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Treatment of motoneuron degeneration by intracerebroventricular delivery of VEGF in a rat model of ALS

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Neurotrophin treatment has so far failed to prolong the survival of individuals affected with amyotrophic lateral sclerosis (ALS), an incurable motoneuron degenerative disorder. Here we show that intracerebroventricular (i.c.v.) delivery of recombinant vascular endothelial growth factor (Vegf) in a *SOD1*^{G93A} rat model of ALS delays onset of paralysis by 17 d, improves motor performance and prolongs survival by 22 d, representing the largest effects in animal models of ALS achieved by protein delivery. By protecting cervical motoneurons, i.c.v. delivery of Vegf is particularly effective in rats with the most severe form of ALS with forelimb onset. Vegf has direct neuroprotective effects on motoneurons *in vivo*, because neuronal expression of a transgene expressing the Vegf receptor prolongs the survival of *SOD1*^{G93A} mice. On i.c.v. delivery, Vegf is anterogradely transported and preserves neuromuscular junctions in *SOD1*^{G93A} rats. Our findings in preclinical rodent models of ALS may have implications for treatment of neurodegenerative disease in general.

Amyotrophic lateral sclerosis (ALS) is a paralyzing disorder that kills individuals within 3–5 years of onset[1–3]. Symptoms result from the degeneration of motoneurons in the spinal cord and brain stem. The disease affects healthy individuals in the midst of their life, and more than 90% of those affected lack a family history of the disorder. Many individuals with ALS first notice muscle weakness in their limbs ('limb onset'), but in about 25% of affected individuals motoneurons first degenerate in motor nuclei of the brain stem ('bulbar onset'), causing dysarthria and dysphagia. Bulbar-onset ALS generally progresses faster and causes life-threatening respiratory problems sooner than limb-onset ALS, although the latter also eventually leads to bulbar symptoms.

The cause of motoneuron degeneration remains largely enigmatic[1,2]. Mutations in superoxide dismutase 1 (SOD1) cause motoneuron degeneration in humans and mice, and mice expressing human SOD1 with a Gly93Ala mutation (termed *SOD1*^{G93A} mice) have become the standard model in which to evaluate drug candidates[4]. *SOD1*^{G93A} rats develop a form of ALS that progresses even more rapidly, but they have not been widely used[5,6]. We previously reported that low levels of the angiogenic factor VEGF[7] cause ALS-like motoneuron degeneration in gene-targeted mice and increase the risk of ALS in humans[8–10]. In addition, intramuscular transfer of the *VEGF* gene delays onset and prolongs the survival of *SOD1*^{G93A} mice[11]. These observations indicate that VEGF is an attractive candidate for ALS treatment.

No effective cure for ALS is available. Halting or delaying the life-threatening degeneration of bulbar motoneurons might have the greatest clinical impact but is a formidable challenge. Because ALS results from the degeneration of motoneurons, neurotrophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), insulin-like growth factor 1 (IGF-1), glial-derived neurotrophic factor (GDNF), leukemia-inhibitory factor (LIF) and others have been evaluated for ALS therapy[12]. Gene transfer of *Gdnf*, and particularly *Igf1*, via a retrogradely axon-transported viral vector, prolongs the survival of *SOD1*^{G93A} mice[13], but the clinical utility of this therapy remains to be determined.

Delivery of recombinant neurotrophic factors offers flexible control of the dose and duration of the administered protein. So far, however, intrathecal infusion of BDNF, i.c.v. delivery of GDNF, and systemic administration of BDNF or CNTF have not resulted in substantial clinical improvement in individuals affected with ALS, although IGF-1 was found to reduce disease progression in one study but not in another[12]. In *SOD1*^{G93A} mice, LIF improves motoneuron survival without prolonging lifespan in one study[14], but had no effect in another study[15]. Unfortunately, delivery of BDNF, CNTF or IGF-1 was never evaluated in *SOD1*^{G93A} mice, whereas GDNF was administered only to healthy

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Table 1 Distribution of ^{125}I-VEGF after i.c.v. injection[a]

Time after i.c.v. injection of ^{125}I-VEGF (h)	^{125}I-VEGF recovered cpm (%)[b]	Percentage of ^{125}I-VEGF recovered[c] CSF	Brain	Spinal cord	Peripheral organs
1	667,224 ± 61,050 (61 ± 9)	9 ± 3	70 ± 1	10 ± 3	11 ± 6
3	348,852 ± 52,449 (31 ± 4)	1.1 ± 0.5	51 ± 5	15 ± 6	33 ± 7
24	118,641 ± 42,224 (10 ± 4)	0	50 ± 11	12 ± 7	38 ± 18

[a]Distribution of ^{125}I-VEGF at three different time points after stereotactic injection of 40 ng of ^{125}I-VEGF into the left lateral ventricle. With a specific activity of 1,000–2,500 μCi/mmol, the amount of injected ^{125}I-VEGF corresponded to 1,201,550 ± 48,62 cpm. [b]Data are the mean ± s.e.m. counts for each time point (n = 3); the percentage is given in parentheses. [c]The relative proportion of the total amount of ^{125}I-VEGF recovered in CSF, brain, spinal cord and peripheral organs is given as a mean ± s.e.m. percentage.

animals[16]. At least part of the failure can be ascribed to the short half-life, immunogenicity, undesired toxicity, limited ability to cross the blood-brain barrier after systemic delivery, and insufficient penetration from the cerebrospinal fluid (CSF) into the spinal parenchyma of these proteins after their central delivery[17–19].

Here we report that i.c.v. delivery of recombinant Vegf is particularly effective in prolonging the survival of rats suffering with the most life-threatening form of ALS caused by the degeneration of bulbar and cervical motoneurons.

RESULTS

i.c.v. delivery of Vegf

We first determined the optimal route, dose and duration of administration of Vegf. On systemic delivery, Vegf caused an immune response, was trapped by circulating soluble Flt1 (the extracellular ligand-binding domain of Vegf receptor-1) and did not cross the blood-brain barrier (data not shown). We therefore explored whether Vegf could be delivered via an i.c.v. route, which we favored over intrathecal delivery because it results in higher levels of neurotrophin in bulbar than in lumbar regions[19] and might therefore have a greater effect on bulbar and cervical motoneurons, which control vital functions such as swallowing and respiration. For i.c.v. delivery of Vegf, we stereotactically positioned a catheter, connected to a subcutaneously implanted osmotic minipump, in the lateral ventricle of *SOD1*^{G93A} rats. The Vegf^{164} isoform, which shows optimal biological properties in terms of stimulating motoneuron survival[8], was used throughout our studies in rats. Recombinant rat Vegf^{164} was expressed in *Pichia pastoris* and purified to near homogeneity (data not shown).

To study the distribution of VEGF after i.c.v. delivery, a bolus of 10^6 cpm of VEGF labeled with ^{125}I (^{125}I-VEGF) was stereotactically injected into the left lateral ventricle. After 1, 3 and 24 h, 61 ± 9%, 31 ± 4% and 10 ± 4% of the protein was recovered in the whole rat indicating that ^{125}I-VEGF was largely cleared by 24 h (**Table 1**). Quantification of subfractions in various brain regions showed that the ^{125}I-VEGF injected was cleared from the CSF within 3 h. After 1 h, up to 70% of ^{125}I-VEGF was recovered in the brain hemispheres, suggesting that after i.c.v. delivery VEGF diffuses and accumulates in the neural parenchyme (**Table 1**). Autoradiography confirmed that ^{125}I-VEGF diffused from the ventricular site of injection into the adjacent brain parenchyme (**Fig. 1a–e**).

Notably, ^{125}I-VEGF was distributed along a rostro-caudal gradient, with largest amounts at the site of injection and progressively smaller amounts from the brain stem to the lumbar spinal cord. At 1 h after injection, 74 ± 4% of ^{125}I-VEGF in the central nervous system was detected in the brain hemispheres, 12 ± 2% in the brain stem, and 7 ± 2%, 3 ± 1% and 1 ± 1% in the cervical, thoracic and lumbar spinal cord, respectively. A similar pattern was found at 3 h after i.c.v. injection (data not shown).

To determine the stability of the injected ^{125}I-VEGF, extracts from various organs were precipitated with trichloroacetic acid (TCA), and the fraction of TCA-precipitated (intact) ^{125}I-VEGF was expressed as a percentage of the total radioactivity per organ. In contrast to ^{125}I-VEGF in the plasma and urine, a substantial fraction of ^{125}I-VEGF in the CSF, brain and spinal cord remained intact for at least 3 h (**Table 2**). Electrophoresis confirmed that ^{125}I-VEGF in the CSF recovered at 3 h after injection had the expected molecular weight of intact VEGF (45 kDa), whereas ^{125}I-VEGF in the urine (**Fig. 1f**) or plasma (data not shown) was degraded.

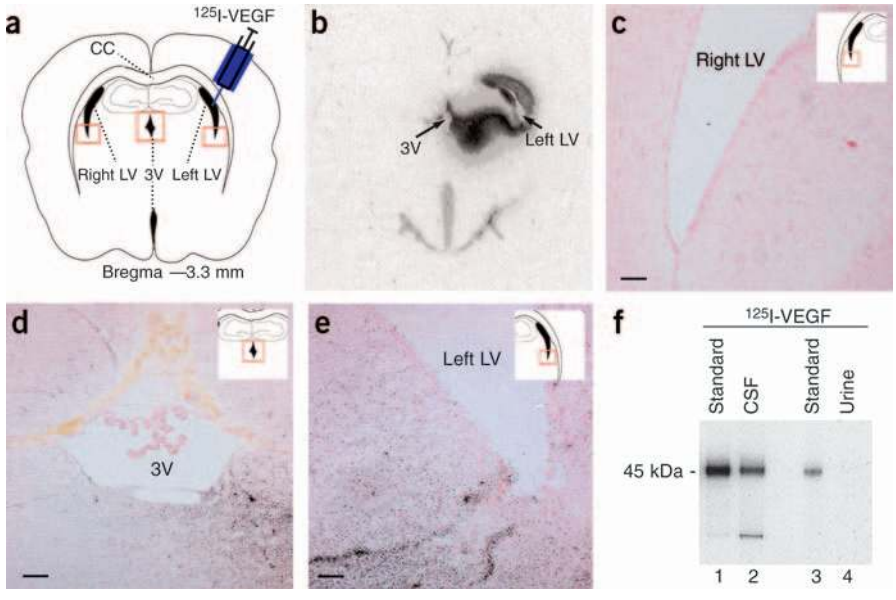

Figure 1 Distribution of i.c.v.-delivered ^{125}I-VEGF. (**a**) Injection site of ^{125}I-VEGF in the left lateral ventricle (LV). Boxes indicate the regions shown in **c–e**. 3V, third ventricle; CC, corpus callosum. (**b**) Autoradiogram of brain section 1 h after i.c.v. administration of ^{125}I-VEGF, showing penetration of ^{125}I-VEGF into the brain parenchyme adjacent to the injection site (section at –3.3 mm posterior to bregma, injection site at –0.8 mm posterior to bregma). (**c**–**e**) Micro-autoradiogram showing distribution of ^{125}I-VEGF (black grains) in the parenchyma surrounding the third (**d**) and left lateral (**e**) ventricles and, only minimally, surrounding the right lateral ventricle (**c**). Scale bars, 100 μm (**d**); 50 μm (**c**,**e**). (**f**) Autoradiogram taken after SDS-PAGE gel electrophoresis showing that ^{125}I-VEGF remains largely intact in the CSF but is degraded in the urine at 3 h after i.c.v. delivery. Lanes 1 and 3, ^{125}I-VEGF standard; lane 2, CSF (10 μl); lane 4, urine (10 μl). Equal amounts of radioactivity were loaded in lanes 1 and 2, and 3 and 4.

Table 2 Stability of ^{125}I-VEGF after i.c.v. injection

	Percentage of intact ^{125}I-VEGF[a]						
Time (h)	CSF	Cervical spinal cord	Lumbar spinal cord	Brain	Plasma	Lung	Urine
1	89 ± 2	76 ± 7	77 ± 9	80 ± 17	13 ± 5	48 ± 11	1 ± 1
3	63 ± 5	58 ± 0	61 ± 5	79 ± 3	15 ± 2	25 ± 10	7 ± 5

[a]The fraction of intact ^{125}I-VEGF (as a percentage of the total ^{125}I-VEGF) in different tissues at 1 and 3 h after stereotactic injection of 40 ng ^{125}I-VEGF into the left lateral ventricle of rats was determined by TCA precipitation. Data are the mean ± s.e.m (n = 3).

Taken together, these findings show that after i.c.v. delivery VEGF diffuses rapidly from the CSF into the neural parenchyme of the brain and spinal cord, where it remains intact for several hours. Because Vegf was cleared from the CSF within 3 h, in subsequent studies we delivered Vegf continuously via osmotic minipumps, which were replaced every 4 weeks. Up to 70–80% of the Vegf in the pumps remained active after 4 weeks (data not shown). At a dose of 0.2–0.6 μg per kg (body weight) per day, Vegf was well tolerated without inducing edema, leakage, vessel growth or an immune response, even when it was administered chronically for more than 100 d (data not shown).

Effect of Vegf in the *SOD1*$^{G93A/LSd}$ rat model of ALS

SOD1$^{G93A/LSd}$ rats (L refers to a low, 2.5-fold increase in SOD1^{G93A}, Sd to a Sprague-Dawley background) have a very rapid disease progression and die within 10 d of disease onset[6]. *SOD1*$^{G93A/LSd}$ rats showed, however, a large interlitter variability in disease onset, ranging from 95 to 145 d. To reduce this variability, we analyzed the results from *SOD1*$^{G93A/LSd}$ littermates in a paired manner (n = 17 rats analyzed in six litter pairs). As compared with the administration of control artificial CSF (aCSF), treatment of *SOD1*$^{G93A/LSd}$ rats with 0.6 μg of Vegf per kg per day starting in rats aged 60 d significantly delayed by 10 d the onset of limb paralysis, which was scored as dragging of a hindlimb or failure to use a forelimb during walking or righting (144 ± 9 d versus 134 ± 6 d; $P < 0.05$). After Vegf delivery, rats remained spontaneously active at an older age (**Supplementary Fig. 1** online). Vegf also prolonged the survival of the rats by 10 d (153 ± 8 d versus 143 ± 5 d; $P < 0.01$; **Fig. 2a**).

Thus, despite the very rapid disease progression and the large interlitter variability of disease onset in this model, Vegf treatment delayed onset, improved motor performance and prolonged the survival of *SOD1*$^{G93A/LSd}$ rats without causing adverse effects. The therapeutic effect of Vegf was specific, because i.c.v. delivery of a denatured (inactive) Vegf preparation was ineffective (**Supplementary Note 1** online). Vegf treatment did not affect spinal levels of SOD1 protein or activity (**Supplementary Note 2** online).

Disease subtype in the *SOD1*$^{G93A/HWr}$ rat model of ALS

To confirm our results in *SOD1*$^{G93A/LSd}$ rats, we also used *SOD1*$^{G93A/HSd}$ rats[5] (H refers to a high, 8.6-fold increase in SOD1^{G93A}). These rats also showed a large variability in disease onset (ranging from 102 to more than 175 d). The Sprague-Dawley strain has been reported to show a large inter-individual disease variability for other neurodegenerative phenotypes[20]. When this strain was backcrossed for five generations onto a Wistar (Wr) background, the resulting *SOD1*$^{G93A/HWr}$ rats showed earlier disease onset and a smaller variability in disease duration (data not shown). Indeed, although motor performance and motoneuron counts were normal in rats aged 50 d, by 85 d *SOD1*$^{G93A/HWr}$ rats had started to walk around their cages significantly less (**Fig. 2b**) and had lost a third of their largest lumbar motoneurons (data not shown). The survival of *SOD1*$^{G93A/HWr}$ rats varied very little (129 ± 2 d and 128 ± 2 d for *SOD1*$^{G93A/HWr}$ rats, backcrossed for three and five generations, respectively, onto the Wistar background; n = 14–39).

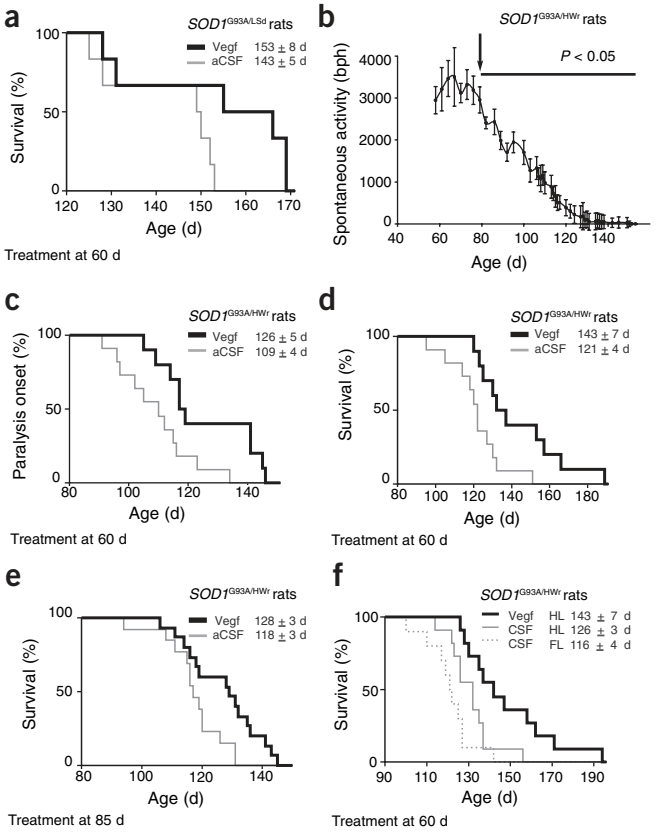

Figure 2 Effects of Vegf delivery in rat models of ALS. (**a**) Vegf prolongs the survival of *SOD1*$^{G93A/LSd}$ rats ($P < 0.05$). (**b**) Analysis of spontaneous walking activity shows that *SOD1*$^{G93A/HWr}$ rats become less active at 85 d of age. Disease onset (arrow) is defined as the age when walking activity differs significantly from the average presymptomatic activity observed between 60 and 75 d. bph, beam crossings per hour. (**c**,**d**) Vegf delivery in rats starting at 60 d of age delays the onset of paralysis by 17 d (**c**; $P = 0.014$) and increases survival by 22 d (**d**; $P = 0.008$). (**e**) Delivery of Vegf at disease onset (85 d) increases survival by 10 d ($P = 0.01$). (**f**) Rats treated with aCSF show onset in forelimbs (FL) and hindlimbs (HL), whereas those treated with Vegf show only hindlimb onset and live 27 and 17 d longer than aCSF rats with forelimb FL ($P = 0.009$) and hindlimb ($P = 0.027$) onset, respectively. **a** and **c**–**f** are Kaplan-Meier survival curves.

SOD1$^{G93A/HWr}$ rats also showed two forms of ALS symptoms. In 45% of the rats, the hindlimbs were the first to become paralyzed ('hindlimb onset' group); in the rest, the forelimbs became affected first ('forelimb onset' group). Disease symptoms occurred earlier in *SOD1*$^{G93A/HWr}$ rats in the forelimb than in the hindlimb onset group: they showed earlier onset of paralysis, lost grip strength sooner, performed more poorly on the rotarod at a younger age, and died sooner (**Table 3** and **Supplementary Fig. 1** online). Notably, *SOD1*$^{G93A/HWr}$ rats with hindlimb onset started to lose weight 3 d after limb paralysis, whereas those with forelimb onset began to lose weight 9 d before limb paralysis (**Table 3**). The weight loss that preceded the onset of limb paralysis by such a long period in rats with forelimb onset is unlikely to be due to atrophy of forelimb muscles. Humans with bulbar onset initially have swallowing difficulties and, subsequently, life-threatening respiratory problems[2,3]. The weight loss before limb disease in *SOD1*$^{G93A/HWr}$ rats with forelimb onset may thus be caused by reduced food intake owing

Table 3 Disease characteristics of *SOD1*$^{G93A/HWr}$ rats

	SOD1$^{G93A/HWr}$ rats	
Age (d)	Forelimb onset[a] ($n = 6$)	Hindlimb onset[a] ($n = 5$)
Body weight loss	99 ± 4	116 ± 4*
Loss of grip strength	102 ± 3	121 ± 4*
Paralysis	105 ± 4	113 ± 7
Loss of rotarod activity	107 ± 4	120 ± 4*
Death	113 ± 4	132 ± 5*
Disease duration[b]	8 ± 1	19 ± 3*
Δ (paralysis–weight loss)[c]	9 ± 2	–3 ± 3*

[a]Data are the mean ± s.e.m. age (in days) of the rats at which the phenotype was observed. [b]Difference (in days) between the age of death and the onset of paralysis, calculated by averaging the difference measured for each rat. [c]Difference (in days) between the onset of limb paralysis and weight loss, calculated by averaging the difference measured for each rat. *$P < 0.05$ versus forelimb group.

to dysphagia that is secondary to the bulbar and cervical motoneuron pathology. Thus, the premature loss in body weight, the earlier onset and the faster progressing disease in *SOD1*$^{G93A/HWr}$ rats with forelimb onset suggest that these rats suffered from a 'bulbar-onset-like' form of ALS.

Effect of Vegf in the *SOD1*$^{G93A/HWr}$ rat model of ALS

As compared with aCSF administration, treatment of *SOD1*$^{G93A/HWr}$ rats with i.c.v. delivery of 0.2 μg of Vegf per kg per day starting in rats aged 60 d delayed disease onset and improved motor performance and overall clinical outcome ($n = 11$–10). Vegf-treated rats had a higher body weight than control rats for a period of 37 d, namely, from day 113 to day 150 (**Supplementary Fig. 2** online), and Vegf treatment also delayed the onset of paralysis (**Fig. 2c**).

Two statistical methods (Kaplan-Meier and repeated-measures analysis of variance (ANOVA), assigning a zero value for deceased rats) were used to analyze the duration and magnitude of the effect of Vegf on spontaneous walking and rotarod performance. For spontaneous activity, Vegf-treated rats consistently performed better than controls for as long as 35–42 d, and the magnitude of the effect of Vegf on motor performance was as much as 18 d (**Supplementary Fig. 2** online). Grip strength was also significantly improved by Vegf treatment (**Supplementary Fig. 2** online). In addition, the survival of *SOD1*$^{G93A/HWr}$ rats was prolonged by 22 d after Vegf treatment (**Fig. 2d**). As a consequence, by the time that the first Vegf-treated rat died, more than half (6 out of 11) of the aCSF-treated rats had died already. Calculation of the hazard ratio by Cox regression analysis showed that the survival rate was 3.5-fold greater for Vegf-treated than for aCSF-treated rats (95% confidence interval, 1.3–9.8). *SOD1*$^{G93A/HWr}$ rats tolerated the Vegf treatment well without adverse effects.

We also evaluated whether i.c.v. delivery of Vegf would be capable of prolonging survival when initiated at the time of disease onset. Therefore, 0.2 μg of Vegf per kg per day was administered to rats aged 85 d, when the first signs of motor impairment emerge ($n = 15$ for aCSF, $n = 13$ for Vegf). Despite the rapidly progressing course of disease in this model, treatment of *SOD1*$^{G93A/HWr}$ rats with i.c.v.-delivered Vegf significantly improved survival by 10 d (**Fig. 2e**). As might be expected for a treatment that was initiated only at the onset of paralysis, Vegf treatment did not significantly delay disease onset, although there was a positive trend towards a delay: aCSF-treated rats developed paralysis at 108 ± 3 d, whereas Vegf-treated rats developed disease at 115 ± 2 d ($P = 0.045$, one-sided t-test). Vegf treatment also prolonged disease duration, as defined as the period between onset of paralysis and death (9 ± 1 d for aCSF versus 13 ± 1 d for Vegf; $P < 0.05$). Taken together, these findings show that i.c.v. delivery of Vegf prolongs the survival of *SOD1*$^{G93A/HWr}$ rats when treatment is initiated both before and at the onset of disease.

Effect of i.c.v. delivery of Vegf on the ALS disease subtype

Vegf treatment in rats aged 60 d also changed the disease subtype from a severe to a much milder form. Indeed, significantly fewer *SOD1*$^{G93A/HWr}$ rats suffered with the severe forelimb-onset type of disease after Vegf than after aCSF delivery (1 of 10 Vegf-treated rats versus 12 of 24 aCSF-treated rats showed forelimb onset; $P < 0.05$). As compared with aCSF-treated rats with hindlimb or forelimb onset, Vegf-treated rats survived 17 or 27 d longer, respectively ($P < 0.05$ or $P < 0.001$; **Fig. 2f**). This finding is relevant because an involvement of brain stem and cervical motoneuron disease results in a worse prognosis in rats (see above) and humans[1–3].

When started at 85 d, i.c.v. delivery of Vegf did not significantly alter the disease subtype, although there was a clear tendency towards change (4 of 15 Vegf-treated rats versus 12 of 24 aCSF rats showed forelimb onset; $P = 0.07$, one-sided t-test), probably because the Vegf treatment was initiated only when some rats were already affected by forelimb disease. The more pronounced therapeutic effect of Vegf on forelimb than on hindlimb muscles is probably due to higher levels of Vegf in the bulbar and cervical spinal cord than in the lumbar spinal cord after i.c.v. delivery (see above).

Vegf prolongs motoneuron survival in *SOD1*^{G93A} rats

We used stereology to quantify the numbers and volumes of motoneurons in the whole facial nucleus on Nissl-stained sections of the

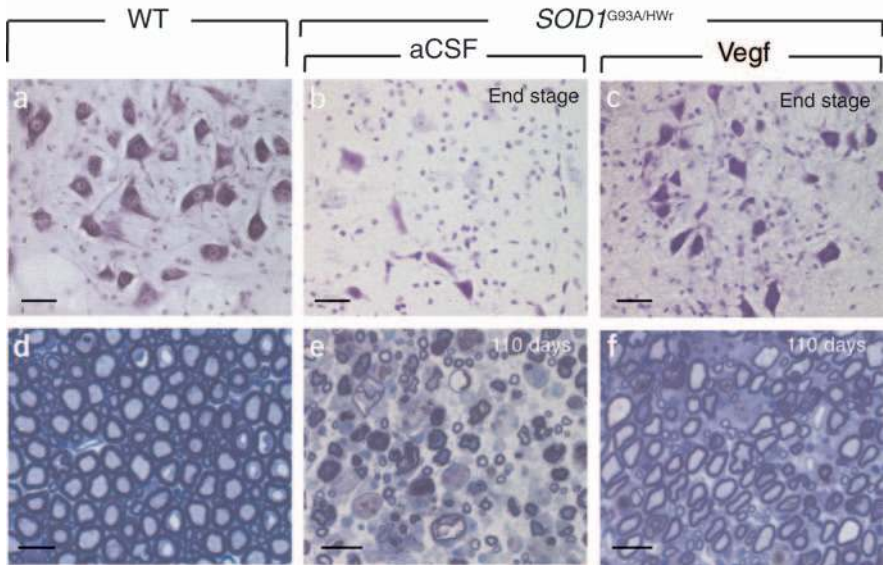

Figure 3 Effects of Vegf on motoneuron degeneration and axonal loss. (**a–c**) Nissl staining in the facial nucleus shows that, in comparison to healthy age-matched wild-type (WT) rats (**a**), terminal aCSF-treated *SOD1*$^{G93A/HWr}$ rats (**b**) show more severe motoneuron loss than do terminal Vegf-treated *SOD1*$^{G93A/HWr}$ rats (**c**). (**d–f**) Toluidine blue staining of L5 ventral roots from wild-type (**d**), aCSF-treated (**e**) and Vegf-treated (**f**) *SOD1*$^{G93A/Hwr}$ rats shows that aCSF-treated rats have more severe axonal loss than Vegf-treated rats. Scale bars, 50 μm (**a–c**); 25 μm (**d–f**).

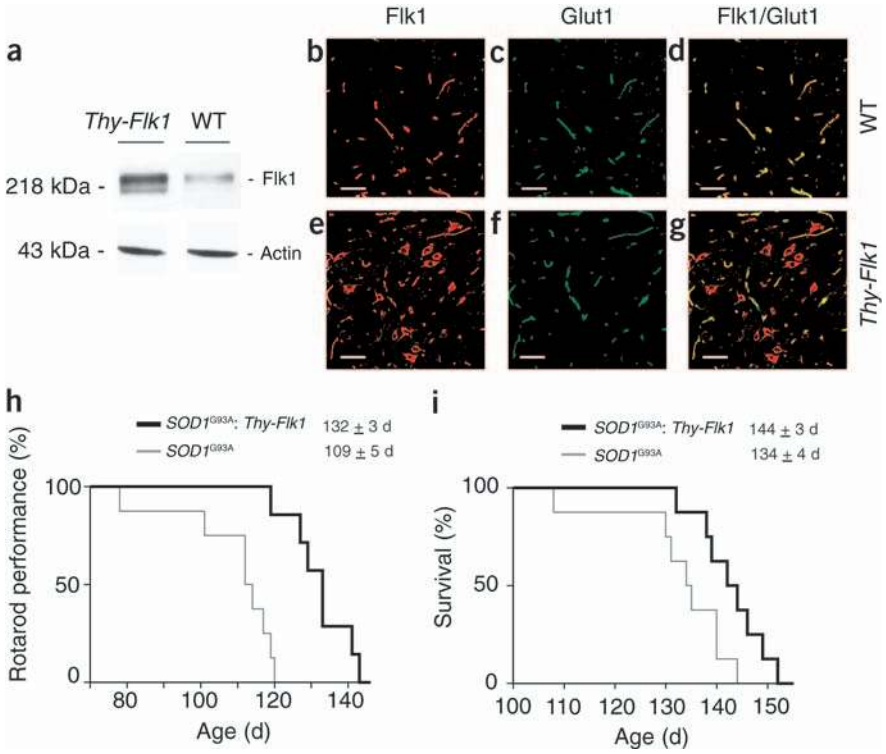

Figure 4 Overexpression of *Flk1* in motoneurons improves the motor performance and increases the lifespan of *SOD1*^{G93A} mice. (**a**) Immunoblot analysis shows that Flk1 is more abundant in the spinal cord of *Thy-Flk1* transgenic mice than in that of wild-type (WT) mice. (**b–g**) Double immunostaining for Flk1 and Glut1 shows that *Flk1* is expressed in blood vessels in the spinal cord of control mice (WT; **b–d**), but in both blood vessels and motoneurons in *Thy-Flk1* mice (**e–g**). Scale bars, 50 μm. (**h**) Neuronal expression of a *Flk1* transgene delays rotarod failure of *SOD1*^{G93A} mice by 23 d ($P = 0.004$). (**i**) Neuronal expression of *Flk1* prolongs the survival of *SOD1*^{G93A} mice by 10 d ($P = 0.033$).

brain stem of terminal *SOD1*$^{G93A/HWr}$ rats (Methods and **Supplementary Table 1** online). The facial nucleus was analyzed because it is affected in mouse models of ALS. Preterminal Vegf-treated rats had twice as many motoneurons as did aCSF-treated control rats (6,730 ± 472 motoneurons per facial nucleus after Vegf treatment versus 3,310 ± 299 after aCSF treatment; $n = 4$, $P < 0.001$; **Fig. 3a–c**), and the number of residual motoneurons in Vegf-treated rats was only slightly lower than in wild-type rats (7,726 ± 34; $n = 4$, $P = 0.13$). Vegf treatment had no effect on the perikaryal volume of motoneurons (data not shown).

Stereology was also used to count the number of healthy motoneurons in the cervical spinal cord of Vegf- and aCSF-treated *SOD1*$^{G93A/HWr}$ rats at 110 d. Sections (20-μm thick) of the whole cervical spinal cord were stained with Nissl, and motoneurons were counted in a defined spinal cord region of interest (Methods). Because large motoneurons preferentially degenerate in ALS, we counted the number of α-motoneurons with a perikaryal projection area of 300 μm^2 or more. Vegf treatment preserved these large α-motoneurons in the cervical spinal cord (9,160 ± 952 after Vegf treatment versus 5,320 ± 679 after aCSF treatment; $n = 3$, $P < 0.05$). Similar results were obtained when all α-motoneurons, irrespective of their size, were counted (data not shown). In addition, in L5 ventral roots of *SOD1*$^{G93A/HWr}$ rats at 110 d, more motor axons remained intact after i.c.v. delivery of VEGF (700 ± 147 axons per ventral root after Vegf treatment versus 390 ± 78 after aCSF treatment; $n = 5$, $P < 0.05$; **Fig. 3d–f**).

These results show that i.c.v. treatment of Vegf delays motoneuron degeneration in the brain stem and cervical and lumbar spinal cord in rats with ALS. Further histological analysis showed no changes in vessel density, size or leakage in the brain stem and spinal cord (data not shown), thus confirming that Vegf treatment is well tolerated by rats with ALS.

Molecular mechanism of the neuroprotective effect of Vegf

We further explored the neuroprotective mechanisms of Vegf. *In vitro* studies have indicated that Vegf protects motoneurons against stress-induced cell death by binding Vegf receptor-2 (also known as Flk1)[8], but a direct neurotrophic activity of Vegf *in vivo* has not been shown. To address the latter issue, we generated transgenic mice by using the *Thy1.2* expression cassette to drive expression of murine *Flk1* in postnatal neurons[21]. As compared with non-transgenic littermates, *Thy-Flk1* mice expressed more *Flk1* mRNA transcripts (466 ± 54 versus 56 ± 9 copies of *Flk1* per 10^3 copies of *Hprt*; $n = 5$, $P < 0.05$) and protein (**Fig. 4a**). Double immunolabeling showed that Flk1 was detectable only in Glut1-positive blood vessels in control mice, but in both blood vessels and neurons in *Thy-Flk1* mice (**Fig. 4b–g**). The *Thy-Flk1* mice were healthy and were intercrossed with *SOD1*^{G93A} mice. Levels of Vegf in the spinal cord were comparable in mice of both genotypes aged 60 d (9.4 ± 1.0 and 11 ± 1.4 pg of Vegf per mg of protein in *SOD1*^{G93A} mice and *Thy-Flk1*:*SOD1*^{G93A} mice, respectively; $n = 5$, not significant).

Neuronal overexpression of *Flk1* in *SOD1*^{G93A} mice delayed the onset of motor impairment by 23 d ($n = 8$, $P < 0.001$; **Fig. 4h**), and *Thy-Flk1*:*SOD1*^{G93A} mice performed better than *SOD1*^{G93A} mice for 26 d ($P < 0.01$; **Supplementary Fig. 3** online). In addition, *Thy-Flk1*:*SOD1*^{G93A} mice survived for 10 d longer than their *SOD1*^{G93A} littermates ($P < 0.05$; **Fig. 4i**). Thus, although survival was prolonged, the disease course was accelerated in *Thy-Flk1*:*SOD1*^{G93A} mice, possibly because the neuroprotective effect of Flk1 is more pronounced during the initial stages of the disease. Using stereology, we also found that fewer large α-motoneurons with a perikaryal projection area of more than 200 μm^2 had degenerated in the lumbar spinal cord of *SOD1*^{G93A}:*Thy-Flk1* mice at 110 d (2,150 ± 194 in *Thy-Flk1*:*SOD1*^{G93A} mice versus 1,210 ± 114 in *SOD1*^{G93A} mice; $n = 4$, $P < 0.01$). Similar results were obtained when all α-motoneurons, irrespective of their size, were counted (data not shown). These results show that overexpression of *Flk1* in motoneurons delays the degeneration of spinal motoneurons in *SOD1*^{G93A} mice by transmitting survival signals of endogenous Vegf.

Anterograde axonal transport of Vegf

Muscle weakness and paralysis develop when axons degenerate and neuromuscular junctions disintegrate. Thus, therapies that target axons and neuromuscular junctions, in addition to motoneuron cell bodies, offer extra benefit. We therefore examined whether Vegf is axonally transported after ligation of the sciatic nerve[22]. Immunostaining of longitudinal sciatic nerve sections at 6 and 20 h after nerve ligation showed that Vegf accumulated in the nerve segments that were distal (closest to the muscle) and proximal (closest to the spinal cord) to the ligation,

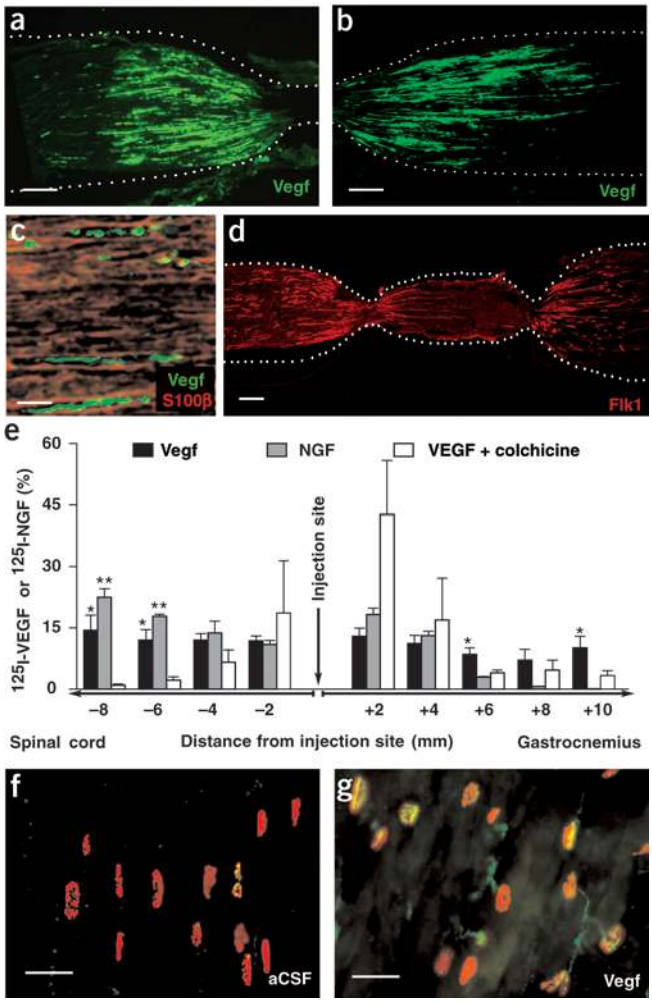

Figure 5 Axonal transport of Vegf and effects on neuromuscular junctions. (**a–c**) Immunostaining of endogenous Vegf (**a**,**b**) and endogenous Vegf and S100β (**c**) on longitudinal sections of the rat sciatic nerve located proximal (**a**,**c**) and distal (**b**) to the ligation site at 20 h after ligation. (**d**) Immunohistochemical staining of endogenous Flk1 on a longitudinal section of the rat sciatic nerve located proximal and distal to the ligation site at 20 h after ligation. (**e**) Axonal transport of ^{125}I-VEGF or ^{125}I-NGF at 6 h after their injection into the rat sciatic nerve. Data (mean ± s.e.m.) show the amount of radioactivity (as a percentage of the total amount retrieved), counted in 2-mm-wide nerve segments located proximal and distal to the injection site. ^{125}I-NGF is transported retrogradely, whereas ^{125}I-VEGF is transported both anterogradely and retrogradely. Coinjection with colchicine (green) blocks transport of ^{125}I-VEGF, resulting in an accumulation of ^{125}I-VEGF at the injection site. $^{*}P < 0.05$ versus ^{125}I-VEGF plus colchicine; $^{**}P < 0.05$ versus ^{125}I-NGF in segments +6 and +8. (**f**,**g**) Double staining of skeletal muscle sections with bungarotoxin (red) and anti-synaptophysin (green) to label endplates and axon terminals, respectively. Innervated endplates are yellow, denervated endplates are red. More endplates remain innervated in Vegf-treated rats (**g**) than in aCSF-treated rats (**f**). Scale bars, 200 μm (**a**,**b**,**d**); 50 μm (**f**,**g**); 25 μm (**c**).

indicating that Vegf was transported both retrogradely and anterogradely (**Fig. 5a,b**). Single and double labeling for Vegf, the Schwann cell marker S100β and the axon-marker neurofilament 200 (Nf-200) showed that Vegf was present in axons (**Fig. 5c** and **Supplementary Fig. 3** online).

Relative to the proximal ligature site, Vegf had accumulated over a distance of 420 ± 12 μm and 900 ± 78 μm after 6 and 20 h, respectively ($n = 3$). Accumulation of Vegf at these sites was specific, because immunoreactive Vegf was undetectable in nerve segments at sites further from the ligation (ventral roots at L5), in unligated control nerves or in ligated nerves injected with colchicine, an inhibitor of axonal transport (data not shown). Enzyme-linked immunosorbent assay (ELISA) showed that sciatic nerve Vegf levels were low in the unligated control nerve (2 pg of Vegf per mg of protein), but had increased to 190 and 120 pg of Vegf per mg of protein in the proximal and distal nerve segments, respectively, at 20 h after double ligation. Similar to other neurotrophic factors[23–25], Vegf seemed to be cotransported with its receptor. Indeed, Flk1 accumulated both proximally and distally to the ligatures in the sciatic nerve, indicating that Flk1 was also transported anterogradely and retrogradely in axons (**Fig. 5d** and **Supplementary Fig. 3** online). Flk1 was undetectable in unligated nerves or in L5 ventral roots of ligated nerves (data not shown).

We corroborated these immunohistochemical results by analyzing the axonal transport of ^{125}I-VEGF injected into an unligated sciatic nerve[22]. At 6 h after intraneural injection, ^{125}I-VEGF had accumulated significantly in nerve segments that were proximal and distal to the injection site, indicative of both anterograde and retrograde transport (**Fig. 5e**). Axonal transport of VEGF was specific, because coinjection of the axon transport blocker colchicine (25 μg) blocked anterograde and retrograde transport of ^{125}I-VEGF; in addition, injection of ^{125}I-labeled nerve growth factor (NGF), which is known to be transported primarily retrogradely[22], resulted in a predominant accumulation of ^{125}I-NGF in the proximal nerve segment. Taken together, these results show that VEGF and Flk1 are axonally transported in both the anterograde and the retrograde direction.

Vegf preserves neuromuscular junctions in *SOD1*^{G93A} rats

We next analyzed whether ^{125}I-VEGF, after i.c.v. delivery, was detectable in the hypoglossal or sciatic nerve at the site of ligation because its presence in this region would suggest that the protein diffuses from the CSF to motoneurons in the brain stem and lumbar spinal cord and is then transported anterogradely within these axons. At 20 h after i.c.v. injection of 10^6 cpm of ^{125}I-VEGF, levels of ^{125}I-VEGF were more than twofold higher in ligated than in unligated nerve segments (94 ± 8 and 212 ± 4 cpm in the ligated versus 34 ± 14 and 82 ± 8 cpm in the unligated hypoglossal and sciatic nerve, respectively; $n = 3$, $P < 0.05$ and 0.001).

Because these findings suggested that Vegf transported anterogradely after i.c.v. delivery might affect neuromuscular junctions, we analyzed whether i.c.v. delivery of Vegf affected the innervation of endplates in *SOD1*$^{G93A/HWr}$ rats aged 110 d, the stage at which aCSF-treated rats became paralyzed. Gastrocnemius and biceps muscles from rats with hindlimb or forelimb onset, respectively, were stained with tetramethylrhodamine isothiocyanate (TRITC)-bungarotoxin (red) and rabbit antibodies to synaptophysin (green) to label the endplates and axon terminals, respectively. In this assay, 'yellow' double-labeled end-plates are innervated, whereas 'red' endplates are denervated. Only 12 ± 7% of the endplates were innervated after aCSF treatment, whereas 25 ± 9% of the endplates were innervated in Vegf-treated rats ($n = 5$, $P < 0.05$; **Fig. 5f**,**g**). Thus, Vegf treatment preserves neuromuscular junctions in a preclinical rat model of ALS.

DISCUSSION

The most significant finding of this study is that i.c.v. delivery of Vegf delays disease onset, ameliorates motor performance and prolongs survival in two rat models of ALS. To our knowledge, this is the first preclinical study to show that i.c.v. delivery of a neuroprotective factor has a therapeutic effect on ALS. Several neurotrophins have been evaluated in individuals affected with ALS and ALS mouse models, but none has consistently yielded a substantial benefit[12]. Why, then, is Vegf effective?

One explanation relates to the fact that Vegf itself has biological activities that differ substantially from those of other neurotrophic factors. Over- or underexpression studies have shown that *Bdnf*, *Gdnf*, *Cntf*, *Lif* or *Igf1* affect motoneurons during development and when motoneurons are injured, after birth[17,19,26–28]. Gene transfer of some of these factors even stimulates motoneuron survival in mouse models of ALS[13]; however, loss of these neurotrophins does not cause adult-onset ALS-like motoneuron degeneration with clinical signs of paralysis in mice[26,27,29,30], and only limited human genetic evidence supports a role for these factors in human ALS[29,31], raising the issue of whether they participate significantly in the pathogenesis of chronic motoneuron degeneration. By contrast, genetic studies in mice and humans have implicated VEGF in motoneuron degeneration[8,9], and intramuscular transfer of the *VEGF* gene prolongs survival in mouse models of ALS[11]. In addition, we have shown here that overexpression of a Vegf receptor in postnatal neurons prolongs the survival of motoneurons *in vivo*. Apart from these neural effects, Vegf may also have vascular effects and stimulate perfusion, which would further promote motoneuron survival. Thus, the pleiotropic biological profile of Vegf might contribute to its overall beneficial effect.

Another explanation for the therapeutic effect of Vegf observed here might relate to its route of administration, that is, its continuous and direct infusion into the CSF. When delivered systemically, Vegf is immunogenic, trapped by soluble Flt1, and not likely transported across the blood-brain barrier. By contrast, our data show that VEGF rapidly diffuses from the CSF into the spinal cord parenchyma, reaches the spinal motoneurons and is transported anterogradely in peripheral axons. Notably, VEGF remains intact for several hours in the neural parenchyme. The close correlation between the distribution of Vegf (higher in brain stem than in lumbar spinal cord) and its therapeutic effect (shifting the disease type from forelimb/bulbar to hindlimb/lumbar onset) suggests that Vegf induces its greatest effect at sites where its levels are highest. Because Vegf was rapidly cleared from the central nervous system (similar to other neurotrophins[19]), the continuous delivery of Vegf also may have contributed to its beneficial effect.

Although little is known about axonal transport of Vegf and its receptor Flk1, anterograde axonal transport of endogenous Vegf may be involved in maintaining the integrity and function of the neuromuscular junctions. Axonal transport of exogenously administered VEGF may have similar effects. Our findings suggest that endogenous Vegf and Flk1 are indeed transported anterogradely and that after i.c.v. injection ^{125}I-VEGF is also transported anterogradely. Notably, i.c.v. delivery of Vegf also preserves the innervation of endplates. It remains to be determined whether this results from an effect of Vegf on the motoneuron cell body and/or an effect of anterogradely transported Vegf on the neuromuscular junction.

When motoneurons in the brain stem and cervical spinal cord degenerate, vital functions such as swallowing and respiration are affected. Thus, a treatment that is particularly effective in prolonging the survival of these motoneurons would be beneficial not only for individuals with bulbar-onset ALS but also for those with limb onset at more advanced stages. This goal, however, is challenging to achieve with gene therapy. An advantage of i.c.v. delivery of Vegf is that the protein is better able to reach the bulbar and cervical motoneurons than the lumbar motoneurons. Because i.c.v. delivery of Vegf stimulated motoneuron survival in the facial nucleus and cervical spinal cord, other brain stem nuclei may be also preserved, a possibility that is likely to be relevant for bulbar nuclei in humans. Our observations that Vegf has a larger effect on the disease subtype with forelimb onset than on that with hindlimb onset imply that Vegf can affect the most severe type of motoneuron degeneration.

What are the medical implications of our findings? First, i.c.v. delivery of Vegf prolongs survival in a preclinical rodent model of a severe form of ALS; this result may revive the therapeutic use of a neuroprotective growth factor such as VEGF after the failure of previous clinical trials[12]. Second, use of recombinant VEGF facilitates control of the dose, duration and frequency of delivery; this possibility provides an attractive clinical option and may be relevant for tailoring VEGF treatment, including dosage, to the individual patient. Third, the correlation between the spatial gradient of Vegf levels and the therapeutic benefit on the disease subtype indicates that Vegf acts in relative proximity to its injection site; thus, individuals suffering from ALS with bulbar onset would benefit more from i.c.v. delivery of Vegf, whereas those with lumbar onset might benefit more from an intrathecal infusion of Vegf at the lumbar level.

Fourth, chronic i.c.v. delivery has been applied previously; the discomfort of a surgical intervention to position the pump may be outweighed by the advantages of controlling the dose and duration of Vegf delivery and the lack of systemic side-effects and immune response. Fifth, because genetic data indicate that low levels of VEGF affect both sporadic and familial ALS[9], VEGF administration may be also effective in sporadic cases of ALS; indeed, VEGF has survival effects on various types of neurons, regardless of the sort of stress (hypoxia, excitotoxicity, serum deprivation or mutant SOD1-related toxicity among others) encountered and is thus an attractive candidate[10]. Last, Vegf was effective at a dose of 0.2 μg per kg per d and toxic only above 2 μg per kg per d, yielding a satisfactory therapeutic safety window; in short, Vegf therapy seems to be well tolerated for protracted time periods without causing vascular side-effects when used at a dose that is at least fivefold lower than that needed to stimulate angiogenesis in ischemic tissues[32].

In summary, our data indicate that VEGF may assist in slowing down motoneuron degeneration and warrant further studies to clarify the relevance of VEGF delivery for the ALS community.

METHODS

Biodistribution of VEGF. Human ^{125}I-VEGF^{165} (Amersham Pharmacia) was injected into the left lateral ventricle of Wistar rats. After i.c.v. injection, organs were weighed and the radioactivity was measured. We used cryosections of the brain and spinal cord for autoradiography.

Rodents. Rats expressing high levels of human *SOD1*^{G93A} (line 26H in a Sprague-Dawley background) were provided by D. Howland[5] and back-crossed for five generations onto the HAN Wistar background (Charles River). Sprague-Dawley rats expressing lower levels of human *SOD1*^{G93A} (line G93A-39) were provided by Y. Itoyama[6]. *Thy-Flk1* mice, expressing a murine *Flk1* transgene in postnatal neurons, were generated by pronuclear injection of a mouse *Thy1.2* expression cassette[33] and were intercrossed with human *SOD1*^{G93A} mice that had been back-crossed for more than ten generations onto a FvB background (provided by C. Kunst). All procedures involving experimental rodents were approved by the Institutional Animal Care and Research Advisory Committee of the KU Leuven, Belgium (P02047 and P02046).

Surgical procedures. Recombinant rat Vegf^{164} (expressed using *Pichia pastoris* and purified on heparin-agarose columns) was infused into the left lateral ventricle of the rats by using Alzet osmotic pumps connected with a catheter to a brain infusion cannula. Minipumps, delivering Vegf^{164} at a rate of 0.25 μl/h, were replaced every 28 d.

Behavioral analysis. Three times a week, an investigator who was blind to the treatment measured motor performance. Measurements were started in rats aged 60 d to obtain baseline values before disease onset at 85 d. A rat-specific rotarod (Ugo Basile), constantly rotating at a speed of 15 rotations per minute, was used to score rats for their ability to stay on the rotarod during a 3-min period. Rats were judged to fail when, on average in five separate trials, they fell

off the rotarod before 2 min. Paralysis of the hindlimb was scored when the rat dragged one of its hindlimbs, and paralysis of a forelimb was scored when the rat failed to use its forelimb for walking or righting. In previous studies, failure of the rat to right itself after being turned on either side for a maximum of 30 s was scored as 'clinical death'[5]. Our initial experiments showed, however, that *SOD1*^{G93A} rats that were unable to right themselves could survive for several days. We therefore scored the time of death as the day when the rats had lost 40% of their presymptomatic body weight, because experiments indicated that the rats died 24–36 h after this point.

Histology and stereology. Perfusion-fixed spinal cord and brain were embedded in paraffin, and serial sections were stained using antibodies to Smi32, Smi31, Gfap, Cd68 or Glut1. Glut1-positive vessels were counted on five equally spaced sections over a distance of 350 μm and are expressed as vessels/mm^2. Gfap- and Cd68-positive areas were measured as a ratio of the total area. Quantification of intact L5 ventral root axons was done on semi-thin cross-sections stained with toluidine blue. Motoneurons were counted using high-precision design-based stereology (see **Supplementary Methods** online).

Transport and neuromuscular junctions. Ligated nerves[22] were stained for Vegf, Flk1, S100β and neurofilament-200. A Quantikine rat Vegf ELISA kit (R&D Systems) was used to measure Vegf. To evaluate axonal transport of exogenously applied factors, we injected 1.25 μCi of human ^{125}I-VEGF or ^{125}I-NGF (1,000–2,500 μCi/mmol; Amersham) into the sciatic nerve. The ipsilateral and contralateral sciatic nerves were cut into 2-mm segments and the radioactivity was counted 6 h after the injection. To study neuromuscular junctions, the gastrocnemius and biceps muscles of rats were fixed and stained with TRITC-bungarotoxin (diluted 1: 200; Molecular Probes) and rabbit anti-synaptophysin antibodies (1:100; DAKO).

Statistics. We used SPSS v.10 for statistical calculations. Cumulative survival statistics of paralysis, spontaneous activity, dynamometer, rotarod and survival were calculated by using Kaplan-Meier statistics. Log-rank *P* values were calculated to compare survival curves. Spontaneous activity, rotarod performance, grip strength and body weight data were analyzed by using repeated-measures ANOVA. For body weight analysis, deceased rats were assigned their last-observed value, that is, 40% of their original body weight, for the remaining period of the analysis. To analyze rotarod performance, grip strength and spontaneous activity, we assigned a zero value to deceased rats.

Cox regression analysis was used to calculate the hazard risk after Vegf treatment. Data obtained with the *SOD1*$^{G93A/LSd}$ rats were analyzed using an ANOVA-paired *t*-test. When testing the effect of Vegf, we used 17 *SOD1*$^{G93A/LSd}$ rats from six different litters, of which 8 rats received Vegf and 9 rats received aCSF. When two rats from a single litter received the same treatment, the values obtained with these rats were averaged and analyzed by a paired *t*-test. All data are reported as the mean ± s.e.m. Additional details of the methods used in this study are given in the **Supplementary Methods** online.

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

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Neuroscience* website for details).

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