intracellular anti-oxidant, which converts superoxide radicals in the cytoplasm to hydrogen peroxide (which is then further converted by peroxidases to water). Its known activity initially lead to the hypothesis that reduced SOD1 activity could drive motor neuron degeneration in ALS via increased oxidative stress (2). However, transgenic mice develop ALS-like motor neuron degeneration through ubiquitous expression of either dismutase *active* or *inactive* mutant SOD1 forms. Although neither increased expression of wild-type SOD1 nor deletion of endogenous SOD1 cause motor neuron disease (1), evidence has lead to the conclusion that disease is caused by a novel acquired toxicity of mutant SOD1 independent of its dismutase activity (1).

Prominent models proposed for the nature of SOD1-linked ALS toxicity, include intraastrocytic damage from it leading to loss of the EAAT2 glutamate transporter (3), aberrant association with mitochondria (4), aberrant cosecretion with chromogranin (5), sustained activation of NADPH oxidase (6, 7), and inhibition of the ERAD pathway for removal of misfolded proteins (8). Whichever of these are correct, evidence from many directions has demonstrated that toxicity is non-cell-autonomous, with mutant-mediated damage generated within multiple cell types, including the affected motor neurons but also by their neighboring astrocytes and microglia. Initial evidence for non-cell autonomy came from analysis of chimeric mice, which demonstrated a protective effect of wild-type cells surrounding mutant SOD1 expressing motor neurons (9). By using cell type specific Cre-mediated gene excision, it was then shown that reducing mutant SOD1 expression in motor neurons delayed disease initiation (10), although similar mutant reduction in either microglia (10) or astrocytes (11) slowed disease progression. A contribution of mutant expressing astrocytes to driving death of human (7, 12) or mouse (13, 14) motor neurons has also been seen in vitro.

Because in the spinal cord, microglia and astrocytes represent the major nonneuronal cell types associated with motor neurons, a contribution from them in driving disease mechanism may not, in retrospect, be surprising. In the periphery, however, although mutant synthesis solely in muscle, the motor neuron targets, can provoke damage to those muscles (15), this apparently plays little role in disease mechanism. Reduction of mutant SOD1 synthesis in the skeletal muscle of ALS mice does not affect disease onset or progression (16).

Possible damage within or to the Schwann cells, the myelinating glia of the peripheral nervous system, has not been examined so far, despite the fact that they are associated with the full length of peripheral axons (which represent 90% of the volume of motor neurons). Highlighting the potential for a contribution from them in ALS is the fact that in contrast to a 15:1–20:1 ratio of central nervous system glia surrounding the motor neuron perikaryon, the ratio of Schwann cells to a single motor axon is 1,000:1 (17). Schwann cells form an intimate bidirectional relationship with their neuronal partners: during development Schwann cells are essential for the survival of motor neurons, whereas neuron-derived factors guide survival and differentiation of Schwann cells along axons (18). These interactions become again important during neuronal regeneration (19, 20). Schwann cells distal to the injury do not die, rather they shed off and phagocytose (together with macrophages) their myelin sheets and then enter a dedifferentiation/redifferentiation program to guide and support the regrowing axon followed ultimately by remyelination of it. Further, in

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The authors declare no conflict of interest.

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certain forms of Charcot-Marie-Tooth peripheral neuropathies, disease toxicity is directly linked to mutations in genes encoding Schwann cell myelin components (21), resulting in demyelination and even secondary axonal degeneration.

However, surprisingly little is known about a contribution, if any, of Schwann cells in ALS. Studies from human ALS have described myelin alterations along the peripheral nerves (22), most likely secondary due to massive axonal degeneration. At neuromuscular junctions in ALS mice, a recent study reported induction of the axon repellent semaphorin 3A in terminal Schwann cells (23) (a subpopulation of Schwann cells important for synaptic support and regeneration), suggesting either a detrimental or physiologic response to ongoing denervation (24). All of this raises the question of whether Schwann cells in ALS actively contribute to motor neuron degeneration or whether they are merely bystanders to the ongoing neurodegenerative process.

To assess the role of Schwann cells in ALS in a first global approach, we focus here on their contribution to non-cell-autonomous mutant SOD1-linked ALS toxicity. With the known capacity of Schwann cells to respond to axonal damage and produce various neurotrophic factors (19), we hypothesized that mutant SOD1 expression disturbs normal Schwann cell functions, especially during disease progression, which is defined by axonal degeneration and regenerative and/or compensatory attempts of surviving motor neurons (24, 25). Therefore, we have used Cre-mediated gene excision in ALS mice (10) to remove mutant SOD1 specifically from Schwann cells.

Results

Efficient Cre-Mediated Mutant *SOD1* Gene Excision in Schwann Cells of ALS Mice. To assess the contribution of Schwann cell-expressed mutant SOD1 to non-cell-autonomous ALS toxicity, we mated mice heterozygous for a mutant human $SOD1^{G37R}$ transgene flanked by loxP sites (LoxSOD1^{G37R} mice) that develop progressive and fatal ALS-like motor neuron degeneration (10) with mice expressing the Cre recombinase under the control of mouse myelin-protein-zero (P_0) regulatory sequences (26). P_0-cre mice are well-established to excise floxed genes almost exclusively from peripheral myelinating or nonmyelinating Schwann cells without targeting central nervous system myelinating glia (oligodendrocytes) or other central or peripheral neurons or glia (26, 27). By mating P_0-cre mice with Cre-inducible ROSA26-β-galactosidase (β-Gal) reporter mice (10, 11), we confirmed this selectivity, demonstrating β-Gal activity in most sciatic nerve Schwann cells (Fig. 1*A* and *B*) but none in spinal cord cells (Fig. 1*D*). Because one report suggested the potential involvement of terminal Schwann cells in ALS (23), we also confirmed P_0-cre activity in many of these specialized, S100-positive, unmyelinating Schwann cells, which are closely apposed to nerve terminal branches at neuromuscular junctions (Fig. 1*C*) (30–50% were β-Gal positive; D. Hess, R. Balice-Gordon, M.L. Feltri, and L. Wrabetz, personal communication).

P_0-cre-mediated $SOD1^{G37R}$ transgene excision in Schwann cells was highly efficient. By using real-time PCR (qPCR) on sciatic nerve tissue extracts (containing genomic DNA derived almost exclusively from Schwann cells), a 70% reduction of $SOD1^{G37R}$ transgene levels in LoxSOD1^{G37R}/P_0-cre mice was identified, with no significant reduction in spinal cord tissue (Fig. 1*E*). Mutant $SOD1^{G37R}$ mRNA levels were also reduced by a comparable 70% (Fig. 1*F*). Considering that the nerve also contains some (P_0-cre negative) perineurial and endothelial cells (18), the actual excision rate in Schwann cells is probably nearly complete.

Schwann Cell-Expressed Dismutase Active Mutant SOD1 Is Neuroprotective as Its Removal Reduces Survival. To assess the effect of Schwann cell-specific removal of mutant SOD1 in ALS mice, we

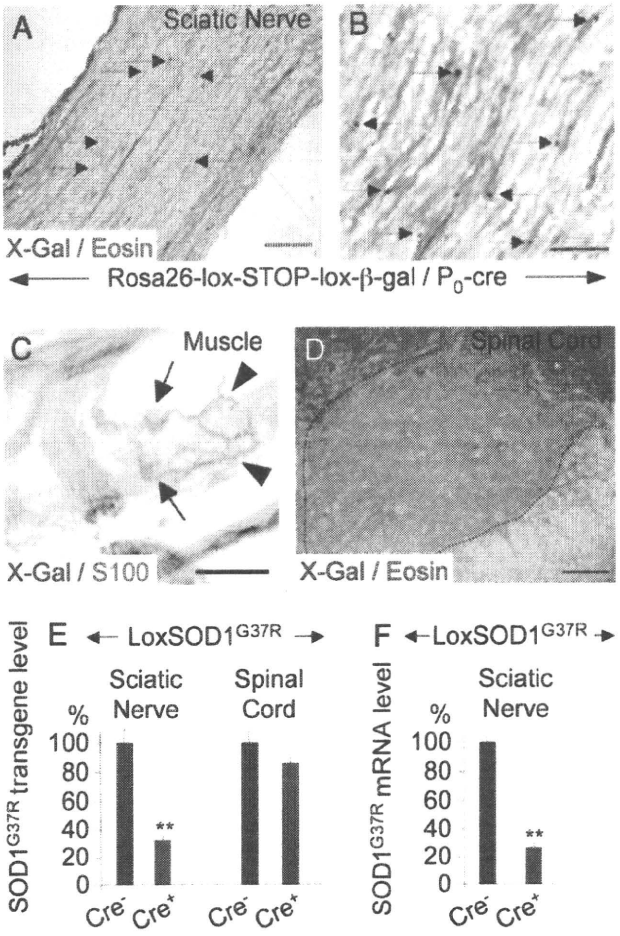

Fig. 1. Targeted P_0-cre-mediated gene excision in LoxSOD1^{G37R} ALS mice was used to specifically reduce Schwann cell-expressed mutant SOD1. (*A–D*) β-galactosidase (β-Gal) activity in Schwann cells (*A* and *B*, arrows) of longitudinal sciatic nerve sections, as well as in terminal Schwann cells (*C*, arrows; visualized with S100 immunostaining) at the neuromuscular junction (arrowheads) of gastrocnemius muscles but not in (*D*) spinal cord glia or neurons of (*A–D*) adult ROSA26/P_0-Cre reporter mice, visualized with X-Gal (*A–D*) and counterstained with eosin (*A*, *B*, and *D*). [Scale bars: 100 μm (*A* and *D*); 30 μm (*B* and *C*).] (*E* and *F*) qPCR showing a 70% reduction of human $SOD1^{G37R}$ transgene (*E*) and mRNA (*F*) levels ($P < 0.01$; Student's *t* test) in sciatic nerves of LoxSOD1^{G37R}/P_0-cre (Cre$^+$) mice relative to LoxSOD1^{G37R} (Cre$^-$) littermates, without significant spinal cord $SOD1^{G37R}$ transgene reduction (*E*) ($n = 3$ mice per group; presymptomatic, 4 months old; error bars, SEM).

compared disease courses between LoxSOD1^{G37R}/P_0-cre mice and LoxSOD1^{G37R} littermates. Both LoxSOD1^{G37R} and P_0-cre lines were in congenic C57BL/6 grounds. A presymptomatic phase was defined by the period of weight gain typical for young adult mice. Earliest disease onset was defined by the age at the inflection point in the weight curve. An early symptomatic phase was characterized by gait alterations, reduced grip strength, and denervation-induced muscle atrophy (10, 11, 28), that last of which was responsible for weight loss. A simple, objective early disease point was defined by the time at which weight loss reached 10% of peak weight, a measure repeatedly used previously in ALS mice (10, 11, 28, 29). Further symptomatic progression resulted in paralysis at end stage disease (29).

Despite efficient removal of mutant SOD1 from Schwann cells, disease onset was not significantly different between LoxSOD1^{G37R}/P_0-cre (8.2 months; 249 ± 11.2 days (d); $n = 20$) and LoxSOD1^{G37R} (8.5 months; 259 ± 9.9 d; $n = 19$) mice (Fig.

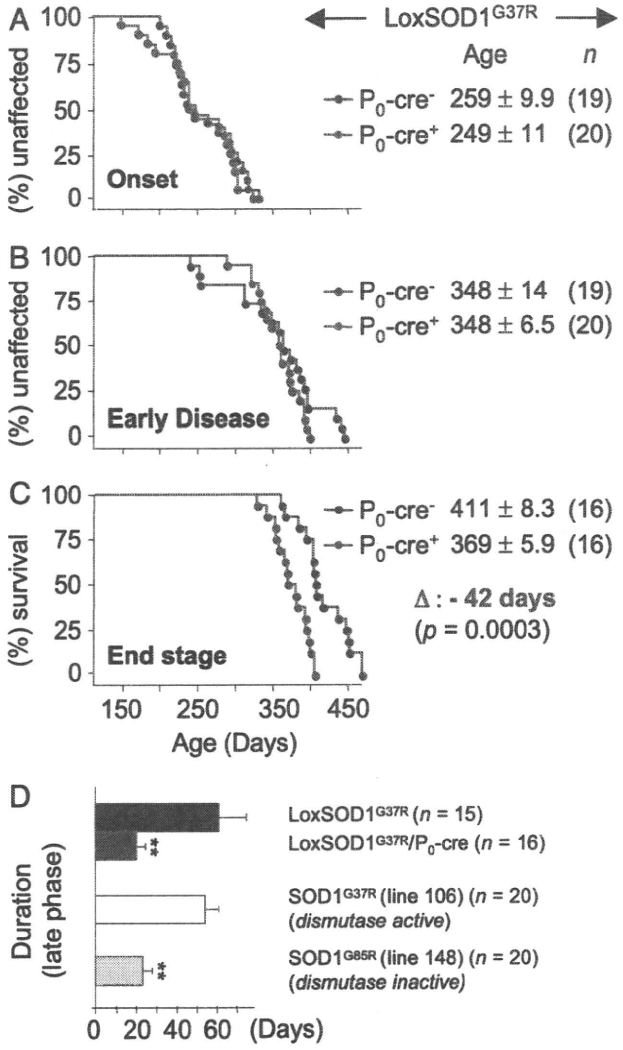

Fig. 2. Selective mutant *SOD1* gene excision from Schwann cells accelerates disease progression in ALS mice. (*A–C*) Plots of ages (in days) at which disease onset (*A*), early disease (*B*), and end stage (*C*) are reached in LoxSOD1^{G37R} ALS mice with (blue) and without (red) mutant SOD1 in Schwann cells. (*D*) Correlation between dismutase activity of Schwann cell-localized SOD1 mutants with slow disease progression in ALS mice: removal of dismutase *active* mutant SOD1^{G37R} from Schwann cells leads to a 3-fold faster late phase (from early disease to end stage) in LoxSOD1^{G37R}/P$_0$-cre mice than in LoxSOD1^{G37R} mice ($P < 0.01$; Student's *t* test). This difference correlates well with a faster late phase in dismutase inactive mutant SOD1^{G85R} mice (line 148; end stage at 12.5 months) as compared to a slow late phase in dismutase active mutant SOD1^{G37R} mice (line 106; end stage at 13.5 months) ($P < 0.001$; Student's *t* test) (see *Discussion*).

2*A*). Likewise, the age at which disease progressed to early phenotypic disease was similar in LoxSOD1^{G37R}/P$_0$-cre (11.5 months; 348 ± 6.5 d; n = 20) and LoxSOD1^{G37R} (11.5 months; 348 ± 14.0 d; n = 19) mice (Fig. 2*B*). Very surprisingly, however, removal of mutant SOD1^{G37R} from Schwann cells significantly reduced survival by accelerating disease progression after this early disease stage. End stage for LoxSOD1^{G37R}/P$_0$-cre mice was reached 42 days (1.4 months) earlier than for LoxSOD1^{G37R} mice (12.1 months; 369 ± 5.9 d; n = 16 versus 13.5 months; 411 ± 8.3 d; n = 16; P = 0.0003; Student's *t* test) (Fig. 2*C*). An "early phase" of disease progression (from onset through early phenotypic disease) was unchanged between LoxSOD1^{G37R}/P$_0$-cre

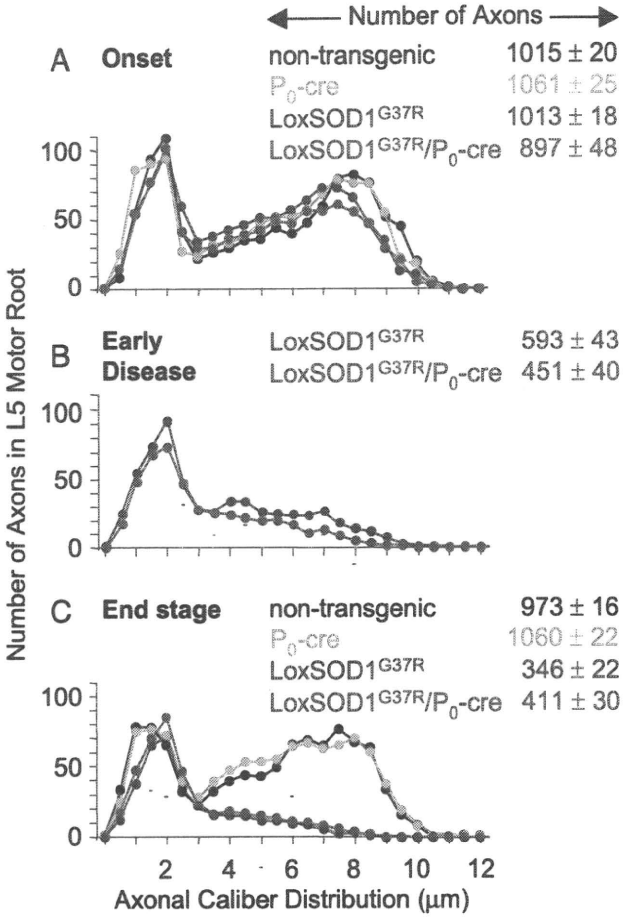

Fig. 3. Assessment of axonal degeneration in lumbar motor roots of ALS mice with or without Cre-mediated mutant *SOD1* excision in Schwann cells. Numbers and distributions of axonal diameters in L5 motor roots of LoxSOD1^{G37R} and LoxSOD1^{G37R}/P$_0$-cre mice at onset (8.5 months) (*A*), early disease (*B*), and end stage (*C*). No axonal degeneration was seen in nontransgenic or single transgenic P$_0$-cre control mice at ages matched to onset (8.5 months) or end stage (13.5 months) (*A* and *C*) (n = 4 mice per group; error, SEM).

(99 ± 9.8 d; n = 20) and LoxSOD1^{G37R} mice (89 ± 13.8 d; n = 19) (Fig. 2 *A* and *B*). However, progression through the "late phase" of disease was significantly accelerated (by almost 3 fold; Fig. 2 *C* and *D*) in LoxSOD1^{G37R}/P$_0$-cre mice (20.9 ± 3.6 d, n = 16) as compared with LoxSOD1^{G37R} mice (61.7 ± 12.9 d, n = 15; $P < 0.01$; Student's *t* test) (Fig. 2*D*), suggesting a link between slow disease progression in ALS mice and a protective effect of dismutase active mutant SOD1 in Schwann cells (Fig. 2*D*).

To determine how removal of mutant SOD1^{G37R} from Schwann cells influenced degenerative processes in ALS mice, we first confirmed motor axon integrity (Fig. 3 *A* and *C* and supporting information (SI) Fig. S1) and observed normal axon calibers and myelin thickness (Fig. S2) in young and aged P$_0$-cre control mice. We then compared the extent of motor axonal degeneration at similar disease-stages in mutant SOD1 mice. We found that in LoxSOD1^{G37R} mice (with or without P$_0$-cre), major axonal degeneration started after onset (Fig. 3 and Fig. S1) and was accompanied by secondary myelin alterations (Fig. S2 and Fig. S3). At all 3 disease stages analyzed (onset, early disease, and end stage) there were no significant differences between LoxSOD1^{G37R}/P$_0$-cre and LoxSOD1^{G37R} mice, either with respect to axonal loss or caliber reduction (Fig. 3 and Fig. S1) or for overall axonal or myelin pathology (Fig. S2 and Fig. S3). This

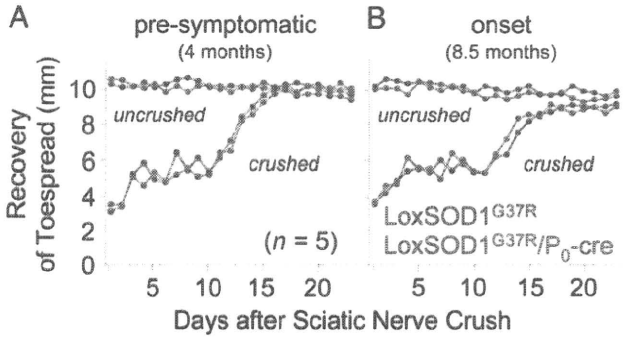

Fig. 4. The inherent regenerative capacity of motor neurons after crush injury is not influenced by Schwann cell-expressed mutant SOD1. The speed of nerve regeneration (measured by the toe spread) after unilateral crush injury was assessed at 2 time points [at 4 months (*A*), presymptomatic, and at 8.5 months (*B*), onset] before the appearance of overall symptoms (at 11.5 months). No significant differences were detected between LoxSOD1^{G37R}/P_0-cre and LoxSOD1^{G37R} ALS mice (n = 5 mice per group; only males used), indicating that removal of mutant SOD1^{G37R} from Schwann cells did not influence the sciatic nerve's inherent regenerative capacity (similar results were obtained with females; Fig. S4*A*).

is consistent with a true faster axonal degeneration in the late phase of disease progression in LoxSOD1^{G37R}/P_0-cre mice, as they reached end stage (after early disease) 3 times more quickly than LoxSOD1^{G37R} mice but showed a similar high degree of axonal degeneration at each stage. There was even a trend of increased axonal loss already at early disease in LoxSOD1^{G37R}/P_0-cre mice (Fig. S1), suggesting that acceleration of progression starts at or just before our definition of early disease.

The Inherent Regenerative Capacity of Motor Neurons After Crush Injury Is Not Influenced by Schwann Cell-Expressed Mutant SOD1. Schwann cells are involved in successful axonal regeneration (19) and during disease progression in ALS mice, there are active attempts at neuronal regeneration and/or compensation (24, 25). As wild-type SOD1 has been shown to be neuroprotective in neural injury paradigms (30, 31), we hypothesized that the decrease in overall dismutase activity from removal of dismutase active mutant SOD1^{G37R} from Schwann cells could thereby decrease the nerve's inherent capacity for regeneration. Therefore, we crushed sciatic nerves at 2 time points (4 and 8.5 months of age), before appearance of overall symptoms (at ≈11.5 months), and compared functional regeneration between LoxSOD1^{G37R}/P_0-cre and LoxSOD1^{G37R} mice. To avoid bias due to known neuroprotective effects of estrogen (32), genders were analyzed separately. No significant differences were found in regeneration when comparing LoxSOD1^{G37R}/P_0-cre and LoxSOD1^{G37R} mice (Fig. 4 and Fig. S4 *A* and *B*). In both, however, there was a trend to slower regeneration when comparing the 8.5- to the 4-month-old ALS mice (Fig. 4*A* and *B*; Fig. S4*C*), consistent with recent data showing a disease-linked slowing of nerve regeneration in a different line of SOD1 mutant mice (24).

More Aggressive Disease Progression in ALS Mice with Reduced Schwann Cell-Expressed Mutant SOD1 is Accompanied by Reduced IGF-1. To test if the presence or absence of dismutase active mutant SOD1 within Schwann cells affected the known regeneration-associated capacity of Schwann cells to induce diverse neurotrophic factors (19), we assessed the expression of 4 potent motor neurotrophic factors: insulin-like growth factor 1 (IGF-1), pleiotrophin (PTN), ciliary neurotrophic factor (CNTF), and glial-derived neurotrophic factor (GDNF). Analysis of sciatic nerve mRNAs by RT-qPCR (normalized to LoxSOD1^{G37R} mice) revealed that

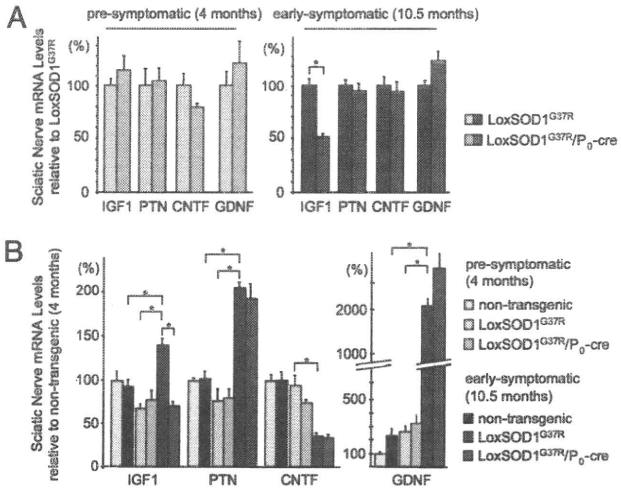

Fig. 5. More aggressive disease progression in ALS mice with reduced Schwann cell-expressed mutant SOD1 is accompanied by reduced IGF-1. (*A*) By using RT-qPCR analysis, whole sciatic nerve mRNA expression levels of IGF-1, PTN, CNTF, and GDNF are compared between LoxSOD1^{G37R}/P_0-cre and LoxSOD1^{G37R} ALS mice at presymptomatic and early symptomatic disease stages. At an early symptomatic time point (10.5 months), removal of dismutase active mutant SOD1^{G37R} from Schwann cells leads to a 50% reduction ($P < 0.05$; Student's t test) selectively of IGF-1 in LoxSOD1^{G37R}/P_0-cre ALS-mice. (*B*) Comparison of mRNA levels from presymptomatic and early symptomatic ages in LoxSOD1^{G37R} ALS mice: disease-associated inductions are found for IGF-1 (2.0-fold), PTN (2.7-fold), and GDNF (8.6-fold), whereas CNTF is reduced (2.3-fold) ($P < 0.05$; Student's t test). There is no disease-associated IGF-1 induction after removal of mutant SOD1 from Schwann cells. Absolute inductions of IGF-1 and PTN in early symptomatic LoxSOD1^{G37R} ALS mice as compared with age-matched (nontransgenic) control mice are 1.5-fold and 2.0-fold, respectively ($P < 0.05$; Student's t test) (n = 3 mice per group; error bars, SEM).

removal of mutant SOD1^{G37R} from Schwann cells did not lead to changes in inherent IGF-1, PTN, CNTF, or GDNF expression levels presymptomatically (at 4 months) (Fig. 5*A*), consistent with similar regenerative capacities after crush injury (Fig. 4*A*). However, at an early symptomatic age (10.5 months) and shortly before reaching our early phenotypic disease mark (10% weight loss at ≈11.5 months), sciatic nerve Schwann cell-derived IGF-1 levels were reduced almost 50% ($P < 0.05$; Student's t test) in LoxSOD1^{G37R}/P_0-cre mice as compared with age-matched LoxSOD1^{G37R} mice (Fig. 5*A*). This loss was selective for IGF-1, as no differences were detected for PTN, CNTF, or GDNF (Fig. 5*A*).

Relative to the levels in presymptomatic LoxSOD1^{G37R} mice, significant ($P < 0.05$; Student's t test) disease-associated inductions were identified in early symptomatic LoxSOD1^{G37R} mice for IGF-1 (2.0-fold), PTN (2.7-fold), and GDNF (8.6-fold), as well as a decrease in CNTF (2.3-fold), alterations that are comparable to those known to occur also after sciatic nerve injury (17, 19, 33, 34) (Fig. 5*B*). Importantly, whereas removal of mutant SOD1^{G37R} from Schwann cells did not influence disease-associated PTN, GDNF, or CNTF regulation, there was a complete lack of IGF-1 induction in sciatic nerves of LoxSOD1^{G37R}/P_0-cre mice as compared to LoxSOD1^{G37R} mice (Fig. 5*B*).

Discussion

In contrast to prior efforts with selective removal of a mutant *SOD1* gene from astrocytes (11) or microglia (10), each of which sharply slowed disease progression, our current findings have shown that similar removal of the same dismutase active mutant SOD1^{G37R} from Schwann cells accelerated the late phase of disease progression resulting in a disease course as aggressive as

that seen in a dismutase inactive mutant SOD1^{G85R} ALS mouse line. Our gene excision targeted 70% of axonal Schwann cells as well as 40% of terminal Schwann cells. When taken together with a prior demonstration that reduction of mutant SOD1 synthesis in muscle had no effect on any aspect of disease (16), our findings offer no support for an important role for mutant-derived toxicity within the non-neuronal cells at the neuromuscular junction. A further insight is that, in contrast to astrocytes and microglia, both muscle (16) and Schwann cells seem to be protected against the buildup of mutant SOD1 toxicity.

The most straightforward explanation for the unexpected protective role of Schwann cell-expressed mutant SOD1 in slowing disease progression is that the mutant we have tested (SOD1^{G37R}) retains fully functional dismutase activity (35). Indeed, ubiquitous overexpression of wild-type SOD1 in transgenic mice is neuroprotective against both cerebral ischemia (30) and spinal cord injury (31), whereas systemic SOD1 deletion in mice increased axotomy-induced motor neuron death (36). Dismutase inactive SOD1 mutant ALS mice would lack any Schwann cell-derived neuroprotective effect and should, therefore, have a more aggressive late disease phase. Indeed, retrospective analysis of survival data from our different ALS mouse cohorts revealed that dismutase inactive mutant SOD1^{G85R} (line 148) mice had a 2.5-fold shorter late phase (23.8 ± 4.6 d; $n = 20$) (29) compared with dismutase active mutant SOD1^{G37R} (line 106) mice (54.7 ± 6.4 d; $n = 20$) (28), despite similar survival times (12.5–13.5 months) (Fig. 2*D*). This more rapid disease progression in the dismutase inactive mutant was statistically highly significant ($P < 0.001$; Student's t test). A search of the literature revealed further support for such a correlation between dismutase activity and slow disease progression: disease in mice from dismutase inactive SOD1^{G127X} has a 2.5-fold shorter symptomatic disease phase than does a dismutase active SOD1^{G93A} line with similar survival times (37).

Although toxicity from a dismutase inactive mutant (SOD1^{G85R}) proceeds independent of overall dismutase activity provided by endogenous SOD1 (38), we suggest that during mutant SOD1-induced axonal degeneration, a cascade is triggered that produces reactive oxygen species at least some of which can be detoxified via SOD1 synthesized by Schwann cells. Potential sources of such reactive species include extracellular superoxide produced by mutant SOD1-dependent stimulation of NADPH oxidase in microglia/macrophages (6) and astrocytes (7) or from mutant SOD1 damage to mitochondria (4, 39, 40), especially in neurons. Indeed, mutant SOD1 damage within microglial/macrophages (10) or astrocytes (11) is known to drive rapid disease progression, findings that correlate well with our evidence here that Schwann cells depleted of a dismutase active mutant substantially shorten disease course. However, as Schwann cells are localized outside the spinal cord, the most likely sources for reactive oxygen species are (1) peripheral macrophages (which are known to invade degenerating peripheral nerves) (20), (2) Schwann cells themselves, or (3) the degenerating axons along which the Schwann cells are aligned.

Our hypothesis might be expected to predict a protective effect of (ubiquitously) overexpressing wild-type SOD1 in ALS mice, whereas no change (38) or a disease acceleration (41) have actually been reported. However, as toxicity of mutant SOD1 aggregates can be increased by wild-type SOD1 overexpression (41), protective effects within (nonsensitive) Schwann cells could have been masked in these prior efforts by increased toxicity within (sensitive) motor neurons, astrocytes, and/or microglia. Likewise, deletion of mouse SOD1 did not speed up disease in ALS mice (38), most probably due to similar opposing effects that neutralized each other or that the endogenous level of dismutase activity in Schwann cells was insufficient to provide a protective benefit.

Interestingly, increased disease duration in human ALS is correlated with increased stability of ALS-linked SOD1 mutants (42). As protein stability in general correlates with dismutase activity (35, 42), it is tempting to speculate that dismutase activity of Schwann cell-expressed SOD1 mutants is an important determinant of slowed disease progression in ALS. As a direct consequence, therapeutic down-regulation of dismutase active mutant SOD1 forms in familial ALS (43) should avoid Schwann cells.

A direct test of our hypothesis could be undertaken by selectively increasing wild-type SOD1 levels within Schwann cells, especially in ALS mice with a dismutase inactive mutant, such as SOD1^{G85R}. Such activity should prolong disease progression. Further, removal of mutant SOD1^{G85R} from Schwann cells would be predicted not to affect disease course. Interestingly, genetic deletion of *CCS1* (the enzyme responsible for loading SOD1 with the catalytic copper essential for dismutase activity) in mutant SOD1^{G37R} (line 29) ALS mice, led to slightly reduced survival (44) (and P.C. Wong; personal communications), consistent with a protective action of dismutase activity, although this effect was not visible in mutant SOD1^{G37R} (line 42) mice with very high levels of transgene expression (44).

Although linking the protective effect of Schwann cell-derived mutant SOD1^{G37R} to dismutase driven reduction of toxic superoxide is the most obvious explanation for our findings, reactive oxygen species, especially hydrogen peroxide (the product of the dismutase reaction), could also directly influence intracellular or intercellular signaling processes, including modulating transcription (45). Indeed, we found a disease-associated induction of IGF-1 transcripts in sciatic nerve Schwann cells of ALS mice, but this was completely abolished upon removal of mutant SOD1^{G37R} from those Schwann cells. Lack of IGF-1 induction was surprisingly selective after mutant SOD1 removal from Schwann cells, with responses of pleiotrophin, GDNF, and CNTF unaffected.

Moreover, although we detected much stronger disease-associated induction of GDNF than IGF-1, recent viral-mediated delivery of GDNF in ALS mice was much less efficient than IGF-1 (46). Thus, even a small loss of IGF-1 might have strong effects in ALS. Previous studies have already established the motor neuron protective potential of IGF-1 in ALS mice (46, 47) and IGF-1 synthesis by Schwann cells is known to be induced during axonal regeneration (34). As the degenerating sciatic nerve also contains invading macrophages (20), we cannot exclude them as a source of IGF-1 induction. In this case, mutant SOD1^{G37R} action (including damage) within Schwann cells could trigger secretion of paracrine acting factors (e.g., cytokines), which could initiate growth factor production in invading macrophages.

Nevertheless, what our evidence establishes is that this IGF-1 induction is dependent on mutant SOD1^{G37R} expression in Schwann cells, a finding that identifies Schwann cells—long overlooked in ALS—as participants in pathogenesis in familial SOD1-linked ALS and therefore establishing them as potential targets for therapeutic intervention in ALS.

Materials and Methods

Animals. All transgenic mouse lines were on a pure C57Bl/6 background. *Po-cre mice*: Mice heterozygous for a mP$_0$TOTA-Cre transgene that contains a complete mouse P$_0$-gene (*mpz*) with 6 kb of promotor, in which the ATG start of translation has been mutated and substituted with the Cre-recombinase gene (48). For additional details, see *SI Materials and Methods*.

Survival Analysis. Mice were weighed weekly as an objective and unbiased measure of disease course (10, 11, 28, 29). Additional details, see *SI Materials and Methods*.

For qPCR for mutant *SOD1* transgene levels, immunohistochemistry, morphometric analysis of axons, sciatic nerve regeneration measurements and RT-qPCR for sciatic nerve mutant *SOD1* and growth factor mRNA levels, see *SI Materials and Methods*.

Note. A study published after the manuscript from Keller et al. (40) provided additional evidence for the involvement of Schwann cells in ALS pathogenesis.

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1. Boillee S, Vande Velde C, Cleveland DW (2006) ALS: A disease of motor neurons and their nonneuronal neighbors. *Neuron* 52:39–59.
2. McNamara JO, Fridovich I (1993) Human genetics. Did radicals strike Lou Gehrig? *Nature* 362:20–21.
3. Rothstein JD, Martin LJ, Kuncl RW (1992) Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. *N Engl J Med* 326:1464–1468.
4. Liu J, et al. (2004) Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria. *Neuron* 43:5–17.
5. Urushitani M, et al. (2006) Chromogranin-mediated secretion of mutant superoxide dismutase proteins linked to amyotrophic lateral sclerosis. *Nat Neurosci* 9:108–118.
6. Harraz MM, et al. (2008) SOD1 mutations disrupt redox-sensitive Rac regulation of NADPH oxidase in a familial ALS model. *J Clin Invest* 118:659–670.
7. Marchetto MC, et al. (2008) Non-cell-autonomous effect of human $SOD1^{G37R}$ astrocytes on motor neurons derived from human embryonic stem cells. *Cell Stem Cell* 3:649–657.
8. Nishitoh H, et al. ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev* 22:1451–1464, 2008.
9. Clement AM, et al. (2003) Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. *Science* 302:113–117.
10. Boillee S, et al. (2006) Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* 312:1389–1392.
11. Yamanaka K, et al. (2008) Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis. *Nat Neurosci* 11:251–253.
12. Di Giorgio FP, Boulting GL, Bobrowicz S, Eggan KC (2008) Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. *Cell Stem Cell* 3:637–648.
13. Di Giorgio FP, Carrasco MA, Siao MC, Maniatis T, Eggan K (2007) Non-cell autonomous effect of glia on motor neurons in an embryonic stem cell-based ALS model. *Nat Neurosci* 10:608–614.
14. Nagai M, et al. (2007) Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nat Neurosci* 10:615–622.
15. Dobrowolny G, et al. (2008) Skeletal muscle is a primary target of $SOD1^{G93A}$-mediated toxicity. *Cell Metab* 8:425–436.
16. Miller TM, et al. (2006) Gene transfer demonstrates that muscle is not a primary target for non-cell-autonomous toxicity in familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci USA* 103:19546–19551.
17. Hoke A (2006) Neuroprotection in the peripheral nervous system: rationale for more effective therapies. *Arch Neurol* 63:1681–1685.
18. Woodhoo A, Sommer L (2008) Development of the Schwann cell lineage: from the neural crest to the myelinated nerve. *Glia* 56:1481–1490.
19. Chen ZL, Yu WM, Strickland S (2007) Peripheral regeneration. *Annu Rev Neurosci* 30:209–233.
20. Martini R, Fischer S, Lopez-Vales R, David S (2008) Interactions between Schwann cells and macrophages in injury and inherited demyelinating disease. *Glia* 56:1566–1577.
21. Scherer SS, Wrabetz L (2008) Molecular mechanisms of inherited demyelinating neuropathies. *Glia* 56:1578–1589.
22. Perrie WT, et al. (1993) Changes in the myelinated axons of femoral nerve in amyotrophic lateral sclerosis. *J Neural Transm Suppl* 39:223–233.
23. De Winter F, et al. (2006) The expression of the chemorepellent Semaphorin 3A is selectively induced in terminal Schwann cells of a subset of neuromuscular synapses that display limited anatomical plasticity and enhanced vulnerability in motor neuron disease. *Mol Cell Neurosci* 32:102–117.
24. Pun S, Santos AF, Saxena S, Xu L, Caroni P (2006) Selective vulnerability and pruning of phasic motoneuron axons in motoneuron disease alleviated by CNTF. *Nat Neurosci* 9:408–419.
25. Schaefer AM, Sanes JR, Lichtman JW (2005) A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. *J Comp Neurol* 490:209–219.
26. Feltri ML, et al. (2002) Conditional disruption of beta 1 integrin in Schwann cells impedes interactions with axons. *J Cell Biol* 156:199–209.
27. Bolis A, et al. (2005) Loss of Mtmr2 phosphatase in Schwann cells but not in motor neurons causes Charcot-Marie-Tooth type 4B1 neuropathy with myelin outfoldings. *J Neurosci* 25:8567–8577.
28. Lobsiger CS, Garcia ML, Ward CM, Cleveland DW (2005) Altered axonal architecture by removal of the heavily phosphorylated neurofilament tail domains strongly slows superoxide dismutase 1 mutant-mediated ALS. *Proc Natl Acad Sci USA* 102:10351–10356.
29. Lobsiger CS, Boillee S, Cleveland DW (2007) Toxicity from different SOD1 mutants dysregulates the complement system and the neuronal regenerative response in ALS motor neurons. *Proc Natl Acad Sci USA* 104:7319–7326.
30. Saito A, Hayashi T, Okuno S, Ferrand-Drake M, Chan PH (2003) Overexpression of copper/zinc superoxide dismutase in transgenic mice protects against neuronal cell death after transient focal ischemia by blocking activation of the bad cell death signaling pathway. *J Neurosci* 23:1710–1718.
31. Sugawara T, Lewen A, Gasche Y, Yu F, Chan PH (2002) Overexpression of SOD1 protects vulnerable motor neurons after spinal cord injury by attenuating mitochondrial cytochrome c release. *FASEB J* 16:1997–1999.
32. Islamov RR, et al. (2002) 17Beta-estradiol stimulates regeneration of sciatic nerve in female mice. *Brain Res* 943:283–286.
33. Sendtner M, Stockli KA, Thoenen H (1992) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *J Cell Biol* 118:139–148.
34. Sullivan KA, Kim B, Feldman EL (2008) Insulin-like growth factors in the peripheral nervous system. *Endocrinology* 21:21–31.
35. Borchelt DR, et al. (1994) Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. *Proc Natl Acad Sci USA* 91:8292–8296.
36. Reaume AG, et al. (1996) Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. *Nat Genet* 13:43–47.
37. Jonsson PA, et al. (2004) Minute quantities of misfolded mutant superoxide dismutase-1 cause amyotrophic lateral sclerosis. *Brain* 127:73–88.
38. Bruijn LI, et al. (1998) Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1 *Science* 281:1851–1854.
39. Vande Velde C, Miller TM, Cashman NR, Cleveland DW (2008) Selective association of misfolded ALS-linked mutant SOD1 with the cytoplasmic face of mitochondria. *Proc Natl Acad Sci USA* 105:4022–4027. Epub 2008 Feb 4022.
40. Vijayvergiya C, Beal MF, Buck J, Manfredi G (2005) Mutant superoxide dismutase 1 forms aggregates in the brain mitochondrial matrix of amyotrophic lateral sclerosis mice. *J Neurosci* 25:2463–2470.
41. Deng HX, et al. (2006) Conversion to the amyotrophic lateral sclerosis phenotype is associated with intermolecular linked insoluble aggregates of SOD1 in mitochondria. *Proc Natl Acad Sci USA* 103:7142–7147.
42. Sato T, et al. (2005) Rapid disease progression correlates with instability of mutant SOD1 in familial ALS. *Neurology* 65:1954–1957.
43. Smith RA, et al. (2006) Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest* 116:2290–2296.
44. Subramaniam JR, et al. (2002) Mutant SOD1 causes motor neuron disease independent of copper chaperone-mediated copper loading. *Nat Neurosci* 5:301–307.
45. Veal EA, Day AM, Morgan BA (2007) Hydrogen peroxide sensing and signaling. *Mol Cell* 26:1–14.
46. Kaspar BK, Llado J, Sherkat N, Rothstein JD, Gage FH (2003) Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science* 301:839–842.
47. Dobrowolny G, et al. (2005) Muscle expression of a local Igf-1 isoform protects motor neurons in an ALS mouse model. *J Cell Biol* 168:193–199.
48. Feltri ML, et al. (1999) P0-Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. *Ann NY Acad Sci* 883:116–123.
49. Keller AF, Gravel M, Kriz J (2008) Live imaging of amyotrophic lateral sclerosis pathogenesis: Disease onset is characterized by marked induction of GFAP in Schwann cells. *Glia*, in press.

4. 神経変性疾患における細胞死研究のパラダイムシフト

山中宏二

神経変性疾患における神経細胞死の研究の焦点は，ここ10年ほどで大きく変貌している．当初，精力的に研究されていたアポトーシスより，むしろ現在では非アポトーシス型の細胞死の関与が示されている．また，筋萎縮性側索硬化症（ALS）の研究からは，神経細胞とグリア細胞の細胞間ネットワークの破綻による非細胞自律性の神経細胞死という生命現象が示され，細胞死研究の面からも大きな転換点を迎えている．本稿では，神経変性疾患における細胞死研究の歴史を踏まえて，神経細胞死研究のトピックスや今後の方向性を概説する．

研究背景

本稿では，神経変性疾患における神経細胞死研究の流れと，最近のトピックスを概説する．臨床神経学でいう神経変性疾患は，中年以降に異常タンパク質が病巣に蓄積することが特徴とされ，特定の細胞群に神経細胞死が起こる疾患と考えてよいといえる．具体的には，アルツハイマー病，パーキンソン病，ポリグルタミン病や，われわれが研究をすすめている筋萎縮性側索硬化症（ALS）[※1]などがその代表的な疾患である．それに対して，先天的にまたは実験的にある遺伝子の機能を欠失することに起因する神経細胞死は本稿では扱わない．

神経変性疾患における神経細胞死のメカニズムにつ

[キーワード＆略語]
筋萎縮性側索硬化症，SOD1，神経変性疾患，運動ニューロン死，非細胞自律性

ALS：amyotrophic lateral sclerosis（筋萎縮性側索硬化症）
COX-2：cyclooxygenase-2（シクロオキシゲナーゼ2）
EAAT2：excitatory amino acid transporter 2（glutamate transporter）（興奮性アミノ酸トランスポーター2）
iNOS：inducible Nitric Oxide Synthase（誘導型一酸化窒素合成酵素）
IL-1β：interleukin-1β（インターロイキン1β）
IL-6：interleukin-6（インターロイキン6）
PGE_2：prostaglandin E2（プロスタグランジンE2）
PINK1：PTEN induced putative kinase 1
RNAi：RNA interference（RNA干渉）
SOD1：superoxide dismutase 1（Cu/Zn superoxide dismutase）（スーパーオキシドジスムターゼ1）
TNFα：tumor necrosis factor α（腫瘍壊死因子α）

Neuronal cell death in neurodegenerative diseases：a paradigm shift
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いての研究の焦点は，ここ10年で大きく変貌している．1990年代のアポトーシスの実行因子群の発見を契機に神経変性疾患においてもアポトーシスの関与が積極的に研究されてきたが，細胞死が必ずしもアポトーシスによって説明できないことがわかってきており，むしろ現在では非アポトーシス型の細胞死の分子機構が精力的に研究されている．

1 神経変性疾患における細胞死研究の流れ

神経変性疾患において，原因遺伝子が次々と発見された'90年代以前は，死後脳の病理標本により神経細胞死を形態学的に研究することが主流であった．その過程で，先述の異常タンパク質がまずは封入体という形で発見，記載されてきた．封入体の構成タンパク質の同定と前後して，遺伝性神経変性疾患の遺伝子が次々と同定されたのが'90年代であった．先述の4つのカテゴリーの疾患群では，ポリグルタミン病を例外として，疾患の大半が非遺伝性のものである．しかし，数%の患者においては優性遺伝性変異により，その遺伝子産物が封入体の主要構成成分として疾患病巣に蓄積している．アルツハイマー病におけるアミロイド前駆体タンパク質，パーキンソン病におけるαシヌクレイン，ALSにおいて最近同定されたTDP-43がその実例である．

これらの疾患について，原因遺伝子の発現によるモデルマウスが作製され神経変性がそれぞれ再現されているが，現在においても，これらの神経変性疾患にアポトーシスの機序が関与しているのかという疑問に対して明確に答える研究結果はないように思われる．具体例として，培養神経細胞を用いた細胞死研究や，遺伝性ALSモデルとして広く知られる変異SOD1※2マウスの病巣の生化学的解析では，活性型カスパーゼ3などの発現がみられアポトーシスの分子機構が発動していることを示唆する報告や[1]，それを否定する報告が混在している[2]．また，ハンチントン病のモデルマウスにおいても明らかな神経変性の表現型にもかかわらず，神経細胞死がはっきりしない[3]．

さらに，ヒト剖検標本ではアポトーシス関連分子の検出は困難であることが多い．また，孤発性ALSの剖検標本においてもアポトーシス関連分子の検討がなされているが，典型的なアポトーシス形態の検出は非常にまれであった[4]．アポトーシスが比較的短時間に起こる細胞死であるのに対し，緩徐な細胞死が起こると考えられる神経変性疾患においては，アポトーシスの分子機構が作動しているものの，不完全であり，典型的なアポトーシス経路による細胞死が起こっていないのではないかと考えられる．未だ結論が出ていない問題であり，今後の研究の進展が待たれるが，その一方で非アポトーシス型の細胞死の研究が急速に進展しており，以下でそのトピックスを概説する（**図1**）．

※1　筋萎縮性側索硬化症（ALS）

運動ニューロンに選択的に細胞死が起こる神経変性疾患．中高年に発症し，発症後1～5年で，進行性の全身の筋力低下，麻痺をきたし，呼吸筋の障害により人工呼吸器を装着しない限り死に至る．現在，本邦では約6,000人が闘病している神経難病である．

※2　SOD1（スーパーオキシドジスムターゼ1）

酸素に依存する生物の細胞内で発生する有害な活性酸素であるスーパーオキシドを解毒する反応系を触媒する酵素がスーパーオキシドジスムターゼである．遺伝型のALSでは，この遺伝子に変異がみられる．

2 神経変性疾患における非アポトーシス細胞死

1）ミトコンドリアの関与

長年にわたり非分裂細胞として生存する神経細胞にとって，ミトコンドリアの機能は，エネルギー産生，カルシウムイオン制御，膜電位維持，軸索輸送などに重要な役割を果たしている．特に，パーキンソン病の病態において，ミトコンドリアの関与が指摘されている．電子伝達系のcomplex Iの機能低下は，孤発性患者や，パーキンソン病モデルとしてよく知られるMPTP投与モデルにおいて明らかとなっている．ミトコンドリア障害がアポトーシスに関与することは推察されるが，劣性遺伝性パーキンソン病においてはその遺伝子産物であるparkin, DJ-1, PINK-1がミトコンドリア外膜に局在し，その機能喪失がミトコンドリア障害やアポトーシスを惹起することが細胞モデル，ショウジョウバエモデルでよく示されている[5]．これらは，最初に定義した異常タンパク質の蓄積による神経変性を特徴とした神経変性疾患の範疇から逸脱するものであるが，研究トピックスとして紹介した．

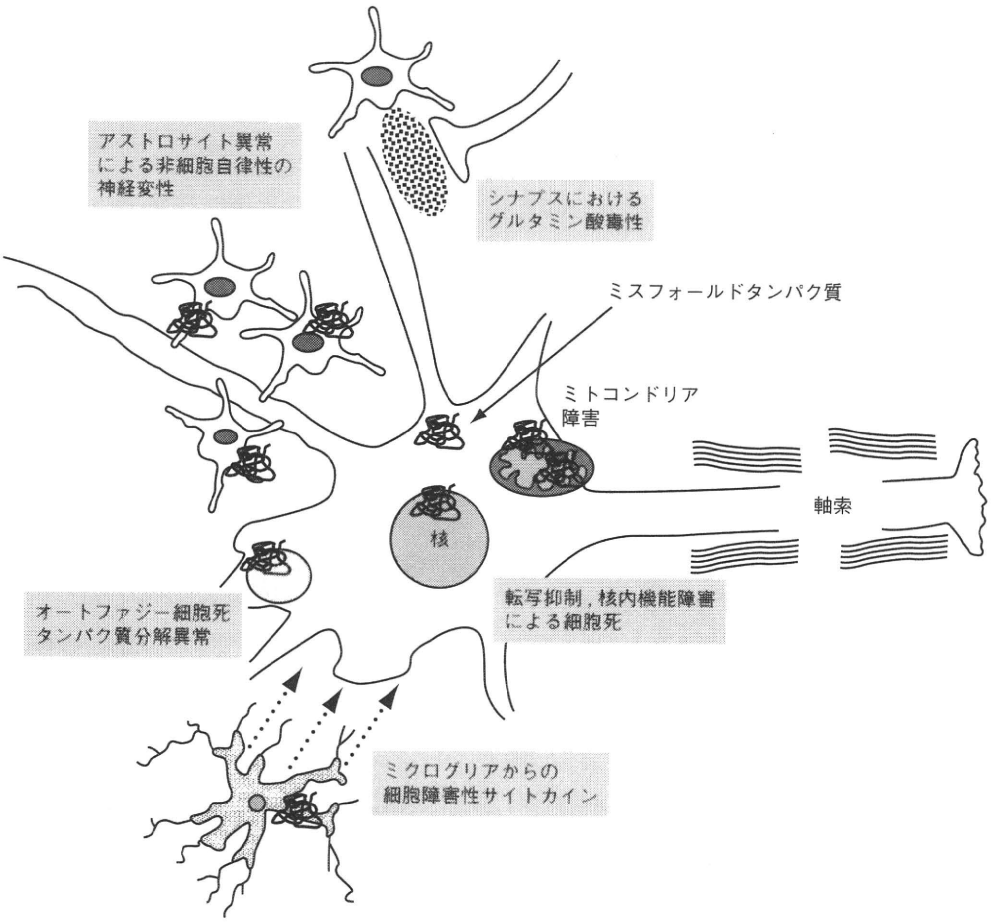

図1　神経変性疾患における細胞死：多様なメカニズム
アルツハイマー病，パーキンソン病，ポリグルタミン病，ALSでは，ともに異常タンパク質が神経細胞内の小器官や周囲のグリア細胞に蓄積する過程で，機能障害をきたし，神経細胞死に至る

異常タンパク質蓄積によっても，ミトコンドリア障害が惹起され神経変性に至ることは，アルツハイマー病，ポリグルタミン病，ALSにおいて広く研究されている．これらの疾患によるミトコンドリア障害は，①ミスフォールドした変性タンパク質が，ミトコンドリア外膜から内部へのタンパク質輸送を阻害する，②変異タンパク質が何らかの機序でミトコンドリア膜間に入り，電子伝達系を直接障害し，活性酸素の異常産生をきたす，③ミトコンドリアによるカルシウムイオンの緩衝作用（buffering）を障害する，などの機序が提唱されている[6]．ミトコンドリア障害は神経変性に重要な役割を果たしているが，その下流の細胞死に至る分子機構は明らかではなく，その解明が待たれる．

2）オートファジーと神経細胞死

オートファジー細胞死の詳細については，他総説を参照（**第1章-3，第8章-1**を参照）されたいが，ここでは，神経変性疾患におけるオートファジーの関与について簡潔にまとめておきたい．ポリグルタミン病研究において，オートファジーの関与は積極的に研究されてきた．具体的に，ラパマイシン投与によるオートファジーの促進がハンチントン病モデルマウスの症状改善をきたした報告[7]などが代表例である．また，アルツハイマー病モデルにおいてもオートファジー様形態変化が神経細胞に起こっていることが示されている[8]．さらにアルツハイマー病早期では，オートファジー制御タンパク質ベクリン1の低下が起こってい

る[9]．オートファジーの抑制により，神経細胞における異常タンパク質蓄積と神経変性が起こることと考え合わせると，オートファジー経路は，ユビキチン／プロテアソーム経路にて分解できない異常タンパク質を分解することを通じて神経保護的な役割を果たしており，神経変性疾患では，その機能低下が病態に関与している可能性が考えられる．

3）ネクローシス様細胞死，第3の細胞死について

細胞質小胞形成，ミトコンドリア膨張などを伴う，古典的ネクローシスと類似した形態変化を伴う細胞死が報告されている．神経変性疾患では，ポリグルタミン病モデルにおいて岡澤らが報告した転写抑制が引き起こす細胞死がその代表例である[10]．先述したミトコンドリアの関与する細胞死も，形態学的観点から見直すとこのカテゴリーに属するのかもしれない．例えば，ALSモデルの変異SOD1マウスでは，病巣における空胞を伴った病理像が特徴的所見として報告されている[11]．今後の研究の展開が期待される分野であり，このカテゴリーに属する細胞死が，神経変性疾患において重要である可能性がある．

4）細胞間ネットワークの破綻による細胞死

神経細胞が，細胞内の病的変化で自己完結的に細胞死に至る（細胞自律性の細胞死）という考え方がこれまで優勢であった．多くの神経変性疾患の病態研究は，選択的に障害される神経細胞における分子病態の解明を目指してきた．一方で，周囲に存在するグリア細胞の病態に着目した研究が，近年急速に広がりをみせている．以下で述べるALSにおける非細胞自律性の神経細胞死の研究に代表されるように，アルツハイマー病やポリグルタミン病モデルにおいてもその病態におけるグリア細胞の積極的関与が明らかになっている．

3 ALSにおける非細胞自律性の神経細胞死：研究の流れと新たな細胞死概念の確立

1）ALSモデルを用いた神経細胞死研究

ALSにおける神経細胞死研究は，'93年の優性遺伝性ALSの原因遺伝子SOD1の発見により急速に加速した．SOD1変異を再現したトランスジェニックマウスでは，運動ニューロン特異的な神経細胞死が再現され，ALSモデル動物として広く研究に用いられている．これまでの研究により，変異SOD1は本来の酵素活性と無関係な毒性※3を発揮することにより神経変性をきたすことが知られている[11]．当初，神経細胞死の機序にアポトーシスの関与が考えられ，このモデルマウスを用いたBcl2の過剰発現[12]やカスパーゼ阻害剤[13]などによるアポトーシス経路の抑制による治療実験が行われ，生存期間の延長効果を示したが，その効果は限定的であり，2000年ごろには神経細胞のみを標的とした細胞死抑制の研究に限界が見えていた．神経変性研究におけるこのような状況が，神経細胞死における非神経細胞の関与を考える契機となった．

2）ALSにおける非細胞自律性の神経細胞死

SOD1変異を有するALS患者やマウスモデルでは，変異タンパク質はすべての細胞群に発現しているが，運動ニューロン特異的に変性が起こる．またALS以外の多くの遺伝性神経変性疾患においても，原因遺伝子がほとんどの細胞群に発現するが系特異的な神経変性を認める．非神経細胞に発現する病因遺伝子産物の役割は不明であり，ALS病巣でみられるアストロサイトの増殖やミクログリアの活性化が，神経変性に伴う二次的変化なのか，または神経変性に直接貢献する一次的病変であるのかは未解明であった．神経細胞のみに変異SOD1を発現したマウスが神経変性をきたさなかった[14]ことから，運動ニューロン変性に非神経細胞が関与している可能性が高まった．その後，野生型マウスとALSを発症するSOD1マウスとのキメラマウスを用いた研究で，野生型細胞が多い環境にある変異SOD1を発現する運動ニューロンは長期生存することが示され，SOD1マウスで起こる運動ニューロン変性は非細胞自律性（non-cell-autonomous）に起こる，つまり神経変性は神経細胞由来の変異SOD1毒性※3のみで自律性に起こる訳ではなく周囲の非神経細胞由来の変異SOD1毒性も必要であることが示唆された[15][16]．

※3　変異型SOD1による毒性

遺伝型のALSではSOD1遺伝子に変異がみられるが，この酵素の活性が失われるために運動神経の細胞死が起こるのではなく，変異型のSOD1が酵素活性とは無関係の毒性を発揮することが神経細胞死の原因と考えられている．しかし，現時点でその毒性の詳細は不明である．

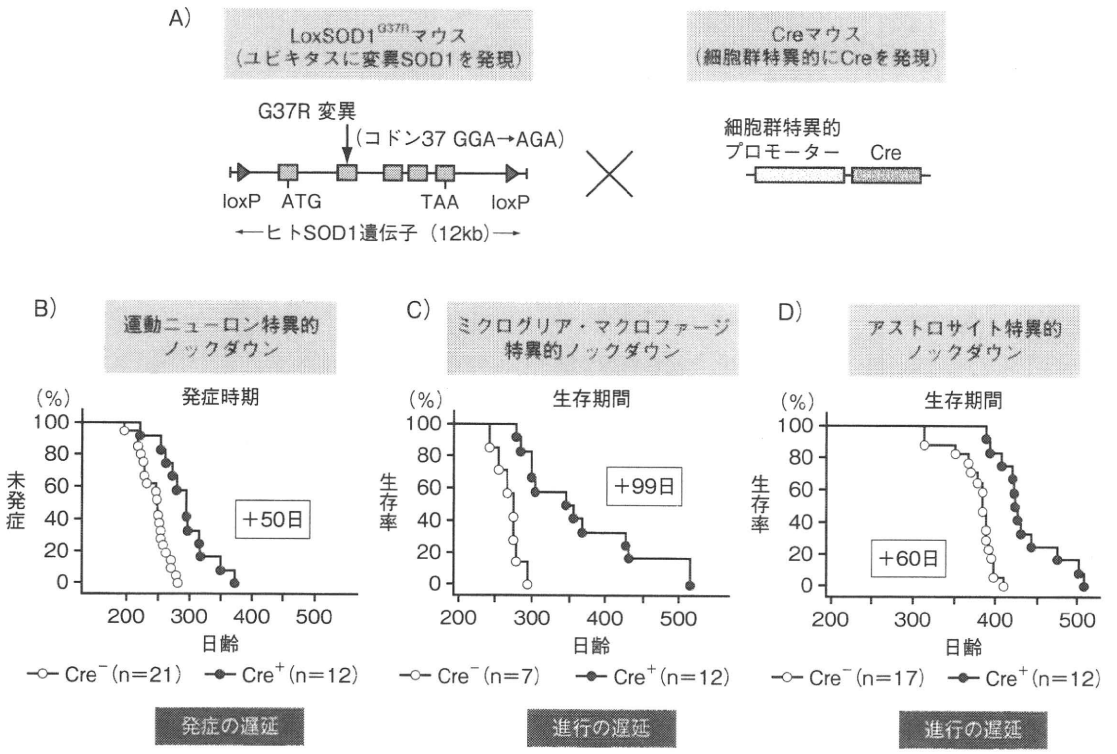

図2　ALSモデルでは，神経細胞とグリア細胞における病的変化が疾患の発症と進行に深く関与する
A）新規ALSモデルLoxSOD1^{G37R}と細胞群特異的Creマウスの交配実験．LoxSOD1^{G37R}マウスはユビキタスに変異SOD1を発現し，進行性の運動麻痺と運動ニューロン変性と再現し，Creマウスとの交配でそのトランスジーンが細胞群特異的に除去できる．B）～D）運動ニューロン特異的な変異SOD1ノックダウン（B），ミクログリア，マクロファージ特異的ノックダウン（C），アストロサイト特異的ノックダウン（D）による疾患の発症時期の遅延（B）と生存期間の延長（C，D）．Cre陽性群において，変異SOD1遺伝子のノックダウンによる発症時期の遅延や生存期間の延長がみられる

3）ALS発症および進行に関する細胞群の同定

i）運動ニューロンにおける変異SOD1毒性はALSの発症を規定する

これらを踏まえて私たちは，どの細胞群に由来する変異SOD1毒性が神経変性に最も重要であるのかを解明するため，Cre-Loxシステムを用い，細胞群特異的に除去可能な変異SOD1を発現する新たなモデルマウスLoxSOD1^{G37R}を樹立した（**図2A**）[17]．まず運動ニューロンにおける変異SOD1毒性の役割を解明するため，運動ニューロン特異的にCreタンパク質を発現するマウスとLoxSOD1^{G37R}マウスを交配し，運動ニューロン特異的に変異SOD1を除去すると，変異SOD1の除去の効率は部分的であったが，ALSの発症時期※4を遅延させることで，生存期間を延長した（**図2B**）[17) 18)]．またこの研究結果は，運動ニューロン特異的にRNA干渉（RNA interference）を用いて変異SOD1の発現を抑制した場合，SOD1^{G93A}マウスの発症時期が著明に遅延した結果と一致しており，双方の実験結果から運動ニューロンにおける病的変化がALSの発症時期を規定していると考えられる．

ii）ミクログリアとアストロサイトはALSの進行を規定する細胞群である

ミクログリアは マクロファージに由来する中枢神経系の貪食細胞であり，ALSの進行につれてその活性化が病巣でみられる．ミクログリアは神経栄養性の因子を分泌する一方，炎症反応に関与する多くのサイトカインなども放出することが知られ，神経細胞にとって保護的である一方，神経傷害もきたすという両面性をもつ細胞群である[19) 20)]．そこで先述のLoxSOD1^{G37R}マウスをCD11b-Creマウスと交配することにより，ミ

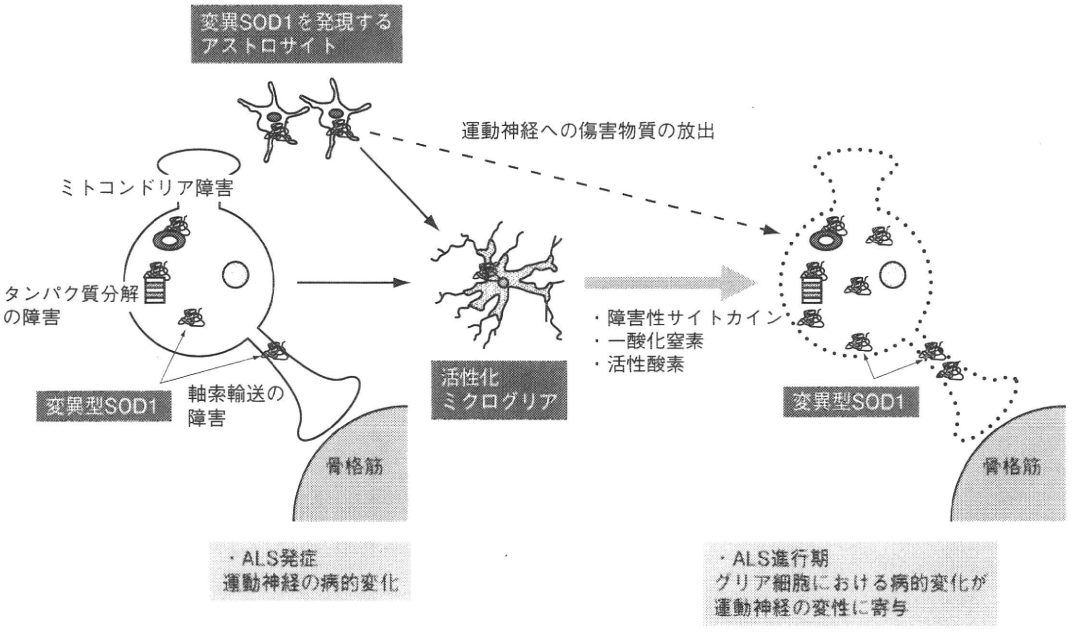

図3　ALSの発症と進行は運動ニューロンとグリア細胞における変異SOD1の毒性により規定される
ALS発症は運動ニューロン内で起こるさまざまな病的変化の集積によって起こると考えられる（図左）．その後，疾患の進行期にはニューロン周囲のグリア細胞（ミクログリアやアストロサイト）に起こる病的変化が神経変性に強く関与する（図右）．神経変性が主にグリア細胞からの栄養因子が失われるために起こるのか，あるいは有害なサイトカインなどの放出によるものであるのかは今後解明すべき課題である

クログリアとマクロファージに発現する変異SOD1を除去するとマウスの疾患進行※4を遅延することにより，生存期間※4は著明に延長した（**図2C**）[17]．また，Appelらは変異SOD1マウスに野生型の骨髄を移植することによりミクログリアおよびマクロファージを置換し，疾患進行を遅延させた[21]．これらの研究からミクログリアにおける病的変化がALSの疾患進行を規定することが示され，ミクログリアを正常化することがALS治療のターゲットとなることが期待される．

ミクログリアは死細胞や病原体の貪食にかかわる細胞であるが，アストロサイトは神経細胞に栄養因子を供給するのみならず，シナプス活動の調節など多岐にわたる機能をもつグリア細胞である．そこで，Lox-SOD1^{G37R}を用いてアストロサイトの関与を検討した．アストロサイト特異的に変異SOD1を除去すると，ミクログリアの異常な活性化を抑制し，発症後の進行を延長して生存期間を著明に延長した（**図2D**）[18]．また*in vitro*の実験系で，変異SOD1を発現するアストロサイトは運動ニューロンに対して毒性を発揮することも示されており[22,23]，グリア細胞が実行する非細胞自律性の神経細胞死という概念が，多くの研究により実証されている．つまりALSの発症は，運動ニューロンに蓄積するさまざまな病的変化に起因するが，その進行はアストロサイトとミクログリアに起因する病的変化が深く関与し，アストロサイトがミクログリアをより活性化させ，細胞障害性サイトカインなどの放出を促進して運動ニューロン変性を加速すると考えられる（**図3**）．

※4　発症時期／生存期間／疾患の進行（罹病期間）

発症時期：ALSの症状を示し始めた時期
（本研究では体重減少の開始点）
生存期間：モデルマウスの誕生から死亡までの期間
罹病期間：発症時期から死亡までの期間
（＝生存期間—発症時期）
罹病期間が延長すると，病気の進行が遅延したと考えられる．

iii）ALSのグリア細胞にみられる分子病態

変異SOD1マウスでは，TNFα，IL-1β，IL-6などのサイトカインの産生亢進，プロスタグランジン合成

系の酵素COX-2やPGE$_2$，NO産生酵素iNOSなどの発現亢進が報告され[19)20)]，神経系におけるこれらの主要な産生細胞であるミクログリアのALS病態への積極的な関与を支持している．他にも多くのサイトカインの発現異常が報告されており，これらを統合的に制御することでミクログリアの機能を正常化することが可能かもしれない．アストロサイトに関しても，われわれが示したミクログリア活性化の調節以外にも，グルタミン酸トランスポーターEAAT2を介してシナプス間隙におけるグルタミン酸興奮毒性の軽減に関与していることなどで神経保護作用をもつことが示されているが，まだ不明な点が多く今後の解明が待たれる．

4）非細胞自律性の神経細胞死は，他の神経変性疾患においてもみられる

ALS以外の神経変性疾患においても，近年グリア細胞の病態への関与が示されている．小脳変性を示すポリグルタミン病のSCA7モデルにおいては，変異型のSCA7をバーグマングリア（Bergmann Glia）に発現したトランスジェニックマウスでは近接する神経細胞であるプルキンエ細胞に神経変性が起こることが示された[24)]．また，オリゴデンドロサイトにおいてαシヌクレインを発現したモデルマウスでは，神経変性が起こることが示され[25)]，パーキンソン病類縁疾患としての多系統変性症モデルとして知られる．さらに，アルツハイマー病モデルマウスでは，骨髄移植によりマクロファージ，ミクログリアを野生型に置換するとアミロイドβタンパク質が効率的に除去されることが示された[26)]．このようにグリア細胞が実行する非細胞自律性の神経変性は，ALSに限定された生命現象ではなく，神経変性疾患に広く見られる現象として今後のさらなる研究の展開が期待される分野である．仕様書

おわりに

神経難病として知られ，克服が難しいと考えられていた神経変性疾患における細胞死の研究は，非アポトーシス細胞死の研究やグリア細胞が実行する非細胞自律性の神経細胞死の研究へと急速に展開している．孤発性神経変性疾患の治療は発症後に行われ，疾患の進行を遅延させることが治療目標となるため，疾患の進行を規定する因子の検索は非常に重要である．ALSにおいて疾患進行速度を規定する細胞群として同定されたグリア細胞はまさに神経変性疾患治療の標的として有望である．今後も神経細胞死が，多様な観点から研究されることにより神経変性のメカニズムが明らかとなることが期待される．

文献

1）Pasinelli, P. et al. : Proc. Natl. Acad. Sci. USA, 97 : 13901-13906, 2000
2）Migheli, A. et al. : Nat. Med., 5 : 966-967, 1999
3）Mangiarini, L. et al. : Cell, 87 : 493-506, 1996
4）Yamazaki, M. et al. : Neuropathology, 25 : 381-387, 2005
5）Schapira, A. H. : Lancet Neurol., 7 : 97-109, 2008
6）Lin, M. T. & Beal, M. F. : Nature, 443 : 787-795, 2006
7）Ravikumar, B. et al. : Nat. Genet., 36 : 585-595, 2004
8）Boland, B. et al. : J. Neurosci., 28 : 6926-6937, 2008
9）Pickford, F. et al. : J. Clin. Invest., 118 : 2190-2199, 2008
10）Hoshino, M. et al. : J. Cell Biol., 172 : 589-604, 2006
11）Bruijn, L. I. et al. : Annu. Rev. Neurosci., 27 : 723-749, 2004
12）Kostic, V. et al. : Science, 277 : 559-562, 1997
13）Li, M. et al. : Science, 288 : 335-339, 2000
14）Pramatarova, A. et al. : J. Neurosci., 21 : 3369-3374, 2001
15）Clement, A. M. et al. : Science, 302 : 113-117, 2003
16）Yamanaka, K. et al. : Proc. Natl. Acad. Sci. USA, 105 : 7594-7599, 2008
17）Boillee, S. et al. : Science, 312 : 1389-1392, 2006
18）Yamanaka, K. et al. : Nat. Neurosci., 11 : 251-253, 2008
19）Sargsyan, S. A. et al. : Glia. 51 : 241-253, 2005
20）Yamanaka, K. & Yamashita, H. : Brain Nerve., 59 : 1163-1170, 2007
21）Beers, D. R. et al. : Proc. Natl. Acad. Sci. USA, 103 : 16021-16026, 2006
22）Nagai, M. et al. : Nat. Neurosci., 10 : 615-622, 2007
23）Di Giorgio, F. P. et al. : Cell Stem Cell, 3 : 637-648, 2008
24）Custer, S. K. et al. : Nat. Neurosci., 9 : 1302-1311, 2006
25）Yazawa, I. et al. : Neuron, 45 : 847-859, 2005
26）Simard, A. R. et al. : Neuron, 49 : 489-502, 2006

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