Astrocytes carrying the superoxide dismutase 1 (SOD1^{G93A}) mutation induce wild-type motor neuron degeneration in vivo

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Recent studies highlight astrocytes as key drivers of motor neuron (MN) degeneration and disease propagation in mutant human superoxide dismutase 1 (mSOD1)-mediated amyotrophic lateral sclerosis. However, in vivo analysis of specific astrocytic influence in amyotrophic lateral sclerosis has proven difficult because mSOD1 is ubiquitously expressed throughout the CNS of rodent models studied. Here, we transplanted SOD1^{G93A} glial-restricted precursor cells—glial progenitors capable of differentiating into astrocytes—into the cervical spinal cord of WT rats to reveal how mutant astrocytes influence WT MNs and other cell types (microglia and astrocytes) in an in vivo setting. Transplanted SOD1^{G93A} glial-restricted precursor cells survived and differentiated efficiently into astrocytes. Graft-derived SOD1^{G93A} astrocytes induced host MN ubiquitination and death, forelimb motor and respiratory dysfunction, reactive astrocytosis, and reduced GLT-1 transporter expression in WT animals. The SOD1^{G93A} astrocyte-induced MN death seemed in part mediated by host microglial activation. These findings show that mSOD1 astrocytes alone can induce WT MN death and associated pathological changes in vivo.

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Amyotrophic lateral sclerosis (ALS), an adult-onset neurodegenerative disease affecting ~30,000 individuals in the United States, is characterized by motor neuron (MN) loss leading to paralysis and eventually death (1). Although most ALS cases are sporadic, 5–10% are inherited, with 20% of those cases linked to dominant mutations in the gene encoding for Cu/Zn superoxide dismutase 1 (SOD1) (2). Transgenic rodents engineered to carry mutant human SOD1 (mSOD1) genes broadly recapitulate the human disease (3, 4).

Despite the relative selectivity of MN loss in ALS, studies in mSOD1 rodent and tissue culture models implicate nonneuronal (glial) cell types in the disease process (5, 6). Astrocytes in particular are hypothesized to play a role in both mSOD1 and sporadic forms of ALS (5–8). Recently, coculture studies showed selective degeneration of embryonic stem cell-derived MNs by mSOD1-expressing astrocytes, possibly via toxic astrocyte-secreted factors acting through a variety of proposed mechanisms (9–12). These studies shed light on mSOD1 astrocyte-specific influences on MN degeneration independent of mSOD1 expression in MNs. However, it is unknown whether these astrocyte-specific toxicities translate in vivo.

Here, glial-restricted precursors (GRPs)—lineage-restricted astrocyte precursors derived from the developing spinal cord (13, 14)—were transplanted into rodent spinal cord to determine whether mSOD1 astrocyte-mediated toxicity to MNs translates in vivo. Specifically, GRPs harboring the human G93A mutation (SOD1^{G93A}) were transplanted into the cervical spinal cord ventral horn of WT rats to assess the basic biology of mSOD1 glial lineages in vivo and to address whether SOD1^{G93A} GRP-derived astrocytes can induce WT MN death or dysfunction in this setting. Findings show that SOD1^{G93A} astrocytes alone can induce MN death and associated pathological changes in vivo, perhaps in part by modulating deleterious microglial activity.

Results

Transplanted SOD1^{G93A} GRPs Differentiate Efficiently into Astrocytes.

Transplanted GRPs were found in cervical ventral horn of rats after immunohistochemical detection with an antibody to M2, a mouse cell membrane marker (Fig. 1*A* and *B*). M2⁺ cells harboring human SOD1^{G93A} or those overexpressing the WT form of SOD1 (SOD1^{WT}) were distinguished from WT cells by immunolabeling with an antibody to human SOD1 (hSOD1; Fig. 1*B*, *Inset*). Before transplant, hSOD1 levels did not significantly differ between SOD1^{G93A} and SOD1^{WT} GRPs ($P > 0.05$ for SOD1^{WT} vs. SOD1^{G93A}; $*P < 0.001$ vs. WT; Fig. 1*C*). After transplant, hSOD1 levels in cervical spinal cord were similar in SOD1^{WT} and SOD1^{G93A} GRP-transplanted rats [$n = 3$ per group; SOD1^{WT}: 0.5 ± 0.2 arbitrary units (A.U.); SOD1^{G93A}: 0.6 ± 0.3 ; $P > 0.05$ for SOD1^{WT} vs. SOD1^{G93A}], yet roughly 19-fold less than transgenic SOD1 animals (TG-SOD1^{WT}: 13.4 ± 3.2 ; TG-SOD1^{G93A}: 11.1 ± 2.1 ; $*P < 0.001$; Fig. 1*D*). At 3 mo, 41.2% $\pm$ 1.4%, 35.3% $\pm$ 0.9%, and 37.3% $\pm$ 7.7% of transplanted GRPs survived for SOD1^{G93A} ($n = 3$), WT ($n = 3$), and SOD1^{WT} ($n = 3$) groups, respectively ($P > 0.05$). At this time, GRP-derived cells no longer proliferated (Fig. S1) and remained primarily localized within 1 mm of transplant sites in gray matter, as previously described (Fig. S1) (15).

Quantification of the differentiation profile of GRPs at 1 wk ($n = 4$ per group; Fig. 1*E*) and 3 mo ($n = 6$ –8 per group; Fig. 1*F*) after transplant revealed an efficient transition from immature to mature phenotypes. At 1 wk, most SOD1^{G93A} GRPs and WT GRPs immunostained for the immature neural precursor marker nestin ($47.3\% \pm 2.8\%$ and $39.2\% \pm 3.5\%$; Fig. 1*E* and *G*) or the astrocyte-restricted precursor cell marker CD44 ($47.6\% \pm 1.3\%$ and $52.6\% \pm 2.5\%$; Fig. 1*E*). Few glial fibrillary acidic protein (GFAP⁺) astrocytes constituted the remaining population of transplanted SOD1^{G93A} ($8.4\% \pm 2.3\%$) and WT ($12.7\% \pm 1.0\%$) GRPs (Fig. 1*E*). Notably, CD44⁺ cells differentiate almost exclusively into GFAP⁺ astrocytes, and CD44⁺ astrocyte-restricted precursors subsequently mature to acquire GFAP⁺ immunoreactivity (16). SOD1^{WT} GRPs differentiated more rapidly into astrocyte lineages ($*P < 0.001$; Fig. 1*E*). By 3 mo, nearly all GRP types expressed CD44 (SOD1^{G93A}: $93.8\% \pm 0.8\%$; WT: $92.6\% \pm 1.4\%$; SOD1^{WT}: $93.3\% \pm 2.0\%$; Fig. 1*F* and *H*) and had primarily differentiated into GFAP⁺ astrocytes (SOD1^{G93A}: $87.3\% \pm 2.0\%$; WT: $91.4\% \pm 1.5\%$; SOD1^{WT}: $91.5\% \pm 2.0\%$; Fig. 1*F* and *I*). Few GRPs remained positive for oligodendrocyte precursor marker NG2 or mature oligodendrocyte marker RIP (Fig. 1*F*). Transplanted cells did not give rise to neuronal phenotypes (Fig. 1*E* and *F*).

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SOD1^{G93A} GRP-Derived Astrocytes Induce Ubiquitinated Inclusions and Loss of Host MNs. At 3 mo, SOD1^{G93A} GRP-derived astrocytes not only localized in the vicinity of host MNs but spatially interacted with host MN soma and dendritic fields (Fig. 2). Confocal microscopy revealed that M2⁺ cells were closely apposed to cell bodies of host MNs immunolabeled with the nonphosphorylated neurofilament marker SMI-32 (Fig. 2A–C and Fig. S2) and showed localization of cells at synapsin⁺ synaptic sites (Fig. S2). Only MNs in the vicinity of SOD1^{G93A} GRP-derived astrocytes (and not those from WT, SOD1^{WT} GRP-derived astrocytes) contained ubiquitin⁺ aggregates (Fig. 2A–C), a characteristic pathological feature of mSOD1-mediated ALS in rodents (17, 18) and humans (19, 20). Neuronal ubiquitin accumulation occurred concomitantly with migrating SOD1^{G93A} cells and was not exclusive to MNs whose soma came into direct contact with transplant-derived cells (Fig. S2). Aggregates of endogenous SOD1 or TDP-43, a protein whose mislocalization and aggregation is implicated in the pathogenesis of ALS (21), were not found in host cervical MNs and did not colocalize with ubiquitin aggregates (Fig. 2D and E).

Transplanted SOD1^{G93A} cells caused a temporal loss in the number of cervical MNs. At 1 wk, when most grafted GRPs expressed immature cell markers, quantification of the average number of SMI-32⁺ MNs did not reveal differences among transplant groups ($n = 3$ per group; at transplant sites, SOD1^{G93A}: 9.1 ± 2.0 average MNs; WT: 9.0 ± 1.2 ; and SOD1^{WT}: 9.6 ± 0.9 ; dead SOD1^{G93A}: 10.2 ± 0.5 ; Media: 10.0 ± 1.2 ; $P > 0.05$ for all comparisons). However, by 3 mo (when $\approx 90\%$ of grafted GRPs were GFAP⁺ astrocytes) rats transplanted with SOD1^{G93A} GRPs ($n = 8$) demonstrated an average of 3.6 ± 0.4 MNs at transplant sites (Fig. 2F), $>65\%$ less than the average number of MNs seen in rats transplanted with WT GRPs ($n = 6$; 11.9 ± 0.9), SOD1^{WT} GRPs ($n = 6$; 10.5 ± 0.4), dead SOD1^{G93A} GRPs ($n = 6$; 11.5 ± 0.6), or injected with media ($n = 6$; 11.5 ± 0.9) ($*P < 0.0001$ for SOD1^{G93A} vs. Media). This MN loss corresponded to that observed in transgenic SOD1^{G93A} rats injected with media ($n = 4$; TG-Media; $*P < 0.001$ for TG-Media vs. Media; Fig. 2D). However, unlike the ex-

tensive loss seen in TG-Media rats, MN loss in SOD1^{G93A} GRP-transplanted rats was spatially limited, occurring up to 1.7 mm past transplant sites ($*P < 0.05$ for SOD1^{G93A} vs. Media; Fig. 2F). We confirmed our use of SMI-32 as a measure for MN loss with choline acetyltransferase (ChAT) immunostaining performed on adjacent tissues (Fig. S3).

Next, we determined whether the MN loss was an astrocyte-specific effect by transplanting SOD1^{G93A} and WT mouse-derived fibroblasts into cervical ventral horn. No MN loss occurred in rats engrafted with SOD1^{G93A} or WT fibroblasts ($n = 5$ per group; FIB-SOD1: 9.5 ± 1.8 average MNs; FIB-WT: 9.8 ± 1.6) compared with rats injected with media (Fig. S4). Similarly, MN loss was not seen after cervical transplantation of frozen-thawed dead SOD1^{G93A} GRPs (Fig. 2F). These findings suggest that observed effects rely on the expression of SOD1^{G93A} in neural cell subtypes rather than the presence of the SOD1^{G93A} protein itself.

Declined Forelimb Motor and Respiratory Physiological Functions. Because GRPs were transplanted near MNs that innervate forelimb muscles, the effect of SOD1^{G93A} GRP transplants on forelimb motor function was assessed via grip strength testing ($n = 8$ –12 per group). Hindlimb motor function was assessed as a control, because MNs of the lumbar spinal cord that innervate the hindlimb muscles were not targeted by transplants. Although SOD1^{G93A} GRP-derived astrocytes had no effect on hindlimb grip strength ($P > 0.05$ for all comparisons; Fig. 3A) or body weight ($P > 0.05$ for all comparisons; Fig. 3B), they produced a significant decline in forelimb grip strength across time ($*P < 0.05$ vs. Media; Fig. 3C). In contrast, TG-Media rats ($n = 4$) demonstrated limb weakness that progressed from hindlimb (Fig. 3A) to forelimb (Fig. 3C) and lost weight over time (Fig. 3B) ($*P < 0.05$ vs. Media, $*P < 0.05$ relative to SOD1^{G93A}) (15).

Because cervical MNs innervate the diaphragm via the phrenic nerve, we examined whether physiological function in relevant MN populations to the phrenic nerve were influenced by engrafted SOD1^{G93A} GRPs. This was assessed by phrenic nerve compound muscle action potentials (CMAPs), a functional electrophysiolog-

Fig. 2. SOD1^{G93A} GRP-derived astrocytes induce cervical host MN ubiquitin inclusions and MN death. (A–C) Host SMI-32⁺ MNs surrounded by M2⁺ SOD1^{G93A} GRP-derived astrocytes (A), but not M2⁺ WT (B) or SOD1^{WT} (C) GRP-derived astrocytes, contained ubiquitinated aggregates. (D and E) Ubiquitin⁺ aggregates in MNs did not colocalize with endogenous SOD1 (D) or TDP-43 (E). (F) SOD1^{G93A} GRP-derived astrocytes caused extensive MN loss, an effect not seen with WT or SOD1^{WT} GRP-derived astrocytes, dead SOD1^{G93A} cells, or media. The letter x denotes transplant sites. Similar MN loss occurred in SOD1^{G93A} rats injected with media (TG-Media) (F), although the loss was more extensive in these animals. Error bars represent SEM. (Scale bars in A–E, 10 μm.)

ical assay of diaphragm function (Fig. 3D) (15, 22). Compared with control transplants ($n = 8$ –10 per group), SOD1^{G93A} GRP transplants ($n = 12$) significantly decreased peak response amplitudes of CMAPs by $\approx 50\%$ after phrenic nerve stimulation at 3 mo ($*P < 0.0001$ for SOD1^{G93A} vs. Media) (Fig. 3D and E). This was accompanied by a modest loss (10%) in phrenic nerve myelinated axons and the presence of rare degenerating axons (Fig. S3). TG-Media rats ($n = 4$) showed a $\approx 94\%$ decline in CMAP amplitude at endstage as previously reported ($*P < 0.0001$ vs. Media, $^{#}P < 0.05$ vs. SOD1^{G93A}; Fig. 3E) (22). Transplants had no effect on the latency of response ($P > 0.05$ for all comparisons; Fig. 3E).

Fig. 3. SOD1^{G93A} GRP-derived astrocytes impair forelimb grip strength and decrease phrenic nerve CMAPs. (A–C) In contrast to TG-Media rats, transplantation of SOD1^{G93A} GRPs in WT rats had no effect on hindlimb grip strength (A) or on weight (B). However, SOD1^{G93A} transplants produced a significant decline in forelimb grip strength over time (C). (D) Representative recordings of CMAPs taken from individual WT and SOD1^{G93A} GRP-transplanted rats. (E) CMAPs recorded at 3 mo revealed a significant decrease in peak response amplitude in rats transplanted with SOD1^{G93A} GRPs, although this effect was not as pronounced as that seen in TG-Media rats. No differences in response latency were found among the various transplant groups. Error bars represent SEM.

Reduced Host Astrocytic Glutamate Transporter Expression Accompanies MN Loss. Dysfunction of astrocyte glutamate transport, specifically GLT-1 (EAAT2), is found in ALS patients and in mSOD1 rodents (4, 23) and may be an underlying factor in disease progression. In the present study, qualitative immunohistochemical examination of GLT-1 revealed that the punctate staining typically seen surrounding MNs was diminished in rats transplanted with SOD1^{G93A} GRPs, whereas transplants themselves expressed GLT-1 (Fig. S5). Immunoblots confirmed GLT-1 loss in cervical C6 (C6) region of rats engrafted with SOD1^{G93A} GRPs ($n = 4$ –5 per group; SOD1^{G93A}: 0.9 ± 0.2 A.U.; WT: 2.7 ± 0.4 ; SOD1^{WT}: 2.9 ± 0.3 ; Media: 2.8 ± 0.2 ; $*P < 0.05$ vs. Media; Fig. S5). This loss was comparable to that measured in TG-Media rats ($n = 2$; 1.6 ± 0.2 A.U., $P > 0.05$ vs. SOD1^{G93A}; Fig. S5) and was accompanied by a marked host astrocyte response (Fig. S6). Although no differences in GLT-1 were found among transplanted WT rats when lumbar L5 level tissues were tested, GLT-1 loss occurred in L5 of SOD1^{G93A} rats, consistent with previous studies (Fig. S5) (4). To address whether the effects seen on GLT-1 were specific to this protein or a nonspecific response to altered host astroglial biology, we examined whether the astroglial glutamate transporter GLAST was altered. Levels of GLAST remained unchanged among transplant groups in both C6 and L5 levels of spinal cord (Fig. S5).

Host Microgliosis Is a Feature of SOD1^{G93A} GRP-Derived Astrocyte Induced Pathology. SOD1^{G93A} GRP-derived astrocytes induced host microgliosis in cervical ventral gray matter (Fig. 4). Microglial activation was first determined by Iba-1 immunohistochemistry (Fig. 4A–C and Fig. S7) (15, 24) and further confirmed by

Fig. 4. SOD1^{G93A} GRP-derived astrocytes induce ventral horn microgliosis. (A–C) Low-magnification images show Iba-1⁺ microgliosis in cervical ventral horn of rats transplanted with SOD1^{G93A} GRPs (A) but not WT GRPs (B) or media (C). (D) Quantification of Iba-1⁺ in ventral gray matter (denoted by region within dotted line) revealed significant microgliosis in SOD1^{G93A} GRP-transplanted rats compared with controls at 3 mo. Iba-1⁺ microgliosis occurred up to 480 μ m past transplant sites, only one-third of the distance from where MN loss occurred. However, CD68 immunostaining (E) performed on the same tissue sections revealed a response that was more directly correlated to MN loss. (F and G) Immunoblot analysis of Iba-1 (F) and CD68 (G) expression confirmed microgliosis at 3 mo in C6 of rats transplanted with SOD1^{G93A} GRPs. Levels, however, were only $\approx$ 50% of that seen in TG-Media rats. Representative immunoblots of Iba-1 and CD68 protein expression in C6 are shown in F and G. All bands were normalized to corresponding actin level. Error bars represent SEM. (Scale bars in A–C, 100 μ m.)

µm past SOD1^{G93A} GRP transplant sites, only one-third the distance from where MN loss occurred (Fig. 4D). However, analysis of CD68⁺ immunostaining performed on the same tissue sections revealed activated microglia up to 1.7 mm past SOD1^{G93A} GRP transplant sites, coinciding with MN loss in these animals ($^*P < 0.05$ relative to Media; Fig. 4E). Because Iba-1 stains both resting and activated microglia, we speculate that Iba-1⁺ immunostaining of microglia was not a sensitive enough measure to detect activated microglia in regions where this response was less prominent. Immunoblot analysis of Iba-1 and CD68 expression in C6 confirmed transplant-induced microglial activation at 3 mo (Fig. 4F and G). Still, microglial activation in SOD1^{G93A} GRP transplanted rats was only ~50% of that measured in TG-Media rats ($n = 2-4$ per group; $^*P < 0.05$ vs. Media, $^{\#}P < 0.001$ for TG-Media vs. SOD1^{G93A}).

Minocycline Inhibits SOD1^{G93A} Astrocyte-Induced MN Loss and Phenotypic Deficits.

notypic Deficits. To elucidate the contribution of microgliosis to MN toxicity, we administered the microglial inhibitor minocycline to rats transplanted with SOD1^{G93A} GRPs. Rats received 50 mg/kg minocycline ($n = 8$) or saline ($n = 8$) (i.p.) daily beginning at 1 wk after transplant until their time of killing. This dose and route of minocycline administration was selected on the basis of previous studies demonstrating efficacy in mSOD1 mice (28, 29) and in rats after spinal cord (30) or ischemic injury (31). We began minocycline administration at 1 wk after transplant owing to the lack of MN loss in SOD1^{G93A} GRP-transplanted rats at this time point and to allow for recovery time from surgery. Some rats received minocycline ($n = 4$) or saline ($n = 4$) (i.p.) starting on the day of surgery to control for any unknown

Fig. 5. Minocycline inhibits host microgliosis, MN loss, and phenotypic deficits in rats transplanted with SOD1^{G93A} GRPs. (A–C) Compared with vehicle (Saline), minocycline administered at 1 wk after transplant (1 wk MINO) or on the day of transplant (d/o MINO) to rats transplanted with SOD1^{G93A} GRPs inhibited Iba-1⁺ microgliosis. Red dotted line indicates original baseline levels measured in media-injected control rats. (D) Although minocycline did not prevent MN loss at transplant sites, it inhibited MN loss at distances past transplant sites that contained fewer numbers of SOD1^{G93A} cells. (E and F) Minocycline blocked the forelimb decline in SOD1^{G93A} GRP-transplanted rats (E) but had no effect on hindlimb grip strength (F). (G) Minocycline also restored CMAP amplitudes to near baseline levels (denoted by red dotted line), with no effect on the latency of response. Error bars represent SEM. (Scale bars in A and B, 100 μ m.)

acute effects of transplanted SOD1^{G93A} cells possibly facilitating MN loss at later time points.

Compared with vehicle (Saline; Fig. 5*A*), minocycline administered at 1 wk after transplant (1 wk MINO; Fig. 5*B*) or on the day of transplant (d/o MINO) reduced Iba-1⁺ immunostaining in cervical gray matter ($*P < 0.05$ for MINO groups vs. Saline; Fig. 5*C*). Minocycline treatment reduced Iba-1 levels to below baseline levels previously measured in our control groups, likely accounting for the significant effect seen at 1.4 mm past transplant sites. CD68⁺ immunostaining was reduced as well (Fig. S8). Although SOD1^{G93A} GRP-transplanted rats administered minocycline still had MN loss at the site of transplantation (1 wk MINO: 5.5 ± 0.6 average MNs; d/o MINO: 4.7 ± 0.4 ; Saline: 5.6 ± 0.4), minocycline blocked MN loss at distances past the transplantation site ($*P < 0.05$ for 1 wk MINO and d/o MINO vs. Saline; Fig. 5*D*). Minocycline had no effect the migratory behavior of transplanted SOD1^{G93A} GRPs or their differentiation into GFAP⁺ astrocytes (Fig. S8).

Further, minocycline blocked the decline in forelimb grip strength ($*P < 0.05$ relative to 1 wk MINO at multiple time points; Fig. 5*E*), whereas hindlimb grip strength remained unaffected (Fig. 5*F*). Because rats receiving minocycline on the day of surgery took slightly longer to recover than rats receiving minocycline at 1 wk after transplant of SOD1^{G93A} GRPs, they had significantly lower forelimb grip strength measures at early time points ($*P < 0.05$ for d/o MINO vs. 1 wk MINO at 1, 2, and 3 wk after transplantation). Additionally, minocycline partially blocked the decrease in phrenic nerve CMAPs (1 wk MINO: 7.6 ± 0.9 mV; d/o MINO: 7.6 ± 1.6 ; vs. Saline: 4.3 ± 0.6 ; $*P < 0.05$ relative to Saline; Fig. 5*G*) but had no effect on the response latency of CMAPs ($P > 0.05$; Fig. 5*G*).

Discussion

Here, we used a unique cell transplantation strategy to show that astrocytes carrying the human ALS-linked mutation SOD1^{G93A} can induce focal MN pathology and death, forelimb motor and respiratory dysfunction, marked reactive astrocytosis, and reduced GLT-1 transporter expression in WT animals. We also provide evidence that graft-derived SOD1^{G93A} astrocytes initiate a host microglial response that facilitates MN death in cervical regions beyond the sites of SOD1^{G93A} GRP-derived astrocyte integration. Findings suggest that mSOD1-containing astrocytes alone can induce WT MN death accompanied by WT astrocytic and microglial responses in vivo.

Previous studies using chimeric mice carrying mixtures of WT and mSOD1 cells showed that mSOD1-containing “nonneuronal” cells randomly distributed throughout the spinal cord were sufficient to induce ubiquitinated inclusions in WT MNs (32). However, the precise nonneuronal cell type(s) responsible for this effect could not be distinguished. A study using the Cre-loxP system to selectively excise mSOD1 from astrocytes in SOD1^{G37R} mice showed that astrocytes determine late disease progression and duration (7). More recently, selective excision of mSOD1 from astrocytes in SOD1^{G85R} mice demonstrated that astrocytes determine disease onset as well as early disease progression (8). Although both studies revealed astrocytes as potential propagators of ALS, there may be SOD1 mutation-specific effects accounting for the differences observed phenotypically. One limitation of these studies is that other cell types that have not undergone targeted recombination still possess mSOD1, thus not allowing for the isolation of the specific influences of mSOD1 astroglia in a WT milieu. Additionally, because these models have mSOD1 expression in other cell types, animals continue to develop disease along a continuum (albeit more slowly), making subtle longer-term analyses of cell-specific influences challenging. In the present work, transplanted SOD1^{G93A} GRPs differentiated almost exclusively into astrocytes, and thus findings could be more definitively attributed to astrocytic (rather than microglial, oligodendroglial, or neuronal) influences. Furthermore, because SOD1^{G93A} GRPs

were focally introduced and not randomly integrated, the anatomical relationship of these cells to host MN pathology could be delineated.

Our finding that SOD1^{G93A} GRP-derived astrocytes cause WT MN pathology in vivo corroborates studies showing mSOD1 astrocyte-mediated MN toxicity in vitro (9–12). Although in vitro studies showed that SOD1^{G93A} astrocytes can induce MN loss over days, we did not observe significant MN loss until 3 mo after transplantation. This difference may reflect the fact that grafted SOD1^{G93A} cells took longer to mature into astrocytes in vivo and suggests that a spatial relationship between graft-derived SOD1^{G93A} astrocytes and MNs may first need to be established. Additionally, neuroprotective effects could arise from other cell types to overcome any toxicity exerted by the SOD1^{G93A} transplants at earlier time points. Although MN loss and ubiquitin aggregation was noted in areas containing few or no graft-derived SOD1^{G93A} astrocytes, we could not establish whether this was caused by a toxic astrocyte-secreted factor from graft-derived cells, as suggested by in vitro studies. Indeed, graft-derived astrocytes could have apposition to the dendritic arbors of host MNs outside of the field of view in accounting for pathology. Ubiquitin deposits seen outside MNs could reflect remnants of degenerated MNs, although their presence in other host or transplanted cell types could not be excluded. Because neither SOD1^{G93A} fibroblasts nor frozen-thawed dead SOD1^{G93A} GRPs induced pathological change, the presence of SOD1^{G93A} protein in the parenchyma does not seem to be a contributor to MN pathology. Therefore, in this model, it seems that the mSOD1 effect is astrocyte initiated.

In contrast to our study, an earlier attempt by Gong et al. (33) to selectively express mSOD1 in astrocytes failed to produce any MN degeneration or motor impairment in mice, even though astrocytosis was observed. Several explanations could account for the absence of MN pathology in their study. First, they used the GFAP promoter to overexpress mSOD1 in mature astrocytes, whereas in our study mSOD1 was expressed under the native SOD1 promoter. Second, they used a different SOD1 mutation (murine SOD1^{G86R}), which could result in a varying toxicity. Finally, adaptive mechanisms might have arisen from the expression of GFAP promoter-driven SOD1^{G86R} during postnatal periods.

We found a host glial inflammatory response (astrocytosis and microgliosis) in rats transplanted with SOD1^{G93A} GRPs. Reduced GLT-1 expression occurred concomitantly with astrocytosis, a feature typical of ALS in humans and rodent models of the disease (34). Because impaired GLT-1 transport may lead to MN degeneration by causing the accumulation of excitotoxic levels of extracellular glutamate (23), GLT-1 loss might be one factor that underlies the SOD1^{G93A} GRP transplant-induced MN demise. Microgliosis was most evident at regions near transplant sites and became more modest at distant sites. This is in line with previous observations in LoxSOD1^{G37R}/GFAP-Cre⁺ mice, in which microgliosis was greatest in areas containing the highest concentration of mSOD1-expressing astrocytes (7), and may suggest cross-talk between mutant astrocytes and microglia. Studies have shown that the release of superoxide and production of nitric oxide by activated microglia can augment MN death in vitro (35).

Minocycline, an antibiotic that inhibits microglial activation (31) and confers neuroprotection in ALS mice (28, 29, 36), was used as a pharmacological tool to reduce the contribution of host microgliosis to SOD1^{G93A} GRP transplant-induced MN death. Because minocycline treatment suppressed microgliosis in SOD1^{G93A} GRP-transplanted rats, we believe that at least part of the basis for minocycline's neuroprotective effects was related to its ability to inhibit microglial activation. However, minocycline-mediated inhibition of caspase-independent and -dependent mitochondrial cell death pathways in distressed MNs could be an additional mechanism of action (28). Our findings suggest that the activation of microglia by SOD1^{G93A} GRP-derived astrocytes is essential for the MN injury observed beyond sites of GRP-

derived astrocyte integration and could suggest a mechanism for anatomical disease spread.

In sum, many features of our model (i.e., the presence of ubiquitinated inclusions in MNs, MN degeneration and death, GLT-1 transporter loss, and glial activation) are consistent with those in SOD1^{G93A} rodents. However, at time points studied our effects remained primarily within the anatomical region of transplantation and unlike transgenic models, did not result in extensive MN loss across spinal cord regions. Our observations suggest that although the presence of SOD1^{G93A} in astrocytes is sufficient to cause MN death, the recapitulation of the complete ALS phenotype is not observed and may be dependent on other cell-specific pathways for execution of the phenotype. Alternatively, the lack of a severe phenotype could simply be a practical issue, given that focal transplantation at few select sites targets only a subpopulation of MNs, whereas limb and respiratory function is controlled by a much larger region of the spinal cord. Future studies using this focal transplantation paradigm may allow for more detailed analysis of temporal changes occurring over longer time points and allow for a more detailed assessment of mutant/host astrocyte as well as mutant astrocyte/host microglial and MN interactions.

Materials and Methods

GRP Transplantation. Mouse SOD1^{G93A} GRPs, WT (B6SJL) GRPs, SOD1^{WT} GRPs, frozen-thawed dead SOD1^{G93A} GRPs, WT fibroblasts, and SOD1^{G93A} fibro-

blasts were suspended at a concentration of 7.5×10^4 cells/ μ L (in basal media). Seven groups of animals were used: SOD1^{G93A} GRPs, WT GRPs, SOD1^{WT} GRPs, dead SOD1^{G93A} GRPs, WT fibroblasts, SOD1^{G93A} fibroblasts, and media. At 90 d of age, laminectomized rats received six grafts (bilaterally at C4, C5, and C6) of 1.5×10^5 cells (in 2 μ L basal media) into ventral horn. Some rats received only laminectomy to serve as "naïve" controls. Transplants focally targeted the cervical spinal cord at segments containing MNs innervating the forelimbs and diaphragm, as previously described (15).

Minocycline Administration. Rats receiving SOD1^{G93A} GRPs were treated daily with an i.p. injection of saline (vehicle) or minocycline hydrochloride (Sigma) (28, 29).

Statistical Analysis. For weight and grip strength results, treatment groups were compared across time using a random-effects model to control for correlation due to repeated measures (SAS). All other results were analyzed using ANOVA, repeated-measures ANOVA, and the Fisher protected least significant difference post hoc test to determine the significance of differences between individual groups (StatView). Data are presented as mean $\pm$ SEM, and significance level was set at $P < 0.05$.

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