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Effect of combined treatment with methylprednisolone and soluble Nogo-66 receptor after rat spinal cord injury

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Abstract

Methylprednisolone (MP) is a synthetic glucocorticoid used for the treatment of spinal cord injury (SCI). Soluble Nogo-66 receptor (NgR) ectodomain is a novel experimental therapy for SCI that promotes axonal regeneration by blocking the growth inhibitory effects of myelin constituents in the adult central nervous system. To evaluate the potential complementarity of these mechanistically distinct pharmacological reagents we compared their effects alone and in combination after thoracic (T7) dorsal hemisection in the rat. Treatment with an ecto-domain of the rat NgR (27–310) fused to a rat IgG [NgR(310)ecto-Fc] (50 μ M intrathecal, 0.25 μ L/h for 28 days) or MP alone (30 mg/kg i.v., 0, 4 and 8 h postinjury) improved the rate and extent of functional recovery measured using Basso, Beattie, Bresnahan (BBB) scoring and footprint analysis. The effect of MP treatment on BBB score was apparent the day after SCI whereas the effect of NgR(310)ecto-Fc was not apparent until 2 weeks after SCI. NgR(310)ecto-Fc or MP treatment resulted in increased axonal sprouting and/or regeneration, quantified by counting biotin dextran amine-labeled corticospinal tract axons, and increased the number of axons contacting motor neurons in the ventral horn gray matter caudal to the lesion. Combined treatment with NgR(310)ecto-Fc and MP had a more pronounced effect on recovery of function and axonal growth compared with either treatment alone. The data demonstrate that NgR(310)ecto-Fc and MP act in a temporally and mechanistically distinct manner and suggest that they may have complementary effects.

Keywords

axon; injury; Nogo; Nogo-66 receptor; regeneration; spinal cord

Introduction

Methylprednisolone (MP) is a synthetic glucocorticoid, widely used for its anti-inflammatory and immunosuppressive actions, which is mediated via binding to a cytosolic receptor to modulate gene expression (Gold *et al.*, 2001). Short-term, high-dose MP treatment has been shown to be efficacious in animal models of spinal cord injury (SCI) when administered soon after injury (Oudega *et al.*, 1999; Nash *et al.*, 2002). Proposed mechanisms of action of MP include antioxidant effects (Hall, 1992) and potent anti-inflammatory effects mediated by suppression of Nuclear Factor kappa-B binding (Hsu & Dimitrijevic, 1990; Nadeau & Rivest,

2003). Glucocorticoid receptor expression is rapidly but only transiently induced after SCI (Yan *et al.*, 1999), perhaps explaining the very limited therapeutic window of MP.

The inability of central nervous system axons to regenerate after injury has been attributed, in part, to the inhibitory effects of myelin components Nogo (Chen *et al.*, 2000; GrandPré *et al.*, 2000; Prinjha *et al.*, 2000; Filbin, 2003), myelin-associated glycoprotein (McKerracher *et al.*, 1994) and oligodendrocyte myelin glycoprotein (Wang *et al.*, 2002a). A Nogo-66 receptor (NgR) was identified (Fournier *et al.*, 2001) that is now known to also mediate the neuronal growth inhibitory actions of oligodendrocyte myelin glycoprotein (Wang *et al.*, 2002b) and myelin-associated glycoprotein (Domeniconi *et al.*, 2002; Liu *et al.*, 2002). The NgR is a 473 amino acid glycoprophosphatidylinositol-linked protein containing eight leucine-rich repeats that is expressed in postnatal neurons (Fournier *et al.*, 2001). The localization of NgR, its coreceptors/signaling components, the p75 neurotrophin receptor (Wang *et al.*, 2002b; Wong *et al.*, 2002), LINGO-1 (Mi *et al.*, 2004) and TAJ(TROY) (Park *et al.*, 2005; Shao *et al.*, 2005), and its ligands in the central nervous system provide compelling circumstantial evidence for a role of this pathway in axonal regeneration/plasticity after injury (Huber *et al.*, 2002; Wang *et al.*, 2002; Filbin, 2003; Mi *et al.*, 2004). Furthermore, the fact that NgR mediates the growth inhibitory actions of at least three myelin inhibitory proteins makes it an attractive target for the development of drugs to promote axonal regeneration after SCI (McGee & Strittmatter, 2003; Lee *et al.*, 2003).

Recombinant forms of NgR and its ligands have been used to investigate the growth-permissive effects of soluble NgR constructs on neurite outgrowth in neuronal cells grown on myelin (GrandPré *et al.*, 2002). One such recombinant protein is an ecto-domain of the rat NgR (27–310) fused to a rat IgG [NgR(310)ecto-Fc]. This protein inhibited Nogo-66, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein binding to immobilized NgR *in vitro* and NgR(310)ecto-Fc delivered into the intrathecal space significantly improved histological and functional recovery after experimental SCI (Li *et al.*, 2004).

We undertook the present studies to compare the effect of a Nogo receptor inhibitor, NgR(310)ecto-Fc and MP in a rat model of SCI to evaluate the complementarity of these reagents. Both pharmacological agents improved functional and histological recovery after SCI when treatment was initiated immediately after injury. The effect of MP on functional recovery was apparent as early as the day after SCI whereas the effect of NgR(310)ecto-Fc was not manifest until 2 weeks after SCI, consistent with the respective proposed anti-inflammatory/anti-oxidant and pro-regenerative actions of these agents. Combined treatment with NgR(310)ecto-Fc and MP increased the rate and, in some instances, the extent of recovery. *In vitro* and *in vivo* findings suggest that these agents act by temporally and mechanistically distinct means.

Materials and methods

Recombinant rat soluble Nogo receptor 1-Fc fusion protein

Soluble rat NgR (residues 27–310) fused to the hinge and Fc region of rat Ig1 was expressed in chinese hamster ovary (CHO) cells [NgR(310)ecto-Fc] in BCM16. Conditioned media were concentrated 10-fold by ultrafiltration and filtered. Tris-HCl (pH 8.9), NaCl and glycine were added to final concentrations of 100 mM, 3 and 1.5 M, respectively. The concentrated medium was loaded onto a Protein A-sepharose column (Amersham Biosciences, Piscataway, NJ, USA). The column was washed with two column volumes of binding buffer (100 mM Tris-HCl, pH 8.9, 3 M sodium chloride, 1.5 M glycine) followed by one column volume of 5 mM Tris-HCl, 3 M sodium chloride, pH 8.9. NgR(310)ecto-Fc was eluted with 25 mM phosphate, pH 2.8, 100 mM sodium chloride and neutralized. The eluted protein was dialyzed against phosphate-buffered saline, filtered, aliquoted and stored at –70 °C. Purity, as assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and size exclusion

chromatography, was greater than 95%. Under reducing and nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis, NgR(310)ecto-Fc had apparent masses of 60 and 120 kDa, respectively. The endotoxin level in the product was <4 endotoxin units/mg.

Neurite outgrowth assay

Myelin was dried overnight in poly-L-lysine-precoated plates (Becton Dickinson, Bedford, MA, USA) at 80 or 400 ng/well (2.5 and 12.7 ng/mm², respectively). Wells were then coated with 10 µg/mL laminin (Calbiochem, La Jolla, CA, USA) for 1 h at room temperature (22–24 °C). Embryonic day 13 chick dorsal root ganglion neurons were dissociated and plated for 6–8 h as previously described (GrandPré *et al.*, 2000; Fournier *et al.*, 2001). Neurons were treated with 8 µM NgR(310)ecto-Fc in the presence or absence of 10 µg/mL MP (Pharmacia, Kalamazoo, MI, USA) for the entire outgrowth period. Neurons were then fixed and stained with βIII tubulin antibody (Covance, Princeton, NJ, USA) and neurite outgrowth was quantified using an automated cellular imaging and analysis system (Axon Instrument, Union City, CA, USA). Neurite outgrowth per cell was normalized to the average of duplicate control wells for each experiment ($n = 3$).

Spinal cord injury and corticospinal tract tracing

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Biogen Idec Inc. Institutional Animal Care and Use Committee.

Female Long Evans rats (7 weeks old; Charles River, Wilmington, MA, USA) were anesthetized using 2.5 mg/kg Midazolam i.p. (Abbott Laboratories, Chicago, IL, USA) and 2–3% Fluothane (Baxter, Deerfield, IL, USA) in O₂ and a dorsal laminectomy performed at spinal level T6 and T7. General anesthesia was maintained at 1.5–2% Fluothane in O₂. A dorsal hemisection was performed completely interrupting the main dorsomedial and the minor dorsolateral corticospinal tract (CST) components. A micro-scalpel was used to stereotactically transect the cord at a depth of 1.8 mm from the surface of the cord. Immediately after CST transection an intrathecal catheter was inserted into the subarachnoid space at T7 and connected to a primed mini-osmotic pump (Alzet model 2004; Alza Corp., Cupertino, CA, USA) inserted into the subcutaneous space. Mini-osmotic pumps delivered rat IgG isotype control protein or phosphate-buffered saline (5 mg/mL, $n = 8$; Pharmingen, San Diego, CA, USA) or NgR(310)ecto-Fc (50 µM, $n = 19$) at a rate of 0.25 µL/h. A cohort of NgR(310)ecto-Fc-treated rats ($n = 8$) were also treated with MP (Pharmacia; 30 mg/kg i.v.) and a separate cohort treated with MP alone (30 mg/kg i.v.) immediately after injury and again 4 and 8 h later. Following surgery the laminectomy site was sutured and the skin wound stapled closed. Postoperative care comprised analgesia (Buprenorphine/Buprenex, 0.05 mg/kg s.c.; Reckitt Benckiset Healthcare Ltd, Hull, UK) every 8–12 h for 3 days and antibiotic treatment (Ampicillin, 100 mg/kg s.c. twice daily; Bristol Myers Squibb, New York, NY, USA) for 7 days after surgery. Bladders were expressed manually twice a day for the duration of the study (28 days) or until return of function (the time of which was noted).

For histological tracing of the CSTs, 2 weeks after CST transection animals were re-anesthetized and an incision made in the scalp. The area around the skin incision was injected with a local anesthetic (Marcaine; Abbott Laboratories), the left sensorimotor cortex exposed via a craniotomy and 7 µL 10% biotin dextran amine (BDA; 10 000 MW; Molecular Probes, Eugene, OR, USA) in phosphate-buffered saline injected using a nanoliter injector and micro4 controller (World Precision Instruments) at 12 points 0–3.5 mm posterior to Bregma and 0–2.5 mm lateral to the midline at a depth of 1 mm below the surface of the cortex. After needle removal the craniotomy was covered with gel foam and the scalp stapled closed. In some instances the CST was labeled bilaterally using the same procedure.

At the termination of the *in vivo* phase of the experiment, Alzet osmotic pumps were removed and any remaining contents of the pumps removed for analysis. Stability of the infused protein was confirmed in all instances.

Behavioral analysis

All animals were scored using the open-field BBB scoring system (Basso *et al.*, 1995). Rats were evaluated the day after CST transection (day 2) and weekly thereafter for 4 weeks with observers blinded to the treatment regimen.

Footprint analysis was performed 4 weeks after injury. The plantar surface of the paws was painted with nontoxic dye and footprints marked over consecutive steps. Stride length was quantified as the mean distance between hindlimb prints for at least three strides per animal.

Histological analysis

At 28 days after CST transection rats were anesthetized with Inactin (100–110 mg/kg i.p.; Sigma, St Louis, MO, USA) and transcardially perfused with heparinized saline (100 mL, 10 iu heparin) followed by 4% paraformaldehyde (150 mL). Spinal cords were removed, post-fixed in 4% paraformaldehyde and then impregnated with 30% sucrose for 48 h; 25-mm lengths of spinal cord, 10 mm rostral and 15 mm caudal to the transection site, were embedded in optimal cutting temperature compound (OCT) with transverse segments of cord taken 10–15 mm rostral and 15–20 mm caudal to the lesion. Frozen sections (50 μ m) were serially cut and stained with streptavidin-conjugated AlexaFluor-594 (1: 200; Molecular Probes) to visualize labeled CST axons. Axon counts were performed on transverse sections taken 10 and 15 mm caudal to the transection site. All measurements were performed blind. Every eighth section, i.e. sections 400 μ m apart, was counted for each animal at each level of the cord and values expressed as mean number of axons per section.

Anti-vesicular glutamate transporter 1 (vGLUT1) antibody (dilution 1: 2500; Chemicon, Temecula, CA, USA) was used to stain for neuronal cell bodies and α - and γ -motor neurons in lamina 9 were identified by their size and morphology. All immunostaining was visualized using Alexa-conjugated secondary antibodies (Molecular Probes).

Statistical analysis

Multiple group comparisons were made using two-way repeated measures ANOVA with Tukey's posthoc test or one-way ANOVA with Dunnett's posthoc test. Frequency data were compared using the Fischer exact test. A probability of < 0.05 was defined as a statistically significant result.

Results

Ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG but not methylprednisolone reversed the neurite outgrowth inhibitory effect of myelin in dorsal root ganglion cells

As a first step towards investigating combined treatment with MP and NgR(310)ecto-Fc for SCI, we sought to verify that these reagents have independent mechanisms of action. The activity of NgR(310)ecto-Fc is based on its ability to reverse the inhibition of axon growth by myelin (Fig. 1A and B). In contrast, MP alone had no effect on neurite outgrowth from dorsal root ganglion neurons on a myelin substrate and the presence of MP did not alter axon growth stimulation by NgR(310)ecto-Fc. These data indicate that MP does not directly influence myelin-induced neurite outgrowth inhibition and that MP and NgR(310)ecto-Fc have independent actions. These *in vitro* data support the hypothesis that MP and NgR(310)ecto-Fc will enhance SCI recovery in a sequentially effective manner.

Ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG and methylprednisolone treatment had a temporally distinct effect on functional recovery after spinal cord transection

Both MP and NgR(310)ecto-Fc treatments were initiated immediately after SCI and functional recovery was assessed using the BBB open-field scoring method (Basso *et al.*, 1995) the following day and weekly thereafter. Control animals recovered hindlimb function over the course of the study reaching a mean BBB score of 12 ± 0.87 after 4 weeks. Mean BBB scores for treated groups at the same time-point were: MP, 14.9 ± 0.23 ; NgR(310)ecto-Fc, 14.8 ± 0.24 and NgR(310)ecto-Fc plus MP, 15.63 ± 0.18 (Fig. 2A). A statistically significant increase in BBB score was observed in MP- and MP plus NgR(310)ecto-Fc-treated rats the day after surgery compared with control animals or animals treated with NgR(310)ecto-Fc alone (Fig. 2B). This observation indicated an early effect of MP treatment on recovery. Given this very early effect of MP, BBB scores were ‘normalized’ to day 2 to subtract out this early effect of MP (Fig. 2C) thus illustrating the much later onset of effect of NgR(310)ecto-Fc. In the combined treatment group ‘normalized’ BBB scores abrogated the enhancing effect of MP on NgR(310)ecto-Fc treatment (Fig. 2C) illustrating that (i) in the combined treatment group the effect of MP occurred early after SCI and (ii) by subtracting out this effect the rate and extent of functional recovery in the combined treatment group and the NgR(310)ecto-Fc group were identical and more pronounced than MP treatment alone.

A discriminating point on the BBB score is a score of 14, corresponding to consistent weight supported plantar steps and consistent hindlimb–forelimb co-ordination (M. Basso, personal communication). Accordingly, results were expressed as the frequency with which rats attained a score of 14 or greater; 50% of control rats attained a score of 14 or greater by 4 weeks after injury (Fig. 2D). All rats (100%) treated with NgR(310)ecto-Fc or MP or combined therapy demonstrated consistent plantar stepping and coordinated movement by 4 weeks. Combination therapy increased the rate of recovery of coordinated function as a significantly higher proportion of this treatment group reached a score of 14 or greater by 3 weeks compared with controls or either NgR(310)ecto-Fc or MP treatment alone (Fig. 2D). Improved functional recovery was also demonstrated as significantly improved mean stride length in NgR(310)ecto-Fc- and NgR(310)ecto-Fc plus MP-treated groups compared with controls (Fig. 2E). MP treatment alone did not significantly improve stride length measured 4 weeks after SCI.

Ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG and methylprednisolone treatment enhanced axonal plasticity/regeneration after spinal cord transection

Biotin dextran amine-labeled axons were observed in the dorsal and ventral CSTs (Fig. 3A). The majority of CST fibers (>95%) projected via the dorsal tract and dorsolateral CSTs with less than 5% of labeled fibers observed in the ventral region of the cord. The number of BDA-labeled axons in the ventral CST of injured rats was enhanced compared with that observed in uninjured rats (data not shown). Axons projecting down the dorsal CST branched into the adjacent gray matter (Fig. 3B). In the majority of rats BDA-labeled axons were observed caudal to the site of transection. In all treatment groups a greater number of BDA-labeled axons were observed caudal to the site of transection than in the control group. Treatment with NgR(310)ecto-Fc or combined treatment with MP and NgR(310)ecto-Fc resulted in significantly greater numbers of axons counted 15 mm caudal to the injury site (Fig. 4A). BDA-labeled axons appeared to sprout from both the dorsal columns into the dorsal horn gray matter and the spared ventral CST, projecting into the ventral gray matter. Axon counts in discrete regions of the cord revealed the largest increase in axon number in the gray matter (Fig. 4B). These data suggest that treatment with NgR(310)ecto-Fc with or without MP promotes plasticity in the spinal cord after injury. In some instances BDA-labeled axons were observed to project from the transected dorsal CST into the lesion site (Fig. 4C). These axons were not observed in vehicle-treated control rats and were more prominent in cords from animals that received combined treatment with MP and NgR(310)ecto-Fc. This latter observation provides evidence

that combined treatment with NgR(310)ecto-Fc and MP provided the most permissive environment for regeneration.

Combined treatment with ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG and methylprednisolone increased the number of axonal connections between biotin dextran amine-labeled corticospinal tract fibers and lumbar motor neurons

As treatment increased the number of BDA-labeled axons observed caudal to the site of spinal cord transection (Fig. 5A), we quantified the number of BDA-labeled projections forming synaptic contacts with motor neurons in the ventral horn. Counts were performed on transverse sections 15 mm caudal to the site of injury (T7) in the region of the lumbar motor neuron pool (L1). Motor neurons were identified as vGLUT1-positive cells with a typical α - or γ -motor neuron morphology. Axons contacting motor neurons were determined to be Alexa 594-conjugated streptavidin-positive BDA-labeled axons (red) that abutted an Alexa-488-stained vGLUT1-positive cell (green) yielding clear double staining (yellow) (Fig. 5B). The number of axons contacting α - and/or γ -motor neurons was significantly increased in all treatment groups compared with control animals, with the most marked and significant effect observed in animals receiving combined treatment with NgR(310)ecto-Fc and MP (Fig. 5C).

Discussion

Promoting recovery after spinal cord injury

Dorsal over-hemisection of the rat spinal cord results in immediate functional deficits that recover spontaneously over time and the mechanisms underlying this recovery have been attributed to compensatory collateral sprouting of surviving axons (Weidner *et al.*, 2001). In the present study vehicle-treated rats recovered significant function to a mean BBB score of 12. It is clear that the rat spinal cord has a substantial inherent capacity for reorganization (Fouad *et al.*, 2001; Bareyre *et al.*, 2004) and elegant studies have demonstrated plasticity in discrete components of the rat CST after transection and the relative functional significance of such plasticity (Weidner *et al.*, 2001; Bareyre *et al.*, 2004). These studies highlight the particularly important role of the ventral CST and intraspinal circuits in recovery of function in the untreated spinal cord-injured rat. An alternative or additional mechanism for recovery of function in treated rats is axonal regeneration. The experiments described here were undertaken to evaluate whether the pro-regenerative effect of NgR(310)ecto-Fc (Li *et al.*, 2004) was evident and/or enhanced when administered concurrently with MP.

Methylprednisolone improved recovery after spinal cord transection

Consistent with previous findings, MP improved functional and histological outcome after SCI in the rat (Oudega *et al.*, 1999; Nash *et al.*, 2002). MP had an early effect on recovery of function after SCI with rats showing improved BBB scores as early as the day after surgery (day 2). Reports suggest that high-dose MP can have anti-inflammatory (Hsu & Dimitrijevi, 1990; Nadeau & Rivest, 2003), antioxidant (Hall, 1992) and neuroprotective effects, reducing axonal dieback (Oudega *et al.*, 1999). These effects may serve to provide an environment more conducive to regeneration as MP treatment alone resulted in an increased number of axons observed caudal to the lesion, as has been reported elsewhere (Nash *et al.*, 2002). MP had no effect on the ability of dorsal root ganglion cells to extend neurites on a myelin substrate *in vitro*. The early effect of MP treatment *in vivo* and the lack of effect of MP on neurite outgrowth *in vitro* suggest that it has no direct pro-regenerative effects at the level of the axon.

Ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG improved recovery after spinal cord transection

As shown previously, intrathecal administration of NgR(310)ecto-Fc significantly improved functional and histological outcome after spinal cord transection in the rat (Li *et al.*, 2004). The beneficial effect of NgR(310)ecto-Fc treatment on BBB score was significant 2 weeks after injury, the timing of which is consistent with enhanced plasticity and/or regeneration. Histological analysis showed an increased number of BDA-labeled axons caudal to the transection site. The ventral CST is known to give rise to collateral arborizations that project into the gray matter where they branch extensively (Brösamle & Schwab, 1997). In our studies, projections from the ventral CST into the ventral horn, specifically into the lumbar motor neuron pool, appeared to be targeted towards motor neurons. This observation is consistent with previous studies showing an increased number of CST projections into the medial motor neuron pool in the cervical cord after transection (Weidner *et al.*, 2001). Although few in number, such connections are purported to be of great functional significance (Weidner *et al.*, 2001). The increased number of axons contacting motor neurons in the NgR(310)ecto-Fc-treated group compared with controls indicates that administration of this decoy receptor results in a more permissive environment for axonal remodeling.

The demonstration that NgR(310)ecto-Fc can reverse the inhibitory effect of myelin on neurite outgrowth in dorsal root ganglia supports the concept that NgR(310)ecto-Fc can promote axonal growth *in vivo* by inhibiting the interaction of the myelin associated inhibitors Nogo-66, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein with the NgR, as it is now well established that these proteins actively inhibit axonal growth after injury (Filbin, 2003).

Effect of combined treatment with ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG and methylprednisolone on recovery after spinal cord transection

Combined treatment with NgR(310)ecto-Fc and MP tended to have a greater effect on functional and histological recovery than either treatment administered alone. Substantial improvements in BBB score and the ability to attain coordinated hindlimb–forelimb movement and increase stride length were observed in the combined treated group compared with controls. In all behavioral tests the highest level of function was attained in the combined treatment group compared with either MP or NgR(310)ecto-Fc treatment alone. Subtraction of the early effect of MP on functional recovery, by normalization of BBB scores to day 2, illustrated the more pronounced effect of NgR(310)ecto-Fc 2, 3 and 4 weeks after SCI compared with MP treatment alone. Presentation of the data in this form highlights the temporally distinct effects of MP and NgR(310)ecto-Fc in this model.

Axonal growth was enhanced after combined treatment with MP and NgR(310)ecto-Fc and observed to be due to both increased plasticity and regeneration as defined previously (Steward *et al.*, 2003). It can be inferred from the data presented herein that MP acts to reduce secondary damage to the spinal cord by one or several reported mechanisms of action (Oudega *et al.*, 1999; Gold *et al.*, 2001; Nash *et al.*, 2002; Nadeau & Rivest, 2003) and NgR(310)ecto-Fc increases the ability of axons to remodel and regenerate by blocking the effects of the inhibitory components of myelin (McGee & Strittmatter, 2003; Filbin, 2003). The pharmacological effects of these reagents occurred in a temporally distinct manner.

Conclusions

The present data demonstrate that a pro-regenerative therapy, NgR(310)ecto-Fc, can be combined with MP to provide robust recovery in an experimental model of SCI. The apparently different and complementary mechanisms of action of MP and NgR(310)ecto-Fc indicate that they are sequentially effective at limiting damage and promoting recovery.

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Abbreviations

BBB	Basso, Beattie, Bresnahan
BDA	biotin dextran amine
CST	cortico-spinal tract
MP	methylprednisolone
NgR	Nogo-66 receptor
NgR(310)ecto-Fc	ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG
SCI	spinal cord injury
NgR	NgR(310)ecto-Fc

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Fig. 1.

Effect of ecto-domain of the rat Nogo-66 receptor (NgR) (27–310) fused to a rat IgG [NgR (310)ecto-Fc] and methylprednisolone (MP) on myelin-induced inhibition of neurite outgrowth in chick dorsal root ganglia (DRGs) *in vitro*. NgR, not MP, significantly rescues the inhibitory effect of myelin. (A) Dissociated embryonic day 13 chick DRG neurons were plated on phosphate-buffered saline (PBS) or myelin (400 ng/well) in the presence of NgR(310)ecto-Fc or MP. (B) Quantification of neurite outgrowth per cell ($n = 3$) expressed as a percentage of PBS control $\pm$ SEM ($n = 3$). Scale bar, 200 μ m. * $P < 0.05$ compared with PBS control.

Fig. 2. Effect of ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG [NgR(310)ecto-Fc] and methylprednisolone (MP) on functional recovery after spinal cord injury (SCI). (A) BBB score was recorded weekly for 4 weeks. All treatment groups showed improved BBB scores compared with controls over the course of the study. $*P < 0.05$ vs. control, two-way repeated measure ANOVA with Tukey's posthoc test. (B) BBB score was significantly improved in MP-treated rats 2 days after SCI. $*P < 0.05$ vs. control, two-way repeated measure ANOVA with Tukey's posthoc test. (C) BBB scores normalized to day 2 for individual animals illustrate a significant improvement in functional recovery in NgR(310)ecto-Fc-treated rats $\pm$ MP 2, 3 and 4 weeks after SCI. $*P < 0.05$ vs. control, two-way repeated measure ANOVA with Tukey's posthoc test. (D) Frequency of consistent plantar stepping and hindlimb–forelimb coordination, illustrating the proportion of rats in each group that attained a score of 14 or higher 3 and 4 weeks after SCI. Unlike control rats, all treated animals were able to walk with hindlimb–forelimb coordination by 4 weeks after injury. Combined treatment with NgR(310)ecto-Fc and MP significantly improved the rate of functional recovery. $*P < 0.05$ vs. control Fischer exact test. (E) Mean stride length was significantly improved in NgR(310)ecto-Fc- and MP + NgR(310)ecto-Fc-treated groups compared with controls. $*P < 0.05$, one-way ANOVA with Dunnett's posthoc test.

Fig. 3.

Biotin dextran amine (BDA)-labeled axons project beyond the site of transection and sprout into the gray matter. Strong BDA labeling of the dorsal corticospinal tract (CST) was observed rostral to the lesion site (A and B) and ventral and lateral CST components could also be identified (A and B). Axons projected from the dorsal CST into adjacent gray matter. Highly arborized axonal sprouts were identified in and around the gray matter 10 and 15 mm caudal to the lesion. (C) BDA-labeled axons observed 10 mm caudal to the lesion site in a rat treated with ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG.

Fig. 4. Effect of ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG [NgR(310)ecto-Fc] and methylprednisolone (MP) treatment on axon number caudal to the spinal cord lesion. (A) Treatment with NgR(310)ecto-Fc and MP + NgR(310)ecto-Fc significantly increased the total number of biotin dextran amine (BDA)-labeled axons per section counted 15 mm caudal to the site of injury. (B) The largest increase in axon numbers was observed in the gray matter (GM) compared with ventral white matter (vWM) and dorsal white matter (dWM). * $P < 0.05$ vs. control, one-way ANOVA with Dunnett's posthoc test. (C) In treated animals BDA-labeled axons were observed to project from the severed dorsal corticospinal tract into the lesion area. This example was from a rat treated with NgR(310)ecto-Fc and MP.

Fig. 5. Effect of ecto-domain of the rat Nogo-66 receptor (27–310) fused to a rat IgG [NgR(310)ecto-Fc] and methylprednisolone (MP) treatment on the number of biotin dextran amine (BDA)-labeled axons contacting motor neurons in the ventral horn. BDA-labeled fibers represent corticospinal tract (CST) axons projecting from above the site of spinal cord transection. BDA-labeled axons projected from the ventral CST towards and into the gray matter of the ventral horn (A); vGLUT1-positive cells represent motor neurons in the ventral horn of the low thoracic/high lumbar region of the spinal cord (15 mm below the site of the lesion). BDA-labeled axons (red) abutted vGLUT1-positive cells (green) shown here by double staining (yellow) (B). The number of axons contacting motor neurons in the ventral horn was significantly increased in the MP + NgR(310)ecto-Fc-treated group compared with controls (C). * $P < 0.05$, one-way ANOVA with Dunnett's posthoc test.