BRIEF COMMUNICATIONS

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Astrocytes as determinants of disease progression in inherited amyotrophic lateral sclerosis

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Dominant mutations in superoxide dismutase cause amyotrophic lateral sclerosis (ALS), an adult-onset neurodegenerative disease that is characterized by the loss of motor neurons. Using mice carrying a deletable mutant gene, diminished mutant expression in astrocytes did not affect onset, but delayed microglial activation and sharply slowed later disease progression. These findings demonstrate that mutant astrocytes are viable targets for therapies for slowing the progression of non–cell autonomous killing of motor neurons in ALS.

ALS is an adult-onset neurodegenerative disease, characterized by a progressive and fatal loss of motor neurons. Dominant mutations in the gene for superoxide dismutase (*SOD1*) are the most frequent cause of inherited ALS. Ubiquitous expression of mutant *SOD1* in rodents leads to progressive, selective motor neuron degeneration as a result of acquired toxic properties. The exact mechanism responsible for motor neuron degeneration in ALS, however, is not known[1,2]. Mutant damage in the vulnerable motor neurons is a key determinant of disease onset[3], whereas accumulating evidence supports an active role of non-neuronal cells in motor neuron degeneration[3–7]. Evidence with selective gene excision[3] or bone-marrow grafting[5] has demonstrated that mutant SOD1-derived damage in microglia accelerates later disease progression. Despite the importance of astrocyte function, the role of mutant action in astrocytes in disease has not been tested *in vivo*.

To examine whether mutant SOD1 damage in astrocytes contributes to disease, *loxSOD1*^{G37R} mice[3], carrying a mutant *SOD1* gene that can be deleted by the action of the Cre recombinase, were mated with *GFAP-Cre* mice (**Fig. 1** and **Supplementary Fig. 1** online), which express both Cre recombinase and β-galactosidase (*LacZ*) under the control of the human GFAP promoter[8]. Mice from these matings that carry the *GFAP-Cre* transgene are denoted as Cre$^+$, whereas mice without it are referred to as Cre$^-$. To determine the cell-type specificity of Cre expression in the spinal cord, *GFAP-Cre* mice were mated to Rosa26 mice, which ubiquitously express a *LacZ* gene that encodes functional β-galactosidase only after Cre-mediated recombination. Although this *GFAP-Cre* transgene is expressed in a subset of neurons in the cerebellum and hippocampus during embryogenesis[9], measurement of β-galactosidase activity (by deposition of a blue reaction product after addition of the X-gal substrate) demonstrated that Cre expression and Cre-mediated recombination was restricted in the spinal cord to GFAP-reactive astrocytes (**Fig. 1a**,**b**). The efficiency of mutant gene excision in cultured astrocytes from newborn *loxSOD1*^{G37R}*/GFAP-Cre*$^+$ mice was ~76% (**Fig. 1d**,**e**), determined by quantitative PCR for human SOD1 transgene number (**Fig. 1d**) and immunoblotting for mutant SOD1 levels (**Fig. 1e**). We observed neither detectable Cre activity nor mutant gene excision in microglia (**Fig. 1c** and **Supplementary Fig. 2** online).

A simple, objective measure of disease onset and early disease was applied by initiation of weight loss, itself reflecting denervation-induced muscle atrophy. Reduction of SOD1^{G37R} in astrocytes did not slow disease onset nor early disease (GFAP-Cre$^+$, 341.6 ± 48.9 d; GFAP-Cre$^-$, 337.0 ± 35.8 d; **Fig. 1f**,**h**). However, late disease progression (from early disease to end stage) was sharply delayed, providing a mean extension of survival by 48 d (Cre$^+$, 87.4 d; Cre$^-$, 39.5 d; **Fig. 1j**). Progression from onset to early disease was more modestly slowed by 14 d (Cre$^+$, 99.3 d; Cre$^-$, 85.2 d; **Fig. 1i**). Overall survival was extended by 60 d (Cre$^+$, 436.5 ± 38.8 d; Cre$^-$, 376.5 ± 26.9 d; **Fig. 1g**). This contrasts with delayed disease onset from diminished mutant synthesis solely within motor neurons (with a *VAChT-Cre* transgene carrying the motor neuron–specific vesicular acetylcholine transporter promoter) without affecting disease progression (**Supplementary Results**, **Supplementary Methods** and **Supplementary Fig. 3** online), just as reported previously with an *Isl1* (*Islet1*)*-Cre* transgene that is expressed in motor neurons and some peripheral tissues[3].

Astrocytic and microglial cell activation is a well-accepted feature of SOD1 mutant–mediated ALS[1,2]. An elevated proportion of GFAP-positive astrocytes appeared before disease onset (**Fig. 2a**) in *loxSOD1*^{G37R} mice. This astrogliosis was progressive, readily apparent by onset (**Fig. 2b**) and more prominent during disease progression (**Fig. 2c**). Despite substantial mutant reduction, astrogliosis was not, however, different in comparing disease-matched *loxSOD1*^{G37R}*/GFAP-Cre*$^+$ mice (**Fig. 2d**,**e**) and *loxSOD1*^{G37R}*/GFAP-Cre*$^-$ mice (**Fig. 2b**,**c**).

Microglial activation occurred at earliest disease onset in *Cre*$^-$ mice (**Fig. 2g**) and was progressively more prominent during disease progression (**Fig. 2h**). Microglial activation was, however, substantially delayed from onset through early disease in the *GFAP-Cre*$^+$ mice when mutant SOD1 levels were reduced only in astrocytes (**Fig. 2i**,**j**). By exploiting the presence of β-galactosidase to mark astrocytes with diminished SOD1 mutant synthesis, examination of sections throughout lumbar spinal cords of symptomatic *loxSOD1*^{G37R}*/GFAP-Cre*$^+$ mice

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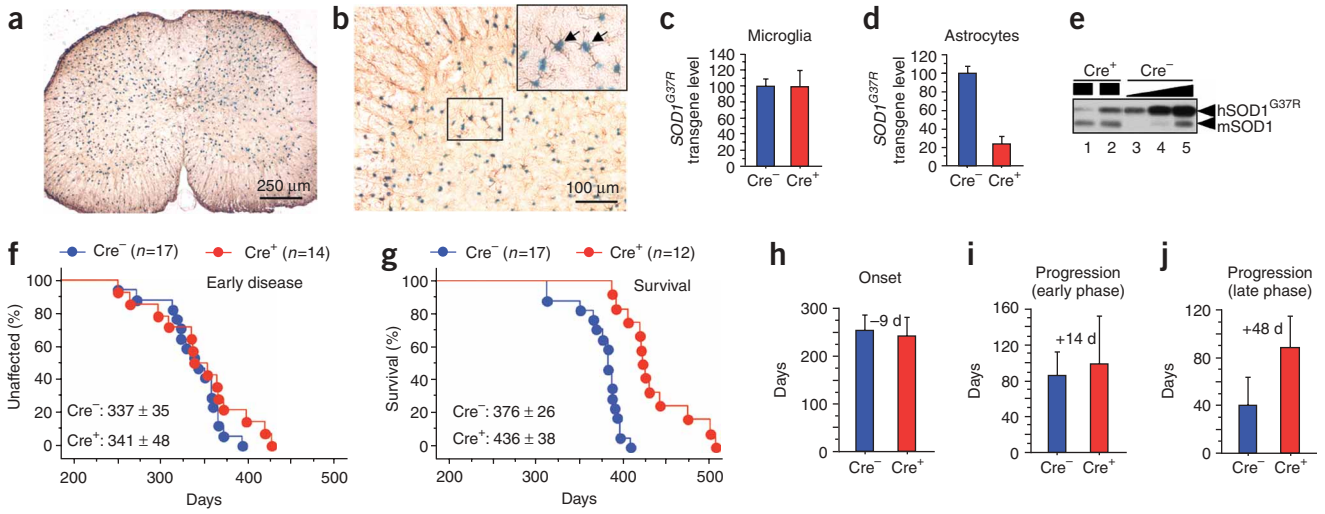

Figure 1 Selective Cre-mediated gene excision shows that mutant SOD1 action in astrocytes is a primary determinant of late disease progression. (**a**,**b**) β-galactosidase (β-gal) activity in astrocytes in whole (**a**) or in the anterior horn region (**b**) of the lumbar spinal cord section of *GFAP-Cre*/Rosa26 reporter mice visualized with X-gal and immunostaining with GFAP antibody. Inset, magnified image of the boxed area in **b**. Arrows indicate β-gal/GFAP-Cre–expressing astrocytes. (**c**,**d**) *loxSOD1*^{G37R} transgene levels ($n = 3$ for each group) in primary microglia (**c**) or astrocytes (**d**) from *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ and *loxSOD1*^{G37R} mice using real-time PCR. (**e**) We determined SOD1^{G37R} and mouse SOD1 levels by immunoblotting extracts from isolated primary astrocytes of *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ (lanes 1, 2) and a dilution series of a comparable extract from LoxSOD1^{G37R} astrocytes representing 25%, 50% and 100% of the protein amounts loaded in lanes 1 and 2 (lanes 3–5). (**f**,**g**) Ages at which early disease phase (to 10% weight loss, $P = 0.76$; **f**) or end-stage disease ($P < 0.0001$; **g**) were reached for *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ mice (red) and *loxSOD1*^{G37R} littermates (blue). Mean ages ± s.d. are provided. (**h**–**j**) Mean onset ($P = 0.47$) (**h**), mean duration of early disease (from onset to 10% weight loss, $P = 0.35$; **i**) and a late disease (from 10% weight loss to end stage, $P < 0.0001$; **j**) for *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ (red) and *loxSOD1*^{G37R} littermates (blue). At each time point, *P* value was determined by unpaired *t*-test. Error bars denote s.d.

revealed an inverse relationship (**Fig. 3a–g**) between the number of astrocytes with reduced mutant SOD1 (*Cre*$^{+}$) and activated microglia (correlation coefficient, $r = -0.868$, $P < 0.001$), despite comparable astrocytic activation. Thus, microglial activation was most prominent in areas with the highest mutant SOD1–expressing astrocyte concentration.

Elevated production of nitric oxide by upregulated inducible nitric oxide synthase (iNOS) has been reported in mutant SOD1 mice[10], although deletion of the iNOS gene has modest[11] or no[12] effect on SOD1-mediated disease. It is not known in which glial cells this nitric oxide is produced in *in vivo* models of ALS, although both microglia and astrocytes have an ability to produce it when stimulated *in vitro*[13]. Triple staining of lumbar spinal cord sections with iNOS, Mac2 and GFAP antibodies (**Fig. 3h–r**) revealed that almost all iNOS-positive cells were Mac2-positive microglia (**Fig. 3n–r** and **Supplementary Fig. 4** online), indicating that activated microglia are the primary cell type producing nitric oxide in this SOD1 mouse model. Diminishing mutant synthesis in astrocytes inhibited iNOS induction in disease-matched, symptomatic SOD1 mice (**Fig. 3h**,**k**), consistent with substantial inhibition of microglial activation (**Fig. 3i**,**l**).

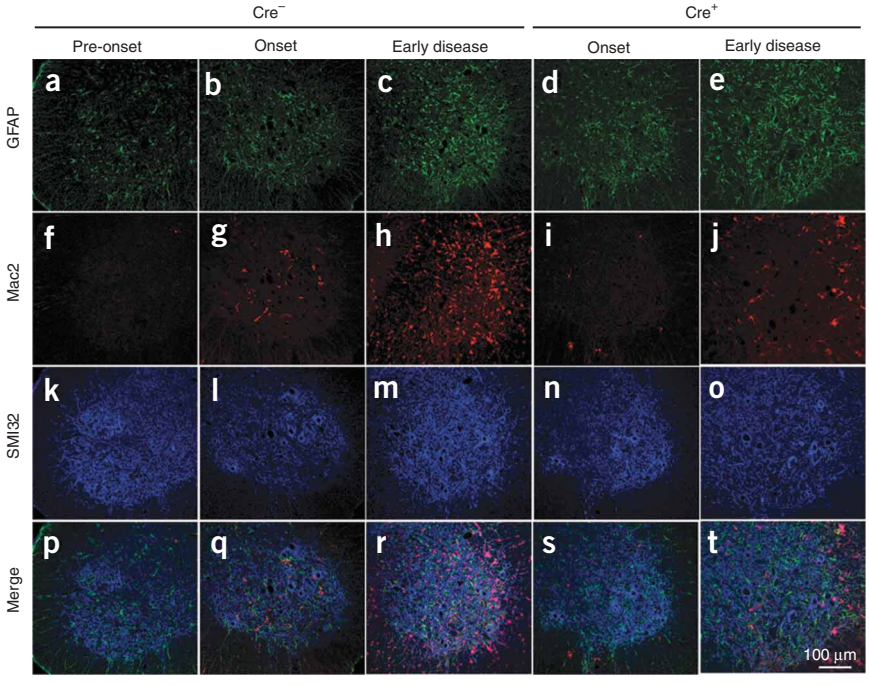

Figure 2 Selective downregulation of mutant SOD1 in astrocytes significantly inhibits microglial activation. (**a**–**t**) GFAP-positive astrocytes (**a**–**e**), Mac2-positive activated microglia (**f**–**j**) and motor neurons identified with the neurofilament antibody SMI-32 (**k**–**o**) in the lumbar spinal cord of a *loxSOD1*^{G37R} mouse before disease onset (**a**,**f**,**k**,**p**), at disease onset (**b**,**g**,**l**,**q**) or during early disease (**c**,**h**,**m**,**r**), together with *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ mice at disease onset (**d**,**i**,**n**,**s**) or during early disease (**e**,**j**,**o**,**t**). Merged images are shown in **p**–**t**.

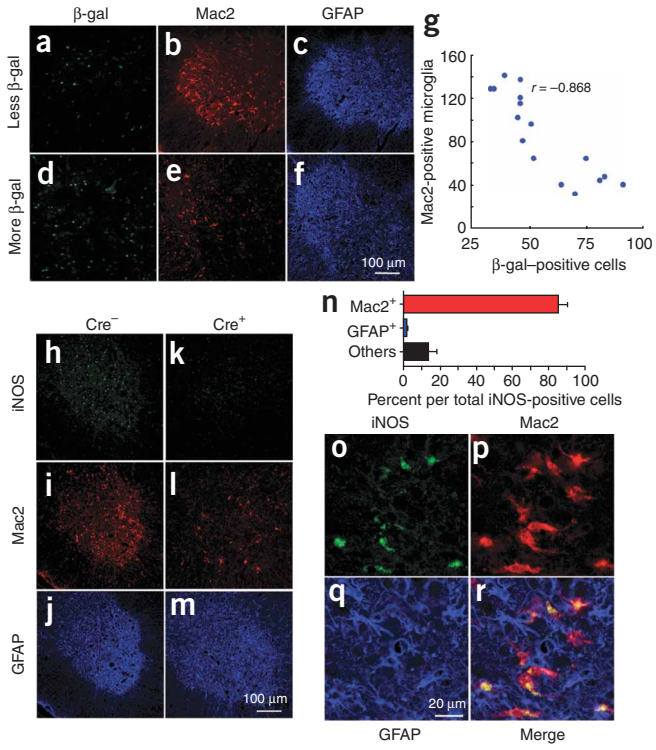

Figure 3 Mutant-expressing astrocytes enhance microglial activation and induction of iNOS. (**a**–**f**) Images of β-galactosidase (**a**,**d**), Mac2 (**b**,**e**) and GFAP (**c**,**f**) staining from a left (**a**–**c**) and right (**d**–**f**) lumbar spinal cord section from a 12-month-old *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ mouse. *GFAP-Cre*$^{+}$ astrocytes are marked by β-galactosidase (**a**,**d**). (**g**) Inverted correlation between the number of Cre-positive astrocytes and Mac2-positive microglia in *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ mice lumbar spinal cord sections (correlation coefficient, $r = -0.868$, $P < 0.001$). (**h**–**m**) Lumbar spinal cord sections from *loxSOD1*^{G37R} (**h**–**j**) and *loxSOD1*^{G37R}/*GFAP-Cre*$^{+}$ (**k**–**m**) mice at the early disease stage immunostained with antibodies to iNOS (**h**,**k**), Mac2 (**i**,**l**), and GFAP (**j**,**m**). (**n**) Quantification of iNOS-positive cells in the anterior horn from lumbar spinal cord of symptomatic *loxSOD1*^{G37R} mice. We plotted the averaged percent of iNOS^{+}/Mac2^{+} (red), iNOS^{+}/GFAP^{+} (blue) and iNOS^{+}/other cell type (black) per total iNOS^{+} cells. (**o**–**r**) Magnified images of anterior horn from lumbar spinal cord of symptomatic *loxSOD1*^{G37R} mice stained with iNOS (**o**), Mac2 (**p**) and GFAP (**q**). Merged image illustrates that iNOS-positive cells are Mac2-positive microglia (**r**).

A role for astrocytes in inherited ALS has been previously considered in several contexts. Mutant-expressing astrocytes produce and release one or more as yet uncharacterized components that can accelerate motor neuron death *in vitro*[6,7]. Focal loss of the astrocytic EAAT2 glutamate transporter in affected regions[14] (**Supplementary Fig. 5** online) and the failure of normal glutamate uptake of *SOD1*^{G93A} astrocytes *in vitro*[15] support glutamate-dependent excitotoxicity as a component of disease. Nevertheless, diminished mutant SOD1 synthesis in most astrocytes did not affect disease-dependent loss of EAAT2 from those astrocytes (**Supplementary Fig. 5**), indicating that a reduction in glutamate transport reflects non–cell autonomous damage to astrocytes, in part, from mutant SOD1 synthesized by other cells. Our use of selective gene excision has now demonstrated that mutant SOD1 damage in both microglia[3] and astrocytes (**Fig. 1g**–**j**) accelerates later disease progression without affecting the initiation of motor neuron degeneration and phenotypic disease onset. Discovery that damage in astrocytes determines the timing of microglial activation and infiltration provides further evidence that, beyond any direct effect of mutant astrocytes on motor neurons, such astrocytes amplify an inflammatory response from microglia (including enhanced production of nitric oxide and possibly of toxic cytokines), leading to further damage to the motor neurons and accelerated disease progression through a non–cell autonomous mechanism (**Supplementary Fig. 6** online). These findings validate therapies, including astrocytic stem cell–replacement approaches, that aim to slow disease progression in ALS by supplementing healthy astrocytes or modulating toxicity in astrocytes to control an inflammatory response of microglia.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

K.Y., S.J.C., S.B., N.F.-T. and H.Y. conducted the experiments. D.H.G., R.T. and H.M. provided essential experimental tools and advice. K.Y., S.B., and D.W.C. were responsible for the overall design of the project, analyses of the results and writing the manuscript.

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