NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans

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In the adult mammalian CNS, chondroitin sulfate proteoglycans (CSPGs) and myelin-associated inhibitors (MAIs) stabilize neuronal structure and restrict compensatory sprouting following injury. The Ngroceptor family members NgR1 and NgR2 bind to MAIs and have been implicated in neuronal inhibition. We found that NgR1 and NgR3 bind with high affinity to the glycosaminoglycan moiety of proteoglycans and participate in CSPG inhibition in cultured neurons. Ngroceptor triple mutants (NgR1−/−; NgR2−/−; NgR3−/−; which are also known as Rtn4r, Rtn4rl2 and Rtn4rl1, respectively), but not single mutants, showed enhanced axonal regeneration following retro-orbital optic nerve crush injury. The combined loss of NgR1 and NgR3 (NgR1−/−; NgR3−/−), but not NgR1 and NgR2 (NgR1−/−; NgR2−/−), was sufficient to mimic the triple mutant regeneration phenotype. Regeneration in NgR1−/−; NgR3−/− mice was further enhanced by simultaneous ablation of Rptpir (also known as Ptprs), a known CSPG receptor. Collectively, our results identify NgR1 and NgR3 as CSPG receptors, suggest that there is functional redundancy among CSPG receptors, and provide evidence for shared mechanisms of MAI and CSPG inhibition.

In the adult mammalian CNS, structural neuronal plasticity is restricted by a number of extrinsic (environmental) and cell-intrinsic growth inhibitory mechanisms1–2. Although such mechanisms are believed to be important for stabilization of intricate networks of neuronal connectivity in CNS health, they also limit adaptive neuronal growth and sprouting following brain or spinal cord injury. Spontaneous repair following severe CNS injury is incomplete and is commonly associated with permanent neurological deficits. Thus, a detailed understanding of the mechanisms that block neuronal growth and repair is of great interest, both biologically and clinically.

A large number of CNS inhibitory cues have been identified2–4. In experimental animal models of spinal cord injury, acute blockage of MAIs5,6 or enzymatic degradation of CSPGs with chondroitinase ABC (ChaseABC)7–9 promotes neuronal sprouting and correlates with improved behavioral outcomes.

The best characterized MAIs are the reticulon family member Nogo, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp)2. Three isoforms of Nogo have been identified, all of which contain a 66 amino-acid loop (Nogo66) that signals neuronal inhibition. Mechanistic studies have identified the Nogo66 receptor-1 (NgR1) and paired immunoglobulin (Ig)-like receptor B (PirB) as functional receptors for MAI5,10,11. NgR1 is comprised of 8.5 leucine-rich repeats (LRRs), flanked by N-terminal (NT) and C-terminal (CT) LRR capping domains. The NT-LRR-CT cluster of NgR1 is fused to a ~100 amino-acid residue stalk region and connected to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor11. NgR1 and its close relative NgR2 show overlapping, yet distinct, binding preferences toward MAIs. Nogo66 and OMgp bind selectively to NgR1 (ref. 2), whereas MAG associates with NgR1 and NgR2 (ref. 12). The related molecule NgR3 is poorly characterized, and no functional NgR3 ligand(s) have been identified to date. In vitro, loss of NgR1 renders neurons more resistant to Nogo66-, MAG- and OMgp-induced growth cone collapse, but not to longitudinal neurite outgrowth inhibition on substrate-bound inhibitors13–15. MAIs activate RhoA, RockII and conventional isoforms of protein kinase C (PKC) to destabilize the neuronal cytoskeleton16,17. Similar to NgR1, PirB supports binding of Nogo66, MAG and OMgp. In culture, functional ablation of Pirb (also known as Lirb3) promotes neurite outgrowth on substrate-bound MAIs and crude CNS myelin. Notably, the combined perturbation of PirB and NgR1 signaling leads to a further release of neurite outgrowth inhibition on crude CNS myelin, but not on recombinant Nogo66 or MAG18.

CSPGs are a diverse class of extracellular matrix molecules that influence axonal growth and guidance of developing neurons19.
Following injury to the adult CNS, CSPG expression is upregulated and CSPG is abundant in reactive astrocytes associated with glial scar tissue. CSPGs are comprised of a protein core with covalently attached glycosaminoglycan (GAG) side chains. GAG chains are large, unbranched polymers composed of ~20–200 repeating disaccharide units. Chondroitin sulfate (CS)-GAGs contain alternating units of N-acetylgalactosamine and glucuronic acid. Most commonly, the hydroxyl groups at position 4 (CS-A) or position 6 (CS-C) of these units are sulfated. Chondroitin sulfate (CS) inhibition of neurite outgrowth is a prototype of MAI-mediated inhibition of neurite outgrowth. Because the loss of NgR1 and NgR3, but not NgR2, in mice lacking GAGs yields a phenotype indistinguishable from wild-type mice, this suggests that there is some degree of functional redundancy among these two receptors.

**RESULTS**

**NgRs participate in prototypic MAI-independent inhibition**

To determine the role of Nogo receptor family members in CNS myelin inhibition, we generated Ngr1−/−, Ngr2−/−, Ngr3−/−, and Ngr1−/−; Ngr2−/−; Ngr3−/− triple mutants, p75−/− (also known as Ngfr) single mutants, and mice lacking three NgR family members (Ngr1, Ngr2, and Ngr3) (Supplementary Fig. 1). Ngr1−/−, Ngr2−/−, or Ngr3−/− mice showed a significant (P < 0.001, one way ANOVA, Tukey’s post hoc) yet incomplete, release of growth inhibition (Fig. 1 and Supplementary Fig. 2). Compared with CGNs isolated from wild-type, Ngr1−/−, Ngr2−/−, or Ngr3−/− mice, CGNs from Ngr1−/−; Ngr2−/−; Ngr3−/− mutants grew significantly longer neurites on myelin (P < 0.001, one-way ANOVA, Tukey’s post hoc). Notably, in two different types of neurons, CGNs and DRGs, the combined loss of Ngr1 and Ngr2 did not result in enhanced neurite growth on crude CNS myelin (Fig. 1 and Supplementary Fig. 2). Because only Ngr1 and Ngr3, but not Ngr2, are expressed in P7 CNS of Ngr1−/− or Ngr3−/− mice, this suggests that Ngr3 participates in myelin inhibition. This is somewhat surprising, as Ngr3 does not associate with recombint Nogo, MAG, or OMgp.

To directly test whether Ngr3 participates in the neurite outgrowth inhibition induced by endogenously expressed Nogo, MAG, or OMgp, we repeated our experiments with CNS myelin isolated from Nogo (also known as Rtn4), MAG and OMgp (also known as Omgp) triple mutant mice (Ngo−/−; Mag−/−; Omgp−/−)29. Consistent with previous reports, Nogo−/−; Mag−/−; Omgp−/− myelin was less inhibitory than wild-type myelin (Fig. 1 and Supplementary Fig. 2). Notably, on Nogo−/−; Mag−/−; Omgp−/− myelin, CGNs from Ngr1−/−; Ngr2−/−; Ngr3−/− mice continued to extend longer neurites (P < 0.001, one way ANOVA, Tukey’s post hoc) than CGNs from wild-type, Ngr1−/−, Ngr1−/−; Ngr2−/−, or Ngr3−/− mice (Fig. 1). This observation indicates that Ngr3 participates in Nogo-, MAG- and OMgp-independent growth inhibition. Because the loss of Ngr1 or Ngr3 alone were not sufficient to promote growth on myelin, this suggests that there is some degree of functional redundancy among these two receptors.

NgR1 and NgR3, but not NgR2, associate with neural GAGs

To identify candidate NgR3 ligand(s), we generated alkaline phosphatase (AP)-tagged receptor fusion proteins and assayed binding to rat brain tissue sections. Prior to the onset of CNS myelination, NgR1 and NgR3, but not NgR2, bound strongly to numerous fiber tracts in the brain and spinal cord (Fig. 2a). After myelination, a more uniform binding pattern was observed, with a much less pronounced labeling of fiber tracts (data not shown). Notably, brain sections of Nogo−/−; Mag−/−; Omgp−/− and Ngr1−/−; Ngr2−/−; Ngr3−/− triple mutants, p75−/− (also known as Ngfr) single mutants, and mice

**Figure 1** Loss of all three NgRs results in enhanced growth on CNS myelin. (a) P7 CGNs from wild-type (WT), Ngr1−/−, Ngr1−/−; Ngr2−/−, and Ngr3−/− pups were strongly inhibited when plated on crude CNS myelin substrate (40 µg ml−1). In marked contrast, CGNs from Ngr1−/−; Ngr2−/−; Ngr3−/− mice grew longer neurites on CNS myelin. On CNS myelin isolated from Nogo−/−; Mag−/−; Omgp−/− mice (40 µg ml−1), CGNs from wild-type, Ngr1−/−, Ngr1−/−; Ngr2−/−, Ngr2−/−; and Ngr3−/− pups showed enhanced neurite outgrowth. A further release of inhibition was observed when Ngr1−/−; Ngr2−/−; Ngr3−/− neurons were plated on Nogo−/−; Mag−/−; Omgp−/− CNS myelin. On a BSA control substrate, the neurite length of all five genotypes was comparable. Scale bar represents 50 µm. (b) Quantification of neurite length. At least 300 neurites of TuJ1-labeled cells were counted per condition (n = 9 independent experiments). Results are presented as mean ± s.e.m. **P < 0.001 (one-way ANOVA, Tukey’s post hoc)."
Figure 2  NgR1 and NgR3, but not NgR2, contain two discontinuous and evolutionarily conserved sequence motifs that are necessary for binding to brain tissue. (a) Coronal sections of embryonic day 18 (E18) rat brain showing the binding pattern of AP-NgR1 and AP-NgR3. No binding was observed for AP-NgR2. Scale bar represents 250 μm. (b,c) Binding of NgR1-Fc (b) and NgR3-Fc (c) to E18 brain sections was abolished following deletion of a cluster of basic residues (motif 2) in the stalk region. Scale bar represents 250 μm. (d) Schematic of receptor deletion constructs and their relative binding to E18 rat brain tissue compared with soluble NgR1 (NgR1(CT+stalk)). Soluble NgR1 (red) and NgR3 (yellow), but not NgR2 (green), bound strongly to brain tissue sections. The LRRs of NgR1, which were previously shown to participate in myelin inhibitor binding, were dispensable for binding to neural tissue. Deletion of a cluster of basic amino acid residues in the C-terminal region of the NgR1 and NgR3 stalk (motif 2) or replacement of these residues with alanines (NgR1(7ala)) completely abolished binding. (e) Sequence alignment of binding motif 1 and motif 2 of NgR1 and NgR3. In the LRR C-terminal domain, residues F278 and R279 in NgR1 and residues F273 and R274 in NgR3 (motif 1) are important for GAG binding. Motif 2 is comprised of a cluster of basic amino acids, including residues 414–426 in NgR1 and residues 403–415 in NgR3. The basic residues of motif 1 and motif 2 are highlighted.

lacking select gangliosides showed no substantial reduction in soluble receptor binding (Supplementary Fig. 3). In COS-7 cells, components of the NgR1 holoreceptor complex, including p75, TROY and Lingo-1 (ref. 30), failed to support NgR3 binding (Supplementary Fig. 4). This suggests that binding of NgR3 to brain is not mediated by previously identified components of the NgR1 complex. Receptor deletion studies further revealed that the LRRs were not required for binding, and identified two discontinuous and evolutionarily conserved sequence motifs in both NgR1 and NgR3 that were necessary for binding to brain (Fig. 2b–e and data not shown). Motif 1 is located in the C-terminal capping domain and overlaps with the FRG motif, which was previously shown to participate in sialic acid–dependent binding of the ganglioside GT1b to NgR1 (ref. 31). Motif 2, separated from motif 1 by approximately 130 amino acid residues, was located near the juxtamembrane region of the NgR1 and NgR3 stalk domain. In NgR1 and NgR3, motif 2 was comprised of a highly conserved cluster of basic amino acid residues, deletion of which completely abolished binding to brain (Fig. 2b–e). Furthermore, a soluble form of NgR1 in which the basic residues of motif 2 were replaced by seven alanines (NgR1(7ala)-Fc) no longer bound to brain tissue (Fig. 2b–e). Notably, the first three Ig-like domains of RPTPσ (RPTPσ(1–3)) showed very similar GAG-binding profiles (Fig. 3c). At increasing doses, RPTPσ(1–3)-Fc effectively competed with NgR1 for binding to CS-E, indicating that these two receptors complex with, at least in part, overlapping CS-GAG epitopes (Supplementary Fig. 5c). Functional studies with primary neurons revealed that soluble RPTPσ(1–3)-Fc and NgR1-Fc blocked the growth inhibitory activity of CSPGs toward P7 CGNs in vitro. The neutralizing effects of NgR1-Fc critically depended on the presence of the GAG-binding motif 2, as soluble NgR1(7ala)-Fc failed to block CSPG inhibition (Fig. 3d).
To further characterize the relation of CSPG and MAI binding sites on NgR1, we generated a chimeric receptor construct in which the GAG-binding portion of NgR1 (amino acid residues 278–445) was replaced by the corresponding non–GAG-binding sequence of NgR2 (amino acid residues 281–420). Nogo66, MAG and OMgp bound strongly to this chimeric NgR1-NgR2 receptor, indicating that the GAG-binding sequences of NgR1 are not necessary for MAI binding (Fig. 4a). A soluble form of this same chimeric receptor failed to bind to rat brain tissue sections or to GAGs directly (Supplementary Fig. 5d–e). This suggests that MAIs and CSPGs bind to distinct and dissociable sites on NgR1 (Fig. 4b). Moreover, the presence of CS-B, CS-D or CS-E did not substantially influence binding of AP-Nogo66 to NgR1 (Fig. 4c).

Neuronal NgRs participate in CSPG inhibition

To determine whether loss of NgRs leads to disinhibition of neurite growth on substrate-bound CSPGs, we analyzed CGNs from Ngr single and compound mutants (Fig. 5). Loss of Ngr1 or Ngr3 alone, or the combined loss of Ngr1 and Ngr2 (Ngr1<sup>−/−</sup>; Ngr2<sup>−/−</sup>), was not sufficient to attenuate CSPG inhibition. Loss of all three Ngr genes (Ngr1<sup>−/−</sup>; Ngr2<sup>−/−</sup>; Ngr3<sup>−/−</sup>), however, resulted in significant (P < 0.001, one way ANOVA, Tukey’s post hoc) yet incomplete, release of CSPG inhibition. Furthermore, release of inhibition for CGNs isolated from Ngr1<sup>−/−</sup>; Ngr2<sup>−/−</sup> and Rptpσ<sup>−/−</sup> pups was comparable (Fig. 5). Dose-response experiments revealed that, when challenged with high concentrations of CSPGs, loss of NgRs may be compensated for by RPTPg and vice versa. NgRs are not abundantly expressed in P7 DRGs<sup>15</sup> and Ngr1<sup>−/−</sup>; Ngr2<sup>−/−</sup>; Ngr3<sup>−/−</sup> DRG neurons are not disinhibited on CSPG substrate. In a parallel experiment with DRG neurons from Rptpσ<sup>−/−</sup> mice, neurite length was increased on CSPGs (Supplementary Fig. 6b). Collectively, these findings indicate that Ngr1 and Ngr3 bind CS-GAGs directly and participate in CSPG-mediated neurite outgrowth inhibition in a neuronal cell type–specific manner.
NgR1 and NgR3 associate in a ligand-dependent manner

NgRs are GPI-anchored proteins and therefore depend on interactions with transmembrane receptor components to signal growth inhibition across the neuronal plasma membrane. To assess whether NgR1 and NgR3 employ shared signaling mechanisms, we assayed binding of NgR3-Fc to the previously identified NgR1 receptor components p75, TROY and Lingo-1 in COS-7 cells. We observed no binding of soluble NgR3 to p75, TROY, Lingo-1 or NgR1 (Supplementary Fig. 4). There have been conflicting results on whether NgR1 and NgR3 interact32,33. We therefore revisited this issue and found that NgR1 and NgR3 were part of the same immune complex when coexpressed in HEK293T cells. The NgR1-NgR3 association was ligand dependent and was only observed in the presence of exogenously applied CSPGs (Supplementary Fig. 4), suggesting that the two receptors may be part of the same receptor complex. In this same assay, no association of NgR1 with NgR2 was observed, either in the presence or the absence of CSPGs. We next examined whether NgR1, NgR3 and p75 may be part of the same receptor complex. In HEK293T cells co-transfected with NgR1, NgR3 and p75, NgR1 pull-down revealed that the three receptors were present in the same immune complex if cells were treated with CSPGs (Supplementary Fig. 4).

To directly test whether p75 is important for CSPG-mediated neurite outgrowth inhibition, we plated P7 CGNs from p75−/− mice on substrate-bound CSPGs. Loss of p75 did not result in a substantial release of CSPG inhibition (Supplementary Fig. 4). Together, these data indicate that p75, NgR1 and NgR3 interact in the presence of CSPGs, but p75 is not necessary for CSPG-mediated outgrowth inhibition. Our findings confirm and expand on previous work showing that the versican isoform V2 mediates neurite outgrowth inhibition in CGNs and DRG neurons in a p75-independent manner.17

CSPGs in the injured CNS support binding of NgR1 and NgR3

Similar to other CNS fiber tracts, severed retinal ganglion cell (RGC) axons in the rodent optic nerve fail to show spontaneous long-distance axonal regeneration. Retro-orbital crush injury to the optic nerve results in a global upregulation of CSPGs along the nerve34. Injured, but not control, optic nerve sections supported enhanced binding of soluble NgR1-Fc and NgR3-Fc, and the GAG-binding motif 2 of NgR1 and NgR3 was necessary for this binding (Fig. 6). Moreover, binding of soluble receptors was largely abrogated by pretreatment of injured optic nerve sections with Ch'aseABC. Residual binding of NgR1-Fc was likely a result of association with endogenous MAIs (Fig. 6). These findings suggest that CSPGs are endogenous ligands for neuronal NgR1 and NgR3.

Regeneration is enhanced in Ngr1−/−; Ngr3−/− mice

In the adult mouse retina, NgR1, NgR2 and NgR3 were all strongly expressed in RGCs (Fig. 7a). Retinal stratification (Fig. 7b) and optic nerve myelination (Fig. 7c) in Ngr1−/−; Ngr2−/−; Ngr3−/− mice appeared to be normal. To assess RGC axon targeting to the superior colliculus, the suprachiasmatic nucleus and the lateral geniculate nucleus, we injected the right eye of adult wild-type and Ngr1−/−; Ngr2−/−; Ngr3−/− mice with Alexa 594–conjugated cholera toxin β tracer and the left eye with Alexa 488–conjugated cholera toxin β tracer. No defects in RGC axon central projections or target
innervation were observed (Fig. 7d-f). Thus, germline ablation of all three NgRs does not appear to compromise retinal stratification, optic nerve myelination or RGC axonal pathfinding.

To assess whether NgRs contribute to the regenerative failure of injured CNS axons, we performed retro-orbital optic nerve crush injury in Ngr single and compound mutant mice. Compared with injured wild-type controls, Ngr1−/−; Ngr2−/−; Ngr3−/− mice showed a modest, but significant (P < 0.001, one way ANOVA, Tukey's post hoc), increase in RGC axon regeneration (Fig. 8). At 2 weeks post-injury, more GAP43+ fibers were observed 0.2–1.0 mm distal to the injury site in Ngr1−/−; Ngr2−/−; Ngr3−/− mice compared with wild-type mice. Because Ngr1 and Ngr2 are known to associate with MAIs, the Ngr1−/−; Ngr2−/−; Ngr3−/− regeneration phenotype may be a reflection of decreased Nogo, MAG and OMgp inhibition, decreased CSPG inhibition, or a combination thereof. To address this issue, we directly compared regeneration of Ngr1−/−, Ngr2−/− and Ngr3−/− single mutants, as well as Ngr1−/−; Ngr2−/− and Ngr1−/−;
Ngr3−/− double mutants, with that of Ngr1−/−; Ngr2−/−; Ngr3−/− mice. Loss of Ngr1, Ngr2 or Ngr3 alone, or the combined loss of Ngr1 and Ngr2, did not result in substantially enhanced RGC axon regeneration compared with wild-type mice (Fig. 8, Supplementary Fig. 7 and Supplementary Table 1). However, Ngr1−/−; Ngr3−/− mice showed a similar degree of axon regeneration as Ngr1−/−; Ngr2−/−; Ngr3−/− mice. This suggests that Ngr3 is involved in signaling neuronal growth inhibition. When coupled with our findings from neurite outgrowth studies in vitro, that NgR1 and NgR3 operate as functionally redundant CSPG receptors, this suggests that the optic nerve regeneration in Ngr1−/−; Ngr3−/− and Ngr1−/−; Ngr2−/−; Ngr3−/− mice is at least in part a reflection of decreased CSPG inhibition.

As Rptpσ is expressed in adult RGCs27, we examined whether the combined loss of Ngr1 and Ngr3 on an Rptpσ−/− background (Ngr1−/−; Ngr3−/−; Rptpσ−/−) results in a further increase of regenerating axons. Few regenerating axons were observed in Rptpσ−/− single mutants, with no significant difference compared with wild-type controls (P > 0.05). Compared with Ngr1−/−; Ngr3−/− mutants, Ngr1−/−; Ngr3−/−; Rptpσ−/− mutants showed a further increase in the number of regenerating axons (P < 0.001, one way ANOVA, Tukey’s post hoc), suggesting a genetic interaction among these receptors (Fig. 8, Supplementary Fig. 7 and Supplementary Table 1).

An advantage of optic nerve regeneration studies is that the growth potential of RGCs can be sensitized by intraocular injection of the yeast cell wall extract Zymosan, resulting in the release of RGC survival and growth-promoting factors, including oncomodulin35, ciliary neurotrophic factor and leukemia inhibitory factor36. Wild-type mice that received intraocular Zymosan showed greatly enhanced fiber regeneration following crush injury to the optic nerve. Regenerating axons in optic nerve sections were visualized using an antibody to GAP43, 2 weeks after injury. The injury site is marked with an asterisk. (a) Wild-type mice showed very limited regenerative axonal growth following injury. (b) In Ngr1−/−; Ngr2−/−; Ngr3−/− mice, many GAP43+ fibers grew beyond the lesion site. (c) In Ngr1−/−; Ngr3−/−; Rptpσ−/− mice, a further increase of GAP43+ fiber growth was observed. From left to right, lower panels depict high-magnification images of the region 0.5–0.75 mm distal to the lesion site (dashed line in images a, b and c, respectively). Scale bar represents 200 µm. (d) Intracocular injection of Zymosan enhanced regenerative axonal growth in wild-type mice. A further increase was observed in Ngr1−/−; Ngr2−/−; Ngr3−/− mice (e), which was further enhanced in Ngr1−/−; Ngr3−/−; Rptpσ−/− mice (f). From left to right, lower panels depict high-magnification images of the region 0.5–0.75 mm distal to the lesion site (dashed line in images d, e and f, respectively). Scale bar represents 200 µm. (g) Quantification of the number of GAP43+ axons at 0.2–1.2 mm distal to the lesion site (wild type, n = 6; Ngr1−/−, n = 7; Rptpσ−/−, n = 5; Ngr1−/−; Ngr3−/−, n = 8; Ngr1−/−; Ngr3−/−; Rptpσ−/−, n = 41). (h) Quantification of the number of GAP43+ axons at 0.2–1.6 mm distal to the lesion site in Zymosan-injected mice (wild type + Zymosan, n = 6; Ngr1−/− + Zymosan, n = 6; Rptpσ−/− + Zymosan, n = 4; Ngr1−/−; Ngr2−/−; Ngr3−/− + Zymosan, n = 8; Ngr1−/−; Ngr3−/−; Rptpσ−/− + Zymosan, n = 3). Results are presented as mean ± s.e.m. **P < 0.05 (one-way ANOVA, Tukey’s post hoc).
enhanced regeneration of RGC axons, exceeding the regeneration observed in Ngr1−/−; Ngr2−/−; Ngr3−/− mice that were not treated with Zymosan (Fig. 8). Ngr1−/−; Ngr2−/−; Ngr3−/− mice that received intraocular Zymosan showed significantly more (P < 0.05, one way ANOVA, Tukey's post hoc) regenerating axons than wild-type, Ngr1−/−; Ngr2−/−; Ngr3−/− or Rptpsα−/− mice, as well as Ngr1−/−; Ngr2−/− mutants, subjected to intraocular Zymosan. Ngr1−/−; Ngr3−/− and Ngr1−/−; Ngr2−/−; Ngr3−/− mice treated with intraocular Zymosan showed a similar regeneration phenotype. At several distances from the injury site, Ngr1−/−; Ngr3−/−; Rptpsα−/− mutants with intraocular Zymosan showed a further increase in the number of regenerating axons compared to Ngr1−/−; Ngr2−/−; Ngr3−/− mice with intraocular Zymosan (P < 0.05, one way ANOVA, Tukey’s post hoc; Fig. 8, Supplementary Fig. 7 and Supplementary Table 1).

In mice, optic nerve injury leads to the death of ~70% of RGCs by 2 weeks post-injury (Supplementary Fig. 8). The enhanced regeneration observed in Ngr1−/−; Ngr2−/−; Ngr3−/− mice was not a result of increased RGC survival, as similar numbers of injury-induced RGC death were observed in wild-type mice and Ngr1−/−; Ngr2−/−; Ngr3−/− mutants. Intraocular Zymosan administration partially protected RGCs from axotomy-induced cell death; however, the protective effect of Zymosan is similar in wild-type and Ngr1−/−; Ngr2−/−; Ngr3−/− mice (Supplementary Fig. 8). Consistent with the view that a decrease in RGC death was not sufficient to promote axonal regeneration, p53-deficient RGCs were more resistant to injury-induced cell death, but fail to show enhanced regeneration1. To assess whether intraocular Zymosan influences expression of NgRIs or RPTPσ in RGCs, we performed in situ hybridization at 3 and 7 d post-Zymosan injection, but did not observe any obvious changes (Supplementary Fig. 9).

**DISCUSSION**

Here we identified two previously unknown CSPG receptors. We found that Ngr1 and Ngr3 bound directly and with high affinity to select types of CS-GAGs and operated as functionally redundant CSPG receptors. Loss of Ngf family members individually was not sufficient to overcome CSPG inhibition; however, the combined loss of Ngr1 and Ngr3 led to a significant release of CSPG inhibition (P < 0.05). In Ngr1−/−; Ngr2−/−; Ngr3−/− mutants, severed RGC axons showed enhanced regenerative growth. Notably, Ngr1−/−; Ngr3−/−, but not Ngr1−/−; Ngr2−/− mutants, phenocopied the optic nerve regeneration phenotype of Ngr1−/−; Ngr2−/−; Ngr3−/− mice. A further enhancement of axon regeneration was observed in Ngr1−/−; Ngr3−/−; Rptpsα−/− mice. A recent study identified the receptor tyrosine phosphatase LAR as a dominant-negative form of NgR1 in RGCs39 or blocking of RhoA with C3 transferase 39 is not sufficient to promote substantial regeneration of severed optic nerve axons. In a similar vein, removal of one or several MAIs results in inconsistent and often poor regeneration in spinal cord–injured mice38,39. Collectively, mouse genetic studies suggest that germline ablation of multiple growth inhibitory ligands or receptors is not sufficient to promote robust and long-distance regeneration in different fiber tracts of the injured adult CNS.

A substantial effect of environmental inhibitory signals on limiting axon regeneration was revealed, however, when genetic manipulations were combined with activation of RGC intrinsic growth programs. On an Ngr1−/−; Ngr3−/−, Ngr1−/−; Ngr2−/−; Ngr3−/− or Ngr1−/−; Ngr3−/−; Rptpsα−/− background, intraocular Zymosan injection resulted in significantly enhanced axonal growth distal to the injury site compared with wild-type, Ngr1−/−; Ngr2−/− or Rptpsα−/− mutant mice with intraocular Zymosan. Although the additive effects of simultaneous release of growth inhibitory mechanisms and activation of intrinsic growth programs have been reported38,40,41, our data indicate that, in growth-enabled RGCs, members of the NgR family and LAR family collabo rate to negatively affect the number and length of regenerating axons following CNS injury.

**NgR3 participates in neuronal growth inhibition**

Enhanced axon regeneration observed in the optic nerve of Ngr1−/−; Ngr2−/−; Ngr3−/− mice was mimicked by Ngr1−/−; Ngr3−/−, but not by Ngr1−/−; Ngr2−/−, mutants. This suggests that, on an Ngr1−/− background, NgR3, but not NgR2, contributes to the regenerative failure of severed RGC axons. As NgR3 does not directly associate with Nogo, MAG or OMgp, but supports CSPG binding and participates in CSPG inhibition in vitro, our findings suggest that NgR3-CSPG–mediated growth inhibition contributes to the regenerative failure of CNS axons in vivo.

Although CSPGs are the first ligands identified for NgR3, they also bind to NgR1, further underscoring the high promiscuity of NgR1. CSPGs are found in crude CNS myelin preparations17,42 and were present in the CNS myelin that we used (data now shown). Similar to the enhanced neurite outgrowth of Ngr1−/−; Ngr2−/−; Ngr3−/− neurons on CNS myelin (Fig. 1), the enhanced growth of neurons functionally depleted of NgR1 and PirB10 may be, at least in part, a reflection of decreased MAI and CSPG inhibition.

**Implications for experience-dependent neural plasticity**

Although it has been known for some time that MAIs and CSPGs share similar downstream signaling pathways16,17, the level at which MAI and CSPG signaling cascades converge to regulate neuronal...
cytoskeletal dynamics has not yet been determined. We identified NgR1 and NgR3 as functionally redundant CSPG receptors. We provide evidence that Nogo, MAG, OMgp and CSPGs share receptor components and perhaps signal through related receptor complexes to block neuronal plasticity, sprouting and axonal regeneration. In support of this idea, the myelin inhibitor Nogo-A shares structural and sequential similarities with neurocan, an inhibitory CSPG that has been implicated in blocking neuronal regeneration, suggesting a common origin for two seemingly unrelated inhibitors of growth. The newly discovered connection between CSPGs and NgRs is not only relevant for neuronal repair, but may also provide a mechanistic explanation for why two seemingly unrelated manipulations, such as ChAseABC infusion into the mature visual cortex and germline ablation of Ngr1 or Nogo result in enhanced ocular dominance plasticity following monocular deprivation.

Mounting evidence suggests that mechanisms that limit neuronal growth and plasticity following CNS injury and disease resemble those that negatively regulate neuronal growth and synaptic structure under physiological conditions.

The identification of NgRs as shared receptors for MAIs and CSPGs provides new insights into how a diverse group of inhibitory cues regulates neuronal structure and function under physiological conditions and following injury. We propose that NgRs are part of a multicomponent receptor system that serves as a signaling platform to initiate pathways that limit neuronal growth and increase structural stability of synapses. When combined with recent findings that NgR1 and its ligands Nogo and OMgp influence synaptic transmission, experience-dependent network refinement and spatial memory, our results expand the function of these molecules beyond memory.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

ACKNOWLEDGMENTS

We thank M. Tremblay for Rppsα+−/− mice, M. Greenberg for Ngr3−−/− mice, B. Perschala for p75NgR−/− mice, B. Bates, D. Howland and M.L. Mercado for their assistance in the generation and initial analysis of Ngr1−/−; Ngr2−/−; Ngr3−/− mice. U. Rutishauser for Endo-N, D. Figge and Y. Yassu for assistance in ELISA binding assays, Y. Yin for training in optic nerve surgery, Y. Duan for generation of the RPTPα(Δg1–3)-Fc construct, J. Barbari for technical assistance and M.M. Zaleska for project administration. This work was supported by Neuroscience Training Grant T32 EY07178 and the University of Michigan Rackham Merit Fellowship (T.L.D.), Cellular and Molecular Biology Training Grant T32GM07315 (K.T.B. and Y.A.M.), National Research Service Award Ruth Kirschstein Fellowship F31NS061589 (S.I.R.), the New York State Spinal Cord Injury Research Program, the Dr. Miriam and Sheldon G. Adelson Medical Foundation on Neural Repair and Rehabilitation, the US Department of Veterans Affairs (1101RX00229-01), the National Institute of Neurological Disorders and Stroke (R56NS047333, R.J.G.) and the National Eye Institute (L.I.B.).

AUTHOR CONTRIBUTIONS

R.J.G. conceived the study, T.L.D., L.I.B., H.M.G. and R.J.G. designed the experiments. T.L.D., K.T.B., Y.A.M., Y. Koriyama, S.I.R., C.D.L., Y. Katagiri and R.J.G. performed the experiments. T.L.D. and K.T.B. and Y. Koriyama contributed to data analysis and figure preparation. K.L.A., A.W., C.G.G. and B.Z. generated and provided mice or reagents for the study, T.L.D. and R.J.G. wrote the manuscript. All of the authors read and agreed on the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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29. Cafferty, W.B., Duffy, P., Huebner, E. & Strittmatter, S.M. MAG and OMgp synergize with Nogo-A to restrict axonal growth and neurological recovery after spinal cord trauma. *J. Neurosci.* **30**, 6825–6837 (2010).

30. Yamashita, T., Fujitani, M., Yamagishi, S., Hata, K. & Mimura, F. Multiple signals regulate axon regeneration through the Nogo receptor complex. *Mol. Neurobiol.* **32**, 105–111 (2005).

31. Williams, G. *et al.* Ganglioside inhibition of neurite outgrowth requires Nogo receptor function: identification of interaction sites and development of novel antagonists. *J. Biol. Chem.* **283**, 16641–16652 (2008).

32. Barton, W.A. *et al.* Structure and axon outgrowth inhibitor binding of the Nogo-66 receptor and related proteins. *EMBO J.* **22**, 3291–3302 (2003).

33. Zhang, L., Kuang, X. & Zhang, J. Nogo receptor 3, a paralog of Nogo-66 receptor 1 (NgR1), may function as a NgR1 co-receptor for Nogo-66. *J. Genet. Genomics* **38**, 515–523 (2011).

34. Ohlsson, M., Mattsson, P. & Svensson, M. A temporal study of axonal degeneration and glial scar formation following a standardized crush injury of the optic nerve in the adult rat. *Restor. Neurol. Neurosci.* **22**, 1–10 (2004).

35. Yin, Y. *et al.* Oligonucleotides link inflammation to optic nerve regeneration. *Proc. Natl. Acad. Sci. USA* **106**, 19587–19592 (2009).

36. Leibinger, M. *et al.* Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. *J. Neurosci.* **29**, 14334–14341 (2009).

37. Fisher, D. *et al.* Leukocyte common antigen-related phosphatase is a functional receptor for chondroitin sulfate proteoglycan axon growth inhibitors. *J. Neurosci.* **31**, 14051–14066 (2011).

38. Fischer, D., He, Z. & Benowitz, L.I. Counteracting the Nogo receptor enhances optic nerve regeneration if retinal ganglion cells are in an active growth state. *J. Neurosci.* **24**, 1646–1651 (2004).

39. Fischer, D., Petkova, V., Thanos, S. & Benowitz, L.I. Switching mature retinal ganglion cells to a robust growth state in vivo: gene expression and synergy with RhoA inactivation. *J. Neurosci.* **24**, 8725–8740 (2004).

40. Kadoya, K. *et al.* Combined intrinsic and extrinsic neuronal mechanisms facilitate bridging axonal regeneration one year after spinal cord injury. *Neuron* **64**, 165–172 (2009).

41. Fujita, Y., Endo, S., Takai, T. & Yamashita, T. Myelin suppresses axon regeneration by PIR-B/SHP-mediated inhibition of Trk activity. *EMBO J.* **30**, 1389–1401 (2011).

42. Niederost, B.P., Zimmermann, D.R., Schwab, M.E. & Bandtlow, C.E. Bovine CNS myelin contains neurite growth-inhibitory activity associated with chondroitin sulfate proteoglycans. *J. Neurosci.* **19**, 8979–8989 (1999).

43. Shypitsyna, A., Malaga-Trillo, E., Reuter, A. & Stuermer, C.A. Origin of Nogo-A by domain shuffling in an early jawed vertebrate. *Mol. Biol. Evol.* **28**, 1363–1370 (2010).

44. Lee, H. *et al.* Synaptic function for the Nogo-66 receptor NgR1: regulation of dendritic spine morphology and activity-dependent synaptic strength. *J. Neurosci.* **28**, 2753–2765 (2008).

45. Zagrebelsky, M., Schweigreiter, R., Bandtlow, C.E., Schwab, M.E., & Korte, M. Nogo-A stabilizes the architecture of hippocampal neurons. *J. Neurosci.* **30**, 13220–13234 (2010).

46. Raiker, S.J. *et al.* Myelin and Nogo-A negatively regulate activity-dependent synaptic plasticity. *J. Neurosci.* **30**, 12432–12445 (2010).

47. Karlén, A. *et al.* Nogo receptor 1 regulates formation of lasting memories. *Proc. Natl. Acad. Sci. USA* **106**, 20476–20481 (2009).
ONLINE METHODS

Transgenic mice. All handling and surgical procedures were performed in compliance with local and animal handling care guidelines approved by the University of Michigan Committee on Use and Care of Animals. NogoABC(Δ)−/−, Mag(Δ)−/−, Omgp(Δ)−/−, Rptpβ(Δ)−/−, NgR1(Δ)−/−, NgR2(Δ)−/− and p53GsTR−/− mice have been described previously. Ngr3−/− germline mutants were generated by Lexicon Genetics and kindly provided by M. Groenberg (Harvard Medical School). Ngr1 and Ngr2 conditional mutants have been described previously. Ngr3 conditional knockout mice were generated by flanking exon 2 withloxP sites (Supplementary Fig. 1). To generate germline deletion mutants, we crossed conditional knockouts with protemic cre transgenic mice and then intercrossed the progeny with each other, or onto anRptpt+/− background, to generate double and triple mutants.

To confirm that Ngr1−/−, Ngr2−/−; Ngr3−/− mice were deficient for Ngr1, Ngr2 and Ngr3, we analyzed brain extracts of adult wild-type and Ngr1−/−; Ngr2−/−; Ngr3−/− mice by western blotting. To enrich for NgRs, we isolated brain membranes, lysed them in RIPA buffer (Sigma) containing a protease inhibitor cocktail (Sigma), and affinity-purified them with a-garosac-Comcanavalin A beads (Vector Laboratories) overnight at 4°C. Bound glycoproteins were subjected to SDS-PAGE, blotted onto nitrocellulose membranes (Thermo Fisher Scientific) and probed with polyclonal antibodies to Ngr1, Ngr2, Ngr3 or β-actin.

Neurite outgrowth assays. To assay myelin inhibition, we coated 96-well plates with poly-D-lysine hydrobromide (50 μg ml−1). Sigma) overnight, rinsed in water, air dried, and then incubated with a 5-μl spot of BSA (40 μg ml−1) or CNS myelin (40 μg ml−1) prepared from newborn rats. Plates were then incubated with laminin (10 μg ml−1, Sigma) for 1 h at 37°C. P7 CGNs and DRGs were prepared as described previously and cultured for 24 h before they were fixed with 4% paraformaldehyde (wt/vol), blocked in 1% bovine serum (vol/vol) and 0.1% Triton X-100 (vol/vol) in PBS, and stained with antibody to class III β-tubulin (Tuj1, Promega). To visualize the spotted membranes, we also stained wells with antibody to myelin basic protein (Sigma). Alexa Fluor-conjugated secondary antibodies (Invitrogen) were used for fluorescent labeling.

To assay CSPG inhibition, 5-μl spots (1, 2, 10, 100 μg ml−1) of either a mixture of large, extracellular chondroitin sulfate proteoglycans isolated from embryonic chicken brain (Millipore) or BSA were adsorbed on 96-well plates for 3 h at 37°C before coating with poly-D-lysine hydrobromide and laminin. After 24 h in culture, neurite length of CGNs or DRGs was determined as described above. To determine whether NgRs or RPTPβ bind directly to GAG chains, ELISAs were used as described previously. Briefly, GAG chains were biotinylated with EDC and EZ-Link Sulfo-NHS-LC-LC- Biotin (Thermo Scientific) and adsorbed for 15 min to ELISA plates (Immunol, NUNC) precoated with streptavidin (5 μg ml−1, Invitrogen). Plates were blocked (5% BSA), rinsed with HEPES-buffered saline (HBS), and incubated with various amounts of fusion proteins (diluted with 5% BSA) for 2 h at 22°C. Following five washes with HBS, bound alkaline phosphatase activity was monitored with a BluePhos Microwell Substrate Kit (KPL). For competitive binding experiments, immobilized GAG chains were pre-adsorbed with various amounts of RPTPβ (1 μg–3 μg) for 4 h at 4°C, and then incubated with AP-NgR1 (1 nM) for 2 h at 22°C. Bound alkaline phosphatase activity was measured as described above.

Immunoprecipitation. HEK293T cells (in 10-cm culture dishes) were transfected with various combinations of p75NTR, Ngr1, Ngr1-myc, Ngr2-myc and Ngr3-myc expression constructs. After 48 h, the cells were incubated in lysis buffer containing 20 nM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40 (vol/vol), and protease inhibitor mixture. For some assays, cells were incubated with 100 μg ml−1 of CSPG mixture before lysis. Cell lysates were harvested, proteins precipitated with Protein G Plus/Protein A–Agarose (Calbiochem) and washed three times with lysis buffer, and bound proteins were eluted with 2× SDS sample buffer. Precipitates were analyzed by immunoblotting, using antibodies to Ngr1, p75 (Promega), myc (Cell Signaling) or β-actin (Sigma).

Optic nerve surgery. Adult mice (6–8 weeks of age) of either sex were anesthetized with an intraperitoneal injection of ketamine (100 mg per kg body weight, Fort Dodge Animal Health) and xylazine (10 mg per kg, Akorn). The optic nerve was exposed through an incision in the conjunctiva and compressed for 10 s with angle jeweler’s forceps (Dumont #5, Fine Science Tools) at approximately 1 mm behind the eyeball. Care was taken not to damage or rupture the ophthalmic artery. For intraocular injection of Zymosan, 5 μl of a suspension (12.5 μg ml−1 in sterile PBS, Sigma) was injected manually using a Hamilton syringe with a 30 gauge removable needle. Following optic nerve surgery, the operated eye was rinsed with sterile PBS and ophthalmic ointment was applied (Butler AHS). All surgeries were performed under aseptic conditions. Mice were given a lethal dose of anesthesia 14 d after optic nerve injury and perfused through the heart with PBS followed by ice-cold 4% paraformaldehyde.

Histochemical studies. In situ hybridization of mouse retina with cRNA probes specific for Ngr1, Ngr2, Ngr3 and Rptpt was carried out as described previously. For immunohistochemical procedures, cryosections of adult retina were stained with antibody to calbindin (Swant, 1:2,500) or antibody to calretinin (Swant, 1:2,500), and then counterstained with Hoechst 33342 (1:30,000).
To assess axon density and myelination, optic nerves were embedded in epon and stained with Toluidine blue. To assess retinal ganglion cell death at various time points following optic nerve injury, retinal sections were stained with antibody to class III β-tubulin (TuJ1) and, in some instances, with antibody to active caspase-3 (Promega).

For intraocular injections of anterograde tracer, 6-week-old mice received bilateral injections (2 µl) of 1 µg µl⁻¹ Alexa 488– and Alexa 594–conjugated cholera toxin β (Invitrogen) in the left and right eye, respectively. Mice were perfused transcardially 5 d post-injection and their brains were dissected, post-fixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose overnight. Brain tissue was embedded in OCT Tissue-Tek Medium (Sakura Finetek) and coronal sections (50 µm thick) were imaged.

To visualize regenerating axons in the injured optic nerve, eyes with optic nerves attached were dissected, post-fixed and cryoprotected. Optic nerves were embedded and longitudinal sections (14 µm thick) were stained with antibody to GAP-43. The appropriate Alexa Fluor–conjugated secondary antibody (Invitrogen) was then used for fluorescent labeling.

**Statistical analysis.** For quantification of neurite outgrowth, UTHSCSA ImageTool for Windows was used, and processes equal or longer to approximately one cell body diameter were measured. For each condition, at least 150 neurites were quantified, and the mean ± s.e.m. of neurite length for each genotype was determined from multiple independent experiments. For quantification of retinal ganglion cell death, the number of TuJ1-positive cells in the ganglion cell layer per field of view (at least ten sections, three independent experiments per condition) was counted. For quantification of activated caspase-3–positive retinal ganglion cells, the number of cells labeled for activated caspase-3 was calculated as a percentage of the total number of cells (TuJ1 positive) per field of view (at least ten sections, three independent experiments per condition). Quantification of optic nerve binding assays and in situ hybridization (at least 20 sections, four independent experiments per condition) was performed as previously described²⁰, using Microsuite Five (Olympus) quantification software. All data were analyzed using ANOVA followed by Tukey’s post hoc comparisons. All statistics were performed using SigmaStat 3.0 for Windows (Systat Software).

To assess regenerative axonal growth, the number of GAP-43–positive axons at prespecified distances from the injury site was counted in at least three sections per nerve. These numbers were converted into the number of regenerating axons per nerve at various distances as described previously³⁸. All data were analyzed using ANOVA followed by Tukey’s post hoc comparisons. All statistics were performed using GraphPad Prism 5.00 (GraphPad Software). Our finding that loss of all three Ngr genes elicited significant retinal ganglion cell regeneration was based on two independently generated datasets produced by two independent surgeons (K.T.B. and Y. Koriyama). Both datasets were analyzed separately and led to the same conclusions (Supplementary Table 1). In addition, no significant differences in axon regeneration following injury (with or without intraocular Zymosan injection) were observed between mice on three different genetic backgrounds (129, C57BL/6 and BALB/c) (P > 0.05, data not shown).

49. Briani, C., Berger, J.S. & Latov, N. Antibodies to chondroitin sulfate C: a new detection assay and correlations with neurological diseases. *J. Neuroimmunol.* **84**, 117–121 (1998).

50. Robak, L.A. *et al.* Molecular basis of the interactions of the Nogo-66 receptor and its homolog NgR2 with myelin-associated glycoprotein: development of NgROMNI-Fc, a novel antagonist of CNS myelin inhibition. *J. Neurosci.* **29**, 5768–5783 (2009).