Supporting Online Material for

Onset and Progression in Inherited ALS Determined by Motor Neurons and Microglia

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Material and methods
Construction and mating of mice

For construction of the LoxSOD1<sup>G37R</sup> transgene, a pair of 34 base loxP sequences were cloned to each end of the human SOD1 gene carrying the G37R mutation (S1). The final 12kb gene was excised (using Sal I and Not I), purified by agarose gel electrophoresis, and microinjected into one cell stage hybrid (C57BL/6J x C3H/HeJ) F2 mouse embryos. Transgenic founders were identified by immunoblotting of tail protein extracts with an SOD1 antibody that recognizes human and mouse SOD1 with equal affinity (S2). Founders were crossed with C57BL/6 mice five times before mating to the Islet-1-Cre or CD11b-Cre mice.

For generation of the CD11b-Cre mice, a 1.7 kb DNA fragment (position -1584 to +85) of the human CD11b promoter (S3) was directly cloned by PCR from genomic DNA derived from the human leukemic myelomonocytic cell line U937. A sense-strand primer, 5’-CGGGGTACCGGTTCGAAAGTGTCTGC-3’, introduced a Kpn I restriction site, while an antisense primer, 5’-CCGCTCGAGTGGAAGGACCCAGAACC-3’, created a newly formed Xho I restriction site at position +85 of the promoter. The Kpn I-Xho I fragment was cloned into the respective restriction sites of pBluescript (Stratagene) already containing a 1.1 kb Cre recombinase gene with a nuclear localization site (isolated from the pMC-Cre plasmid (kindly provided by Dr V. Episkopolou, MRC Clinical Sciences Centre, Hammersmith Hospital, London, UK)). Finally a polyadenylation signal from the human growth hormone gene was added (kindly provided by Dr R. Palmiter, Howard Hughes Medical Institute and Department of Biochemistry, University of Washington, Seattle, USA). The final gene was isolated for microinjection into fertilized F1:CBAxC57BL/6 eggs as a Kpn I-Not I fragment.

Heterozygous mice for the mutant human SOD1<sup>G37R</sup> transgene (LoxSOD1<sup>G37R</sup>) were crossed either with heterozygous Islet1-Cre mice (S4) (in a C57BL/6 background - the gift from Dr. Thomas Jessel) or heterozygous CD11b-Cre mice (also in a C57BL/6 background). Mice were genotyped by PCR for the presence of the mutant SOD1 transgene, as previously described (S5) and for the Cre transgene using the following primers: sense, CCGGGCTGCGCACGACAA; antisense, GCCGCAGCAAACACATTTT. The CD11b-Cre transgene was identified using an antisense primer, CAGGTATGCTCAGAAAACGCCT, and a sense primer TGGGCCAACCCAAGAAACAAGT.

The feasibility of in vivo excision of the floxed transgene in LoxSOD1<sup>G37R</sup> mice was documented by mating to ZP3-Cre transgenic mice (S6). Resultant doubly transgenic
females were then mated with C57BL/6 males and gene excision rate was tested in the progeny of these females.

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. We previously proposed that decline in peak body weight as the earliest observable measure of disease onset, since there is high degree of correlation between the age at which rotor rod performance declines and initial loss of body weight (S7, S8). Indeed, as subsequently confirmed by others, peak body weight prior to weight loss is an objective, easily measured parameter that defines earliest disease onset, initiating before any observable motor performance decline such as grip strength, rotor-rod performance, and cage activity (S9, S10). The time of early disease was defined at the time when denervation-induced muscle atrophy had produced a 10% loss of maximal weight, and endstage 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., (S2, S11)) and one that was consistent with the requirements of the Animal Care and Use Committee of the University of California. The early or later phase of disease progression was defined by the duration between the onset and early disease or between early disease and endstage, respectively.

**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 (for spinal cords) and 20-micron (for L5 roots) cryosections were stained with the following antibodies: CD11b (1:100, Chemicon), Iba1 (1:500, Wako chemicals), GFAP (1:4000, Dako). β-galactosidase activity was measured by overnight incubation with X-Gal substrate (0.2 mg/ml) in PBS containing 4 mM potassium ferrocyanide and 1mM magnesium chloride.

For motor root morphology, L5 roots transversely sectioned into 5mm blocks were treated with 2% osmium tetroxide in 0.05M cacodylate buffer, washed, dehydrated, and embedded with Epon (Electron Microscopy Sciences). One µm thick cross sections were cut and stained with 1% toluidine blue, 1% sodium borate for 30 sec, rinsed and dried.

Motor neuron numbers were determined from 30 µm serial sections across the entire lumbar spinal cord and counted in every twelfth cresyl violet stained section corresponding to a total of 23-26 sections per animal.

**Immunofluorescence and quantification of fluorescence intensity of human SOD1 in the motor roots**

For immunofluorescence detection, lumbar spinal cord or L5 roots were fixed with 4% paraformaldehyde in phosphate buffer, cryoprotected in 30% sucrose in PBS, and frozen. Thirty-micron (for spinal cords) and 20-micron (for L5 roots) cryosections were incubated with the following antibodies: human specific SOD1 (1:200, (S2, S12)), Myelin Basic Protein (MBP, 1:100, Chemicon) or SMI-32 (1:2000). Bound antibodies were detected with fluorescently tagged anti-rabbit, anti-rat, or anti-mouse antibodies, respectively.
For quantification of fluorescence intensity, L5 root sections from 4 month old presymptomatic mice of each genotype (Cre+ and Cre-), as well as non-transgenic (C57BL/6) mice, were stained contemporaneously using identical conditions, and fluorescence images were obtained with a Bio-Rad confocal microscope (MRC 1024) under the identical image acquisition settings. The power of the laser and the time of the exposure were identical for all samples. Multiple high magnification images covering an entire root section (8-10 images) were collected. Fluorescence intensity was quantified by Image-J software (a public domain Java image processing program inspired by NIH Image) within all axons of an entire motor root. The averaged intensity of axons of the non-transgenic mice was used as a background that was then subtracted from all intensities measured from the LoxSOD1\textsuperscript{G37R} samples. The corrected intensities from both genotypes were further normalized to the mean intensity of all axons from LoxSOD1\textsuperscript{G37R} / Islet1-Cre\textsuperscript{-} mice (mean=1.0). Histograms of normalized intensities of individual axons were plotted in Figure 1C, D and Supplemental Figure 2A.

Lumbar spinal cord sections were stained in an analogous manner and the fluorescence images were collected contemporaneously using identical conditions.

Cell preparation and culture

Microglial cells were harvested as previously described (SI3, SI4). Cortices from 1-day-old mice were dissociated and plated in DMEM (Gibco) containing 10% heat inactivated fetal bovine serum (Gibco). After 2 weeks, non adherent microglial cells were isolated from primary glial cell cultures. Immunostaining with CD11b antibodies and toluidine blue counterstaining demonstrated a purity of 98%. The cells were pelleted and stored at -80°C. Other remaining cells, mainly astrocytes, were trypsinized, pelleted, stored at -80°C and used as enriched astrocytes 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 (SI5), modified as follows. For genomic DNA extraction from peritoneal macrophages and microglial cells, the QIAamp DNA micro kit (Qiagen) was used following the manufacturer’s instructions. For genomic DNA extraction from tails or astrocytes, tissues or cell pellets 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. For tail extracts, 5 μl of the crude extract was diluted with 95 μl of water and heat inactivated at 95°C for 15 min. Genomic DNA was extracted from astrocyte lysates using phenol/chloroform and ethanol precipitation.

DNA (33 ng) was amplified with iQ Supermix (Bio-Rad) and 100 nM of each primer and probe (IDT) in a Bio-Rad iCycler real time PCR machine using the following protocol: 1 cycle 50C, 2 min; 1 cycle 95C, 10 min; 40 cycles 95C, 15 sec, 60C 1 min. Specific primers and probe for the human SOD1 gene were: hSOD1-forward, CAATGTCATGAAAGAG; hSOD1-reverse, GTCGGCCAATGATGCAAT; and hSOD1 probe, fam-CCATGTCATGAAAGATGCTC-BHQ. Primers and probe for the normalizer apolipoprotein B (apoB) were: apoB-forward, CACGTGGCTCCAGCATT; apoB-reverse, TCACCAGTCATTTCTGCCATTG; and apoB probe, Texas Red-CCAATGTCGGCCTGCTCAA-BHQ2.
To establish the sensitivity and linearity of the real-time PCR, genomic DNA extracted from the tail segments from LoxSOD1 mice was mixed in different proportions with comparable DNA C57/Bl6 and the Q-PCR was run with the same amount of total DNA.

**Lasermicrodissection of lumbar motor neurons**

Lumbar spinal cords were isolated, washed, immediately frozen in OCT compound (Fisher) and stored at −80 C. Cryosections (20 µm) were mounted on RNAse-free PEN-Slides (Leica) and immediately frozen at −80 C. Motor neurons were identified by a rapid Nissl-staining protocol (cresyl-violet acetate; Sigma) to preserve the integrity of the RNA. In brief, sections were fixed for 30 sec in 75% ethanol, Nissl-stained for 30 sec, followed by ethanol dehydration and a final step in xylene for 30 sec. All solutions were RNAse-free. Sections were desiccated for 1 hour before microdissection and dissected motor neurons were collected in lysis buffer to protect the RNA (Absolutely RNA-Nanoprep Kit; Stratagene). From each spinal cord, 1500 lumbar (L4-L6) ventral horn motor neurons were excised by laser microdissection (Leica DM-LMD Lasermicrodissection System), recovered, total RNA extracted (Nanoprep Kit), DNase-treated and concentration of the final RNA was determined by absorbance.

**Real-time RT-PCR**

Total RNA from 1500 motor neurons per animal was reverse transcribed using oligo-dT (Superscript-III; Invitrogen) and 1/10 of the resulting cDNA was used for Taqman real-time PCR (parameters for the PCR and primer/probe-set for the human SOD1 gene, as described above). The assay was run in triplicate for n=2 animals per genotype (LoxSOD1G37R/Is11Cre+ and LoxSOD1G37R/Is11Cre−, respectively). No signal was detected in the negative control in which the reverse transcriptase was omitted.

To establish the sensitivity of the real-time RT-PCR, total RNA (25 ng) extracted from spinal cord of LoxSOD1 mice and one from C57/Bl6 mice were mixed in different proportions. Equal amounts of total RNA were reverse-transcribed and a tenth of the resulting cDNA was used for the real-time PCR.

**Protein content analyzed by immunoblotting**

Proteins from spinal cord, peritoneal macrophages, primary microglia, and astrocytes were extracted with 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X100 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 electrophoresed on SDS-polyacrylamide gels, transferred to membranes and developed with ECL (Amersham Biosciences) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) using an anti-peptide antibody (to human SOD1 residues 125-137) that recognizes human and mouse SOD1 with equal affinity (S2, S16).

**Supporting Text**

LoxSOD1G37R transgenic mice develop motor neuron disease from an SOD1 transgene that can be removed by Cre-mediated recombination.
The LoxSOD1G37R line with the highest level of mutant SOD1 expression in spinal cords was identified (Fig. S1B). This line developed fatal progressive motor neuron disease, including progressive weight loss from denervation induced muscle atrophy and paralysis that was essentially indistinguishable from previously described SOD1G37R lines (51). This LoxSOD1G37R line reached end stage disease between 8.5 and 11 months accompanied by death of 55% of spinal motor neurons (Fig. S1C-F). To verify that the SOD1G37R gene could be efficiently excised by Cre expression, this line was mated to ZP3-Cre mice, which express the Cre-recombinase in oocytes (56) (Fig. S1G). No progeny from ZP3-Cre/LoxSOD1G37R females expressed any human SOD1 protein, demonstrating efficient in vivo gene removal in the presence of Cre (Fig. S1H).

**Cre-mediated gene inactivation of mutant SOD1 within motor neurons of LoxSOD1G37R/Islet-1 Cre+ mice.**

Cre recombinase in Islet1-Cre (Is1-Cre) mice is expressed in the nervous system exclusively in progenitors of motor and dorsal root ganglion neurons, and was sufficient to substantially reduce mutant SOD1 accumulation in most motor axons of L5 motor roots of 4 month old Is1-Cre+/LoxSOD1G37R animals, with a mean reduction of mutant SOD1 by ≈50% (Fig. 1A-E, S2A, B). In lumbar spinal cord, this was further confirmed as the ≈30% decrease of human SOD1G37R mRNA by quantitative RT-PCR of pooled laser dissected motor neurons from L4-L6 lower spinal cord (Fig. S2C, D) as well as immunofluorescence staining of human SOD1 in lumbar spinal motor neurons (Fig. S2E, F).

**CD11b-Cre transgenic mice activate Cre exclusively in macrophage/microglial lineages**

Specificity of Cre expression of CD11b-Cre mice was verified by mating 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 (57). Fifty-eight percent (± 4.9%) of peritoneal macrophages (n=3, Fig. S3B) of Rosa26/CD11b-Cre+ mice expressed β-gal, while none did in animals without the CD11b-Cre gene. Although both neurons and astrocytes from Rosa26 mice showed high levels of β-gal activity after germ line Cre expression (Fig. 2B), none could be detected in either of these cell types in mice with the CD11b-encoded Cre (Fig. 2A). In the lumbar spinal cords of Rosa26/CD11b-Cre+ mice β-gal was expressed only in a few, small cells (Fig. 2A), identified as microglial cells by the presence of the microglia specific proteins CD11b or Iba1 (58) (Fig. S3E, G). Subcutaneous injection into Rosa26/CD11b-Cre+ mice of lipopolysaccharide (LPS), known to induce microglial activation and increase CD11b expression (59, 60) (Fig. S3D, E), produced an increase in β-gal expressing cells (Fig. S3E). Thus, CD11b-directed Cre expression within the spinal cord is microglial cell specific and is further induced by activation.
Figure S1. LoxSOD1\(^{G37R}\) transgenic mice develop motor neuron disease from an SOD1 transgene that can be removed by Cre-mediated recombination.

A. Schematic drawing of the LoxSOD1\(^{G37R}\) transgene. The human SOD1 gene with mutation G37R was flanked by loxP sequences. B. Spinal cord extract from a LoxSOD1\(^{G37R}\) mouse immunoblotted with an antibody that recognizes human and mouse SOD1 with equal affinity (S2). B6, extract from a non-transgenic mouse. C. Onset, early disease, and survival times of LoxSOD1\(^{G37R}\) mice. D, E. Ventral horn region of the lumbar spinal cord from 8.5 month old (D) normal and (E) LoxSOD1\(^{G37R}\) mice (stained with cresyl-violet). F. Numbers of ventral horn motor neurons from age-matched normal and endstage LoxSOD1\(^{G37R}\) mice. G. Mating scheme for LoxSOD1\(^{G37R}\) and ZP3-Cre mice. H. Spinal cord extracts from F2 mice in (G) immunoblotted for human and mouse SOD1. Mutant SOD1 was absent from all F2 mice. Bar in D, E: 50\(\mu\)m.

Boillee et al, Supplemental Figure 1 (Figure S1)
Figure S2. Quantification of Cre-mediated gene inactivation of mutant SOD1 within motor neurons.

A. Histograms of relative intensities of mutant SOD1 fluorescence in individual L5 motor axons measured from three LoxSOD1G37R (Cre- #1-3, blue) or LoxSOD1G37R/Isl1Cre+ (Cre+ #4-6, red) mice. B. Total accumulated mutant SOD1 measured as relative total fluorescence intensity in motor axons from entire L5 roots (n=3 for each genotype). Bars indicate standard deviation. C. SOD1G37R mRNA levels determined by real-time PCR on reverse-transcribed spinal cord RNA using various amounts of LoxSOD1G37R RNA.

D. LoxSOD1G37R mRNA contents (n=2 animals for each genotype) in laser micro-dissected lumbar motor neurons from LoxSOD1G37R/Isl1-Cre+ (Cre+) and LoxSOD1G37R (Cre-) mice using RT followed by real-time PCR. E, F. Representative immunofluorescence images of anterior horn regions of lumbar spinal cord from 4 month old presymptomatic LoxSOD1G37R/Isl1Cre+ (F: Isl1-Cre+) and LoxSOD1G37R littermates (E: Isl1-Cre-), as indicated. Spinal cord sections were stained with antibodies to human SOD1 (SOD1; green, left) and neurofilaments (SMI32; red, right) and appropriate fluorescent secondary antibodies. Arrowheads indicate motor neurons. Bars: 40μm.

Boillée et al, Supplemental Figure 2 (Figure S2)
Figure S3: CD11b-Cre-directed excision of SOD1G37R exclusively in macrophage/microglial lineages. A. A CD11b-Cre transgene comprised of the human CD11b promoter, the Cre coding domain including a nuclear localization sequence, and the polyadenylation signal from human growth hormone (hGH). B. β-galactosidase (β-Gal) activity within peritoneal macrophages from Rosa26/CD11b-Cre⁺ mice. (Arrowhead) β-Gal expressing cell. C. Transgene levels determined by real-time PCR, using varying amounts of LoxSOD1G37R tail DNA. D-G. Microglial activation in lumbar spinal cord of Rosa26/CD11b-Cre⁺ mice visualized (brown) with antibodies to CD11b or Iba1 at 48h after sub-cutaneous injection of lipopolysaccharide (LPS) (D, E) or without LPS injection (F, G). E, G (blue); β-gal activity using an X-gal substrate.

Boillee et al, Supplemental Figure 3 (Figure S3)
Supplementary Figure Legends

Figure S1. *LoxSOD1\textsuperscript{G37R}* transgenic mice develop motor neuron disease from an SOD1 transgene that can be removed by Cre-mediated recombination. 
A. Schematic drawing of the *LoxSOD1\textsuperscript{G37R}* transgene. The human SOD1 gene with mutation G37R was flanked by loxP sequences. B. Spinal cord extract from a *LoxSOD1\textsuperscript{G37R}* mouse immunoblotted with an antibody that recognizes human and mouse SOD1 with equal affinity (S2). B6, extract from a non-transgenic mouse. C. Onset, early disease, and survival times of *LoxSOD1\textsuperscript{G37R}* mice. D, E. Ventral horn region of the lumbar spinal cord from 8.5 month old (D) normal and (E) *LoxSOD1\textsuperscript{G37R}* mice (stained with cresyl-violet). F. Numbers of ventral horn motor neurons from age-matched normal and endstage *LoxSOD1\textsuperscript{G37R}* mice. G. Mating scheme for *LoxSOD1\textsuperscript{G37R}* and ZP3-Cre mice. H. Spinal cord extracts from F2 mice in (G) immunoblotted for human and mouse SOD1. Mutant SOD1 was absent from all F2 mice. Bar in D, E: 50\textmu m.

Figure S2. Quantification of Cre-mediated gene inactivation of mutant SOD1 within motor neurons. A. Histograms of relative intensities of mutant SOD1 fluorescence in individual L5 motor axons measured from three *LoxSOD1\textsuperscript{G37R} (Cre\textsuperscript{#1-3}, blue) or LoxSOD1\textsuperscript{G37R}/Is11Cre\textsuperscript{+} (Cre\textsuperscript{#4-6}, red) mice. B. Total accumulated mutant SOD1 measured as relative total fluorescence intensity in motor axons from entire L5 roots (n=3 for each genotype). Bars indicate standard deviation. C. SOD1\textsuperscript{G37R} mRNA levels determined by real-time PCR on reverse-transcribed spinal cord RNA using various amounts of LoxSOD1\textsuperscript{G37R} RNA. D. *LoxSOD1\textsuperscript{G37R}* mRNA contents (n=2 animals for each genotype) in laser micro-dissected lumbar motor neurons from LoxSOD1\textsuperscript{G37R}/Is11-Cre\textsuperscript{+} (Cre\textsuperscript{+}) and LoxSOD1\textsuperscript{G37R} (Cre\textsuperscript{−}) mice using RT followed by real-time PCR. E, F. Representative immunofluorescence images of anterior horn regions of lumbar spinal cord from 4 month old presymptomatic LoxSOD1\textsuperscript{G37R}/Is11Cre\textsuperscript{+} (F: Is11-Cre\textsuperscript{+}) and LoxSOD1\textsuperscript{G37R} littermates (E: Is11-Cre\textsuperscript{−}), as indicated. Spinal cord sections were stained with antibodies to human SOD1 (SOD1; green, left) and neurofilaments (SMI32; red, right) and appropriate fluorescent secondary antibodies. Arrowheads indicate motor neurons. Bars: 40\textmu m.

Figure S3: CD11b-Cre-directed excision of SOD1\textsuperscript{G37R} exclusively in macrophage/microglial lineages. A. A CD11b-Cre transgene comprised of human CD11b promoter, the Cre coding domain including a nuclear localization sequence, and the polyadenylation signal from human growth hormone (hGH). B. \(\beta\)-galactosidase (\(\beta\)-Gal) activity within peritoneal macrophages from Rosa26/CD11b-Cre\textsuperscript{+} mice. (Arrowhead) \(\beta\)-Gal expressing cell. C. Transgene levels determined by real-time PCR, using varying amounts of LoxSOD1\textsuperscript{G37R} tail DNA. D-G. Microglial activation in lumbar spinal cord of Rosa26/CD11b-Cre\textsuperscript{+} mice visualized (brown) with antibodies to CD11b or Iba1 at 48h after sub-cutaneous injection of lipopolysaccharide (LPS) (D, E) or without LPS injection (F, G). E, G (blue); \(\beta\)-gal activity using an X-gal substrate.
References for supporting online materials
