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The neurite outgrowth inhibitor Nogo-A promotes denervation in an amyotrophic lateral sclerosis model

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Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by motor neuron loss and muscle wasting. In muscles of ALS patients, Nogo-A—a protein known to inhibit axon regeneration—is ectopically expressed at levels that correlate with the severity of the clinical symptoms. We now show that the genetic ablation of Nogo-A extends survival and reduces muscle denervation in a mouse model of ALS. In turn, overexpression of Nogo-A in wild-type muscle fibres leads to shrinkage of the postsynapse and retraction of the presynaptic motor ending. This suggests that the expression of Nogo-A occurring early in ALS skeletal muscle could cause repulsion and destabilization of the motor nerve terminals, and subsequent dying back of the axons and motor neurons.

Keywords: amyotrophic lateral sclerosis; G86R mouse; neuromuscular junction; Nogo-A; skeletal muscle

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult human motor neuron disease and causes motor neuron degeneration, progressive skeletal muscle atrophy, paralysis and death within 1–5 years of onset. Although most of the cases occur sporadically, approximately 10% of patients show an autosomal dominant pattern of inheritance and 10–20% of these are associated with missense mutations in the gene encoding Cu/Zn-superoxide dismutase 1 (SOD1), one of the main free radical scavenging enzymes that protects cells against oxidative stress (Bruijn *et al*, 2004). The nature of the selective degeneration of motor neurons in ALS still remains elusive, but several studies postulate that the disease might begin at the distal axon and induce pathological changes earlier in the muscle than in the spinal cord (Frey *et al*, 2000; Fischer *et al*, 2004; Pun *et al*, 2006); however, the contribution of skeletal muscle fibres to initiating such a 'dying back' process has been largely undervalued.

Nogo-A was first identified as a high-molecular-weight membrane protein of spinal cord myelin, in part responsible for the non-permissive properties of the white matter in the central nervous system (Caroni & Schwab, 1988). At the molecular level, three distinct regions of Nogo-A have been shown to trigger neurite growth inhibition, growth cone collapse or inhibition of fibroblast spreading, which makes Nogo-A a key factor in restricting regeneration and repair of injured axons (Prinjha *et al*, 2000; GrandPre *et al*, 2002; Oertle *et al*, 2003). *In vivo* inactivation of Nogo-A by neutralizing antibodies or blockade of some of the components of its cognate signalling pathway leads to enhanced regeneration and improved functional recovery after spinal cord lesion (Li *et al*, 2004; Liebscher *et al*, 2005).

Previously, we have shown that the skeletal muscles of ALS-linked mutant Sod1 mice and patients with sporadic ALS express high amounts of Nogo-A (Dupuis *et al*, 2002). In addition, the levels of muscle Nogo-A in ALS patients are higher in atrophied slow-twitch fibres and correlate with the severity of motor impairment (Jokic *et al*, 2005). To extend our understanding of these findings, we investigated the effects of knocking out *Nogo-A* in ALS mice and evaluated the morphological changes at the neuromuscular junction (NMJ) after overexpressing Nogo-A in non-pathological muscle.

RESULTS

Genetic ablation of Nogo-A delays disease in ALS mice

We first determined Nogo-A expression during ALS pathology in *Sod1(G86R)* mice, a transgenic model that recapitulates many of the characteristics of the human disease (Ripps *et al*, 1995). In the soleus muscle, which in mice consists of slow-twitch fibres, Nogo-A upregulation occurred early in presymptomatic animals

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and persisted after overt hindlimb paralysis (supplementary Fig 1A–C online). This expression accompanied the progressive decrease in muscle fibre size, also noticeable before the onset of apparent motor problems (supplementary Fig 1D online). To analyse the consequences of Nogo-A upregulation, we crossbred *Sod1(G86R)* mice with *Nogo-A*-knockout animals (Simonen *et al*, 2003). We checked that the soleus muscle of *G86R/Nogo-A*$^{-/-}$ mice did not contain Nogo-A and assessed that Nogo-A ablation did not affect the expression of mutant Sod1 at both the messenger RNA and protein levels (supplementary Fig 2 online). We then recorded the survival of double transgenic mice and analysed several parameters that are characteristic of ALS pathology in symptomatic animals at comparable disease stages. This allowed us to recognize true protection effects rather than the age-related delay of symptoms. The mean survival times were 167 ± 11.2 days for *G86R/Nogo-A*$^{-/-}$ mice ($n = 16$) and 142 ± 6.4 days for the control littermates ($n = 17$; $P = 0.0456$, Mann–Whitney test), which accounts for a moderate but significant increase in lifespan as illustrated by the Kaplan–Meier curve representation (Fig 1A). Although the average levels of mutant Sod1 protein were almost identical in *G86R/Nogo-A*$^{+/+}$ and *G86R/Nogo-A*$^{-/-}$ mice (supplementary Fig 2G online), some interindividual variability could be observed (supplementary Fig 2F online). To rule out the possibility that Nogo-A ablation might promote lower mutant Sod1 expression and hence better survival, we analysed the influence of mutant Sod1 protein level variability on survival rates (supplementary Fig 4 online). Correlation analyses between these two parameters showed no significant differences, as determined by Spearman's correlation test ($P = 0.4348$, $n = 11$, *G86R/Nogo-A*$^{+/+}$ mice; $P = 0.1689$, $n = 12$, *G86R/Nogo-A*$^{-/-}$ mice). In addition, analysis of covariance was carried out in the *G86R/Nogo-A*-knockout group of animals, using survival as the dependent variable and mutant Sod1 protein levels as the explanatory variable. The probability corresponding to the *F* value (calculated by Fisher's *F* test) was 0.234 ($n = 12$ mice), which means that the interindividual differences in the expression of mutant Sod1 have no significant influence on the survival of the animal.

To determine the number of large motor neurons in the lumbar spinal cord, we stained sections with toluidine blue and classified cells according to their cross-sectional area. In earlier studies (supplementary Fig 3 online), we found that cells with a cross-sectional area greater than 600 μm^2 correspond to cells positive for the specific motor neuronal marker choline acetyltransferase, which corroborates our toluidine-blue-based estimates. In diseased *Sod1(G86R)* mice, we found a higher number of motor neurons in *Nogo-A*$^{-/-}$ mice than in *Nogo-A*$^{+/+}$ mice (Fig 1B). Furthermore, ubiquitin inclusions in the motor neuron cell bodies, which is a marker of cell stress typically detected in diseased animals, could not be seen in the double transgenic mice (Fig 1C). At the NMJ level, the increased expression of the acetylcholine receptor α-subunit mRNA—a typical marker of denervation in neuromuscular diseases (Duclert & Changeux, 1995)—was prevented in part in *G86R/Nogo-A*$^{-/-}$ mice (Fig 1D). A similar attenuation was observed for the increase of mRNA encoding the muscle-specific receptor tyrosine kinase (MuSK; Fig 1D), which is a receptor kinase involved in acetylcholine receptor clustering (Bezakova & Ruegg, 2003) and the expression of which is coordinated with that of the acetylcholine receptor on denervation (Valenzuela *et al*, 1995). We also carried out immunoprecipitation

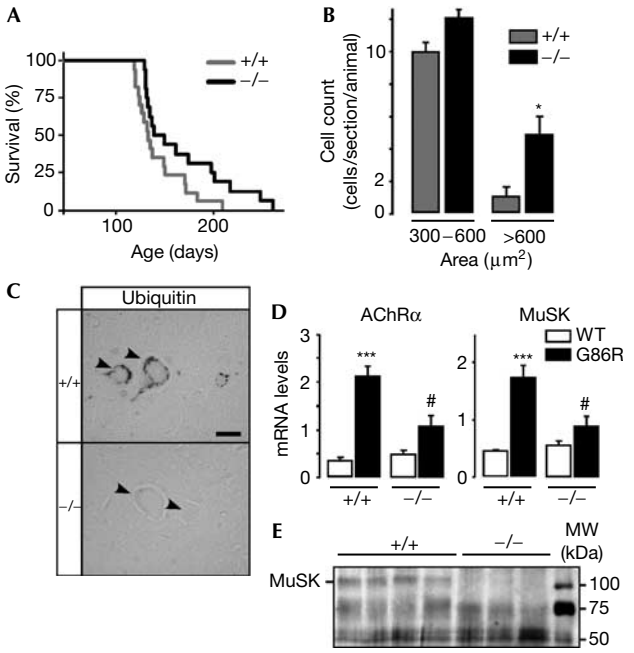

Fig 1 | Genetic ablation of Nogo-A in *Sod1(G86R)* mice. (**A**) Cumulative probability of survival of *G86R/Nogo-A*$^{+/+}$ and *G86R/Nogo-A*$^{-/-}$ mice ($P = 0.04$, $n = 16$–17 mice, log-rank test). (**B**) Cell counts in the ventral horns of the lumbar spinal cord of *G86R/Nogo-A*$^{+/+}$ and *G86R/Nogo-A*$^{-/-}$ mice. Toluidine-blue-stained cells were divided into two groups of 300–600μm^2 and >600 μm^2, the latter clearly representing motor neurons. No differences were observed in the number of cells between non-Cu/Zn-superoxide dismutase 1 (*Sod1)(G86R) Nogo-A*-knockout and wild-type mice (data not shown; $^*P < 0.05$ versus corresponding $+/+$, $n = 3$ mice, Student's *t*-test). (**C**) Ubiquitin immunoreactivity in large motor neurons (cross-sectional area >600 μm^2; arrowheads) of the lumbar spinal cord from *G86R/Nogo-A*$^{+/+}$ and *G86R/Nogo-A*$^{-/-}$ mice. Scale bar, 30 μm. (**D**) Messenger RNA levels of acetylcholine receptor α-subunit (AChRα) and muscle-specific receptor tyrosine kinase (MuSK) in the soleus muscle of wild-type (WT; open bars) and *Sod1(G86R)* mice (filled bars) containing ($+/+$) or not containing ($-/-$) Nogo-A ($^{***}P < 0.001$ versus wild type, $^{\#}P < 0.05$ versus $+/+$, $n = 4$–5 mice, analysis of variance followed by Tukey's test). (**E**) Levels of MuSK protein as determined by western blot of immunoprecipitated MuSK from muscle extracts of *Sod1(G86R)* mice containing ($+/+$) or not containing ($-/-$) Nogo-A. MW, molecular weight markers. In all cases, symptomatic animals reached the end point of the disease. Sod1, Cu/Zn-superoxide dismutase 1.

experiments with whole hindlimb muscles to detect MuSK protein levels, and found that the immunoreactivity specific for MuSK protein observed in the immunoprecipitates from *G86R/Nogo-A*$^{+/+}$ mice had disappeared in *G86R/Nogo-A*$^{-/-}$ mice (Fig 1E). These data clearly show that Nogo-A ablation in ALS mice not only prevented the increase in *musk* gene expression, characteristically observed in these animals, but also reduced the levels of MuSK protein, thereby confirming the attenuated denervation of muscles. Therefore, although the overall effect of Nogo-A deletion on the survival of G86R mice was moderate, these findings show

a protective action for motor neurons at both the cell body and the endplate levels.

Nogo-A overexpression in muscle affects NMJ

The neuroprotective effects of Nogo-A ablation and the increase of Nogo-A in diseased ALS muscle indicate that high levels of ectopic Nogo-A in adult skeletal muscle might influence the NMJ and thereby induce retrograde axon pathology. We tested this hypothesis by analysing the morphology of NMJs *in vivo* in the soleus muscle of wild-type animals on transfection of muscle fibres by electroporation with an expression plasmid encoding Nogo-A fused to a Myc tag. To allow independent identification of Nogo-A-transfected muscle fibres, a plasmid encoding green fluorescent protein (GFP) fused to a nuclear localization signal (NLS–GFP) was co-transfected. First, we examined the expression of Nogo-A in the transfected muscle fibres by staining the soleus muscle with antibodies directed against the Myc tag. As shown in Fig 2A,B, Nogo-A was detected 2 weeks after electroporation in muscle fibres that were also positive for NLS–GFP. This confirms that our experimental design is well suited for the overexpression of Nogo-A in muscle. To examine the influence of Nogo-A overexpression on the morphology of the NMJs, we used *Thy-1* (T-cell antigen)/yellow fluorescent protein mice (Feng *et al*, 2000), which allow direct identification of presynaptic nerve terminals. Postsynaptic acetylcholine receptors were visualized with rhodamine-conjugated α-bungarotoxin. NMJs were examined only 6 weeks after electroporation because we had already observed that changes in morphology on perturbation of MuSK, which is required for the formation of the NMJ (DeChiara *et al*, 1996), require 4–6 weeks (Kong *et al*, 2004). In all experiments, NMJs located on transfected (that is, NLS–GFP-positive) muscle fibres were analysed. As shown in Fig 2C, NMJs on muscle fibres transfected with empty vector (control) looked normal. By contrast, NMJs on muscle fibres expressing Nogo-A were often smaller (Fig 2D), postsynaptic structures were fragmented and the presynaptic nerve terminal only partly occupied the postsynaptic region (Fig 2E). In a few cases, the presynaptic nerve terminal had retracted entirely from the fragmented postsynaptic region (Fig 2F). The effect of Nogo-A on both the pre- and postsynaptic sides was quantified using the groups described above (Table 1). Although most NMJs looked normal in control muscle, approximately 90% of the NMJs located on Nogo-A-expressing muscle fibres had an aberrant structure.

We also quantified the effect of Nogo-A on the size of the postsynaptic apparatus, by measuring the area stained with α-bungarotoxin in flat-mounted muscle preparations. Postsynapses on neighbouring, non-transfected muscle fibres acted as controls. Postsynapses on muscle fibres transfected with empty vector (control) occupied an area of $400 \pm 60\,\mu m^2$ ($n = 4$ mice; a total of 11 NMJs examined), whereas those located on non-transfected muscle fibres in the same muscle were $469 \pm 66\,\mu m^2$ ($n = 3$ mice; six NMJs). Thus, there is no significant difference between transfected and non-transfected muscle fibres in control muscles. By contrast, postsynapses in muscle fibres transfected with the expression construct for Nogo-A were only $241 \pm 15\,\mu m^2$ ($n = 6$ mice; 47 NMJs). Similar to control mice, postsynapses on non-transfected muscle fibres in the same muscles occupied an average area of $452 \pm 35\,\mu m^2$ ($n = 4$ mice; 11 NMJs). Thus, the postsynaptic regions of the muscle fibres that overexpress Nogo-A are significantly ($P = 0.005$) smaller than those in control muscle fibres or neighbouring, non-transfected muscle fibres. Our data are consistent with the idea that increased levels of Nogo-A destabilize the NMJ and eventually lead to retraction of the nerve terminal.

DISCUSSION

Several studies postulate that motor neuron death in ALS is preceded by pathological changes and degeneration of the motor axons and their nerve terminals (Ferri *et al*, 2003; Kieran *et al*, 2005). On the basis of our present findings, we propose that the high expression of Nogo-A in ALS muscle might affect the integrity of the NMJ, leading to its destabilization and retraction of the nerve terminal. This process could trigger a progressive 'dying back' mechanism that ultimately leads to motor neuron degeneration. As muscle Nogo-A upregulation is observed in both mutant Sod1 mice and patients with sporadic ALS (Dupuis *et al*, 2002; Jokic *et al*, 2005), the deleterious effect of muscle Nogo-A on the NMJ might represent a common pathological event and a potential therapeutic target for the maintenance of the neuromuscular connections. Blocking the ability of Nogo-A to inhibit neurite outgrowth promotes axonal sprouting and functional recovery after spinal cord lesion (Li *et al*, 2004; Liebscher *et al*, 2005), which suggests that such an approach could be applicable to alleviate motor impairment in ALS.

Our findings show that specific transcriptional changes in skeletal muscle fibres, that is, increased Nogo-A expression, might hamper the dialogue between motor neurons and muscles. It is now accepted that motor neurons are not the only sites at which mutant Sod1 toxicity acts primarily to trigger ALS (Lino *et al*, 2002; Clement *et al*, 2003). Rather, the disease results from toxicity to the whole neuromuscular unit, as supported by recent studies (Wang *et al*, 2005). Thus, neurogenic injury and also other mechanisms of muscle origin, as discussed here, work together to cause the characteristic phenotype of the diseased NMJ in ALS (Fischer *et al*, 2004). Far from having a passive role in the establishment of neuromuscular connections, muscle-intrinsic signals can initiate the differentiation of neuromuscular synapses independently of neurogenic stimulation (Lin *et al*, 2001) and regulate motor neuron excitability (Nick & Ribera, 2000; Nakanishi *et al*, 2005). In ALS mice, the protective action for motor neurons offered by the intramuscular delivery of retrogradely transported active molecules might also imply benefit to muscle fibres (Kaspar *et al*, 2003; Azzouz *et al*, 2004; Miller *et al*, 2005). Indeed, the expression of a locally acting isoform of insulin-like growth factor 1 in only muscles is sufficient to maintain NMJ integrity, delay motor neuron death and prolong the lifespan of mutant Sod1 mice (Dobrowolny *et al*, 2005). Finally, we recently reported that the skeletal muscles of ALS mice show an unexpectedly augmented metabolic rate before overt motor symptoms appear (Dupuis *et al*, 2004), which further supports the involvement of non-neuronal mechanisms during the course of the disease. Compensating for such a hypermetabolic trait with a highly energetic diet reduces muscle denervation, offers neuroprotection and increases survival (Dupuis *et al*, 2004).

In summary, we provide new evidence for the active role of skeletal muscle in the maintenance of motor neuron function and suggest a role of Nogo-A in synapse integrity and stabilization. The suppression of Nogo-A increased the number of healthy motor neurons in end-stage ALS mice and moderately prolonged their

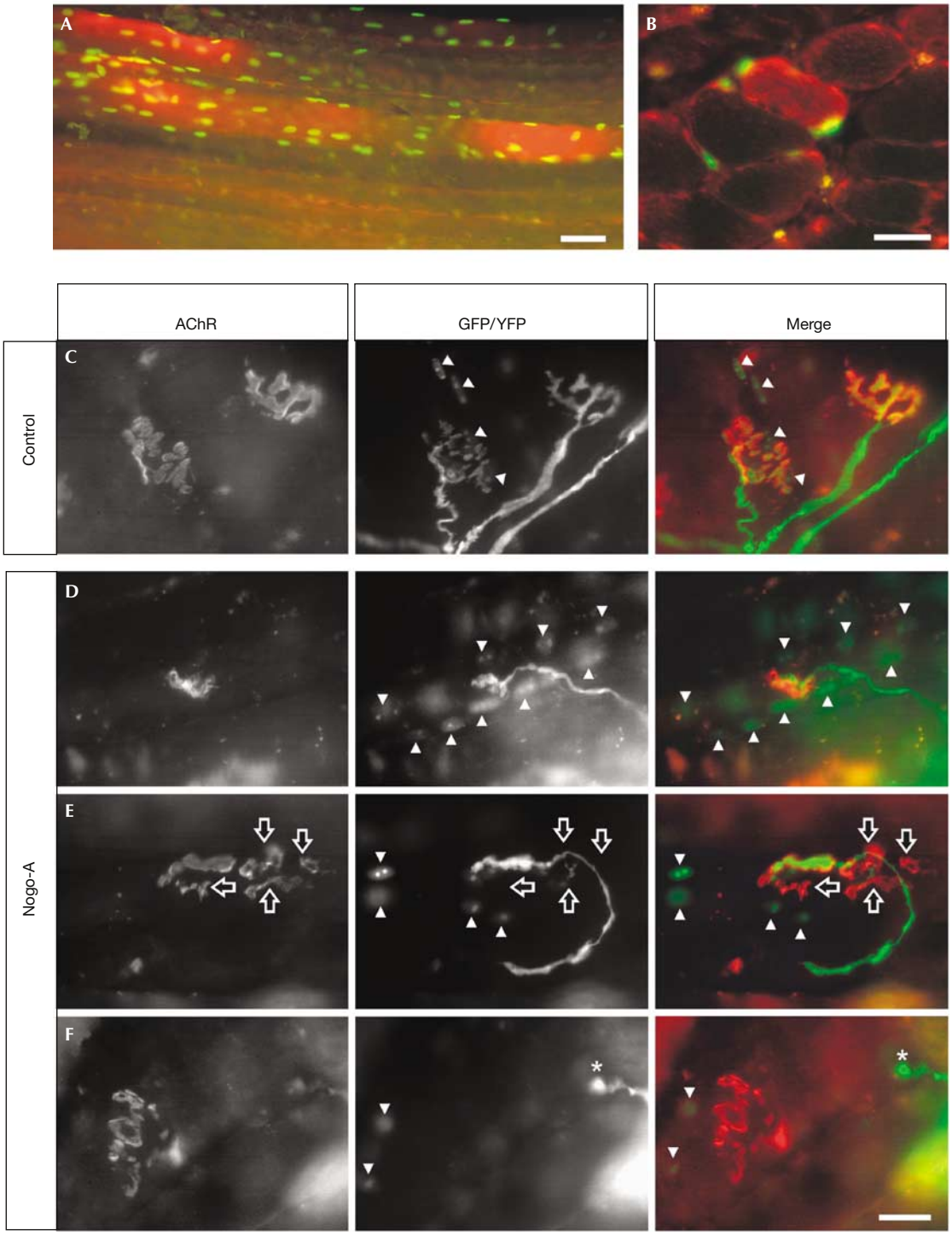

Fig 2 | Overexpression of Nogo-A in muscle causes the disassembly of the neuromuscular junction. Electroporation of the soleus muscle with expression constructs for Nogo-A and nuclear-localized green fluorescent protein (GFP) results in muscle fibres that express high levels of Nogo-A (red) and GFP (green) after 2 weeks. (**A**) Whole-mount and (**B**) cross-section of the soleus muscle. Representative pictures of neuromuscular junctions (NMJs), 6 weeks after electroporation of empty vector (control; **C**) or Nogo-A expression constructs (**D**–**F**). The postsynaptic apparatus was visualized by rhodamine-labelled α-bungarotoxin (acetylcholine receptor; red in merge). Presynaptic nerve terminals were labelled by yellow fluorescent protein (YFP; green in merge) and transfected muscle fibres by nuclear localization signal–GFP (GFP, green in merge). For clarification, GFP-positive myonuclei are indicated by arrowheads. In control transfections, both pre- and postsynaptic structures look normal (**C**). By contrast, overexpression of Nogo-A often results in smaller NMJs (**D**) and fragmentation of the postsynaptic structure including partial (**E**; hollow arrows) or total (**F**; asterisks) retraction of the presynaptic nerve terminal. Scale bar, 25 μm.

Table 1 | Quantification of morphological changes at the neuromuscular junction after overexpression of Nogo-A in the soleus muscle

Experiment	Side of NMJ	Total number of NMJs*	Normal‡	Abnormal‡		
				Smaller	Fragmented	Lost
Control	Postsynaptic	20 (3)	18 (90)	1 (5)	1 (5)	0 (0)
	Presynaptic	8 (3)	7 (87.5)	1 (12.5)	0 (0)	0 (0)
Nogo-A	Postsynaptic	47 (6)	5 (11)	25 (53)	12 (25)	5 (11)
	Presynaptic	45 (4)	7 (17)	19 (45)	12 (29)	4 (9)

The different classes for pre- and postsynaptic structures are as described in Fig 2.
NMJ, neuromuscular junction.
*The total number of NMJs is shown, with the number of mice in parentheses.
‡The data represent the total number of NMJs with a particular phenotype and the relative percentages are in parentheses. Note that in cases in which the phenotype was intermediate between different classes, the less severe class was chosen.

lives. Much effort must now be concentrated on deciphering the molecular mechanisms underlying these effects.

METHODS

Western blot, immunoprecipitation, immunohistochemistry, morphometry, real-time reverse transcription–PCR and analysis of transgene copy number are described in the supplementary information online.

Mice. Transgenic FVB/N males with the G86R murine Sod1 mutation were obtained from our animal facility. *Sod1(G86R)* mice were crossed with C57Bl/6 wild-type or Nogo-A-deficient mice. The resulting F_1 generation animals were crossed again with new C57Bl/6 wild-type and Nogo-A-knockout mice; mice that were homozygous or wild type for the Nogo-A deficiency and heterozygous for the Sod1 mutation were obtained. Genotyping was carried out as described by Ripps *et al* (1995) and Simonen *et al* (2003). Details are given in the supplementary information online.

***In vivo* muscle transfection.** Ectopic expression of Nogo-A in the soleus muscle was obtained by *in vivo* transfection of a pcDNA3/Myc-tagged/Nogo-A plasmid, previously described by Prinjha *et al* (2000). Identification of transfected fibres was achieved by co-transfection of a pGFP-S6 construct encoding a GFP that is targeted to the nucleus (Jones *et al*, 1999). The procedure for transfection has been described previously (Kong *et al*, 2004; for details, see supplementary information online).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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