Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration

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Nogo has been identified as a component of the central nervous system (CNS) myelin that prevents axonal regeneration in the adult vertebrate CNS. Analysis of Nogo-A has shown that an axon-inhibiting domain of 66 amino acids is expressed at the extracellular surface and at the endoplasmic reticulum lumen of transfected cells and oligodendrocytes[1]. The acidic amino terminus of Nogo-A is detected at the cytosolic face of cellular membranes[1] and may contribute to inhibition of axon regeneration at sites of oligodendrocyte injury[2,3]. Here we show that the extracellular domain of Nogo (Nogo-66) inhibits axonal extension, but does not alter non-neuronal cell morphology. In contrast, a multivalent form of the N terminus of Nogo-A affects the morphology of both neurons and other cell types. Here we identify a brain-specific, leucine-rich-repeat protein with high affinity for soluble Nogo-66. Cleavage of the Nogo-66 receptor and other glycophosphatidylinositol-linked proteins from axonal surfaces renders neurons insensitive to Nogo-66. Nogo-66 receptor expression is sufficient to impart Nogo-66 axonal inhibition to unresponsive neurons. Disruption of the interaction between Nogo-66 and its receptor provides the potential for enhanced recovery after human CNS injury.

Assays of Nogo-A function have included growth-cone collapse, neurite outgrowth and fibroblast spreading with substrate-bound and soluble protein preparations[1–5]. The extracellular Nogo-66 domain collapses dorsal root ganglion (DRG) axonal growth cones and inhibits neurite outgrowth[1]. In assays of 3T3 fibroblast morphology, substrate-bound Nogo-66 does not inhibit spreading (Fig. 1b, c). As NI-250 preparations and full-length Nogo-A are non-permissive for 3T3 spreading[2,5], we considered whether different domains of Nogo might subserve this *in vitro* activity. Two pieces of evidence indicate that the N terminus of Nogo-A may contribute to inhibition of axonal regeneration: antibodies directed against this domain reduce the non-permissive qualities of CNS myelin[2]; and this domain alone can reduce cerebellar neurite outgrowth[3].

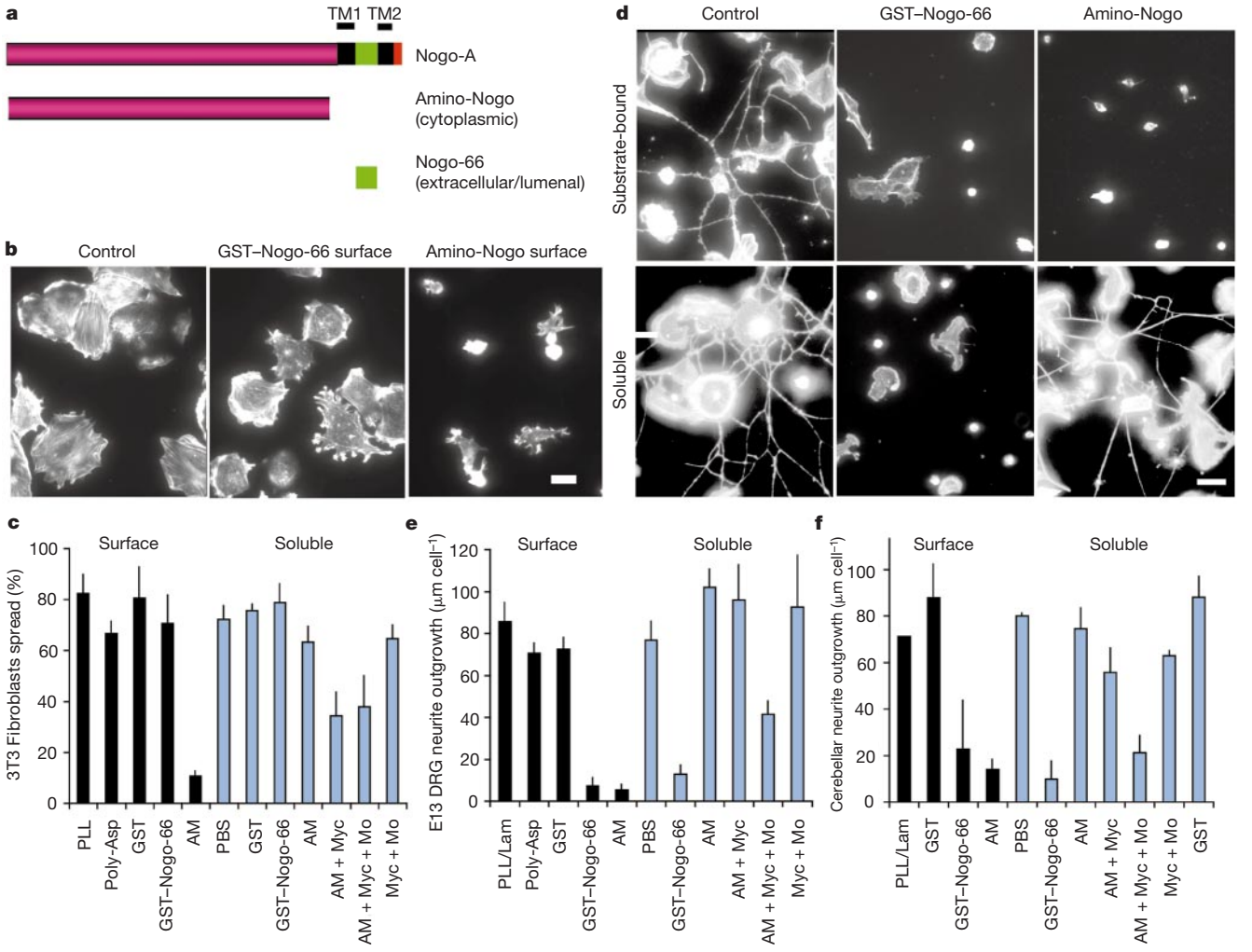

Figure 1 Comparison of Nogo domains. **a**, The Nogo proteins. **b**, NIH 3T3 fibroblasts cultured on different surfaces. Scale bar, 40 μm. **c**, 3T3 fibroblast spreading ($> 1,200\ \mu m^2$) measured on Nogo-coated surfaces (black) or with soluble 100 nM Nogo preparations (blue). AM, Amino-Nogo; AM + Myc, Amino-Nogo pre-incubated with anti-Myc antibody; AM + Myc + Mo, Amino-Nogo plus anti-Myc pre-incubated with anti-mouse IgG; Myc + Mo, anti-Myc antibody plus anti-mouse IgG antibody. **d**, Chick E12 DRGs cultured on surfaces coated with different proteins (substrate-bound) or soluble proteins (soluble). Scale bar, 40 μm. Neurite outgrowth on Nogo-coated surfaces or with soluble Nogo preparations was quantified for E13 DRG (**e**) or cerebellar granule cell cultures (**f**).

To facilitate a comparison between different Nogo-A domains, we expressed the N-terminal 1,040-amino-acid fragment (Amino-Nogo), as a MycHis-tagged protein in HEK293T cells. As predicted from immunolocalization studies[1], the protein is present in cytosolic fractions. Surfaces coated with purified Amino-Nogo protein fail to support 3T3 fibroblast spreading (Fig. 1b, c). Similar results are observed for a kidney-derived cell line, COS-7 (data not shown). The N-terminal domain therefore seems to account for the effects of full-length Nogo-A on fibroblasts.

We also exposed DRG cultures to Amino-Nogo protein (Fig. 1d, e). The fibroblast-like cells in the DRG culture do not spread on the Amino-Nogo substrate. Furthermore, axonal outgrowth is reduced to low levels on surfaces coated with Amino-Nogo. Thus, although the Nogo-66 effects are neural-specific, the inhibitory action of the Amino-Nogo domain is more generalized. When presented in soluble form at 100 nM, the Nogo-66 polypeptide collapses chick E12 DRG growth cones and nearly abolishes axonal extension[1]. In marked contrast, the soluble Amino-Nogo protein seems inactive, and does not significantly modulate DRG growth-cone morphology, DRG axonal extension, or non-neuronal cell spreading (Fig. 1c–e; and data not shown). Cerebellar granule neurons have been studied previously, and soluble Amino-Nogo was presented as an Fc fusion protein—presumably in dimeric form[3]. We therefore considered whether these differences might explain the inactivity of soluble Amino-Nogo.

The response of mouse P4 cerebellar granule neurons and chick E13 DRG neurons to Nogo preparations is indistinguishable (Fig. 1f). Amino-Nogo dimerized with anti-Myc antibody inhibits 3T3 and COS-7 spreading (Fig. 1c; and data not shown) and tends to reduce cerebellar axon outgrowth (Fig. 1f). When further aggregated by the addition of anti-mouse IgG antibody, Amino-Nogo significantly reduces both DRG and cerebellar axon outgrowth (Fig. 1e, f). Although the Amino-Nogo protein is quite acidic, electrostatic charge alone does not account for its inhibitory effects, as poly-Asp does not alter cell spreading or axonal outgrowth (Fig. 1c, e). Both of the Nogo domains can potently inhibit axon growth under certain circumstances, but they possess different mechanisms of action.

On the basis of the neuronal-selective action of Nogo-66 and its high potency as a soluble monomeric ligand, we sought to identify a neuronal receptor for this Nogo domain. We chose the placental alkaline phosphatase (AP) fusion protein approach[6]. An AP–Nogo fusion protein can be purified from the conditioned medium of transfected cells in milligram amounts. This protein is biologically active as a growth-cone-collapsing agent, with an effective concentration for half-maximal response (EC_{50}) of 1 nM (Fig. 2). AP–Nogo is slightly more potent than glutathione *S*-transferase (GST)–Nogo-66, perhaps because the protein is synthesized in a eukaryotic

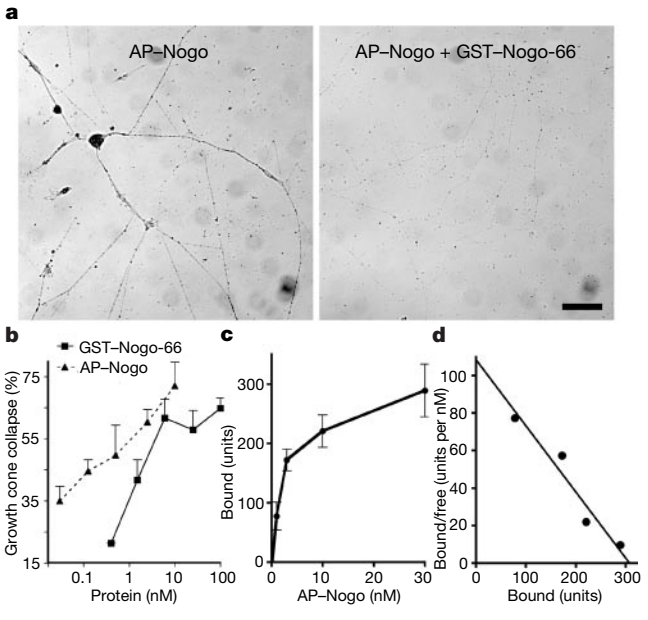

Figure 2 Ligand-binding assay for axonal Nogo-66 receptors. **a**, Dissociated chick E12 DRG neurons incubated with 10 nM AP–Nogo or 10 nM AP–Nogo + 160 nM GST–Nogo-66, and stained for bound AP. Scale bar, 40 μm. **b**, Potency of AP–Nogo and GST–Nogo in E12 chick DRG growth-cone-collapse assays. **c**, AP–Nogo binding to DRG neurons measured as a function of AP–Nogo concentration. Error bars are from individual measurements in one of six experiments with similar results. **d**, Replotted data from **c**. K_d, 3 nM.

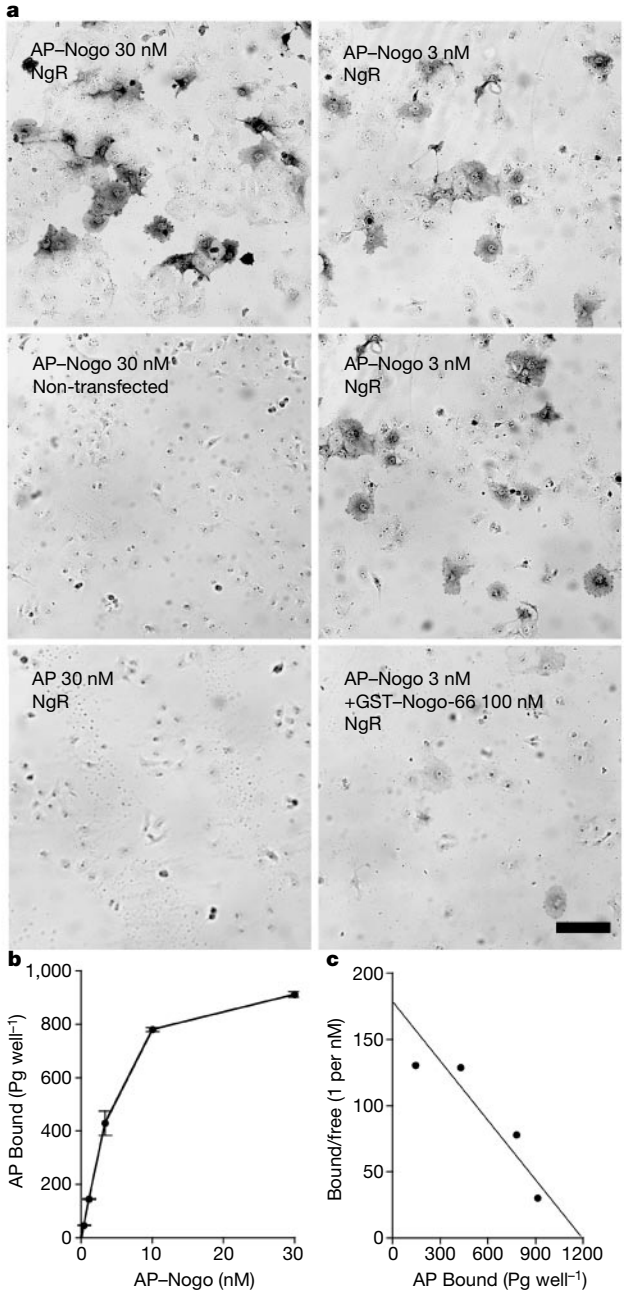

Figure 3 Nogo-66 binding to COS-7 cells expressing the Nogo-66 receptor. **a**, COS-7 cells transfected with an expression vector encoding mouse NgR or a vector control. Two days later, binding of AP–Nogo or AP was assessed. Scale bar, 200 μm. **b**, AP–Nogo binding to NgR expressing COS-7 cells measured as a function of AP–Nogo concentration. Error bars are from individual measurements in one of six experiments with similar results. **c**, Replotted data from **b**. K_d, 7 nM.

rather than a prokaryotic cell. AP–Nogo binds to saturable, high-affinity sites on chick E12 DRG axons. Binding is blocked by excess GST–Nogo-66, consistent with competitive binding to a neuronal receptor site. As the apparent dissociation constant (K_d, 3 nM) for these sites is close to the EC_{50} of AP–Nogo in the collapse assay, the sites are probably physiologically relevant Nogo-66 receptors.

We used the AP–Nogo binding assay for expression cloning of a Nogo-66 receptor. Pools of a complementary DNA expression library from the adult brain of a mouse, representing 250,000 independent clones, were transfected into non-neuronal COS-7 cells. Non-transfected COS-7 cells do not bind AP–Nogo (Fig. 3), but transfection with two pools of 5,000 clones showed a few cells with strong AP–Nogo binding. Single cDNA clones encoding a Nogo-binding site were isolated by sib selection from each of the two positive pools. The two independently isolated clones are identical to one another except for a 100 base-pair (bp) extension of the 5′ untranslated region in one clone. Transfection of these cDNAs into COS-7 cells yields a binding site with an affinity for AP–Nogo similar to that observed in E13 DRG neurons; the K_d for binding is about 7 nM (Fig. 3). AP alone does not bind with any detectable affinity to these transfected cells, indicating that the affinity is due to the 66 residues derived from Nogo. Furthermore, excess GST–Nogo-66 displaces AP–Nogo from these sites.

The cDNA directing the expression of a Nogo-binding site in COS-7 cells encodes a protein of 473 amino acids, which we have called Nogo-66 receptor (NgR). The predicted protein contains a signal sequence followed by eight leucine-rich-repeat (LRR) domains, an LRR carboxy-terminal flanking domain that is cysteine rich, a unique region and a glycosylphosphatidylinositol (GPI) anchorage site (Fig. 4). There is no full-length cDNA in GenBank with significant nucleotide sequence similarity (expectation value, $E \leq 0.05$ by BLASTN analysis); several mouse and human expressed sequence tags (ESTs) match fragments of the sequence precisely. In the LRR domains, there is moderate amino-acid sequence similarity (up to 35% amino-acid identity) to many other proteins containing this domain. The LRR proteins sharing the greatest sequence similarity are slit1–3 and the acid-labile subunit of the insulin-like growth-factor-binding protein complex. We have identified a human homologue of the mouse NgR cDNA that shares 89% amino-acid identity. The existence of this cDNA was predicted from the mouse cDNA structure and from analysis of human genomic sequence deposited in GenBank as part of the human genome sequencing effort. The exons of the human NgR gene on chromosome 22q11 are separated by nearly 30 kilobases (kb), and the messenger RNA was not previously recognized in the genomic sequence. The predicted protein structure is consistent with a cell-surface protein capable of binding Nogo.

We next verified that the protein was associated with the cell surface through a GPI linkage. A Myc-tagged version of NgR protein with a relative molecular mass of 85,000 (M_r 85K) is found in particulate fractions, and can be released by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment, as expected for a GPI-linked protein (Fig. 4c). The GPI-linked nature of the protein suggests that there may be a second receptor subunit that spans the plasma membrane and mediates Nogo-66 signal transduction.

The simplest model for NgR expression mediating AP–Nogo binding is that the two proteins bind directly to one another. To assess their physical interaction, we incubated extracts from Myc–NgR expressing HEK293T cells with resin-bound GST–Nogo-66 or GST (Fig. 4d). NgR is selectively retained by the Nogo-containing resin, verifying that the two proteins associate as part of a protein complex.

The distribution of the mRNA expression for the NgR is consistent with a function for the protein in regulating axonal regeneration and plasticity in the adult CNS. Northern analysis shows a single band of 2.3 kb in the adult brain, indicating that the isolated NgR clone is full length (Fig. 5). Low levels of this mRNA are observed in heart and kidney but not in other peripheral tissues. In

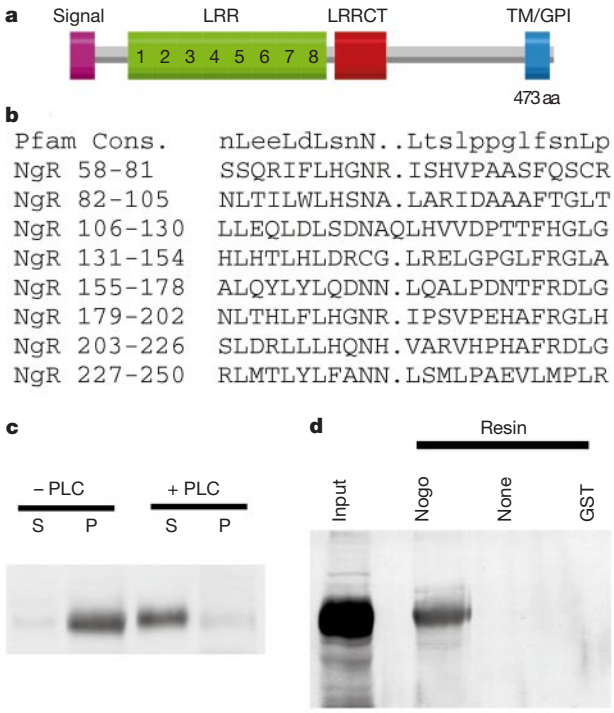

Figure 4 Structure of Nogo-66 receptor. **a**, Main structural features of the NgR protein. Signal, signal peptide; LRR, leucine-rich repeat; LRRCT, LRR C-terminal domain; TM/GPI, predicted transmembrane/glycosylphosphatidylinositol linkage. **b**, Amino-acid sequence of the LRRs is aligned with the Pfam consensus leucine-rich repeat. **c**, Extracts from Myc–NgR-expressing cells treated with or without PI-PLC, and soluble (S) or particulate (P) fractions were analysed by anti-Myc immunoblot. **d**, Myc–NgR PI-PLC extract (Input) was incubated with glutathione-coupled GST–Nogo-66 that contained agarose (Nogo) or buffer (None), or GST (GST). Bound protein was detected by anti-Myc immunoblot.

the brain, expression is widespread and the areas richest in grey matter express the highest levels of the mRNA by northern analysis (data not shown). *In situ* hybridization shows NgR expression in cerebral cortical neurons, hippocampal neurons, cerebellar Purkinje cells and pontine neurons (Fig. 5b). NgR is expressed in those cerebral cortex pyramidal neurons whose regeneration is enhanced by IN-1 treatment[7], and in those cerebellar Purkinje neurons whose sprouting is increased by anti-Nogo-A antibody[8]. Nogo-66 receptor mRNA is not detected in white matter (Fig. 5b), where Nogo-A is expressed by oligodendrocytes[1,2].

To characterize further the expression of the NgR protein, we developed an antiserum to a GST–NgR fusion protein. This antiserum detects selectively an 85K protein in NgR-expressing HEK293T cells (Fig. 5c), and specifically stains COS-7 cells expressing NgR (Fig. 5d). Immunohistological staining of chick embryonic spinal-cord cultures localizes the protein to axons, consistent with the mediation of axon-outgrowth inhibition induced by Nogo-66 (Fig. 5d). NgR expression is not found in the O4-positive oligodendrocytes (Fig. 5d) that express Nogo[1]. Immunoreactive 85K protein is expressed in Nogo-66-responsive neuronal preparations from chick E13 DRGs, but to a much lesser extent in weakly responsive tissue from chick E7 DRGs and chick E7 retina (Fig. 5c). Overall, the pattern of NgR expression is consistent with the protein's mediating Nogo-66 axon inhibition.

We next investigated whether the NgR protein is necessary for Nogo-66 action and not simply a binding site with a function unrelated to inhibition of axonal outgrowth. A first prediction is that PI-PLC treatment to remove GPI-linked proteins from the neuronal surface will render neurons insensitive to Nogo-66. This is true for chick E13 DRG neurons: PI-PLC treatment abolishes both AP–Nogo binding (data not shown) and growth-cone collapse induced by GST–Nogo-66 (Fig. 6a, c). As a control, Sema3A responses in the parallel cultures are not altered by PI-PLC treatment. However, PI-PLC treatment is expected to remove a number of proteins from the axonal surface, so this result leaves open the possibility that other GPI-linked proteins are mediating the Nogo-66 response in untreated cultures.

To show that NgR is capable of mediating Nogo-66 inhibition of axon outgrowth, we expressed the protein in neurons lacking a Nogo-66 response. We examined both DRG and retinal neurons from E7 chick embryos. The Nogo responses in the DRG neurons from this developmental stage are weak[1], but slight responses can be detected in some cultures (data not shown). E7 retinal ganglion cell growth cones are uniformly insensitive to growth-cone collapse induced by Nogo-66 (Fig. 6b, d), do not bind AP–Nogo (data not shown), and do not exhibit 85K anti-NgR immunoreactive protein (Fig. 5c). Expression of NgR in these neurons by infection with recombinant herpes simplex virus (HSV) preparations renders the axonal growth cones of the retinal ganglion cell sensitive to Nogo-66-induced collapse. Infection with a control PlexinA1-expressing

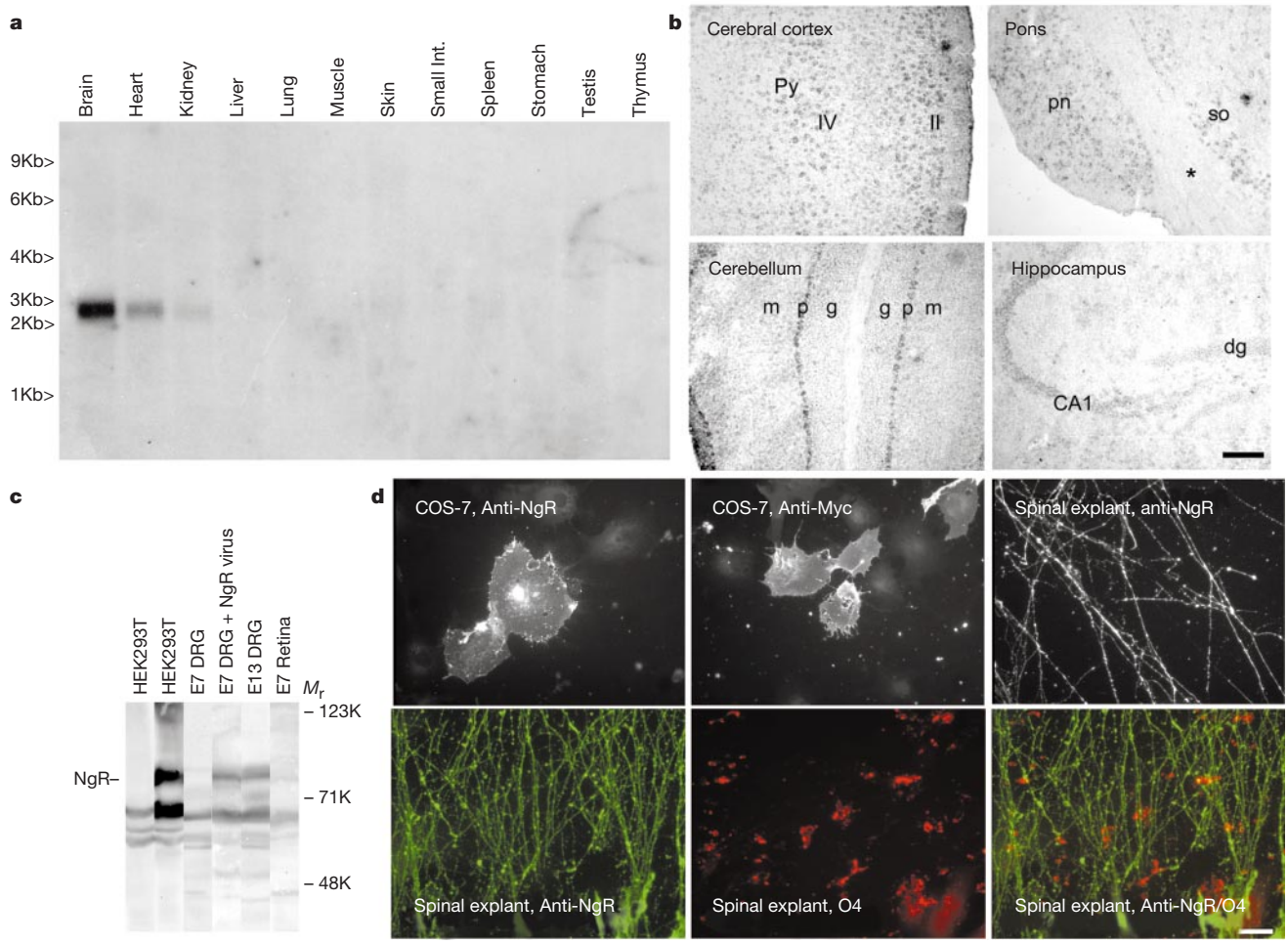

Figure 5 Distribution of Nogo-66 receptor expression. **a**, Northern analysis of NgR in mouse. The positions of RNA size markers (kb) are at the left. **b**, NgR *in situ* hybridization for adult mouse brain. II, IV, cerebral cortical layers; Py, cortical pyramidal cell layer; m, molecular, p, Purkinje cell, and g, granule cell layer of the cerebellum; dg, dentate gyrus of the hippocampus; pn, pontine nucleus of the pons; asterisk, descending pyramidal tract; so, superior olivary nucleus. Scale bar, 500 μm. **c**, Anti-NgR immunoblot analysis of membrane fractions from indicated cells or chick tissues. **d**, COS-7 cells expressing Myc–NgR or chick E5 spinal-cord explants (8 d *in vitro*) were stained with anti-NgR, anti-Myc or the oligodendrocyte-specific O4 antibody. Scale bars, 40 μm (top); 80 μm (bottom).

control HSV preparation does not alter Nogo responses. Together, these data indicate that the Nogo receptor identified here may participate in Nogo-66 inhibition of axon regeneration.

Our study identifies a GPI-linked, LRR protein as a receptor that mediates Nogo-66 inhibition of axonal outgrowth. The expression of this receptor produces a binding site for Nogo-66 with an affinity similar to that for growth-cone collapse induced by Nogo-66, and the two proteins physically associate with one another. Furthermore, expression of NgR protein is sufficient to convert axonal growth cones from a Nogo-66-insensitive to a Nogo-66-responsive state. It is clear that the extracellular 66-residue domain of Nogo and the N-terminal Nogo-A domain have different cellular effects. The biological significance of the Nogo-66 domain is supported by its neuronal specificity, its potent action as a monomeric ligand and the current identification of a high-affinity neuronal receptor for this fragment. The NgR identified here is GPI-linked to the cell surface. This indicates that it may function as part of a receptor complex, serving as the primary Nogo-66 binding site while associated transmembrane receptor subunits are responsible for signal transduction. The Amino-Nogo domain is intracellular, has actions that are not cell-type specific, and functions only in a multivalent state. The two domains may act synergistically to inhibit outgrowth in the injured CNS. Evidence indicates that Nogo expression in the adult brain by oligodendrocytes may serve to limit axonal plasticity and regeneration after injury[9]. Therefore, the current identification of a receptor mediating Nogo-66 action should greatly facilitate the development of agents with pharmaceutical potential in a diverse group of neurological conditions, such as spinal-cord injury, brain trauma, stroke affecting white matter and chronic, progressive multiple sclerosis. □

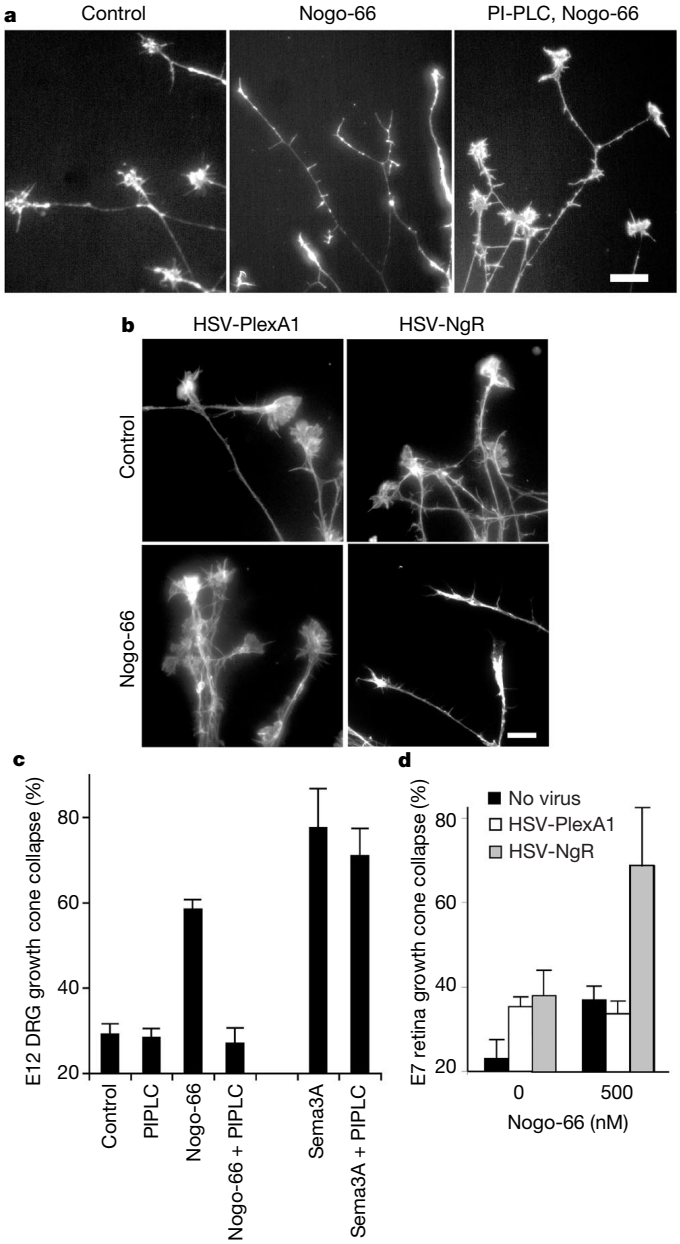

Figure 6 Nogo-66 receptor mediates growth-cone collapse by Nogo-66. **a**, Chick E12 DRG explants exposed to Nogo-66 after pretreatment with PIPLC or buffer. Scale bar, 40 μm. **b**, E7 retinal ganglion cell explants infected with HSV-PlexinA1 or HSV-Myc–NgR, and incubated with or without Nogo-66. Scale bar, 25 μm. **c**, Growth-cone-collapse measurements from experiments as in **a** are shown. DRG cultures treated with or without PI-PLC before exposure to 30 nM GST–Nogo-66 or 100 pM Sema3A. **d**, Quantification of growth-cone collapse in uninfected or viral-infected E7 retinal neurons as in **b**.

Methods

Nogo recombinant proteins

To express Amino-Nogo, the human Nogo-A cDNA for residues 1–1,040 was ligated into pcDNA3.1MycHis (Invitrogen, Burlingame, California) with an in-frame Myc-His tag. We transfected this plasmid into HEK293T cells and Amino-Nogo was purified with a Ni^{2+} resin[10]. The human Nogo-66 sequence was ligated into pcAP-5 (ref. 10) in frame with the signal sequence, 6×His tag and placental AP coding region. This plasmid was transfected into HEK293 cells, and secreted AP–Nogo was purified by Ni^{2+} affinity chromatography. GST–Nogo-66 has been described[1].

Nogo-66 receptor binding assays and expression cloning

To detect AP–Nogo binding, cultures were washed with Hanks balanced salt solution containing 20 mM sodium HEPES, pH 7.05, and 1 mg ml^{-1} bovine serum albumin (BSA) (HBH). The plates were then incubated with AP–Nogo in HBH for 2 h at 23 °C. We detected and quantified bound AP–Nogo as for AP–Sema3A[11]. For saturation analysis of AP–Nogo bound to COS-7 cells, bound AP–Nogo protein was eluted with 1% Triton X-100. After heat inactivation of endogenous AP, we measured AP–Nogo using *p*-nitrophenyl phosphate as substrate.

For expression cloning of a Nogo-66 receptor, pools of 5,000 arrayed clones from a mouse adult-brain cDNA library (Origene Technologies, Rockville, Maryland) were transfected into COS-7 cells, and AP–Nogo binding was assessed. We isolated single NgR cDNA clones by sib selection and sequenced them. A Myc–NgR vector was created in pSecTag2-Hygro (Invitrogen) using the signal peptide of pSecTag2 fused to Myc and residues 27–473 of NgR. Human NgR cDNA was predicted from a human genomic cosmid sequence (AC007663). Oligonucleotide primers based on the predicted human cDNA amplified the cDNA from a human adult-brain cDNA library (Origene Technologies).

To assess binding of Myc–NgR to the cell membrane, particulate fractions were treated with or without 5 U PI-PLC (Sigma, St. Louis, MO) per mg of HEK293T cell protein for 1 h at 30 °C in HBH. After centrifugation at 100,000*g* for 1 h, we analysed equal proportions of the soluble and particulate fractions. To assess the physical interaction of NgR with Nogo-66, we incubated the PI-PLC extract (50 μg total protein) with 10 μg GST–Nogo-66 or buffer, or 10 μg GST for 1 h at 23 °C. We added glutathione-coupled agarose to bind GST and associated proteins. We analysed bound proteins by anti-Myc immunoblot.

RNA analysis

For northern blots, 1 μg poly(A)$^+$ RNA from each adult mouse tissue on a nylon membrane (Origene Technologies) was hybridized with a full-length ^{32}P-labelled probe[1,12]. We used digoxegenin-labelled riboprobes (nucleotides 1–1,222) and adult mouse brain sections[1,12] for *in situ* hybridization. The sense probe produced no signal.

Nogo-66 receptor antibodies

A GST–NgR (residues 27–447) fusion protein was purified from *Escherichia coli* and used to immunize rabbits. We diluted immune serum 3,000-fold for immunoblots and 1,000-fold for immunohistology on tissue-culture samples that had been fixed by formalin. Staining of tissue was totally abolished by addition of 5 μg ml^{-1} GST–NgR.

Cell spreading, neurite outgrowth and viral infection

To measure spreading rates, subconfluent NIH 3T3 fibroblasts or COS-7 cells were plated for 1 h in serum-containing medium before fixation and staining with rhodamine-phalloidin. Glass coverslips were precoated with 100 μg ml^{-1} poly-L-lysine, washed, and then 3 μl drops of PBS containing 15 pmol Amino-Nogo, 15 pmol GST–Nogo-66, 15 pmol poly-Asp (M_r 35 K, Sigma), or no protein were spotted and dried. We added soluble Nogo protein preparations (100 nM) at the time of plating. Amino-Nogo was added alone or after a pre-incubation with a twofold molar excess of anti-Myc 9E10 antibody, or with a twofold excess of anti-Myc plus a twofold excess of purified goat anti-mouse IgG.

Chick E5 spinal cord, chick E7–E13 DRG, chick E7 retina and mouse P4 cerebellar neuron culture, growth-cone-collapse assays and neurite-outgrowth assays have been described[1,10–13]. Here, outgrowth from dissociated neurons was assessed after 12–24 h. For the substrate-bound experiments, glass chamber slides were coated with 100 μg ml^{-1} poly-L-lysine, washed, and then 3 μl drops of PBS containing 15 pmol Amino-Nogo, 15 pmol GST–Nogo-66, 15 pmol poly-Asp, or no protein were spotted and dried. After three PBS washes, we coated slides with 10 μg ml^{-1} laminin. After aspiration of laminin, dissociated neurons were added. For the soluble Nogo experiments, slides were coated with poly-L-lysine and laminin in the same fashion, and then 100 nM Amino-Nogo, 100 nM clustered Amino-Nogo, or 100 nM GST–Nogo-66 was added to the culture medium at the time of plating. After 1 d *in vitro*, some DRG explants were treated for 30 min with 1 unit ml^{-1} PI-PLC (Sigma) before the growth-cone-collapse assay.

An HSV-Myc–NgR stock was prepared as described[10]. We infected E7 retinal explants for 24 h. We stained some cultures infected with HSV-Myc–NgR with anti-Myc antibody to verify protein expression. Error bars reflect the s.e.m. from 4–8 determinations.

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Maize *yellow stripe1* encodes a membrane protein directly involved in Fe(III) uptake

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Frequently, crop plants do not take up adequate amounts of iron from the soil, leading to chlorosis, poor yield and decreased nutritional quality. Extremely limited soil bioavailability of iron has led plants to evolve two distinct uptake strategies: chelation, which is used by the world's principal grain crops[1,2]; and reduction, which is used by other plant groups[3–5]. The chelation strategy involves extrusion of low-molecular-mass secondary amino acids (mugineic acids) known as 'phytosiderophores', which chelate sparingly soluble iron[6]. The Fe(III)-phytosiderophore complex is then taken up by an unknown transporter at the root surface[7,8]. The maize *yellow stripe1* (*ys1*) mutant is deficient in Fe(III)-phytosiderophore uptake[7–10], therefore YS1 has been suggested to be the Fe(III)-phytosiderophore transporter. Here we show that *ys1* is a membrane protein that mediates iron uptake. Expression of YS1 in a yeast iron uptake mutant restores growth specifically on Fe(III)-phytosiderophore media. Under iron-deficient conditions, *ys1* messenger RNA levels increase in both roots and shoots.