# Astrocytes as determinants of disease progression in inherited ALS

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SUPPLEMENTARY TEXT

Slowing disease onset by removing the LoxSOD1^{G37R} transgene from post-natal motor neurons

To examine whether reducing the mutant SOD1 toxicity within post-natal motor neurons affects onset or progression of motor neuron disease, LoxSOD1^{G37R} mice[1] carrying a deletable mutant SOD1 transgene were crossed with VAChT-Cre mice[2], which express Cre recombinase in post-natal motor neurons in spinal cords and brain stem. The specificity of Cre-mediated recombination in the motor neurons of VAChT-Cre mice was confirmed by crossing with Rosa26 reporter mice, revealing Cre-mediated gene excision occurred in approximately 50% of motor neurons (**Supplementary Fig. 3a, b**). Postnatal reduction of SOD1^{G37R} in motor neurons significantly slowed disease onset in LoxSOD1^{G37R} /VAChT-Cre$^+$ mice by an average of 50 days (VAChT-Cre$^+$: 294 ± 41 days; VAChT-Cre$^-$: 244 ± 22 days; mean ± standard deviation, $p<0.0001$, unpaired t test) (**Supplementary Fig. 3c, f**). However, progression from onset through early disease was not delayed in LoxSOD1^{G37R} /VAChT-Cre$^+$ mice with a mean extension of 0 days (VAChT-Cre$^+$: 90.2 ± 42 days; VAChT-Cre$^-$: 90.8 ± 25 days, $p=0.95$, unpaired t test, **Supplementary Fig. 3d, g**), along with no slowing of later disease progression (-7 days; VAChT-Cre$^+$: 36.7 ± 20 days; VAChT-Cre$^-$: 43.5 ±26 days, $p=0.44$, unpaired t test, **Supplementary Fig. 3h**). Overall survival was extended by 42 days (VAChT-Cre$^+$: 421 ± 56 days; VAChT-Cre$^-$: 379 ± 20 days, $p=0.038$, unpaired t test, **Supplementary Fig. 3e**).
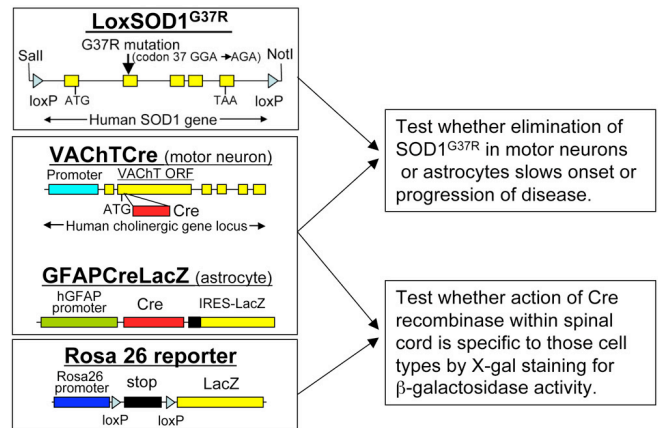

Supplementary Figure 1 Mating scheme for selective excision of a mutant SOD1 encoding gene from astrocytes or motor neurons. Schematic drawings of the LoxSOD1^{G37R}, VAChT-Cre, GFAP-CreLacZ, and Rosa26 reporter transgenes, along with the strategy of mating experiments.
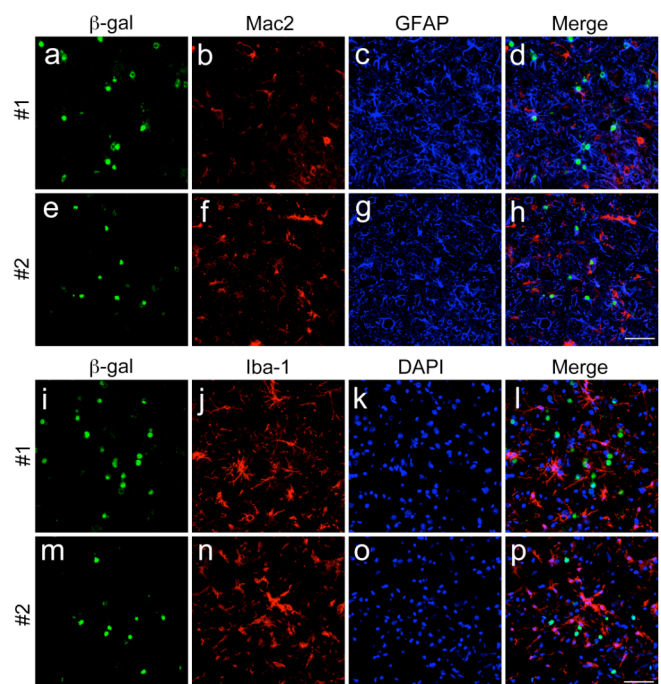
Supplementary Figure 2 Expression of GFAP-CreLacZ is restricted to astrocytes within spinal cord. **(a-h)** Lumbar spinal cord sections of two symptomatic LoxSOD1^{G37R}/GFAP-Cre$^+$ mice stained with **(a,e)** β-galactosidase, **(b,f)** Mac2, and **(c,g)** GFAP antibodies. **(d,h)** Merged images. **(i-p)** The lumbar spinal cord sections of two symptomatic LoxSOD1^{G37R}/GFAP-Cre$^+$ mice were stained with **(i,m)** β-galactosidase and **(j,n)** Iba-1 antibody, followed by staining with **(k,o)** DAPI. **(l,p)** Merged images. Scale bars: 50μm.
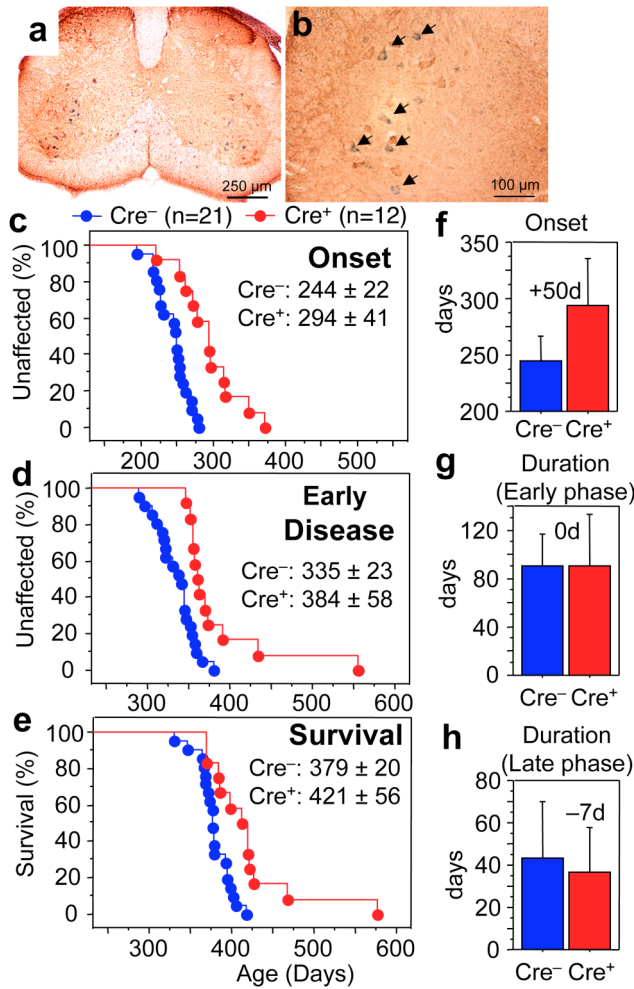
Supplementary Figure 3 Specific reduction of mutant SOD1 in the post-natal motor neurons significantly slowed disease onset. (a, b) β-galactosidase (β-gal) activity within lumbar motor neurons of VAChT-Cre mice. (a) A lumbar spinal cord section of a Rosa26/VAChT-Cre$^+$ mouse was incubated with X-gal followed by the staining with CGRP antibody. (b) Anterior horn section of lumbar spinal cord stained as in (a). Arrows indicate β-gal expressing motor neurons. (c-e) Ages of (c) disease onset ($p < 0.0001$), (d) progression through an early disease phase (to 10% weight loss) ($p=0.019$), and (e) disease endstage ($p=0.038$) of (red) LoxSOD1^{G37R}/VAChT-Cre$^+$ mice and (blue) LoxSOD1^{G37R} littermates. (f-h) Mean onset ($p < 0.0001$) (f), mean duration of an early disease phase ($p=0.95$) (g) (from onset to 10% weight loss) and a later disease phase ($p=0.44$) (h) (from 10% weight loss to endstage) for (red) LoxSOD1^{G37R}/VAChT-Cre$^+$ and (blue) LoxSOD1^{G37R} littermates. At each time point, p value was determined by an unpaired t test.
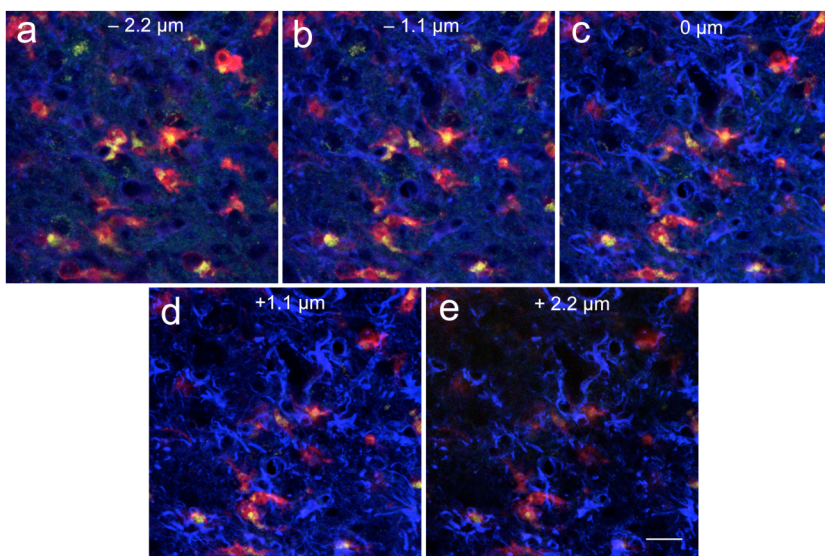
Supplementary Figure 4

Elevated expression of inducible nitric oxide synthase is enriched in microglia of symptomatic LoxSOD1^{G37R} mice. Serial Z-axis confocal images of anterior horn from lumbar spinal cord of symptomatic LoxSOD1^{G37R} mice stained with iNOS (green), Mac2 (red), and GFAP (blue). Merged images at Z-axis positions (a) -2.2 μm, (b) -1.1 μm, (c) 0 μm, (d) +1.1 μm, and (e) +2.2 μm illustrate that most iNOS-positive cells are Mac2-positive microglia. Images at the location 0 μm are provided in main Figure 3o-r. Bar: 20 μm.
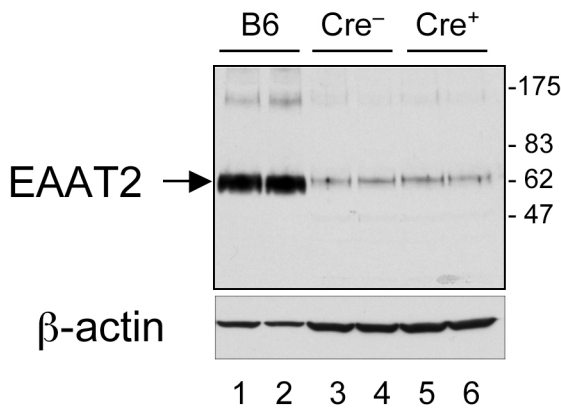
Supplementary Figure 5 Removing mutant SOD1 from astrocytes did not change the loss of EAAT-2 within lumbar spinal cord of end-stage LoxSOD1[G37R] mice. The membrane fraction of lysates prepared from the lumbar spinal cord of 12 months old C57/Bl6 (lanes 1, 2), end-stage LoxSOD1[G37R] (lanes 3, 4), and end-stage LoxSOD1[G37R]/GFAP-Cre[+] (lanes 5, 6) mice was analyzed by immunoblotting with EAAT2 antibody. Equal total protein loading in each lane was verified by immunoblotting with β-actin antibody.
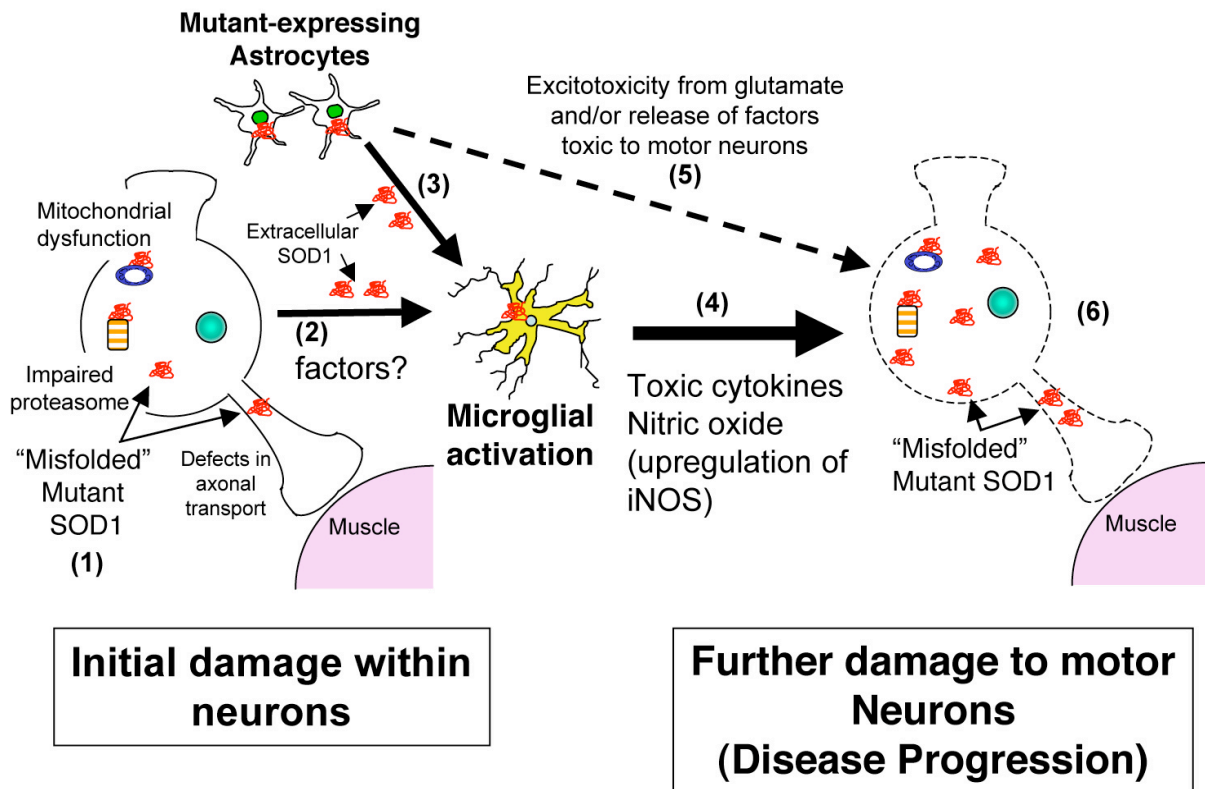
Supplementary Figure 6 Accelerated disease progression from mutant SOD1-mediated toxicity within astrocytes and microglia driving non-cell-autonomous motor neuron death.

(1) Initial damage within motor neurons including age dependent accumulation of an as yet unidentified mutant SOD1-mediated toxicity (hypotheses include mitochondrial dysfunction, impaired proteasome function, and defects in axonal transport). Unidentified factors derived from **(2)** damaged motor neurons or **(3)** astrocytes cause activation of mutant-expressing microglia. Extracellular mutant SOD1 potentially secreted (or released by cell death) from neurons and glia is one of identified factor[10]. Abnormal activated microglia produce high levels of nitric oxide through upregulation of inducible NOS together with secretion of toxic cytokines to motor neurons **(4)**.   A combination of damage from mutant **(4)** microglia and **(5)** astrocytes (including excitotoxicity from glutamate) cause further damage to motor neurons, thereby **(6)** driving rapid disease progression.

SUPPLEMENTARY METHODS

Mating of mice and survival experiments. LoxSOD1[G37R] mice (in a C57BL/6 background), which express a deletable human SOD1 gene carrying the G37R mutation by the action of Cre recombinase, were described previously[1]. For selective expression of Cre recombinase in astrocytes or motor neurons, respectively, GFAP-CreLacZ mice[3] (in a C57BL/6 background) or VAChT-Cre mice[2] (also in a C57BL/6 background) which express Cre within post-natal motor neurons were used. To document the specificity of Cre expression, each line was mated to the Rosa26 mouse that ubiquitously expresses a β-galactosidase (β-gal) transgene that can be translated into functional β-gal only if Cre-mediated recombination removes a premature translation terminator[4].

Mice heterozygous for the mutant human SOD1[G37R] transgene (LoxSOD1[G37R]) were crossed either with heterozygous GFAP-CreLacZ mice or heterozygous VAChT-Cre mice. Mice were genotyped by PCR for the presence of the mutant SOD1 transgene as previously described[5] and for the Cre transgene using the following primers: sense, CCGGGCTGCCACGACCAA; antisense, GGCGCGGCAACACCATTTTT.

For survival experiments, LoxSOD1[G37R]/Cre[+] mice were always compared with their contemporaneously produced littermates without the Cre transgene. Time of disease onset was retrospectively determined as the time when mice reached peak body weight, early disease was defined at the time when denervation-induced muscle atrophy had produced a 10% loss of maximal weight, and end-stage was determined by paralysis so 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.,[1, 6]) and one that was consistent with the requirements of the Animal Care and Use Committee of the University of California and RIKEN Brain Science Institute. Early or later phase of disease progression was defined by the duration between the onset and early disease or between early disease and end-stage, respectively.

Statistical analysis for survival experiments were performed with an unpaired t test and p values were determined.

Immunohistochemistry and antibodies. Mice were perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in phosphate buffer. Before freezing, tissues were cryoprotected for 48h in 30% sucrose in PBS. Thirty-micron cryosections were stained with the following antibodies: GFAP (1:3000, DAKO, Denmark), CGRP (calcitonin gene-related peptide) (1:1000, Peninsula Laboratories, Belmont, CA) overnight followed by biotinylated species-specific secondary antibodies. The staining was revealed by the avidin-biotin complex immunoperoxidase technique (vectastain ABC kit, Vector Laboratories, Burlingame, CA, 1:500 in PBS) and the diaminobenzidine chromogen (Vector Laboratories). Sections were dehydrated and mounted with the toluene-soluble Permount mounting medium (Fisher Scientific, Waltham, MA). β-galactosidase activity was measured by incubation with X-Gal substrate (0.2 mg/ml) in PBS containing 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide and 1mM magnesium chloride.

Immunofluorescence of spinal cord sections. For immunofluorescence detection, lumbar spinal cords were fixed by perfusion with 4% paraformaldehyde in phosphate buffer, cryoprotected in 30% sucrose in PBS, and frozen. Twenty-micron cryosections directly mounted on the slide glasses were incubated with the following antibodies: SMI-32 (1:1000), GFAP (1:1000, DAKO; 1:500, Sigma-Aldrich, St. Louis, MO), β-galactosidase (1:2000, Promega, Madison, WI), Mac-2 (1:500, Cederlane, Ontario, Canada), iNOS (1:500, BD Biosciences, San Jose, CA). Bound antibodies were detected with fluorescently tagged anti-rabbit, anti-rat, or anti-mouse antibodies (Alexa Fluor-405, 488, 546, or 633; Invitrogen, Carlsbad, CA). For some of the antibodies, tissue was incubated with 5% normal goat serum / 0.3% Triton-X100 / PBS for 1 hour at room temperature prior to the incubation with primary antibodies. Sections were mounted in ProLong Antifade reagent (Invitrogen) and analyzed on a confocal microscope (SP2, Leica microsystems, Germany and FV1000, Olympus, Japan).

Quantification of microglia and astrocytes in the spinal cord sections. Transverse sections of lumbar spinal cord from 12-month old LoxSOD1[G37R]/GFAP-CreLacZ mice were stained with antibodies to Mac2, β-galactosidase, and GFAP. Images of the anterior horn area from every 20th lumbar spinal cord section (a total of 16 sections) from each of two mice were photographed in the same conditions, followed by counting of

Mac2-positive cells for activated microglia and β-galactosidase-positive cells for Cre-expressing astrocytes. The values for each sample were plotted and Pearson's correlation coefficient and significance of correlation were determined.

To quantify iNOS-positive cells in spinal cord sections, images of the anterior horn from every 20th lumbar spinal cord section (a total of 16 sections) from each of two symptomatic LoxSOD1[G37R] mice were photographed in the same conditions, followed by counting iNOS$^+$/GFAP$^+$ cells and iNOS$^+$/Mac2$^+$ cells. The averaged percent of iNOS-positive cells that were also GFAP$^+$ or Mac2$^+$ in each section was plotted and p-value was determined by unpaired t test.

Cell preparation and culture. Primary microglia and astrocytes were harvested as previously described[1]. Cortexes from 1-day-old mice were dissociated and plated in MEM supplemented with 10% heat inactivated fetal bovine serum, non-essential amino acids, and L-glutamine (Invitrogen). After 2 weeks, non-adherent microglial cells were removed from primary glial cell cultures. Immunostaining with GFAP antibodies demonstrated a purity of >90% of the astrocytes which remained attached. The cells were pelleted and stored at -80 C before the preparation for DNA or protein extraction.

Quantification of SOD1 transgene content by real-time PCR. To measure SOD1 mutant transgene content, a real time PCR assay was developed from the protocol from Howland et al[7], modified as follows. For genomic DNA extraction from microglial cells, the QIAamp DNA micro kit (Qiagen, Valencia, CA) was used following the manufacturer's instructions. For genomic DNA extraction from astrocytes, pelletted astrocytes were incubated overnight at 55 C in digestion buffer containing 50mM Tris pH8, 50 mM EDTA, 0.5 % SDS and 0.5 mg/ml proteinase K. Debris was pelleted by centrifugation for 10 min at 13,200 rpm. Genomic DNA was extracted using phenol/chloroform and ethanol precipitation.

DNA (33 ng) was amplified with iQ Supermix (Bio-Rad Laboratories, Hercules, CA) and 100 nM of each primer and probe in a Bio-Rad iCycler real time PCR machine using the following protocol: 1 cycle 50 C, 2 min; 1 cycle 95 C, 10 min; 40 cycles 95 C, 15 sec, 60 C 1 min. Specific primers and probe for the human SOD1 gene were: hSOD1-forward,

CAATGTGACTGCTGACAAAG; hSOD1-reverse, GTGCGGCCAATGATGCAAT; and hSOD1 probe, fam-CCGATGTGTCTATTGAAGATTCTG-BHQ. Primers and probe for the normalizer apolipoprotein B (apoB) were: apoB-forward, CACGTGGGCTCCAGCATT; apoB-reverse, TCACCAGTCATTTCTGCCTTTG; and apoB probe, Texas Red-CCAATGGTCGGGCACTGCTCAA-BHQ2.

Protein content analyzed by immunoblotting. Proteins from astrocytes were extracted with 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100 and protease inhibitors (1mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml chymotrypsin). The extract was clarified by centrifugation for 10 min at 13,000 g and supernatants were analyzed by the immunoblotting by using an anti-peptide antibody (to human SOD1 residues 125-137) that recognizes human and mouse SOD1 with equal affinity [8, 9]. For the detection of EAAT2, lumbar spinal cord was homogenized in 50 mM Tris pH 7.4, 150 mM NaCl, 2mM EDTA, and protease inhibitors. Postnuclear supernatant prepared by the centrifugation for 5 min at 800 g was further fractioned by ultracentrifugation for 30 min at 100, 000 g. The pellet (P100 fraction) was dissolved in SDS sample buffer and analyzed by immunoblotting using EAAT2 antibody (Chemicon, Millipore, Billerica, MA). The amount of each sample loaded was verified by immunoblotting with β-actin antibody (Sigma-Aldrich).

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