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nature medicine

Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury

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In the injured central nervous system (CNS), reactive astrocytes form a glial scar and are considered to be detrimental for axonal regeneration, but their function remains elusive. Here we show that reactive astrocytes have a crucial role in wound healing and functional recovery by using mice with a selective deletion of the protein signal transducer and activator of transcription 3 (Stat3) or the protein suppressor of cytokine signaling 3 (Socs3) under the control of the *Nes* promoter-enhancer (*Nes-Stat3*$^{-/-}$, *Nes-Socs3*$^{-/-}$). Reactive astrocytes in *Nes-Stat3*$^{-/-}$ mice showed limited migration and resulted in markedly widespread infiltration of inflammatory cells, neural disruption and demyelination with severe motor deficits after contusive spinal cord injury (SCI). On the contrary, we observed rapid migration of reactive astrocytes to seclude inflammatory cells, enhanced contraction of lesion area and notable improvement in functional recovery in *Nes-Socs3*$^{-/-}$ mice. These results suggest that Stat3 is a key regulator of reactive astrocytes in the healing process after SCI, providing a potential target for intervention in the treatment of CNS injury.

Because the regenerative capability of the mammalian CNS is poor, limited functional recovery occurs during the chronic phase of SCI. At the subacute phase of SCI, however, gradual functional recovery is observed to some extent in both rodents and humans (except in cases of complete paralysis). The mechanism behind this functional recovery remains unclear. Here, we investigated this issue by focusing on the action of reactive astrocytes in a mouse model of SCI.

To interpret the process of paralysis improvement in the subacute phase, we examined serial histological sections of contused spinal cords and followed motor function for 6 weeks after injury in wild-type mice and found that the area of neural cell loss gradually enlarged in a rostral-caudal direction within a few days after SCI (acute phase) and a portion of Hu-expressing neurons were positive for cleaved caspase-3, indicating that the secondary injury process lasted for several days in this model (**Supplementary Fig. 1** online) during which we observed limited functional recovery (**Fig. 1a**). Astrocytes surrounding the lesion underwent a typical change of hypertrophy, process extension and increased expression of intermediate filaments such as GFAP and Nestin by 7 d after SCI (**Fig. 1b**), characteristic of 'reactive astrocytes.' Notably, these astrocytes eventually migrated centripetally to the lesion epicenter and gradually compacted the CD11b^{+} inflammatory cells, contracting the lesion area up until 14 d after SCI (subacute phase; **Fig. 1c,d**). During this process, we observed repair of injured tissue and gradual functional improvement, and reactive astrocytes formed a physical barrier against inflammatory cells, commonly referred to as glial scar. After the migration of reactive astrocytes and completion of glial scar (reactive gliosis), functional improvement reached a plateau around 2 weeks after injury (**Fig. 1a,c**). Although the glial scar has a crucial part in the lack of axonal regeneration in the chronic phase of SCI[1], our data strongly suggest that the emergence and migration of reactive astrocytes have a prominent role in the repair of injured tissue and the restoration of motor function in the subacute phase (before completion of the glial scar).

To confirm that the compaction of the lesion results from migration and not from proliferation of reactive astrocytes, we labeled proliferating cells with bromodeoxyuridine (BrdU). Analysis of mice that received a single injection of BrdU at 7 d after SCI showed that the population of BrdU^{+} cells was composed of reactive astrocytes and inflammatory cells, which were gradually compacted to the lesion center as time progressed (**Fig. 1e** and **Supplementary Fig. 2** online). Analysis of mice that received daily injections of BrdU showed limited astrocyte proliferation after 7 d postinjury, suggesting that the development of reactive gliosis is mainly brought about by cellular hypertrophy and upregulation of GFAP of the astrocytes surrounding the lesion (**Fig. 1f,g**).

To address the regulatory mechanisms behind the reactive response of astrocytes, we investigated the role of Stat3 signaling (**Figs. 2** and **3**). Stat3 is a principal mediator in a variety of biological processes[2–4] including cancer progression, wound healing and the movement of various types of cells. In addition, Stat3 mediates certain aspects of astrogliosis downstream of the action of cytokines such as interleukin (IL)-6, leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) after CNS injury[5–8].

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Received 8 November 2005; accepted 28 April 2006; published online 18 June 2006; doi:10.1038/nm1425

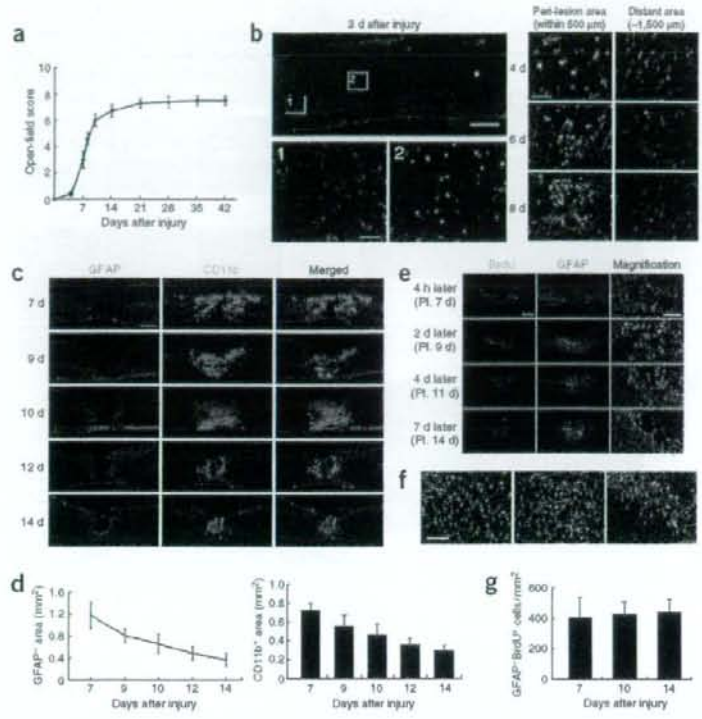

Figure 1 Migration of reactive astrocytes and compaction of inflammatory cells in wild-type mice. (**a**) Time course of lower limb functional recovery after SCI ($n = 10$). There was gradual recovery in the subacute phase. Data are mean ± s.e.m. (**b**) Phosphorylated Stat3 (green) and morphological changes were observed in GFAP$^+$ astrocytes (red) close to the lesion (boxed area 2) but not in distant areas (boxed area 1) at 3, 4, 6 and 8 d after SCI. Scale bar, 500 μm (upper left panel), 100 μm (lower left panel) and 50 μm (right panels). Asterisk indicates the lesion epicenter. (**c**) Time course of GFAP$^+$ reactive astrocytes and CD11b$^+$ cells. Reactive astrocytes gradually confine the area of inflammatory cell infiltration. Scale bar, 500 μm. (**d**) Quantitative analysis of GFAP$^-$ area (surrounded by reactive astrocytes) and CD11b$^+$ area ($n = 3$ per each time point). Data are mean ± s.d. (**e**) BrdU-labeled cells migrated toward the lesion epicenter. Mice were injected with BrdU (100 μg/g body weight) at 7 d after injury and killed 4 h, 2 d, 4 d, 7 d later. Scale bar, 500 μm and 100 μm. (**f**) The proliferating reactive astrocytes were labeled by daily injection of BrdU from the day of injury till killing at 7 (left), 10 (middle) and 14 d (right) after injury. GFAP, red; BrdU, green. Scale bar, 100 μm. (**g**) There were no differences in the number of GFAP$^+$BrdU$^+$ cells from 7 to 14 d after injury ($n = 3$ per group). Data are mean ± s.d.

In the injured spinal cord, phosphorylated Stat3 prominently increased at 12 h after injury, which remained detectable for 2 weeks (**Fig. 3a**). We observed phosphorylation and nuclear translocation of Stat3 mainly in reactive astrocytes surrounding the lesion, but not in distant areas for several days after injury (**Fig. 1b** and **Supplementary Fig. 1**). To elucidate the role of Stat3 in reactive astrocytes, we selectively disrupted the *Stat3* gene under the control of *Nes* gene promoter and second intronic enhancer, which are activated in reactive astrocytes after SCI[9,10]. We created conditional knockout mice (*Nes-Stat3*$^{-/-}$) by crossing *Stat3*$^{lox/P}$ mice[11] with *Nes*-Cre transgenic mice[12], as embryonic lethality ensues in Stat3-null mice. *Nes-Stat3*$^{-/-}$ mice showed no apparent abnormalities in motor function and development, although they showed signs of hyperphagia and leptin resistance[13]. To identify the cells that underwent Cre-mediated recombination, we crossed another transgenic line[14] carrying a reporter gene construct, *CAG* promoter-*lox*P-CAT-*lox*P-EGFP (CAG-CAT$^{lox P/lox P}$-EGFP), which directs the expression of EGFP upon Cre-mediated recombination. After SCI, we observed high Cre-mediated expression of EGFP from reactive astrocytes surrounding the lesion, but not from neurons or oligodendrocytes (**Supplementary Figs. 3** and **4** online), indicating recombination only in reactive astrocytes in both littermates and *Nes-Stat3*$^{-/-}$ mice.

At 2 weeks after injury, *Nes-Stat3*$^{-/-}$ mice showed markedly widespread infiltration of CD11b$^+$ inflammatory cells and demyelination compared to wild-type littermates (**Fig. 2a**). Notably, although the development of glial scar was observed around the injury site several days after injury in *Nes-Stat3*$^{-/-}$ mice (**Fig. 2a,b** and **Supplementary Fig. 3**), the configuration of these cells remained relatively unchanged for the following 6 weeks owing to their limited migration, resulting in widespread demyelination and severe motor deficits (**Fig. 2**). Quantifications of GFAP$^-$ area and CD11b$^+$ area in wild-type littermates and *Nes-Stat3*$^{-/-}$ mice were comparable at 7 d after injury, implying a similar degree of astrocyte loss owing to secondary injury, but marked differences developed during the following 7 d, suggesting that Stat3 has a large impact on the migration of astrocytes rather than their survival after SCI (**Fig. 2c**). The progressive compaction of GFAP$^+$EGFP$^+$ cells toward the lesion center observed in wild-type littermates did not occur in *Nes-Stat3*$^{-/-}$ mice, providing further evidence of Stat3-dependent migration of reactive astrocytes (**Fig. 2d**). Confocal imaging confirmed the emergence of reactive astrocytes without phosphorylated Stat3 in *Nes-Stat3*$^{-/-}$ mice (**Fig. 2e**), indicating that the Stat3 activation is not necessarily indispensable for the appearance of reactive astrocytes or for the upregulation of *Gfap* and *Nes*. These data suggest that Stat3 is a key molecule for the migratory function of reactive astrocytes, which may be deeply involved in tissue repair and functional recovery after SCI (**Fig. 2**).

To further investigate the relationship between Stat3 signaling and function of reactive astrocytes, we analyzed SCI in *Nes-Socs3*$^{-/-}$ mice[15]. Socs3 is the negative feedback molecule of Stat3 and the 'bipolar' relationship between Stat3 and Socs3 has been noted in several selective deletion experiments[13,15,16]. In the injured spinal cord of *Nes-Socs3*$^{-/-}$ mice, phosphorylation of Stat3 was significantly greater and prolonged compared to that in wild-type mice, and immunohistochemistry confirmed greater expression of phosphorylated Stat3 in reactive astrocytes (**Fig. 3a–c**). Notably, the rapid development of reactive gliosis that compacted inflammatory cells in the injured spinal cord of *Nes-Socs3*$^{-/-}$ mice was observed as early as 7 d after injury (**Fig. 3d,e**). GFAP$^-$ area and CD11b$^+$ area were significantly reduced in *Nes-Socs3*$^{-/-}$ mice at 7 d after injury, during which *Nes-Socs3*$^{-/-}$ mice showed marked improvement of motor function compared to littermates (**Fig. 3f,g**). The differences in the area between wild-type

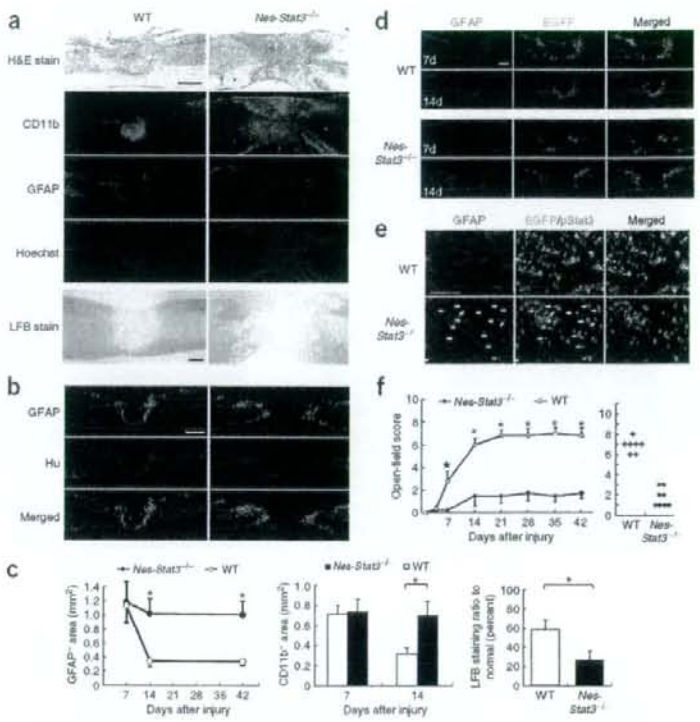

Figure 2 Compaction of inflammatory cells by reactive astrocytes and functional recovery were limited in *Nes-Stat3*$^{-/-}$ mice after SCI. (**a**) At 2 weeks after SCI, the infiltration of CD11b^{+} cells, GFAP^{-} area and demyelination were greater in *Nes-Stat3*$^{-/-}$ mice compared to wild-type (WT) littermates. Scale bars, 500 μm (upper left panel) and 300 μm (lower left panel). (**b**) The areas negative for Hu and GFAP were large in *Nes-Stat3*$^{-/-}$ mice even at 6 weeks after injury. Scale bar, 500 μm. (**c**) Comparison of GFAP^{-} area, CD11b^{+} area and LFB-positive area in both groups ($n = 3$ per each time point). Error bar indicates s.d. $^{*}P < 0.01$. (**d**) The location of GFAP^{+}EGFP^{+} reactive astrocytes shifted toward the lesion epicenter from 1 to 2 weeks in wild-type (WT) littermates, whereas their position remained relatively unchanged in *Nes-Stat3*$^{-/-}$ mice. Scale bar, 300 μm. (**e**) In *Nes-Stat3*$^{-/-}$ mice, Cre-mediated EGFP^{+} cells (green) were colocalized with GFAP^{+} reactive astrocytes (blue; arrows), but colocalization with phosphorylated Stat3 (red) was hardly observed at 4 d after injury. Scale bar, 100 μm. (**f**) Time course of functional recovery of lower limbs and the score of each mouse at 6 weeks after SCI. Whereas gradual recovery was observed in the subacute phase in wild-type (WT) littermates, little improvement was observed in *Nes-Stat3*$^{-/-}$ mice ($n = 7$ *Nes-Stat3*$^{-/-}$; $n = 8$ wild-type). Data are mean ± s.e.m. $^{*}P < 0.01$, $^{\star}P < 0.05$.

littermates and *Nes-Socs3*$^{-/-}$ mice became less pronounced at 2 weeks after injury, but *Nes-Socs3*$^{-/-}$ mice continually showed better motor function. Quantification of demyelination, oligodendrocyte-lost area and distal cord serotonergic innervation showed significant differences between the two groups (**Fig. 3f,h,i** and **Supplementary Fig. 4**). But GAP43^{+} regenerative fibers were comparable irrespective of genotype, and these fibers generally did not colocalize with 5HT^{+} fibers, indicating that the difference in serotonergic innervation did not result from regeneration (**Supplementary Fig. 5** online). These results suggest that the prompt contraction of the lesion spares more myelin, oligodendrocytes and serotonergic fibers, resulting in improved recovery.

Compared to wild-type mice, the development of reactive gliosis that secluded inflammatory cells was enhanced in *Nes-Socs3*$^{-/-}$ mice, whereas it was significantly delayed in *Nes-Stat3*$^{-/-}$ mice, indicating Stat3 signaling as an important factor in the developmental process of reactive gliosis after SCI (**Fig. 4a,b**).

Consistent with *in vivo* results, the *in vitro* migration behavior of astrocytes showed similar properties in a scratch-wounded assay that simulated the postinjury *in vivo* behavior of reactive astrocytes[17]. Astrocytes harvested from *Nes-Socs3*$^{-/-}$ mice showed a higher degree of migration, whereas impaired migration was observed in astrocytes of *Nes-Stat3*$^{-/-}$compared to wild-type mice (**Fig. 4c–e**). As proliferation rates among the three groups were comparable, cell migration activity was not dependent upon cell proliferation activity (data not shown).

A recent report indicates a possible molecular link between Stat3-zinc signaling and cell movement[18]. The zinc transporter LIV1, identified as the transcriptional downstream target of Stat3, was found to be essential for the nuclear localization of the zinc-finger protein Snail, a transcriptional repressor of the *Cdh1* gene (which encodes E-cadherin). Thus, the absence of Stat3 signaling causes dysregulation of cell adhesion and impairs cell movement. Strong support for this theory is given by the result that zinc deficiency impaired compaction of inflammatory cells by reactive astrocytes and increased the number of apoptotic cells after CNS injury[19]. In addition, CNS injury in knockout mice of metallothioneins (zinc-binding proteins involved in zinc ion regulation) had histological characteristics similar to *Nes-Stat3*$^{-/-}$ mice, such as impaired migratory behavior of reactive astrocytes, wide infiltration of inflammatory cells and severe impairment of wound healing[20]. On the other hand, astrocyte-targeted IL-6–expressing transgenic mice showed prompt migration of reactive astrocytes and compaction of inflammatory cells as well as substantial tissue repair after CNS injury[21] similar to *Nes-Socs3*$^{-/-}$ mice (though hyperactivation of IL-6 signaling without a specific cellular target caused considerably more damage owing to robust inflammation after CNS injury[6,22]). Here, expression of *Slc39a6* mRNA (encoding LIV1) in reactive astrocytes 5 d after injury was robust in wild-type mice but was limited in *Nes-Stat3*$^{-/-}$ mice. Consistent with *Slc39a6* mRNA expression, E-cadherin was expressed in reactive astrocytes of *Nes-Stat3*$^{-/-}$ but not in wild-type mice at 2 weeks after SCI. Furthermore, real-time RT-PCR of injured spinal cords showed differences in expression of *Scl39ac* mRNA among the three groups (**Fig. 4f–h**). These data provide insight into the mechanism of astrocytic migration through Stat3 signaling and indicate the significance of reactive astrocytes after CNS injury.

In our SCI model, the area of neuronal cell loss before the emergence of reactive astrocytes was comparable for wild-type and conditional knockout mice, suggesting that the effect of Stat3

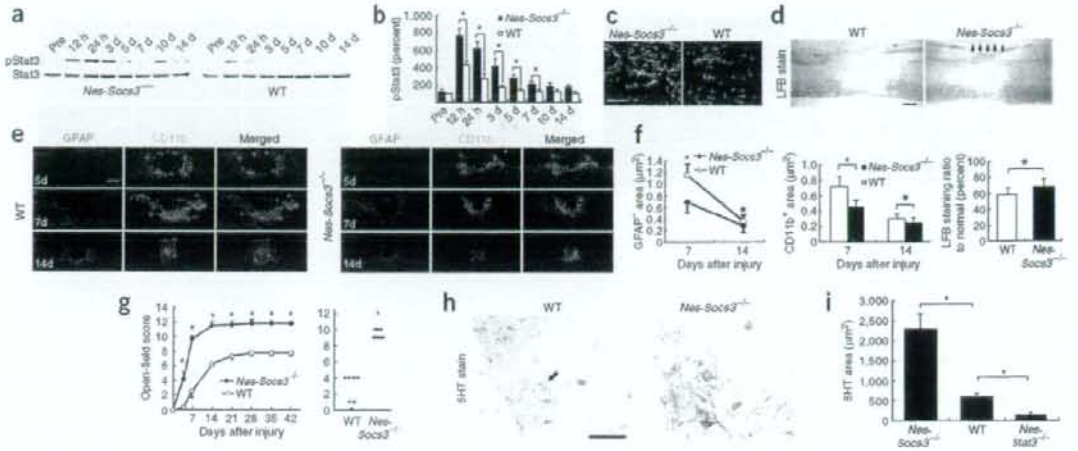

Figure 3 Enhanced activation of Stat3, prompt compaction of inflammatory cells and marked functional improvement in *Nes-Socs3*$^{-/-}$ mice. (**a**) Western blot analysis of Stat3 phosphorylation in injured spinal cord of wild-type (WT) and *Nes-Socs3*$^{-/-}$ mice. pStat3, phosphorylated Stat3. (**b**) Changes in the ratios of pStat3 to total Stat3 after SCI (mean ± s.e.m., $n = 4$ per group). $^{*}P < 0.05$. (**c**) Immunostaining of pStat3 and reactive astrocytes at 7 d after SCI. GFAP, red; pStat3, green. Scale bar, 100 μm. (**d**) Luxol Fast Blue (LFB) staining in littermates and *Nes-Socs3*$^{-/-}$ mice at 2 weeks after SCI. Arrows indicate the spared myelin in the dorsal part of the lesion epicenter. Scale bar, 300 μm. (**e**) Time course analysis of reactive astrocytes and CD11b^{+} cells. The early development of reactive astrocytes and compaction of inflammatory cells in *Nes-Socs3*$^{-/-}$ mice was visible as early as 7 d after SCI. Scale bar, 500 μm. (**f**) Quantitative analysis of GFAP^{-} area, CD11b^{+} area and LFB-positive area (mean ± s.d., $n = 4$). $^{*}P < 0.01$, $^{\star}P < 0.05$. (**g**) Time course of functional recovery and the score of each mouse at 7 d after SCI. Data are mean ± s.e.m. $^{*}P < 0.01$. (**h**) 5HT staining of the ventral horn distal to the lesion in wild-type (WT) littermates and *Nes-Socs3*$^{-/-}$ mice at 6 weeks after SCI. Arrow indicates spared 5HT^{+} fibers. Scale bar, 100 μm. (**i**) Quantitative analysis of serotonergic innervation of the distal cord in the three groups (mean ± s.e.m., $n = 4$ per each group). $^{*}P < 0.01$.

signaling on neuronal survival was minimal compared with its effect on the development and migration of reactive astrocytes (**Supplementary Fig. 1**).

The majority of studies on CNS injury have shown that the glial scar formed in part by reactive astrocytes hinders axonal regeneration. In mice lacking both GFAP and vimentin, reduced astroglial reactivity resulted in improved sprouting of axons and functional restoration after SCI[23]. But reactive astrocytes are also important for supporting repair of the blood-brain barrier. They prevent infiltration of CD45^{+} leukocytes and protect neurons and oligodendrocytes as shown by the selective ablation of dividing astrocytes using ganciclovir and GFAP-TK transgenic mice[24,25]. Here, we showed that Stat3 signaling in reactive astrocytes have a considerable role in the repair of injured tissue and the recovery of motor function. Although these results seem to conflict with one another, consideration of the timeframe in which these events were observed suggests a possible phase-dependent role of reactive astrocytes. In mice lacking both GFAP and vimentin, functional recovery was observed later than 2 weeks after injury[23], whereas substantial recovery was completed within 2 weeks after injury in *Nes-Stat3*$^{-/-}$ and *Nes-Socs3*$^{-/-}$ mice, suggesting that reactive astrocytes in the subacute phase repair tissue and restore function, whereas in the chronic phase of injury they impair axonal regeneration as a physical and chemical barrier.

Together, these results show that reactive astrocytes have a pivotal role in the repair of injured tissue and the recovery of motor function in the subacute phase after SCI, and that the function of reactive astrocytes is largely dependent on Stat3 signaling. This work also raises Stat3 signaling and reactive astrocytes as a potential new therapeutic target for the treatment of traumatic injury in CNS.

METHODS

Generation of conditional knockout mice. *Stat3*$^{loxP/loxP}$ mice, *Stat3*$^{loxP/-}$ mice, *Socs3*$^{loxP/loxP}$ mice, *Nes*-Cre mice and *CAG*-CAT$^{loxP/loxP}$–EGFP transgenic mice were as described elsewhere[3,11,12,14,15,26]. To generate *Nes-Stat3*$^{-/-}$ or *Nes-Socs3*$^{-/-}$ mice, we crossed *Nes*-Cre mice with *Stat3*$^{loxP/loxP}$, *Stat3*$^{loxP/-}$ mice[3,26] or *Socs3*$^{loxP/loxP}$ mice[15]. We used wild-type littermates (*Stat3*$^{loxP/loxP}$ or *Socs3*$^{loxP/loxP}$) as controls in histological and functional evaluations. We performed genotyping with primers described elsewhere[3,12,14,15]. All mice were housed in a temperature- and humidity-controlled environment on a 12-h light-dark cycle.

SCI model. We anesthetized adult C57BL/6J mice, wild-type littermates and conditional knockout mice (female, 8 weeks old) using an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After laminectomy at the tenth thoracic spinal vertebrae, we exposed the dorsal surface of the dura mater and induced SCI using a commercially available SCI device (60 kdyn using Infinite Horizon impactor, Precision Systems & Instrumentation) as previously described[27]. We evaluated motor function of the hind limbs with the locomotor rating test on the Basso-Beattie-Bresnahan (BBB) scale[28] for 6 weeks after injury. The obesity of *Nes-Stat3*$^{-/-}$ mice was not a problem in this study, because their functional recovery was limited to the point at which weight bearing was not an issue. All procedures were approved by the ethics committee of Keio University and were in accordance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

Immunohistochemistry. We anesthetized mice and transcardially perfused them with 4% paraformaldehyde in 0.1 M PBS. We removed spinal cords, embedded them in OCT compound and sectioned them sagittally at 20 μm on a cryostat. We stained tissue sections with primary antibodies to GFAP (DAKO), cleaved caspase-3 (Cell Signaling), CD11b (a marker of monocyte/macrophages and granulocytes; MBL), BrdU (Chemicon), Nestin (Rat 401, Chemicon), GFP (MBL), phosphorylated Stat3 (Cell Signaling), GSTπ (BD Biosciences), serotonin (5HT; DiaSorin, Inc.), GAP43 (Chemicon), E-cadherin

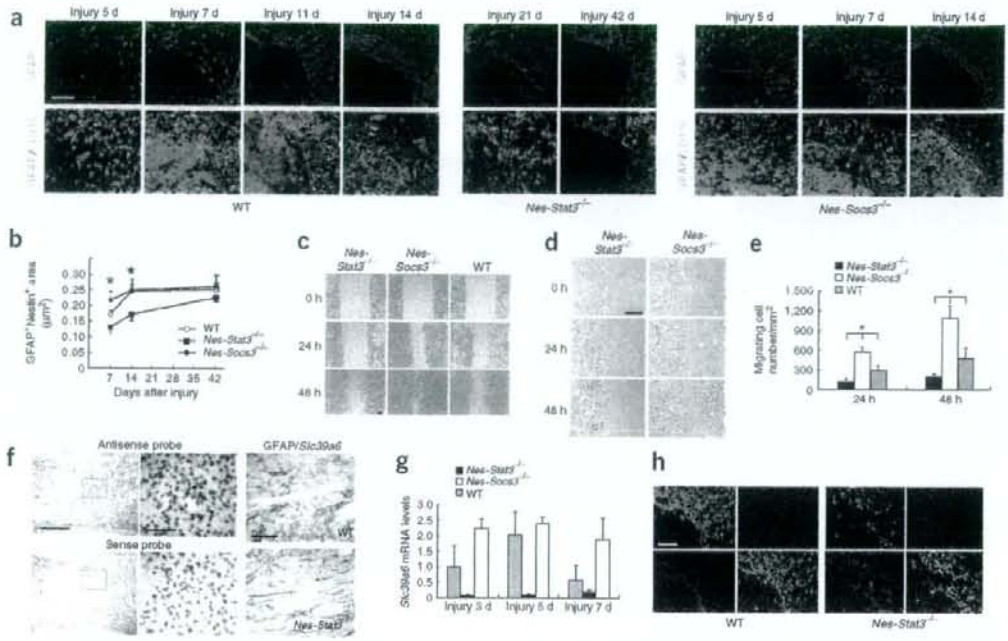

Figure 4 Involvement of Stat3 signaling in the development of reactive gliosis *in vivo*, the migration of reactive astrocytes *in vitro* and the transcriptional activity of LIV1. **(a)** Morphology of reactive astrocytes around the lesion area and infiltration of inflammatory cells in wild-type (WT), *Nes-Stat3*$^{-/-}$ and *Nes-Socs3*$^{-/-}$ mice. Scale bar, 100 μm. **(b)** The amount of reactive gliosis was measured as GFAP^{+}Nestin^{+} (EGFP) area in the three groups ($n = 3$). Error bar indicates s.e.m. $^{*}P < 0.05$. **(c)** *In vitro* scratch injury of monolayer cultured astrocytes derived from wild-type (WT), *Nes-Stat3*$^{-/-}$ and *Nes-Socs3*$^{-/-}$ mice. Scale bar, 20 μm. **(d)** Magnified image of reactive astrocytes derived from *Nes-Stat3*$^{-/-}$ and *Nes-Socs3*$^{-/-}$ mice. Scale bar, 20 μm. **(e)** Migrating cells were quantified as mean number ± s.e.m. per millimeter squared beyond the wound edge ($n = 3$). $^{*}P < 0.01$. **(f)** *In situ* hybridization of *Slc39a6* mRNA in injured spinal cord. *Slc39a6* mRNA was observed in GFAP^{+} reactive astrocytes in wild-type but was rarely found in *Nes-Stat3*$^{-/-}$ mice. Scale bars, 200 μm (upper left panel), 50 μm (upper middle panel) and 50 μm (upper right panel). **(g)** Expression of *Slc39a6* mRNA in injured spinal cords as determined by real-time RT-PCR in the three groups ($n = 3$). Each group was normalized to *Gapdh* values. There are significant differences ($P < 0.01$) among the three groups at each time point. Error bar indicates s.e.m. **(h)** Expression of E-cadherin (red) was not observed in reactive astrocytes of wild-type mice, whereas its active expression was observed in *Nes-Stat3*$^{-/-}$ mice at 2 weeks after SCI. GFAP, green; Hoechst stain, blue. Scale bar, 100 μm.

(Santa Cruz) and Hu[29] (a gift from R. Darnell, The Rockefeller University). We performed nuclear counterstaining with Hoechst 33342 (Molecular Probes). Images were obtained by fluorescence microscopy (Axioskop 2 Plus; Carl Zeiss) or confocal microscopy (LSM510; Carl Zeiss). To quantify the immunopositive and immunonegative area *in vivo*, we selected five representative midsagittal sections in each mouse and measured the area with the MCID system (Imaging Research, Inc.). We quantified the immunopositive area using grain counting and detected the immunonegative area by quantifying the dark area after image binarization. For myelin staining, we performed Luxol Fast Blue (LFB) stain. For comparison of LFB positive area, we selected five representative sagittal sections (3 mm long) of injured spinal cords (2 weeks after SCI) from positions 0.25 mm and 0.5 mm right of the midline, at the midline, and 0.25 mm and 0.5 mm left of the midline and LFB-positive area (blue area), and measured them by MCID system (grain counting), and calculated the ratio to normal section. To count GFAP^{+}BrdU^{+} cells, we selected five representative sagittal sections and randomly captured six regions in each section at ×200 magnification by confocal microscopy. For quantification of 5HT^{+} fibers, we randomly captured ten regions in each axial section of distal cord at ×200 magnification, and we quantified the total 5HT^{+} area using the MCID system. We maintained light intensity and threshold values at constant levels for all analyses. In all sagittal sections shown here, the left side is rostral.

***In vitro* migration assay.** We prepared primary astrocytes from 2-d-old wild-type and conditional knockout mice as previously described[30]. After several passages in DMEM with 10% FBS, we trypsinized cells and plated them to confluency on coverslips coated with poly-L-lysine. After reaching subconfluency, we treated cells with 10 μg/ml mitomycin C for 2 h to avoid the effects of cell proliferation and then subjected them to scratching. We created a cell-free area by scratching the monolayer with a pipette tip and evaluated the migration of cells to the cell-free area from the surrounding area at 24 h and 48 h. We counted the number of migrating astrocytes after taking photographs of ten nonoverlapping fields.

Western blotting. We anesthetized SCI mice, transcardially perfused them with saline, and isolated and lysed a 4-mm long section of the injured spinal cord. We resolved lysates with SDS-PAGE and immunoblotted membranes with antibody to phosphorylated Stat3 (Tyr705) and antibody to Stat3 (Cell Signaling).

***In situ* hybridization.** For detection of *Slc39a6* mRNA, we subcloned a 774-bp DNA fragment corresponding to the nucleotide positions 331–1104 of mouse Slc39a6 into pGEMT-Easy vector (Promega) and used it to generate sense or antisense RNA probes. We hybridized paraffin-embedded spinal cord sections (6 μm) with digoxigenin-labeled RNA probes at 60 °C for 16 h. We detected the bound label using NBT-BCIP, an alkaline phosphate color substrate. We counterstained sections with Kernechtrot.

Real-time quantitative PCR. We isolated total RNA from the injured spinal cord (4 mm long) using the RNeasy Kit (Qiagen) and obtained cDNA by

reverse transcriptase reaction. For quantitative analysis of *Slc39a6* mRNA expression, we used the cDNA as a template in a TaqMan real-time PCR assay using the ABI Prism 7000 sequence detection system (Applied Biosystems) according to the manufacturer's protocol. We performed the amplification using the following primers: forward primer, 5′-TGAAGGCAGCACCAATAG CA-3′; reverse primer, 5′-GGCCTGGATGGTGATCATG-3′; and TaqMan probe, 5′-FAM-CGTCACTGAAATTGTGCAGCCCGTC-TAMRA-3′.

Statistical analysis. We performed statistical analysis with an unpaired two-tailed Student *t*-test for single comparisons, and ANOVA followed by the Tukey-Kramer test for multiple comparisons. For the open-field score, we used repeated-measures ANOVA and the Mann-Whitney *U*-test. In all statistical analyses, significance was accepted at $P < 0.05$.

Accession codes. GenBank: *Mus musculus* solute carrier family 39 (metal ion transporter) number 6 (Slc39a6), NM_139143.

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

ACKNOWLEDGMENTS

This work was supported by grants from Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, the General Insurance Association in Japan, Terumo Foundation Life Science Foundation (to H.O.), and a Grant-in-Aid for the 21st century COE program, Keio Gijuku Academic Development Funds.

AUTHOR CONTRIBUTIONS

S.O. performed most of the experiments to characterize mouse phenotypes. M.N. instructed group members about experimental processes. H.K. helped prepare the manuscript. T.M. and T.S. maintained and prepared knockout mice. K.I. and J.Y. prepared spinal cord–injured animals. A.Y. provided *Nes-Socs3*$^{-/-}$ mice. Y.I. advised experiments by S.O. Y.T. and H.O. designed experiments and prepared the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Journal of Neuroscience Research 83:119–133 (2006)

Disease Progression of Human SOD1 (G93A) Transgenic ALS Model Rats

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The recent development of a rat model of amyotrophic lateral sclerosis (ALS) in which the rats harbor a mutated human SOD1 (G93A) gene has greatly expanded the range of potential experiments, because the rats' large size permits biochemical analyses and therapeutic trials, such as the intrathecal injection of new drugs and stem cell transplantation. The precise nature of this disease model remains unclear. We described three disease phenotypes: the forelimb-, hindlimb-, and general-types. We also established a simple, non-invasive, and objective evaluation system using the body weight, inclined plane test, cage activity, automated motion analysis system (SCANET), and righting reflex. Moreover, we created a novel scale, the Motor score, which can be used with any phenotype and does not require special apparatuses. With these methods, we uniformly and quantitatively assessed the onset, progression, and disease duration, and clearly presented the variable clinical course of this model; disease progression after the onset was more aggressive in the forelimb-type than in the hindlimb-type. More importantly, the disease stages defined by our evaluation system correlated well with the loss of spinal motor neurons. In particular, the onset of muscle weakness coincided with the loss of approximately 50% of spinal motor neurons. This study should provide a valuable tool for future experiments to test potential ALS therapies.

Key words: amyotrophic lateral sclerosis; evaluation system; behavioral analyses; phenotype; variability

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder that mainly affects the upper and lower motor neurons (de Belleroche et al., 1995). It is characterized by progressive muscle weakness, amyotrophy, and death from respiratory paralysis, usually within 3–5 years of onset (Brown 1995). Although most cases of ALS are sporadic (SALS), approximately 10% are familial (FALS) (Mulder et al., 1986). Moreover, 20–25% of FALS cases are due to mutations in the gene encoding copper-zinc superoxide dismutase (SOD1) (Deng et al., 1993; Rosen et al., 1993). More than 100 different mutations in the SOD1 gene have been identified in FALS so far.

Until recently, animal models of FALS have been various transgenic mice that express a mutant human SOD1 (hSOD1) gene. Of these, a transgenic mouse carrying the G93A (Gly-93 → Ala) mutant hSOD1 gene was the first described (Gurney et al., 1994) and is used all over the world because this model closely recapitulates the clinical and histopathological features of the human disease. To evaluate the therapeutic effects of potential ALS treatments in this animal, many motor-related behavioral tasks are used (Chiu et al., 1995; Barneoud et al., 1997; Garbuzova-Davis et al., 2002; Sun et al., 2002; Wang et al., 2002; Inoue et al., 2003; Kaspar et al., 2003; Weydt et al., 2003; Azzouz et al., 2004). However, transgenic mice have innate limitations for some types of experiments because of their small size.

Recently, transgenic rat models of ALS, which harbor the hSOD1 gene containing the H46R (His-46 → Arg) or G93A mutation were generated (Nagai et al., 2001). The larger size of these rat models makes certain experiments easier, such as biochemical analyses that require large amounts of sample, intrathecal administration

Contract grant sponsor: Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST); Contract grant sponsor: Japanese Ministry of Health, Labour and Welfare; Contract grant sponsor: Japanese Ministry of Education, Culture, Sports, Science and Technology.

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Received 22 August 2005; Revised 29 September 2005; Accepted 30 September 2005

Published online 7 December 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20708

of drugs, and, especially, therapeutic trials, including the transplantation of neural stem cells into the spinal cord. The hSOD1 (G93A) transgenic rats typically present weakness in one hindlimb first. Later, weakness progresses to the other hindlimb and to the forelimbs. Finally, the rats usually become unable to eat or drink, and eventually die. Only subjective and ambiguous analyses were made with regard to the clinical progression of this ALS animal model and objective criteria for evaluating the efficacy of these new treatments have not been determined. For these reasons, we assessed the disease progression quantitatively using five different measures (body weight, inclined plane test, cage activity, SCANET, and righting reflex) and established an easy, noninvasive, and objective evaluation system that is sensitive to small but important abnormalities in the hSOD1 (G93A) transgenic rats. In addition, we created a novel scale, the Motor score, to assess disease progression in the transgenic rats without using special apparatuses. We also examined the validity of these measures as assessment tools for the pathology by investigating the number of spinal motor neurons remaining at the disease stages defined by each measure.

MATERIALS AND METHODS

Transgenic Rats

All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine. We used hSOD1 (G93A) transgenic male rats (Nagai et al., 2001) from our colony and their age- and gender-matched wild-type littermates as controls. Rats were housed in a specific pathogen-free animal facility at a room temperature of 23 ± 1°C under a 12-hr light-dark cycle (light on at 08:00). Food (solid feed CE-2, 30kGy; CLEA Japan, Inc.) and water were available ad lib. Transgenic rats were bred and maintained as hemizygotes by mating transgenic males with wild-type females. Transgenic progeny were identified by detecting the exogenous hSOD1 transgene, by amplification of pup tail DNA extracted at 20 days of age by polymerase chain reaction (PCR). The primers and cycling conditions were described previously (Nagai et al., 2001).

Exploration of Assessment Tools to Measure Disease Progression in the hSOD1 (G93A) Transgenic Rats

We evaluated the usefulness of four different measures to assess disease progression in the transgenic rats. All tests were carried out between 12:00–16:00 and in a double-blind fashion.

Body weight. Animals ($n = 9$ for each genotype) were weighed weekly after 30 days of age with an electronic scale. To avoid overlooking the beginning of weight loss, the animals were weighed every second or third day after 90 days of age, the age at which motor neurons are reported to be lost in the lumbar spinal cord (Nagai et al., 2001).

Inclined plane. This test was initially established mainly to assess the total strength of the forelimbs and hindlimbs in a model of spinal cord injury (Rivlin and Tator, 1977). Briefly, rats were placed laterally against the long axis of the inclined plane, and the maximum angle at which they could maintain their position on the plane for 5 sec was measured. To assess the strength of both sides of limbs equally, animals were placed on the inclined plane with the right side of the body to the downhill side of the incline, and then with the left side of the body facing downhill. For each rat, the test was carried out three times for each side, and the mean value of the angles obtained for the right side was compared to that obtained for the left. The lower mean value was recorded as the angle for that rat. Animals ($n = 9$ for each genotype) were tested weekly after 70 days of age and every second to third day after 100 days of age.

Cage activity. Animals ($n = 8$ for each genotype) were housed individually and monitored every day for all 24 hr (except for the days the cages were changed) after they were 70 days old. Spontaneous locomotor activity in the home cage (345 × 403 × 177 mm) was recorded by an activity-monitoring system (NS-AS01; Neuroscience, Inc., Tokyo, Japan) as described previously (Ohki-Hamazaki et al., 1999). The sensor detects the movement of animals using the released infrared radiation associated with their body temperature. The data were analyzed by the DAS-008 software (Neuroscience, Inc., Tokyo, Japan). To eliminate data variability owing to differences in the baseline movement of each rat, the baseline value was calculated as the mean of movement from 70–90 days of age, during which all rats were considered to move normally. We analyzed the data at each time point as the percentage of the baseline value in defining disease onset with this test.

SCANET. For short-term activity, 10 min of spontaneous activity was measured with the automated motion analysis system SCANET MV-10 (Toyo Sangyo Co., Ltd., Toyama, Japan) (Mikami et al., 2002). Animals ($n = 4$ for each genotype) were tested weekly after 30 days of age and every second or third day after 100 days of age. Each rat was individually placed in the SCANET cage for 10 min. Three parameters were measured: small horizontal movements of 12 mm or more (Move 1; M1), large horizontal movements of 60 mm or more (Move 2; M2), and the frequency of vertical movements caused by rearing (RG). To distinguish RG movements from incomplete standing actions, the upper sensor frame was adjusted to 13 cm above the lower sensor frame.

Righting reflex. All affected animals were tested for the ability to right themselves within 30 sec of being turned on either side (righting reflex) (Gale et al., 1985). Failure was seen when animals reached the end-stage of disease (Howland et al., 2002), and was regarded as a generalized loss of motor activity. We used this time point, which we call "end-stage," as "death" rather than the actual death of the animal, to exclude the influence of poor food intake and respiratory muscle paralysis on the survival period. All end-stage animals were sacrificed after being deeply anesthetized.

All statistical analyses were carried out with the two-tailed unpaired Student's t-test. A P-value of <0.05 was considered statistically significant.

Motor Score

To establish our own scoring system for motor function, which could be uniformly applicable to any disease phenotype of this rat model, we examined the common clinical findings

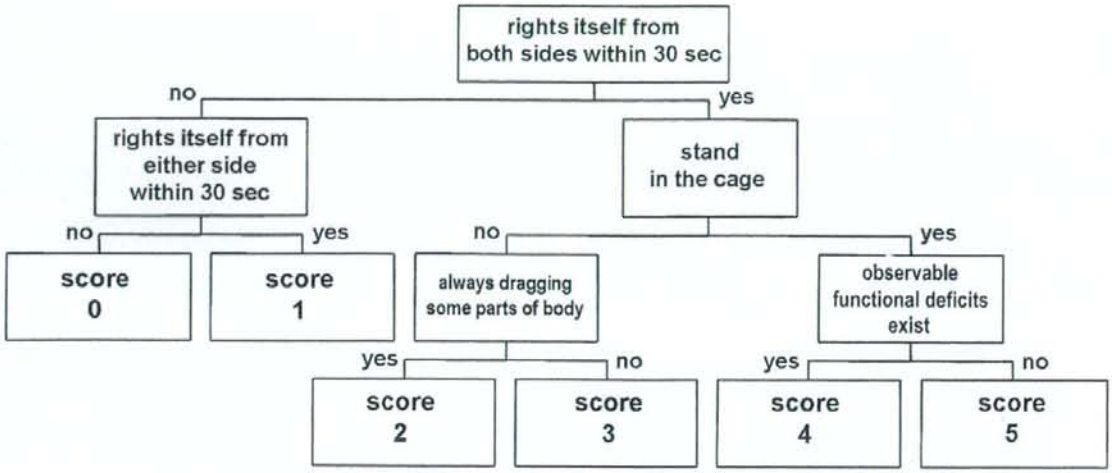

Fig. 1. Chart of Motor score assessment. The degree of motor dysfunction can be assessed by the Motor score as shown in this chart. This scoring system is meant to be used after disease onset, which can be prospectively diagnosed by the inclined plane test (muscle weakness onset). A score of 4 means the same condition as seen for subjective onset (SO). Rats with a score of 5 seem almost as normal as wild-type rats. The detailed testing procedure for the Motor score is described in the text.

of the transgenic rats in detail and assessed their motor functions ($n = 20$). We focused on the following tests: the righting reflex, the ability to stand in the cage, the extent of dragging their bodies when moving, and the existence of observable functional deficits. We evaluated these items sequentially along with the disease progression and classified the rats into six groups by giving them scores between 0 and 5. The scoring chart (Motor score) is shown in Figure 1.

When disease onset in the rats was diagnosed by their scoring $<70°$ on the inclined plane test (muscle weakness onset), the affected rats were tested for righting reflex. If they were unable to right themselves from either side, they were given a score of 0. If they could right themselves from only one side but not the other, they were given a score of 1.

Rats that could right themselves from both sides were examined for the ability to stand in the cage as follows: Rats were observed in the home cage for 1 min to see if they would stand spontaneously (Step 1). When they moved little in the home cage or showed no tendency to stand during Step 1, they were stimulated by being transferred to another cage (Step 2), and then by being returned to their home cage again (Step 3); the transfers were done to activate exploration motivation. During Step 3, the rats were further stimulated by lightly knocking the cage to intensify the motivation to explore. Each step was carried out for 1 min and the test was stopped when the rat stood once. Rats were judged as "unable to stand" if they did not stand, even after all three steps.

Rats that did not stand were subjected to the next test in the open field, where the extent to which they dragged their bodies when moving was assessed. Those who always dragged and could not lift some parts of their bodies except for scrotums and tails at any time were given a score of 2. If they could lift their dragging parts off the ground for even a moment, they were given a score of 3. The phenotype of dragging the forelimbs was different from that of dragging the hindlimbs. As disease progressed, "forelimb-type" rats first began to touch the tips of their noses on the ground, and then began to drag their head and upper trunk as they moved backward with their hindlimbs. "Hindlimb-type" rats dragged their lower trunk and moved forward with their forelimbs.

Finally, rats that had no abnormality in the above-mentioned assessments were examined in detail to see whether they had any observable functional deficits such as paralysis of the limbs or symptoms of general muscle weakness (e.g., walking with a limp, sluggish movement) in the open field. This condition could be judged subjectively and was defined as subjective onset. Rats with any of these symptoms were given a score of 4; otherwise they were given a score of 5.

Because the scores were based on subjective judgment, they might vary depending on the examiner. To examine inter-rater variability, three transgenic rats of different clinical types were examined according to the method described above, recorded on video tape, and subsequently scored by five observers from different backgrounds (Table I). The scores classified by the five observers were statistically analyzed for inter-rater agreement using Cohen's κ statistics (Table II). Kappa values can range from 0 (no agreement) to 1.00 (perfect agreement), and can be interpreted as poor (<0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.00) (Landis and Koch, 1977). The scores for the three transgenic rats were, on the whole, quite consistent among the five observers, suggesting that the Motor score can be used as an objective method for assessing disease progression.

TABLE I. Motor Score of Transgenic Rats Assessed by Five Different Observers

Transgenic rat	Observer	Days after onset (days) 0	1	2	3	4	5	6	7	8
#1407 Eventual hindlimb type										
	A	5	4	4		2	2	1	0	
	B	4	4	4		2	2	1	0	
	C	4	4	4		2	2	1	0	
	D	4	4	4		2	2	1	0	
	E	4	4	4		2	2	1	0	
	Mean	4.2	4	4		2	2	1	0	
#1470 Pure hindlimb type										
	A	5		4	4	2	2	2	2	0
	B	5		4	3	3	2	2	2	0
	C	5		4	3	2	2	2	2	0
	D	4		4	4	2	2	2	2	0
	E	4		4	3	2	2	2	2	0
	Mean	4.6		4	3.4	2.2	2	2	2	0
#1449 Pure forelimb type										
	A	4	3	3	3		2	1	1	0
	B	4	3	3	3		2	1	1	0
	C	3	3	3	3		2	1	1	0
	D	3	3	3	3		2	1	1	0
	E	4	3	2	2		2	1	1	0
	Mean	3.6	3	2.8	2.8		2	1	1	0

TABLE II. The kappa Statistics for Inter-Rater Agreement of Motor Score

Observers	Transgenic rat (clinical type) #1407 Eventual hindlimb	#1470 Pure hindlimb	#1449 Pure forelimb
A vs. B	0.82	0.69	1.00
A vs. C	0.82	0.82	0.83
A vs. D	0.82	0.81	0.83
A vs. E	0.82	0.70	0.69
B vs. C	1.00	0.83	0.83
B vs. D	1.00	0.53	0.83
B vs. E	1.00	0.66	0.69
C vs. D	1.00	0.64	1.00
C vs. E	1.00	0.82	0.54
D vs. E	1.00	0.81	0.54

TABLE III. Clinical Types of hSOD1 (G93A) Transgenic Rats

Clinical type	Subtype	*n*	%
Forelimb	Pure	4	8.2
	Eventual	5	10.2
Hindlimb	Pure	19	38.7
	Eventual	17	34.7
General		4	8.2
Total		49	100

Real-Time RT-PCR and Western Blot Analysis

Tissue specimens were dissected from the cerebral cortices, cerebella, medullae, and spinal cords (cervical, thoracic, and lumbar spinal cords) of the deeply anesthetized rats, and divided into two portions for total RNA and total protein preparation. Total RNA was isolated and first strand cDNA was synthesized as described previously (Okada et al., 2004). The real time RT-PCR analysis was carried out using Mx3000P (Stratagene, La Jolla, CA) with SYBR Premix Ex Taq (Takara Bio, Inc., Otsu, Japan). The primers used for the analysis were human *SOD1* (5′-TTGGGCAATGTGACTGCTGAC-3′, 5′-AGCTAGCAGGATAACAGATGA-3′), rat *SOD1* (5′-ACTTCGAGCAGAAGGCAAGC-3′, 5′-ACATTGGCCACACCGTCCTTTC-3′), and *β-actin* (5′-CGTGGGCCGCCCTAGGCACCA-3′, 5′-TTGGCCTTAGGGTTCAGAGGGG-3′). The results are presented as ratios of mRNA expression normalized to an inner control gene, *β-actin*. Total protein was prepared in lysis buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 20 mM glycerophosphate, 1% Triton X-100, and a protease inhibitor mixture (Complete; Roche Applied Science, Mannheim, Germany). Western blot analysis was carried out by a method established previously. In brief, a 5 μg protein sample of an extract was run on 12% SDS-PAGE, transferred to nitrocellulose, and probed with anti-human SOD1 (1:1,000, mouse IgG, Novocastra Laboratories, Ltd., Benton Lane, UK), and anti-α-tubulin (1:2,000, mouse IgG, Sigma-Aldrich, Inc., Saint Louis, MO). Signals were detected with HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) using an ECL kit (Amersham Bioscience UK limited, Little Chalfont, UK). Quantitative analysis was carried out with a Scion Image (Scion Corporation, Frederick, MD). The amounts of proteins loaded in each slot were normalized to those of α-tubulin.

Immunohistochemical Analysis

Rats were deeply anesthetized (ketamine 75 mg/kg, xylazine 10 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde/PBS (0.1 M PBS, pH 7.4) for histological examination. Spinal cord tissues were dissected out and post-fixed overnight in the same solution. Each spinal cord was dissected into segments that included the C6, T5, and L3 levels, immersed in 15% sucrose/PBS followed by 30% sucrose/PBS at 4°C, and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). Embedded tissue was immediately frozen with liquid nitrogen and stored at −80°C. Serial transverse sections of each spinal segment were cut on a cryostat at a thickness of 14 μm. The sections were pre-treated with acetone for 5 min, rinsed with PBS three times and permeabilized with TBST (Tris-buffered saline with 1% Tween 20) for 15 min at room temperature. After being blocked in the TNB buffer (Perkin-Elmer Life Sciences, Inc., Boston, MA) for 1 hr at room temperature, the sections were incubated at 4°C overnight with an anti-choline acetyltransferase (ChAT) polyclonal antibody (AB144P, Goat IgG, 1:50; Chemicon International, Inc., Temecula, CA). After being washed with PBS three times, the sections were incubated for 2 hr at room temperature with a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Finally, the labeling was developed using the avidin-biotin-peroxidase complex procedure (Vectastain ABC kits; Vector Laboratories, Inc., Burlingame, CA) with 3,3-diaminobenzidine (DAB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) as the chro-

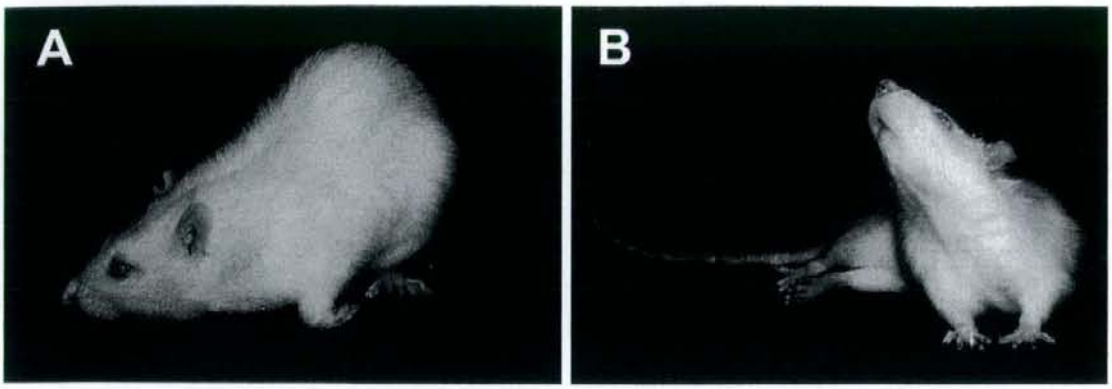

Fig. 2. Characteristic appearance of hSOD1 (G93A) transgenic rats. **A:** Forelimb type. The rat was unable to raise its head and was obligated to take a posture of raising the lumbar region, as indicated, because of the paralyzed forelimbs. **B:** Hindlimb type. The rat showed paraplegia, but was able to raise its head and upper trunk with its non-paralyzed forelimbs.

mogen. Immunohistochemical images were examined with a Zeiss-AxioCam microscope system.

Motor neurons bearing ChAT-immunoreactivity in laminae VII, VIII, and IX of the ventral horn were counted in every tenth section (5 sections total for each segment) for each of the C6, T5, and L3 segments. Only the neurons that showed labeling above background level and were larger than 20 μm in diameter were counted. The numbers of motor neurons in all segments (C6, T5, and L3) were summed for each animal to evaluate not only the local motor neuron loss, but the generalized loss of motor neurons throughout the spinal cord of each animal ($n = 3$ for each genotype at each time point). We next examined the correlation between the number of residual motor neurons and the results of the functional analyses described in this study. Statistical analysis was carried out with two-tailed unpaired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Clinical Types of hSOD1 (G93A) Transgenic Rats

Because we noticed variations in the disease phenotypes expressed by the G93A rats, we classified 49 rats into three clinical categories according to the location of initial paralysis. The clinical types were: the forelimb type, hindlimb type, and general type (Table III). Rats whose paralysis started in the forelimbs and progressed to the hindlimbs were defined as the "forelimb type." In contrast, rats whose paralysis started from the hindlimbs and progressed to the forelimbs were defined as the "hindlimb type." A typical appearance for the forelimb and hindlimb types is shown in Figure 2. Other rats, which showed simultaneous paralysis in the forelimbs and hindlimbs, were categorized as the "general type".

In addition, we classified the forelimb- and hindlimb-type rats into two subtypes, the pure and eventual types, based on the timing of the initial paralysis (Table III). Rats of the pure type showed paralysis that was limited to one or more of the four limbs as the initial observable deficit. Those of the eventual type initially showed symptoms of general muscle weakness (e.g., walking with a limp, sluggish movement), but without unequivocal limb paralysis. In the eventual type animals, paralysis of one of the limbs became apparent later. The ratio of each subtype is shown in Table III.

Evaluation of Disease Progression in the hSOD1 (G93A) Transgenic Rats

Although the transgenic rats varied in their clinical types, all four measures of disease progression (body weight, inclined plane test, cage activity, and SCANET) showed significant differences between the transgenic and wild-type rats (Fig. 3).

In contrast to the continuous weight gain in wild-type rats, the body weight in the affected rats ceased to increase and gradually decreased, with peak body weight attained around 110–120 days of age ($P < 0.05$, after 112 days of age) (Fig. 3A).

In the inclined plane test, initially both the transgenic and wild-type rats uniformly scored 75–80 degrees, after several training trials. However, the transgenic rats showed a significant decline in performance compared to their wild-type littermates from 120 days of age (Fig. 3B).

In the cage activity measurement, the movements of the wild-type rats remained stable, whereas those of the transgenic rats declined rapidly after 125 days of age (Fig. 3C).

In the SCANET test, even the wild-type rats showed decreased movements for all parameters (M1, M2, RG) in the late observation period, though they showed no abnormality in their motor functions. This might be because they had acclimated to the SCANET cage. The movement score of the transgenic rats was consistently worse than that of the wild-type rats after

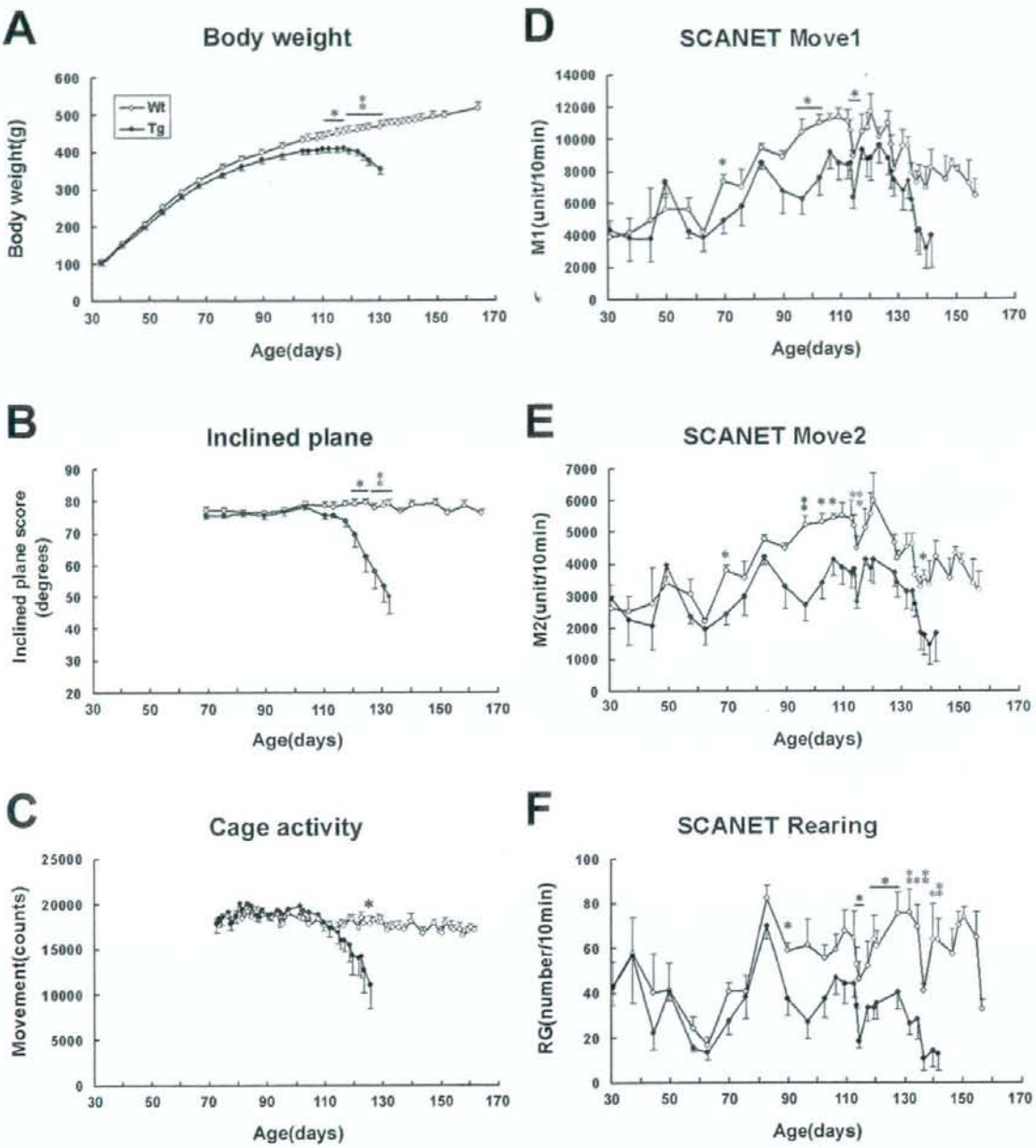

Fig. 3. Disease progression in hSOD1 (G93A) transgenic rats monitored by four effective measures. **A:** Body weight. The weight gain of the transgenic group stopped at around 110–120 days. The difference became statistically significant at 112 days of age ($n = 9$ for each genotype). **B:** Inclined plane. The wild-type group scored 75–80° throughout the period, whereas the score of the transgenic group declined. The difference became statistically significant at 120 days of age ($n = 9$ for each genotype). **C:** Cage activity. The movements of the wild-type group were stable, whereas the scores of the transgenic group declined. Significance was reached at 125 days of age ($n = 8$ for each genotype). **D–F:** SCANET. For all parameters (M1, M2, RG), the movement scores of the transgenic group became constantly worse than those of the wild-type group after 60 days of age. The differences between the groups increased markedly after 90 days of age. Significance was attained beginning at 67 days of age for M1 and M2, and at 87 days of age for RG ($n = 4$ for each genotype). The comparison between the wild-type and transgenic groups was stopped when the first of the transgenic rats reached the end-stage of the disease and was sacrificed. Mean ± SEM. $^{*}P < 0.05$. $^{**}P < 0.01$; two-tailed unpaired Student's *t*-test.

Journal of Neuroscience Research DOI 10.1002/jnr

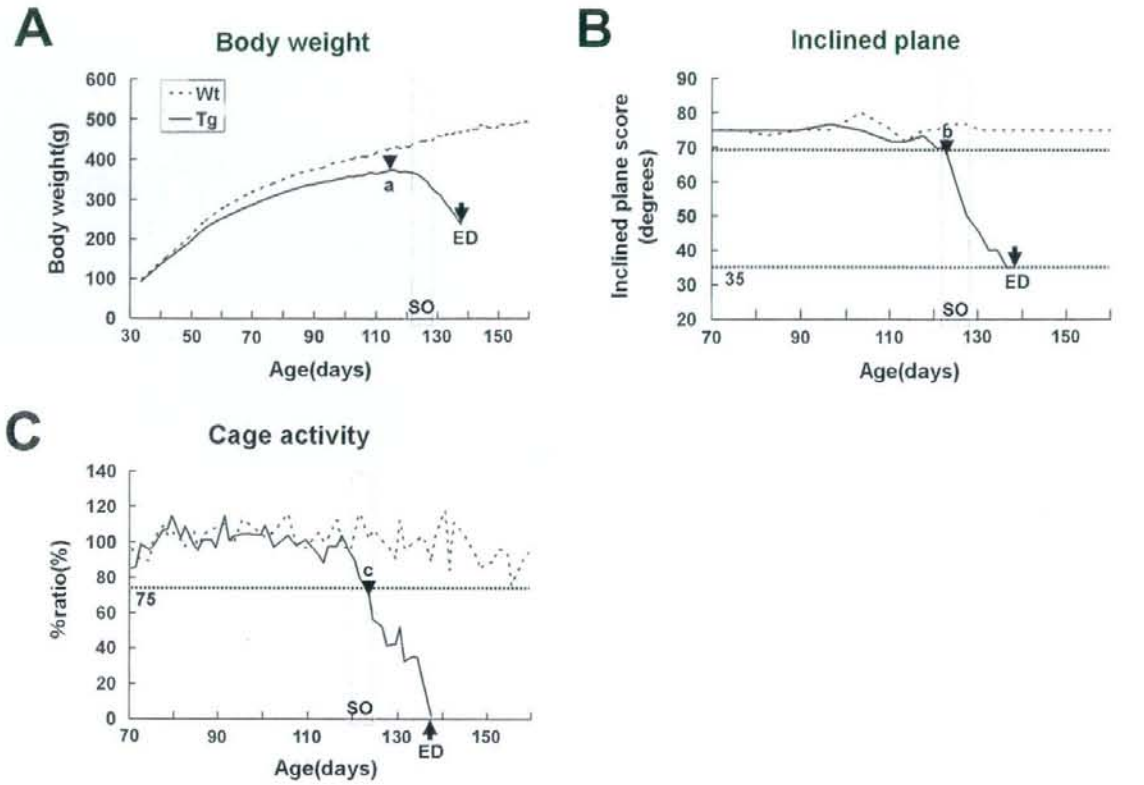

Fig. 4. Schematic presentation of the results from the body weight (**A**), inclined plane test (**B**), and cage activity (**C**) assessments. The onset defined by each measure (black arrowheads) and the end-stage of the disease (ED, black arrows) are indicated in the figures. a, pre-symptomatic onset: the day the transgenic rats scored their maximum body weight. b, muscle weakness onset: the earliest day the transgenic rats scored <70° in the inclined plane test. c, hypo-activity onset: the earliest day the transgenic rats scored <75% of the mean movements from 70–90 days of age in the cage activity measure. SO, subjective onset: the earliest day that observable functional deficits such as paralysis of the limbs or symptoms of general muscle weakness were observed subjectively in the open field (the gray shaded region in A–C).

60 days of age for all parameters (M1, M2, RG), however, even after the wild-type animals showed the decrease in their movement scores. The differences between the two groups increased markedly after 90 days of age for M1, M2, and RG (Fig. 3D–F). The performance of each rat fluctuated so markedly that the SCANET test seems to be inappropriate for statistical analysis.

Onset, End-Stage, and Duration of Disease in hSOD1 (G93A) Transgenic Rats

Using the quantitative analysis of disease progression by body-weight measurement, the inclined plane test, and cage activity, as described above, we defined three time points of "objective onset," as shown in Figure 4. The SCANET results did not allow us to define a time of objective onset, because we could not establish a stable baseline level using the data from the highly variable measurements we obtained, even for wild-type rats. The righting reflex failure was useful for detecting the time point of end-stage disease, which we defined as the generalized loss of motor activity in affected rats. A total of 20 transgenic rats assessed by body weight and the inclined plane test were analyzed for the day of objective onset, end-stage, and duration of the disease. The cage activity data from the eight transgenic rats were obtained simultaneously. The results are shown in Table IV.

The day the transgenic rats reached their maximum body weight was defined as pre-symptomatic onset (113.6 ± 4.8 days of age, black arrowhead in Fig. 4A, Table IV). This onset was judged retrospectively and always preceded the subjective onset (gray shaded region, Fig. 4A), which was determined by observable functional deficits in the open field, such as paralysis of limbs and symptoms of general muscle weakness. The

TABLE IV. Onset, End-Stage, and Duration in Days of Disease in hSOD1 (G93A) Transgenic Rats

Evaluation methods	Body weight and inclined plane (n = 20)	Cage activity (n = 8)
Objective onset		
Pre-symptomatic onset[a]	113.6 ± 4.8 (103–124)	
Muscle weakness onset[b]	125.2 ± 7.4 (110–144)	
Hypo-activity onset[c]		122.8 ± 9.2 (109–139)[c]
Subjective onset (SO)[d]	126.5 ± 7.1 (113–147)	121.3 ± 9.8 (109–140)
End-stage disease (ED)[e]	137.8 ± 7.1 (128–155)	134.1 ± 8.2 (122–149)
Duration[f]		
ED-a[g]	24.3 ± 6.5	
ED-b[h]	12.6 ± 3.5	
ED-c[i]		11.4 ± 1.3

Values are means ± SD.
[a] Maximum of body weight.
[b] Less than 70 degrees in the inclined plane test.
[c] Less than 75% in the mean movements of 70-90 days in the cage activity.
[d] Observable functional deficits.
[e] Righting reflex failure.
[f] Difference in days between ED and each onset;
[g] between ED and pre-symptomatic onset,
[h] between ED and muscle weakness onset,
[i] between ED and hypo-activity onset.

TABLE V. Comparison of the Onset, End-stage, and Duration in Days of Disease in the Forelimb-type and the Hindlimb-type Rats

	Forelimb type (n = 4)	Hindlimb type (n = 14)	General type* (n = 2)
Pre-symptomatic onset[a]	112.5 ± 6.7	114.6 ± 4.3	(108.5)
Muscle weakness onset[b]	125.8 ± 2.8	126.7 ± 7.3	(113.5)
End-stage disease (ED)[c]	134.0 ± 2.4	140.1 ± 7.1	(129.5)
Duration[d]			
ED-a[e]	21.5 ± 8.5	25.5 ± 6.2	(21)
ED-b[f]	8.3 ± 1.0	13.4 ± 3.0	(16)

Values are mean ± SD.
* Values of general-type rats are listed in parenthesis for reference.
[a] Maximum of body weight.
[b] Less than 70 degrees in the inclined plane test.
[c] Righting reflex failure.
[d] Difference in days between ED and each onset;
[e] between ED and pre-symptomatic onset,
[f] between ED and muscle weakness onset.

pre-symptomatic onset was the most sensitive of all the onset measures described in this study (Table IV).

The first day the transgenic rats scored <70° in the inclined plane test was defined as the muscle weakness onset (black arrowhead, Fig. 4B). We could judge this onset prospectively. Muscle weakness onset (125.2 ± 7.4 days of age, Table IV) was usually recorded before or at almost the same time as the subjective onset (8 days before to 1 day after, gray shaded region, Fig. 4B and 126.5 ± 7.1 days of age, Table IV). The day the transgenic rats scored 35° or less on the inclined plane test coincided with the day of righting reflex failure (black arrow, Fig. 4B).

The first day the transgenic rats scored <75% of their baseline movements in the cage activity test was defined as hypo-activity onset (black arrowhead, Fig. 4C and 122.8 ± 9.2 days of age, Table IV). We could also judge this onset prospectively. Hypo-activity onset was recorded 1 day before to 4 days after the subjective onset (SO, shown as the gray shaded region in Fig. 4C and 121.3 ± 9.8 days of age, Table IV). A 0% movement score for cage activity was seen at almost the same time as righting reflex failure (black arrow, Fig. 4C). Although disease onset and end-stage could be objectively defined with these methods, they had a wide range, of about 1 month, because of the diversity of the phenotypes (Table IV).

Differences in Disease Courses Between the Forelimb- and Hindlimb-Type Rats

Because we noticed variability in disease courses among different clinical types of hSOD1 (G93A) rats, we next assessed disease progression in 20 transgenic rats with forelimb- (n = 4), hindlimb- (n = 14), and general- (n = 2) type, using the probability of objective

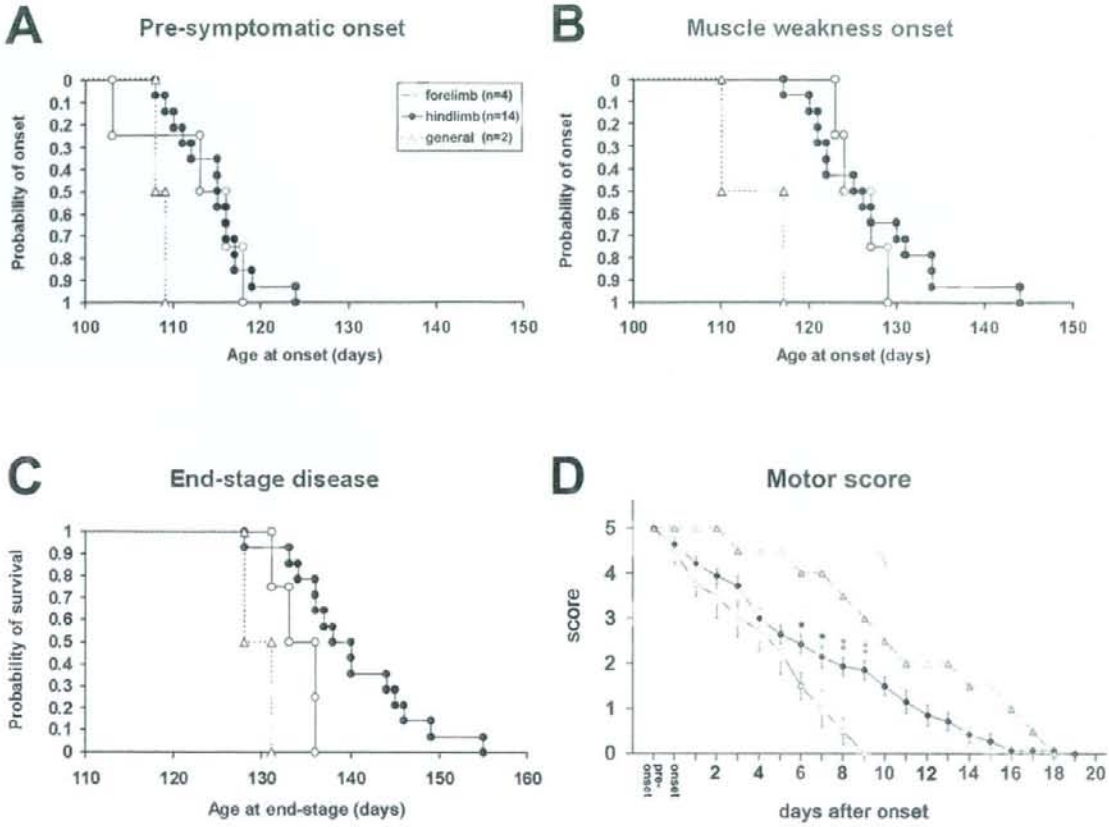

Fig. 5. Comparison of onset, end-stage, and disease progression in the forelimb-type ($n = 4$), and the hindlimb-type ($n = 14$) rats. Data from the general-type rats are also shown as dotted lines. **A,B:** The probability of the objective onsets. We did not see any differences in the probability of the objective onsets defined by body weight measurement (pre-symptomatic onset) and the inclined plane test (muscle weakness onset) between the forelimb- and hindlimb-type rats. **C:** The probability of survival as defined by end-stage disease. Survival was significantly shorter in the forelimb-type than in the hindlimb-type rats ($P < 0.05$, Log-rank test). **D:** Assessment of disease progression using the Motor score. Affected rats were evaluated after muscle weakness onset. The forelimb type worsened more quickly than the hindlimb type. Score decline correlated well with the exacerbation of symptoms in both clinical types, clearly and objectively. Bars = means ± SEM. Statistically significant differences between forelimb and hindlimb types are indicated in the figures. $^{*}P < 0.05$. $^{**}P < 0.01$; two-tailed unpaired Student's t-test.

onsets (pre-symptomatic onset and muscle weakness onset), the probability of survival defined by end-stage disease (failure in righting reflex), and the Motor score (Table V, Fig. 5). We did not see any differences in the objective onsets between the forelimb- and hindlimb-type rats (Fig. 5AB, Table V). However, survival as defined by end-stage disease was significantly shorter in the forelimb-type than in the hindlimb-type rats ($P < 0.05$, Log-rank test, Fig. 5C). Moreover, the duration of the disease calculated from the muscle weakness onset was also significantly shorter in the forelimb-type (8.3 ± 1.0 days) than in the hindlimb-type rats (13.4 ± 3.0 days) (see ED − b, $P < 0.01$, two-tailed unpaired Student's t-test, Table V).

The courses of functional deterioration evaluated by the Motor score after onset (muscle weakness onset) for each clinical type were well represented by the declines in their scores (Fig. 5D). The assessment by the Motor score also showed that disease progression in the forelimb type was more rapid than that in the hindlimb type (Fig. 5D).

Our results raise the question of why this variability in the disease course of each clinical type was observed. We speculated that there might be correlation between clinical type in G93A rats and the amount of locally expressed mutant hSOD1 (G93A) gene product. Therefore, we next investigated expression of the mutant hSOD1 gene in each segment of the spinal cord (cervical, thoracic, and lumbar) in the forelimb- and

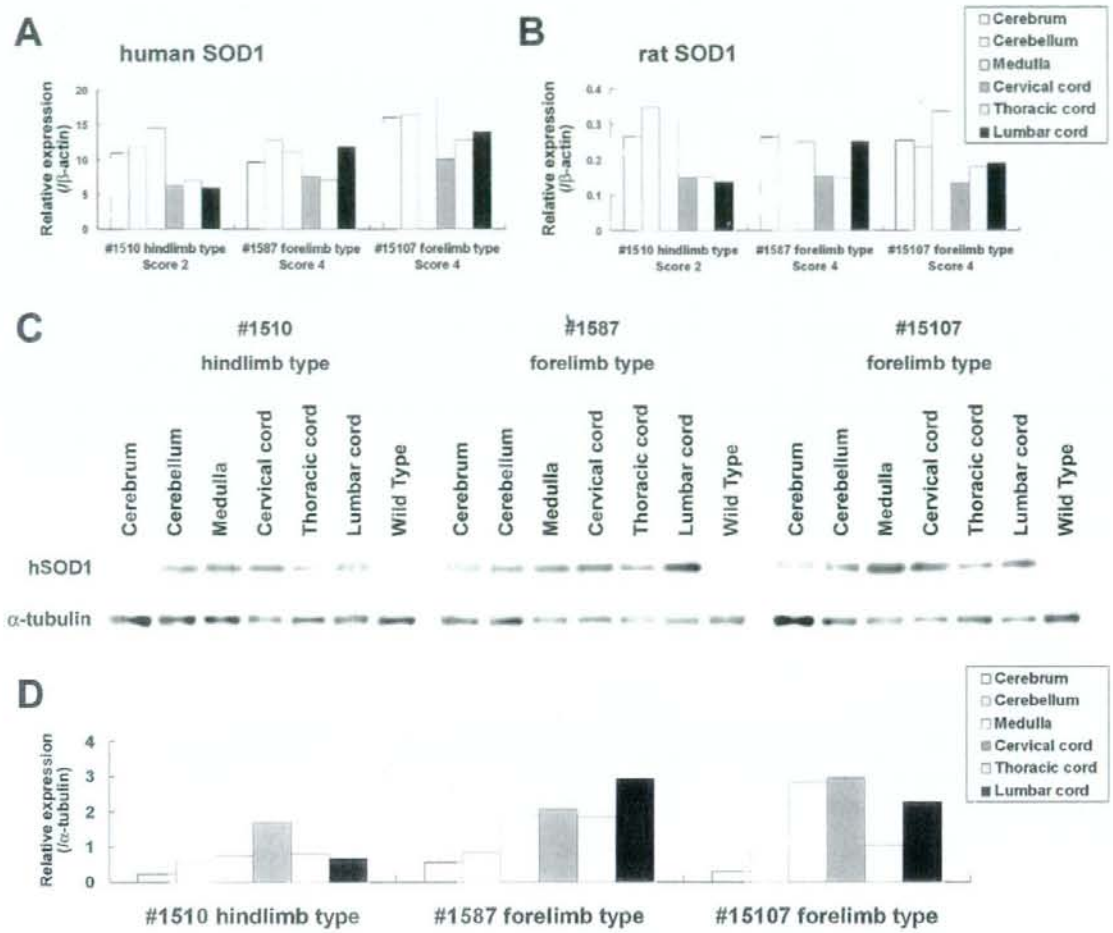

Fig. 6. The expression of mutant hSOD1 mRNA and protein in the cerebral cortex, cerebellum, medulla, and spinal cord (cervical, thoracic, and lumbar) of forelimb- and hindlimb-type rats. **A,B:** The amounts of human (A) and endogenous rat (B) SOD1 mRNA normalized to those of *β-actin* were quantified by real time RT-PCR analysis. **C,D:** Western blot analysis of the mutant hSOD1 protein was carried out in the same rats. Quantitative analysis was carried out with a Scion Image. The amounts of proteins were normalized to those of α-tubulin (D).

hindlimb-type rats by real time RT-PCR and Western blot analysis. However, at least at the stages after the apparent onset of muscle weakness, neither forelimb-type (#1587, Score 4 and #15107, Score 4) nor hindlimb-type rats (#1510, Score 2) necessarily expressed larger amounts of the mutant hSOD1 (G93A) transgene in the cervical cord or in the lumbar cord, respectively, at the mRNA and the protein level (Fig. 6). We also investigated the expression of endogenous rat SOD1 mRNA in the same rats by REAL TIME RT-PCR (Fig. 6B). Distribution of endogenous rat SOD1 mRNA expressed in each segment of the spinal cord showed almost the same pattern as that of mutant hSOD1 mRNA. The expression of endogenous rat SOD1 mRNA was lower than that of mutant hSOD1 mRNA. Thus, we could not detect any definite correlation between the hSOD1 (G93A) transgene local expression profile in the spinal cord and the phenotypes of G93A rats for either the forelimb-type or the hindlimb-type rats (Fig. 6).

Reduction in the Number of Spinal Cord Motor Neurons at Different Disease Stages

We examined histo-pathological changes in the spinal cords of the transgenic rats in comparison with those

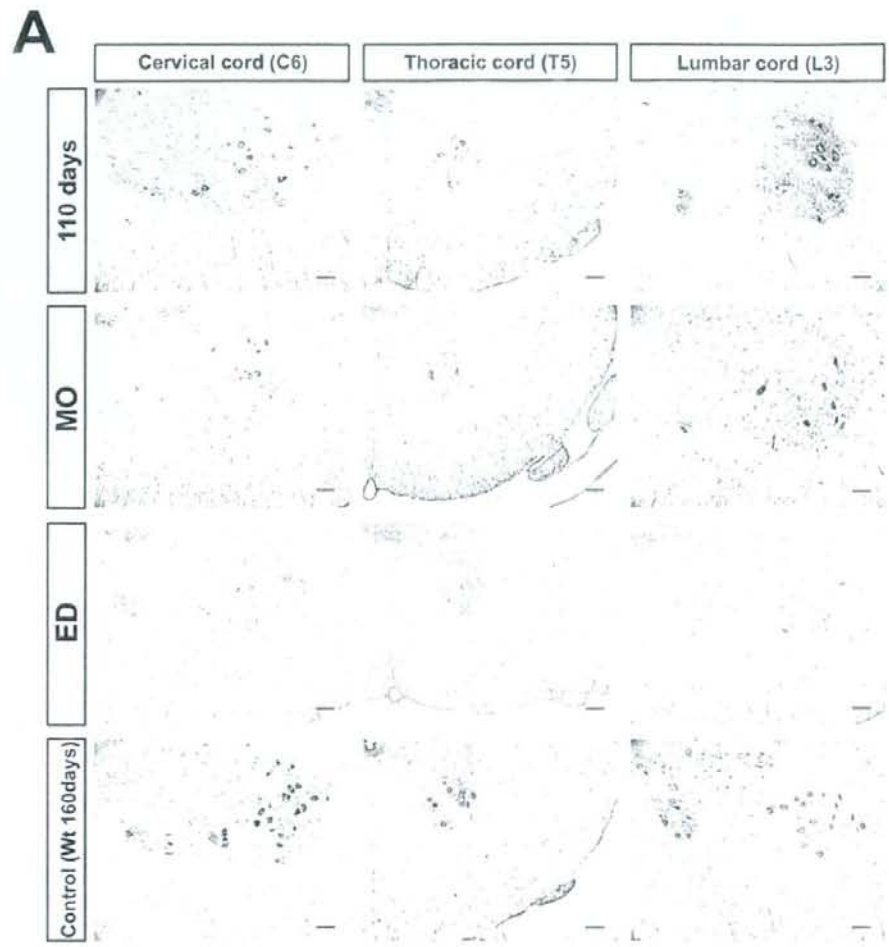

Fig. 7. The loss of motor neurons in the spinal cord of hSOD1 (G93A) transgenic rats at different stages. **A:** Immunohistochemical analysis of the spinal cord of transgenic rats. Transverse sections of the cervical (C6), thoracic (T5), and lumbar (L3) spinal cord of the transgenic rats and their wild-type littermates were stained with an anti-ChAT antibody to label viable motor neurons at the indicated stages (Scale bars = 100 μm). **B:** The number of ChAT immunoreactive motor neurons was counted and is shown in the histograms as the total number of motor neurons in the C6, T5, and L3 segments. This number began to decrease in the transgenic rats at 90 days of age, rapidly declined after 110 days of age, and fell to about 50% and 25% of wild-type rats at the muscle weakness onset (MO, around 125 days) and at end-stage disease (ED, around 140 days), respectively. Bars = means ± SEM ($n = 3$ for each genotype). $^{*}P < 0.05$. $^{**}P < 0.01$; two-tailed unpaired Student's *t*-test.

of their wild-type littermates at 70, 90, and 110 days of age, when the transgenic rats scored <70° in the inclined plane test (muscle weakness onset), and failed the righting reflex. To quantify the number of spinal motor neurons, we stained spinal cord sections of both groups with an anti-ChAT antibody.

As shown in Figure 7A, the numbers of ChAT immunoreactive motor neurons in the cervical (C6), thoracic (T5), and lumbar (L3) segments of the spinal cord decreased with disease progression. Quantitative analysis of the residual motor neurons showed that the total number of motor neurons in the transgenic rats began to decrease at 90 days of age, rapidly declined after 110 days of age, and fell to about 50% and 25% of the numbers in age-matched wild-type littermates at the time the score was <70° in the inclined plane test (muscle weakness onset) and of righting reflex failure, respectively (Fig. 7B).

DISCUSSION

Factors Underlying the Variability in Phenotypes of hSOD1 (G93A) Transgenic Rats

In previous studies of this G93A rat, only the hindlimb-type has been described, and the variety of phenotypes and variable clinical courses have not yet been mentioned (Nagai et al., 2001). Recently, however, another line of G93A rats backcrossed onto a Wistar background ($SOD1^{G93A/HWr}$ rats) was reported to present two phenotypes, including forelimb-type, and a large inter-litter variability in disease onset (Storkebaum et al., 2005). In the same way, commonly used FALS model mice harboring hSOD1 (G93A) gene have been reported to have clinical variability to some extent, and some of them dominantly show forelimb paralysis (Gurney et al., 1994). In this study, we recognized various clinical types, including forelimb-, hindlimb-, and general-type and established quantitative methods to evaluate disease progression that can be applied to any of the clinical types of this ALS model. We have also shown the variability in disease progression to depend on clinical types, that is, disease progression after the onset was faster in forelimb-type than in hindlimb-type rats. This difference may be due to the aggressiveness of the disease per se because we evaluated the time point of "death" (end-stage disease) according to righting reflex failure (Howland et al., 2002) to exclude the influence of feeding problems (bulbar region) and respiratory failure (level C2–C4).

These findings give rise to the next question; why is this variety of phenotypes and variability in the clinical course observed in the same transgenic line? There are at least three possible explanations. One is that the variation is due to the heterogeneous genetic background of the Sprague-Dawley (SD) rat (i.e., the strain used to generate this transgenic line), which might have led to different phenotypes. This idea is supported by the fact that the SD strain shows a large inter-individual disease variability in other models of neurodegenerative disorders, such as Huntington's disease (Ouary et al., 2000). Similar phenotypic variability takes place in human FALS carrying the same mutations in hSOD1 gene (Abe et al., 1996; Watanabe et al., 1997; Kato et al., 2001), which could be explained by heterogeneous genetic backgrounds. Thus, the present transgenic ALS model rats may be highly useful to understand the mechanisms of bulbar onset, arm onset, or leg onset that are seen in human disease. There may be modifier genes of these phenotypes, which should be identified in the future study.

TABLE VI. Adequacy of Evaluation Methods in Regard to Practical Use*

	Body weight	Inclined plane	Cage activity	SCANET	Motor score
Objectivity	A	B	A	A	B
Sensitivity	A	B	C	(A)	-
Specificity	C	B	C	C	A
Motivation independence	A	B	B	D	B
Skill requirements	A	B	A	A	B
Cost of apparatus	B	B	D	D	A

*A, more appropriate; B, appropriate; C, less appropriate; D, inappropriate.

The second is that there is variability in the expression of the mutant hSOD1 protein. The transcriptional regulation of this exogenous gene could be affected by one or more unknown factors, such as epigenetic regulation, and may not be expressed uniformly throughout the spinal cord of each animal. Therefore, some rats might express mutant proteins more in the cervical spinal cord and others might express more in the lumber cord, possibly resulting in the forelimb type and hindlimb type, respectively. However, we found no definite correlation between local expression levels of the mutant hSOD1 mRNA/protein in the spinal cord and the phenotypes of these animals, using real time RT-PCR and western blot analysis after the onset of muscle weakness, when the clinical type of the transgenic rats could be defined (Fig. 6). Moreover, the pathological analysis showed no correlation between the number of residual motor neurons in each segment and the phenotypes of end-stage animals. However, because >50% of spinal motor neurons have already degenerated at the stage of muscle weakness onset, whether local expression of the mutant hSOD1 gene and segmental loss of motor neurons correlate with the clinical types of G93A rats should be further investigated by analyzing younger animals at a stage when motor neuron loss has not progressed as much.

The third explanation involves a structural property of the mutant hSOD1 (G93A) protein itself. It is now thought that mutations in the hSOD1 gene may alter the 3-D conformation of the enzyme and, in turn, result in the SOD1 protein acquiring toxic properties that cause ALS (Deng et al., 1993; Hand and Rouleau 2002). For instance, the hSOD1 (G93A) mutant protein has been reported to be susceptible to nonnative protein–protein interactions because of its mutation site and unfolded structure (Shipp et al., 2003; Furukawa and