Myelin-Associated Glycoprotein Inhibits Schwann Cell Migration and Induces Their Death

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Remyelination of CNS axons by Schwann cells (SCs) is not efficient, in part due to the poor migration of SCs into the adult CNS. Although it is known that migrating SCs avoid white matter tracts, the molecular mechanisms underlying this exclusion have never been elucidated. We now demonstrate that myelin-associated glycoprotein (MAG), a well known inhibitor of neurite outgrowth, inhibits rat SC migration and induces their death via γ -secretase-dependent regulated intramembrane proteolysis of the p75 neurotrophin receptor (also known as p75 cleavage). Blocking p75 cleavage using inhibitor X (Inh X), a compound that inhibits γ -secretase activity before exposing to MAG or CNS myelin improves SC migration and survival *in vitro*. Furthermore, mouse SCs pretreated with Inh X migrate extensively in the demyelinated mouse spinal cord and remyelinate axons. These results suggest a novel role for MAG/myelin in poor SC–myelin interaction and identify p75 cleavage as a mechanism that can be therapeutically targeted to enhance SC-mediated axon remyelination in the adult CNS.

Key words: MAG; myelin; Schwann cell

Significance Statement

Numerous studies have used Schwann cells, the myelin-making cells of the peripheral nervous system to remyelinate adult CNS axons. Indeed, these transplanted cells successfully remyelinate axons, but unfortunately they do not migrate far and so remyelinate only a few axons in the vicinity of the transplant site. It is believed that if Schwann cells could be induced to migrate further and survive better, they may represent a valid therapy for remyelination. We show that myelin-associated glycoprotein or CNS myelin, in general, inhibit rodent Schwann cell migration and induce their death via cleavage of the neurotrophin receptor p75. Blockade of p75 cleavage using a specific inhibitor significantly improves migration and survival of the transplanted Schwann cells *in vivo*.

Introduction

Schwann cells (SCs), the myelin-forming cells of the peripheral nervous system (PNS) exhibit many advantages for cell therapy

(Zujovic and Baron-Van Evercooren, 2013). In animal models of spinal trauma and dysmyelination/demyelination as well as human neurological diseases, the endogenous SCs transgress into the CNS and remyelinate CNS axons, resulting in the restoration of axonal function and even the reversal of neurological deficits (Felts and Smith, 1992; Jasmin et al., 2000; Guest et al., 2005). It is, however, limited to the entry or exit zones of the spinal roots and areas around small blood vessels of the brain (Duncan et al., 1997). Similarly, exogenously transplanted SCs can promote CNS axonal growth, and remyelinate and restore electrophysiological properties, leading to the recovery of neurological function (Duncan et al., 1981; Baron-Van Evercooren et al., 1993, 1996; Honmou et al., 1996; Girard et al., 2005; Zujovic et al., 2011). Unfortunately, SCs do not migrate far enough, and many

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die in the adult CNS. The few SCs that migrate are mostly associated with meningeal cells and basement membranes, but rarely with the white matter tracks (Baron-Van Evercooren et al., 1996 and Zujovic et al., 2010). This poor migration of endogenous and exogenous SCs into the CNS is surprising as SCs migrate extremely well *in vitro* (Dubois-Dalq et al., 1981) during the development or repair of the peripheral nerve (Monk et al., 2015). This restriction is likely due to SC exclusion from astrocytes and/or myelin.

While a few molecular mechanisms regulating the poor SC–astrocyte interaction have been elucidated (Lakatos et al., 2003a, 2003b), those involved in SC–myelin (Iwashita et al., 2000; Bachelin et al., 2010) interaction remain to be understood. CNS myelin contains several inhibitors of neurite outgrowth: Nogo 66, the extracellular domain of Nogo A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPré et al., 2000; Wang et al., 2002a; Filbin, 2003). In neurons, all three inhibitors bind to Nogo receptor (NgR1; Fournier et al., 2001; Domeniconi et al., 2002; Huang et al., 2012), a GPI-linked protein and require p75 neurotrophin receptor as a coreceptor (Wang et al., 2002b) for exerting their action. In the present study, we hypothesized that inhibitors present in CNS myelin play a role in poor SC–myelin interaction. We conducted a series of *in vitro* and *in vivo* experiments to assess SC migration and survival in the presence of MAG/myelin. Previously, it was shown that MAG is a sialic acid binding glycoprotein, a member of the Siglec family of molecules (Mukhopadhyay et al., 1994). Upon binding to NgR1, MAG activates a signaling cascade called regulated intramembrane proteolysis (RIP) or p75 cleavage. p75 cleavage releases two fragments, an ectodomain and a 25 kDa cytoplasmic fragment (p75CTF) formed by the action of α -secretase. The CTF is further cleaved by γ -secretase activity to produce a 20 kDa intracellular domain (p75ICD). p75ICD is necessary and sufficient to activate the small GTPase RhoA and to inhibit neurite outgrowth. Blocking p75 cleavage using inhibitor X (Inh X), a compound that inhibits γ -secretase activity promotes neurite outgrowth (Domeniconi et al., 2005).

We demonstrate that MAG strongly binds to SCs, inhibits migration, and induces their death via p75 cleavage *in vitro*. Blockade of p75 cleavage using Inh X significantly improves the migration and survival of the transplanted SCs *in vivo* in the demyelinated adult mouse spinal cord. Our data suggest that MAG/myelin-mediated p75 cleavage is a mechanism underlying the inefficient SC intervention in the adult CNS and that blocking p75 cleavage using Inh X is a potential therapeutic strategy to enhance SC-mediated remyelination of the adult CNS axons *in vivo*.

Materials and Methods

Schwann cell culture

Primary SC cultures were prepared as described previously (Wei et al., 2009). Briefly, sciatic nerves from postnatal day 2 (P2) to P3 rat pups were enzymatically dissociated and cultured in a medium containing 10% fetal bovine serum and cytosine arabinoside (10 μ M; Sigma-Aldrich) for 3 d to kill fibroblasts, followed by incubation in a serum-free medium, SATO medium, containing NRG1- β 1 (5 nM; R&D Systems) and forskolin (0.5 μ M; Sigma-Aldrich) to stimulate SC growth until confluent.

Viability assay

Schwann cell viability was assessed using the trypan blue dye exclusion method. Briefly, SCs were seeded onto 20 four-well plates at a density of 2.5×10^4 cells in 250 μ l of SATO medium. MAG-Fc and myelin were added at 20 μ g/ml concentration to the medium and incubated for 8 h. The cells were detached using trypsin and resuspended in fresh medium.

Trypan blue dye solution (Invitrogen) was added to 100 μ l of cells in equal volume and incubated for 5 min as per manufacturer instructions. The dead and viable cells were counted using a hemocytometer by viewing under the microscope. The cell viability was determined, first, by obtaining the total cell count in each treatment group (dead + viable count = total count) and then by calculating the percentage of viability with respect to each treatment group.

Migration assay

A migration assay was performed using a previously described protocol with some modifications (Su et al., 2007). This assay used transwell inserts (6.5 mm in diameter with 8.0 μ m pore size; BD Falcon) that fit into the wells of a 24-well plate. The underside of these inserts was precoated with poly-L-lysine (PLL) and placed into wells containing 750 μ l of SATO medium [control (CON)] or SATO medium containing MAG-Fc, MAG (1–3)-Fc, or rat myelin at 20 μ g/ml. Confluent SC cultures were dissociated and seeded into the transwells at a density of 2.5×10^4 cells in 250 μ l of SATO medium. In some experiments, SCs were pretreated with Inh X (1 μ M; Calbiochem) or DMSO (1 μ l/ml; Sigma-Aldrich) for 1 h before dissociation and seeded into the transwells. The cellular chambers served as upper compartments, and the wells containing media plus treatments served as bottom compartments. After 8 h of incubation, nonmigrating SCs that remained in the upper compartments were scraped off using cotton swabs and those that transmigrated and attached to the underside of the chambers were fixed using 4% paraformaldehyde. For quantitation, inserts were stained using Coomassie Brilliant Blue R250, and five independent fields per insert were photographed. The number of cells in each field was manually counted, and the total number of migrating cells per insert was determined.

p75 cleavage assay

Schwann cells were starved in DMEM for 5 h before treatment. Cells were pretreated with the proteasome inhibitor epoxomicin (1 μ M) for 1 h and subsequently stimulated with MAG-Fc, MAG (1–3)-Fc, or myelin at 20 μ g/ml concentration or phorbol 12-myristate 13-acetate (PMA) at 1 μ M concentration for 1 h at 37°C. In some experiments, SCs were incubated with Inh X along with epoxomicin for 1 h, before stimulation with Fc-chimera or myelin. Cells were then washed using cold PBS, lysed in RIPA buffer containing protease inhibitors, and centrifuged for 14,000 $\times$ g. The amount of protein was quantified using BCA assay (Pierce) and immediately used in SDS-PAGE.

RT-PCR Analysis

Total RNA was isolated from purified SC cultures, cerebellar neurons, cortical neurons, and adult rat spinal cord extracts using Qiagen RNA isolation kit following manufacturer instructions. RT-PCRs were performed using standard protocol. The following PCR primers were used: NgR1: 5'-CAGCTCTGCAGTACCTCTAC-3' and 5'-CTCTAAGTCAC-TGGTAGCCAG-3' to amplify a 460 bp fragment of the NgR1 mRNA transcript; and 5'-AGCCTACAGTACCTCTACC-3' and 5'-CTTGG-AAATCGGTGTCGCGCAG-3' to amplify a 486 bp fragment of the NgR2 mRNA transcript. PCR products were then electrophoresed on 1.2% agarose gels.

Immunoblotting

For the detection of protein antigens, SC lysates were separated by 12% SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were blocked in 5% nonfat dry milk in PBS, followed by incubation in rabbit antiserum that specifically binds to the intracellular domain of p75 (clone 9996; a gift from M. Chao, Department of Neuroscience, NYU School of Medicine, New York, NY) to detect p75 cleavage products, anti-NgR1 rabbit polyclonal antibody (1:1000; a gift from R. Giger, Department of Cell and Developmental Biology, University of Michigan School of Medicine, Ann Arbor, MI), or anti-LRP1 (1:1000; Sigma-Aldrich). Membranes were then washed three times in PBS containing 0.05% Tween-20, incubated in goat anti-rabbit HRP secondary antibody (1:1000 to 1:10,000; Millipore) for an hour. Immunoreactive bands were visualized using either Chemiluminescence (Invitrogen) or West Pico Substrate (Thermo Scientific) detection systems. For the detection of actin, membranes were stripped and probed with anti-actin

Figure 1. MAG/myelin (Mye) inhibits SC migration. **A–C**, Photomicrographs showing the Coomassie Brilliant Blue-stained cells that have transmigrated through the pores of transwells to the underside of the membrane in the presence of buffer (control; **A**), MAG-Fc (20 $\mu\text{g}/\text{ml}$; **B**), or myelin (20 $\mu\text{g}/\text{ml}$; **C**). Scale bar, 50 μm . **D**, Quantitation of SC migration in the presence of MAG-Fc and myelin. Cell migration is expressed as a percentage of control. Bars represent the mean $\pm$ SEM of three experiments with two to three replicates for each treatment. * $p < 0.001$, one-way ANOVA, Bonferroni's *post hoc* test.

antibody (1:2000; Sigma-Aldrich), or in some cases anti-actin was combined with other primary antibodies.

Death assay

For qualitative assessment of SC viability, a live/dead kit (Molecular Probes) was used. Purified SCs (3.5×10^3 SCs/well) were cultured on PLL-coated eight-well slide chambers in SC growth medium. After 2 d in culture, SATO medium containing MAG-Fc, MAG (1–3)-Fc, or myelin at a concentration of 20 $\mu\text{g}/\text{ml}$ was added and incubated for 1, 2, or 3 d. At the end of each time point, live/dead assay reagent was added and processed as per manufacturer instructions. The cells were viewed immediately under fluorescence microscopy and photographed. For the detection of apoptosis, cells were fixed using 4% paraformaldehyde and immunostained using anti-S100 (Dako), followed by DAPI to visualize nuclei.

For quantitative analysis of cell death, AlamarBlue assay was performed. Purified SCs (3.5×10^3 SCs/well) were plated onto 96-well microtiter plates. After 2 d in culture, MAG-Fc, MAG (1–3)-Fc, or myelin at a concentration of 20 $\mu\text{g}/\text{ml}$ was added and incubated for 1, 2, or 3 d. At the end of each time point, AlamarBlue reagent was added and incubated for 4 h at 37°C. The viability was assessed by measuring the amount of fluorescence, as described in the manufacturer instruction manual.

Binding assays

Binding of MAG chimera to SC. For qualitative assessment of MAG and MAG (1–3) binding to cells, SCs, CHO cells, and cerebellar neurons (3.5×10^3 cells/well) were cultured on laminin-coated eight-well slide chambers. After 2 d in culture, the cells were washed with PBS and MAG-Fc and MAG (1–3)-Fc chimeras were added at a concentration of 20 $\mu\text{g}/\text{ml}$ and incubated at 37°C for 1 h. The cells were then washed and

fixed using 4% paraformaldehyde. For visualization of chimeric protein, cells were incubated with anti-human IgG-Fc Cy3 conjugate (1:300; Sigma-Aldrich) for 1 h, followed by Hoechst stain for 5 min at room temperature. MUC18-Fc was used as a chimera control, while IgG-Fc served as an Fc control. Cerebellar neurons served as positive control, and CHO cells served as negative control.

Binding of SCs to immobilized MAG chimera. Binding of MAG chimeras to cells was quantitated using a previously published method (Tang et al., 1997). Briefly, 96-well plates were coated with anti-human Fc (Sigma-Aldrich) for an hour at room temperature, followed by incubation with various Fc chimeras at 1–40 $\mu\text{g}/\text{ml}$ overnight in the refrigerator. Live SCs, CHO cells, and cerebellar neurons were prelabeled with Calcein AM (Invitrogen) and then added to coated wells at a density of 1×10^5 cells/well in replicates of six and incubated for 1 h at room temperature. The plates were washed three to five times using Dulbecco's PBS applied under gravity to remove unbound cells. The fluorescence intensity was measured using Typhoon. The percentage of binding was calculated by using the fluorescence emitted by 1×10^5 cells/well as 100%.

Process extension assay

The neurite outgrowth assay protocol described previously for neurons was adapted to measure SC process length. Briefly, dissociated rat dorsal root ganglion (DRG) cultures consisting of DRG neurons and Schwann cells were transiently infected with GFP-encoding adenoviruses and cocultured on confluent cultures of either control CHO or MAG-expressing CHO (MAG-CHO) monolayers for a day. DRG neurons served as internal controls, while SCs are experimental. Cultures were fixed using 4% paraformaldehyde, and SC process lengths were measured from duplicate wells for each condition and quantified as described previously (Mukhopadhyay et al., 1994).

Statistics (in vitro)

Data are represented as the mean $\pm$ SEM from at least three independent experiments. Statistical difference between groups was determined using one-way ANOVA or two-way ANOVA and Bonferroni's *post hoc* analysis or Student's *t* test where appropriate. Values of $p < 0.05$ were considered to be statistically significant.

Demyelination and SC transplantation

Demyelination and SC transplantation were performed as described previously (Zujovic et al., 2010). Three-month-old female nude mice ($n = 22$) were purchased from Janvier. Mice were anesthetized using a ketamine/xylazine mixture. Demyelination was induced by stereotaxic injection of lyssolecithin (LPC; 1%; Sigma-Aldrich) at a rate of 1 μ l/min, and a total volume of 2 μ l was microinjected into the dorsal column white matter of the spinal cord at T8–T9 vertebral levels using a glass micropipette. Forty-eight hours after demyelination, 2 μ l of SCs at a concentration of 5×10^4 cells/ μ l that were pretreated with Inh X (1 μ M) or DMSO (1 μ l) for 1 h followed by a wash were grafted into the dorsal column white matter using a glass micropipette at a distance of one intervertebral space caudal to the lesion site. All animal protocols were performed in accordance with the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Immunohistochemistry

Animals were killed sequentially at 2, 5, and 28 d post-transplantation (d.p.t.) with lethal doses of ketamine/xylazine and were perfused transcardially with 0.1 M phosphate buffer followed by 2% paraformaldehyde. Spinal cords were cryoprotected overnight in 20% sucrose and frozen, and 12- μ m-thick sagittal sections were serially cut. For immunohistochemistry, primary antibodies, including anti-cleaved Caspase 3, to detect cell death (Cell Signaling Technology), anti-GFAP, to identify astrogliosis (1:500; Dako), monoclonal anti-protein zero, P0, to detect PNS myelin (1:5 hybridoma; Yoshimura et al., 1996), and monoclonal anti-myelin oligodendrocyte glycoprotein MOG, to detect CNS myelin (1:200; ascites, Dr. Linnington, University of Edinburgh, UK) were used. Following primary antibody incubation, sections were incubated with appropriate secondary antibodies conjugated to Rhodamine (red) or amino-methylcoumarin acetate (blue). Images were acquired using the Hamamatsu Nanozoomer Slide Scanner or the Zeiss Axio Imager Z1 and Axiovision software.

In vivo quantification and statistical analysis

For evaluation of rostrocaudal SC distribution within the dorsal funiculus, first, we measured the distance between the most rostral and the most caudal GFP⁺ cells on 12 consecutive longitudinal sections of each animal from different groups. Next, we selected for each animal the section with the largest rostrocaudal SC distribution per animal. Data are expressed as the mean of rostrocaudal GFP⁺ SC distribution in micrometers $\pm$ SEM for each group [$n = 10$ for controls; $n = 9$ for SCs pretreated with Inh X (Inh X-SCs)].

Figure 2. MAG induces p75 cleavage in SCs. Blockade of p75 cleavage using Inh X improves SC migration. **A, B**, Immunoblots showing p75 cleavage in SCs treated with buffer (control), MAG (1–3)-Fc (20 μ g/ml), MAG-Fc (20 μ g/ml), or myelin (20 μ g/ml; **A**) and SCs that were pretreated with Inh X for 1 h to block γ -secretase activity and then treated with MAG (1–3)-Fc and MAG-Fc (**B**). MAG-Fc and myelin induce the formation of p75ICD, but not MAG (1–3)-Fc. Pretreatment with Inh X blocks p75ICD formation and leads to accumulation of p75CTF. The numbers below the p75 blots show the fold increase in p75ICD compared with the control bands following densitometric analysis. The p75 blots were stripped and immunolabeled with anti-actin to determine the amount of protein loaded in each lane. **C**, Quantitation of SC migration in which SCs were pretreated with Inh X to block p75 cleavage before treatment with MAG-Fc and myelin. Blockade of p75 cleavage reverses MAG- and myelin-mediated inhibition. Bars represent the mean $\pm$ SEM of three experiments with two to three replicates for each treatment. * $p < 0.001$, two-way ANOVA, Bonferroni's *post hoc* test. $p < 0.001$, Con vs MAG-Fc, Con vs Mye (myelin), MAG-Fc vs MAG-Fc and Inh X, and Mye vs Mye and Inh X.

All other quantifications were performed on 6–12 animals in each group per time point and treatment, using the NIH ImageJ software. Data were averaged from 12 sections per animal with each spaced at 66 μ m. A Mann–Whitney *t* test was used to compare control and treatments.

Schwann cell density was evaluated by measuring the area of GFP⁺ staining on each spinal cord section. Evaluation of GFP–SC interaction with GFAP⁺ astrocytes in the graft site was performed by measuring the percentage of GFP⁺ area present in the GFAP⁺ area, as follows:

Figure 3. MAG/myelin induces SC death via p75 cleavage. Blockade of p75 cleavage using inhibitor X improves SC survival. **A–D**, Photomicrographs of SCs stained with a mixture of Calcein AM and ethidium homodimer-1 dyes to assess viability after treatment with buffer (control; **A**), MAG (1–3)-Fc (20 μ g/ml; **B**), MAG-Fc (20 μ g/ml; **C**), or myelin (20 μ g/ml; **D**) for 2 d. In wells treated with MAG-Fc or myelin (**C**, **D**), SCs died (red) and many cells clumped and/or lifted off the substratum, while those treated with MAG (1–3)-Fc (**B**), however, were viable (green) and remained attached to the substratum, similar to control cells (**A**). Scale bar, 50 μ m. **E**, Quantitation of SC viability using AlamarBlue assay after 24, 48, and 72 h treatment with buffer (control) or MAG-Fc (20 μ g/ml). Bars represent the mean $\pm$ SEM of three experiments with six to eight wells for each treatment. * p < 0.001, one-way ANOVA, Bonferroni's *post hoc* test. **F**, Quantitation of SC viability using AlamarBlue assay after pretreatment with Inh X to block p75 cleavage and subsequently treating with control (buffer) or MAG-Fc for 3 d. Bars represent the mean $\pm$ SEM of three experiments with six to eight wells for each experiment. * p < 0.001, two-way ANOVA, Bonferroni's *post hoc* test. **G–I**, Photomicrographs of SCs immunolabeled with S100 (red) and DAPI (blue) following treatment with buffer (control; **G**), MAG-Fc (20 μ g/ml; **H**), or myelin (20 μ g/ml; **I**) for 2 d. Arrows point to fragmented nuclei in MAG-Fc- and myelin-treated cells. Scale bar, 50 μ m.

$$\frac{\%GFP^{+} \text{ area in GFAP}^{+} \text{ area}}{\text{Total GFP area}}$$

The extent of exogenous SC remyelination was quantified by measuring the percentage of GFP⁺ area colocalizing with P0 area:

$$\frac{\%P0^{+}/GFP^{+} \text{ area}}{\text{Total GFP area}}$$

Evaluation of SC integration in the white matter was quantified by measuring the percentage of the GFP⁺ area in the MOG⁺ area, as follows:

$$\frac{\%GFP^{+} \text{ area in MOG}^{+} \text{ area}}{\text{Total GFP area}}$$

Reagents

MAG-Fc, MAG (1–3)-Fc, and MUC18-Fc were prepared in our laboratory using previously published protocols (Tang et al., 1997).

Results

MAG/myelin inhibits SC migration

To test the effect of MAG/myelin on SC migration, we used a Boyden chamber migration assay. In this assay, cells were placed in the top compartment and separated from treatments in the bottom compartment by a porous membrane. After 8 h of incubation, cells that transmigrated across the pores to the underside of the membrane were fixed, stained, and counted. The results showed that fewer SCs migrated when MAG or myelin was present in the bottom compartment compared with buffer (Fig. 1A–C). Quantitative analysis revealed a significant reduction in the number of SCs migrating in the presence of MAG (60% compared with controls) and myelin (50% compared with controls; Fig. 1D), suggesting that MAG and myelin inhibit SC migration.

To determine whether the reduction in the number of cells migrating across MAG/myelin wells was due to cell death, we assessed cell viability using the trypan blue dye exclusion method. After 8 h of treatment (the length of the migration assay), control, MAG, and myelin-treated SCs exhibited typical SC morphology and appeared normal. There was no difference in the percentage of viability between control and MAG/myelin-treated groups (data not shown). These results clearly established that the reduction in the number of SCs migrating through MAG/myelin-treated wells was not because the treatment killed SCs, but rather because it inhibited their migration across the membrane.

MAG/myelin induces p75 cleavage in SCs

Previously, it was known that MAG induces RIP of p75 or p75 cleavage in neurons to inhibit neurite outgrowth (Domeniconi et al., 2005). To assess whether MAG/myelin induces p75 cleavage in SCs, we treated SCs with MAG, MAG (1–3), and myelin for 1 h and performed immunoblotting using an antibody that specifically binds to the intracellular region of p75 to detect cleavage products of p75. In these experiments, a proteasome inhibitor, epoxomicin, was added before treating with MAG/myelin to prevent rapid degradation of the proteolytic fragments. PMA served as a positive control. Treatment with MAG/myelin resulted in higher levels of p75ICD compared with controls (Fig. 2A). MAG (1–3) did not induce p75 cleavage, and the level of p75ICD was the same or lower than that in the controls. When SCs were pretreated with Inh X, a compound known to inhibit γ -secretase, p75ICD formation was blocked, but, instead, p75CTF accumulated (Fig. 2B). These data clearly demonstrate that MAG and myelin, but not MAG (1–3), induce p75 cleavage in SCs and that this cleavage is specific, as shown by the successful blockade with Inh X.

Figure 4. MAG inhibits SC process extension *in vitro*. **A–D**, Photomicrographs of GFP⁺ dorsal root ganglion neurons and SCs cocultured on control cells, CHO cells (**A**, **C**), or CHO-MAG cells (**B**, **D**) incubated overnight and then fixed. Similar to DRG neurons, SCs extend short processes on MAG-CHO cells compared with control CHO cells. Scale bar, 50 μ m. **E**, Quantitation of the maximum lengths of SC processes on control CHO and CHO-MAG cells. Process length is expressed as the percentage length on control CHO cells. The SC process length is significantly shorter on CHO-MAG cells compared with control CHO cells. Bars represent the mean $\pm$ SEM from three experiments with replicates of two to four wells. * $p < 0.01$ paired *t* test.

p75 cleavage is necessary for MAG/myelin-mediated inhibition of SC migration

Next, we assessed the role of p75 cleavage in SC migration. We preincubated SCs with Inh X to block p75 cleavage and then performed migration assays in the presence of MAG or myelin. Inh X-SCs showed improved migration in the presence of both MAG (122%) and myelin (86.5%) compared with CON untreated SCs (CON-SCs; Fig. 2C). Furthermore, the treatment with Inh X had no adverse effect on SC migration. Both CON-SCs and Inh X-SCs migrated to the same extent. These data suggest that p75 cleavage is necessary for MAG/myelin to inhibit SC migration, and blockade of p75 cleavage reverses inhibition, resulting in significant improvement in SC migration.

MAG/myelin-induced SC death is mediated by p75 cleavage

Previously, it was known that pro-neurotrophin-mediated neuronal death requires p75 cleavage (Kenchappa et al., 2006). The results so far from the present study demonstrate that MAG and myelin induce p75 cleavage in SCs. Combining these two pieces of data, we hypothesized that SCs die upon treatment with MAG/myelin. To test this, we incubated SCs with MAG or myelin for 24, 48, or 72 h. Since 8 h of incubation did not induce cell death, we incubated for longer time periods. At the end of each time period, we assessed SC viability by staining cells with a mixture of Calcein

Figure 5. MAG lacking Ig domains 4–5, MAG (1–3), has no effect on SC migration or survival. **A**, Quantitation of SC migration in the presence of buffer (control), MAG-Fc (20 μ g/ml), myelin (20 μ g/ml), and MAG (1–3)-Fc. Cell migration is expressed as the percentage of control. MAG (1–3)-Fc does not inhibit SC migration. Bars represent the mean $\pm$ SEM of three experiments with two to three replicates for each treatment. * $p < 0.005$, one-way ANOVA, Bonferroni's *post hoc* test. **B**, Quantitation of SC viability using AlamarBlue assay after treatment with buffer (control), MAG-Fc (20 μ g/ml), or MAG (1–3)-Fc (20 μ g/ml) for 24, 48, and 72 h. Cell viability is expressed as the percentage of control at the respective time point. Bars represent the mean $\pm$ SEM of three experiments with six to eight wells for each treatment. * $p < 0.001$, two-way ANOVA, Bonferroni's *post hoc* test.

AM and ethidium homodimer-1b (EtBr). Live cells take up Calcein AM and appear green, while EtBr binds to the nuclei of dead cells, causing them to appear red under fluorescence. One day after treatment, MAG- and myelin-treated SCs appeared rounded and formed small clumps instead of their typical elongated morphology. On day 2, many cells were lifted off the substratum (Fig. 3C,D). The extent of cell death was more dramatic with myelin treatment compared with MAG treatment. While fewer cells were off the substratum after 2 d of treatment with MAG (Fig. 3C), almost all cells were either clumped or off the substratum with myelin treatment (Fig. 3D). For quantitative assessment of cell death, we performed AlamarBlue assay. It seemed like myelin interfered with AlamarBlue assay; therefore, we quantitated cell death only in the presence of MAG. There was significant cell death in wells treated with MAG compared with control starting at 24 h and up to 72 h (Fig. 3E). Nearly 50% of cells were dead by 48 h. These results show that both MAG and myelin induce SC death. Next, we assessed the effect of blocking p75 cleavage on SC survival. We pretreated SCs with Inh X (i.e., Inh X-SCs) for 1 h and subsequently added MAG. We quantitated cell death using AlamarBlue assay at 72 h. SCs pretreated with Inh X showed a significant improvement in survival in the presence of MAG. Inh X pretreatment almost completely reversed the effect of MAG on SC death (Fig. 3F). These data suggest that blocking p75 cleavage is sufficient to block MAG-mediated SC death. The treatment with Inh

X had no adverse effect on control SC viability. To determine the type of cell death, we assessed nuclear morphology following MAG/myelin treatment by staining with DAPI, a nuclear dye. SCs treated with MAG and myelin exhibited nuclear fragmentation, a hallmark of apoptosis, suggesting that MAG/myelin induces apoptosis in SCs (Fig. 3G–I).

MAG inhibits SC process extension

Previous studies have shown that MAG induces growth cone collapse/repulsion as well as inhibit process extension in neurons (Yamashita et al., 2002; Mimura et al., 2006). Since process extension via cytoskeletal rearrangement is a step necessary for migration, in the present study we assessed SC process length upon interaction with MAG. We cocultured SCs and DRG neurons on MAG-expressing CHO cells and control CHO (R2) cells *in vitro* and measured their process lengths. DRG neurons were used as positive controls. SCs cocultured on MAG-CHO cells expressed shorter processes compared with those that were cocultured on control CHO (R2) cells similar to DRG neurons (Fig. 4A–D). Quantitative analysis revealed a significant reduction in the average process length of SCs cocultured on MAG (Fig. 4E). These results suggest that MAG inhibits SC process extension in a manner similar to its action on neurons.

MAG (1–3) does not induce p75 cleavage and has no effect on SC migration or survival

MAG has five Ig-like domains in its extracellular region (Salzer et al., 1987, 1990), and its neurite outgrowth inhibition site is located within Ig-domain five (Cao et al., 2007). Removal of Ig domains four and five generates a truncated MAG, MAG (1–3). MAG (1–3) does not inhibit neurite outgrowth (Cao et al., 2007) and does not induce p75 cleavage, an event that is necessary for MAG to inhibit neurite outgrowth (Domeniconi et al., 2005). Given this background, we reasoned that MAG (1–3) would not induce p75 cleavage in SCs and consequently would have no effect on SC migration or survival.

To test this hypothesis, we first assessed p75 cleavage in SCs treated with MAG (1–3) and compared them with those treated with full-length MAG. Very low levels of p75CTF and p75ICD were present in lysates treated with MAG (1–3) when compared with those treated with full-length MAG, suggesting that MAG (1–3) does not induce p75 cleavage in SCs (Fig. 2A,B). Next, we assessed the effect of MAG (1–3) on SC migration. MAG (1–3) did not inhibit SC migration, whereas both full-length MAG and myelin significantly inhibited SC migration. The number of SCs migrating in the presence of MAG (1–3) was the same as the number of control (untreated) SCs (Fig. 5A). We then assessed SC viability in the presence of MAG (1–3) using AlamarBlue assay. We treated SCs with MAG (1–3) and full-length MAG for 24, 48, and 72 h. Full-length MAG induced significant cell death at all time points tested, whereas MAG (1–3) had no effect on SC

Figure 6. MAG and MAG(1–3) bind to SCs with high specificity. **A–D**, Photomicrographs of SCs (**A**, **C**) and CHO cells (control; **B**, **D**) exposed to MAG-Fc (20 μg/ml; **A**, **B**) and MAG(1–3)-Fc (20 μg/ml; **C**, **D**) chimeras for 1 h in culture and subsequently fixed and stained using anti-human Fc-Cy3 conjugate antibody (red) to visualize the chimera and DAPI (blue) to visualize the nuclei. Scale bar, 50 μm. **E–H**, Quantification of SCs and cerebellar neurons (CGNs) binding to MAG-Fc and MAG(1–3)-Fc using a live cell binding assay. **E**, **F**, Representative photomicrographs of Calcein AM-labeled SCs (**E**) and CHO cells (control; **F**) remaining bound to MAG-Fc-coated wells after five washes. Scale bar, 50 μm. **G**, **H**, Quantitation of SCs and CGN binding to 0, 1, 20, and 40 μg/ml concentrations of MAG-Fc (**G**) and MAG(1–3)-Fc (**H**). Cell binding is expressed as the percentage of control (no chimera). Bars represent the mean ± SEM of three different experiments with six to eight wells for each treatment.

Figure 7. Schwann cells express NgR1 and LRP1. **A, B**, RT-PCR analyses performed on 2-d-old SC lysates showing the presence of NgR1 mRNA (**A**) and the absence of NgR2 (**B**). Lysates of cerebellar neurons, cortical neurons, and spinal cord tissue served as positive controls. **C, D**, Western blots showing NgR1 (**C**) and LRP1 (**D**) expression in control SCs. **C**, NgR1 (75 kDa) is expressed in SCs cultured *in vitro* for 1 d (SC day 1) and 3 d (SC day 3). Hippocampal cells (HP day 21) that express NgR1 served as positive controls, when treated with NgR1 shRNA, NgR1 expression was abolished. Arrow in **A** points to the lane corresponding to NgR1 bands that are present in SC lysates and HP lysate, but not in HP treated with NgR1 shRNA. The bands in the lane corresponding to 50 kDa are nonspecific. **D**, LRP1 (85 kDa) is present in lysates on SC day 1 and SC day 3. Hippocampal cells (HP day 24) express LRP1 and served as a positive control for LRP1.

viability up to 48 h. A slight decrease in viability was observed at 72 h with MAG (1–3), but the percentage of viability with MAG (1–3) at 72 h was significantly higher compared with full-length MAG (Fig. 5B). These data suggest that Ig domains four and five in MAG are necessary for p75 cleavage in SCs. Deleting Ig domains four and five eliminates p75 cleavage, and, as a consequence, SCs are not inhibited and remain viable.

MAG and MAG (1–3) bind to SCs with high specificity

The above results clearly demonstrate that MAG and myelin inhibit SC migration and induce their death, but the question is, does MAG bind to SCs? Previously, it was known that MAG binds to several types of neurons (DeBellard and Filbin, 1999), but it is unknown whether MAG binds to SCs. To test this, we first performed a soluble binding assay. In this assay, various chimera, MAG-Fc, MAG (1–3)-Fc, IgG-Fc (Fc control), and MUC18-Fc (chimera control), were added to cultured SCs or CHO cells for 1 h. The bound chimeras were visualized by incubating with a

cy3-conjugated anti-human-Fc antibody that specifically binds to the Fc portion of the chimeras. The results show that both MAG-Fc and MAG (1–3)-Fc strongly bind to SCs (Fig. 6A,C) but not to CHO cells (Fig. 6B,D). None of the control chimeras showed significant binding to SCs (data not shown).

Next, we performed a solid-phase, nonisotopic binding assay to quantitate SCs binding to MAG chimera. In this assay, MAG-Fc and MAG (1–3)-Fc chimera were immobilized onto 96-well microtiter wells via noncovalent bonding and were presented to Calcein AM-labeled single-cell suspensions of SCs, cerebellar neurons (positive control), or CHO cells (negative control). The results show that SCs bind strongly and specifically to both MAG-Fc and MAG (1–3)-Fc chimeras. The extent of SC binding was similar to that of neurons (Fig. 6E,G,H). CHO cells did not bind strongly to either chimera (Fig. 6F). The percentage binding of SCs and neurons only slightly increased with increase in chimera concentration, and maximum binding occurred at 20 μ g/ml for both MAG-Fc and MAG (1–3)-Fc. Together, the data from soluble binding and solid-phase binding assays demonstrate that MAG-Fc and MAG (1–3)-Fc bind to SCs with high specificity.

Putative MAG binding sites on SCs

We then asked whether SCs express MAG receptors. In neurons, MAG binds to NgR1 (Domeniconi et al., 2002) and NgR2 (Venkatesh et al., 2005), the two isoforms of NgR, as well as to gangliosides (Vyas et al., 2002) and more recently to a low-density lipoprotein receptor-related protein (LRP1; Stiles et al., 2013). SCs express gangliosides (Kamakura et al., 2005) but were reported to not express NgRs (Su et al., 2007). In the present study, we performed RT-PCR and Western blot analyses to test whether SCs used in our experiments express NgRs and LRP1. The results of RT-PCR analysis show that NgR1 mRNA is expressed in SCs cultured for 2 d *in vitro* (Fig. 7A), but NgR2 mRNA is absent (Fig. 7B). Rat cerebellar neurons and cortical neurons cultured *in vitro* and adult rat brain extracts served as positive controls for NgR mRNA. Western blot results showed that SCs express both NgR1 (Fig. 7C) and LRP1 (Fig. 7D) at a level similar to that found in cultured hippocampal neurons (positive control). Hippocampal neurons treated with NgR1 shRNA lenti virus to knock down NgR1 expression were used as a negative control. NgR2 protein was not detected (data not shown). We also assessed the expression of NgR1 in mature, myelinating SCs of the sciatic nerve by immunostaining; however, no expression was detected (data not shown). These results suggest that SCs used in our experiments express NgR1 and LRP1 but not NgR2. Additionally, while NgR1 is expressed in cultured SCs, it may be downregulated in myelinating SCs.

Figure 8. Blockade of p75 cleavage using inhibitor X promotes robust SC migration in the demyelinated mouse spinal cord. **A, B**, Photomicrographs of the longitudinal spinal cord sections illustrating the extent of migration of control (**A**) and Inh X-treated (**B**) GFP⁺ SCs, 5 d after engraftment (G), remotely from the LPC-induced lesion (L) of the dorsal spinal cord funiculus. Scale bar, 1 mm. **C, D**, Visualization of control (**C**) and Inh X-treated (**D**) GFP⁺ SCs at the level of the lesion. Insets are the enlarged areas of dotted squares showing the misalignment of the control SCs over Inh X-treated SCs. Scale bar, 100 μ m. **E, F**, Quantification of the total number of GFP⁺ cells (area) in the dorsal funiculus (**E**) and in the lesion (**F**). Bars represent the mean $\pm$ SEM of 12 sections/animal and 6–12 animals/group (**E**, $*p < 0.01$; **F**, $p < 0.015$ by Mann–Whitney *t* test).

Blockade of p75 cleavage promotes migration and recruitment of transplanted SCs in the demyelinated spinal cord

In vitro experiments established that blockade of p75 cleavage promotes SC migration and improves SC survival. Next, we investigated the effect of blocking p75 cleavage on the ability of SCs to migrate, survive/integrate, and differentiate into myelin-forming cells *in vivo* in the demyelinated adult CNS. LPC-induced demyelination was targeted to the dorsal funiculus of adult nude mice. Forty-eight hours after demyelination, Inh X-SCs ($n = 16$) and CON-SCs ($n = 17$) expressing GFP were grafted caudally in the same tract, at a distance of one intervertebral space (distance = 2 mm) from the LPC-injected site. Grafted animals were killed at 5 d.p.t. ($n = 21$) and

28 d.p.t. ($n = 12$). The spatiotemporal distribution of the transplanted CON-SC and Inh X-SC groups was assessed by scanning longitudinal sections of the spinal cord for a GFP signal throughout the sections. The results show that GFP-SCs migrated more extensively when pretreated with Inh X (7.7 ± 3.5) compared with control (3.2 ± 1.7 ; Fig. 8A,B) as assessed by total GFP signal (Fig. 8E). In addition, we evaluated the rostrocaudal distribution of the grafted SCs. Inh-X-SCs extended significantly more than CON-SCs (6084 ± 399 vs 3300 ± 656 μ m, $p < 0.0026$) along the dorsal funiculus. Next, we analyzed whether Inh X treatment promoted the ability of SCs to be recruited into the lesion site. For this, we first defined the lesion area by immunolabeling spinal cord sections for the my-

Figure 9. Blockade of p75 cleavage using inhibitor X improves SC survival. **A, B**, Photomicrographs of the longitudinal spinal cord sections showing caspase 3⁺ (red) expression in control (**A**) and Inh X-treated (**B**) at the lesions site. Hoechst staining (blue) identifies the nuclei of the entire cell population. Arrow points to GFP⁺ Schwann cell expressing Caspase 3 in control section. Scale bar, 50 μ m. **C**, Quantification of the percentage of Caspase 3⁺ GFP-Schwann cells at the graft and lesion sites. The percentage of dying cells is greater in the control group than in the Inh X-treated group at both graft and lesion sites. Bars represent the mean $\pm$ SEM of 12 sections/animal and five to seven animals/group.

elin protein MOG. We then quantitated GFP positivity per unit area in the lesion site. The results showed a threefold increase in Inh X-SC numbers at the lesion site at 5 d.p.t., suggesting pretreatment with Inh X improved SC recruitment at the lesion site (Inh X-SCs: 3.6 ± 1.6 mm²/lesion; CON-SCs: 1.2 ± 1 mm²/lesion; Fig. 8C,D,F). Moreover, Inh X-SCs had a typical bipolar SC morphology compared with CON-SCs, which were more rounded than elongated (Fig. 8A–D).

Increased GFP positivity could have also resulted from increased survival. To address this, we immunolabeled spinal cord sections for Caspase 3, a marker for dying cells (Fig. 9A,B). While there was no significant difference in SC survival between the two

groups at 5 d.p.t., fewer Caspase 3-positive cells were found at 2 d.p.t. in the Inh X-treated group compared with the control group (lesion site: Inh X-SCs, 0.1 ± 0.1 ; CON-SCs, 0.4 ± 0.2 ; graft site: Inh X-SCs, 0.8 ± 0.4 ; CON-SCs, 1.55 ± 0.7 ; Fig. 9C).

Blockade of p75 cleavage enhances transplanted

SC remyelination

Remyelination of LPC-induced lesion of the mouse spinal cord starts at 7 d.p.t. and is nearly complete by 28 d.p.t. (Gout et al., 1988; Jeffery and Blakemore, 1995). Endogenous oligodendrocyte progenitors and peripheral SCs participate in remyelination (Bachelin et al., 2010; Zujovic et al., 2010). To determine the contribution of exogenously transplanted SCs to remyelination of spinal cord axons, we analyzed the relative amounts of exogenous and endogenous peripheral myelination of CNS axons by staining for GFP and P0 (Fig. 10B,D). We defined lesion borders by immunolabeling for GFAP (Fig. 10A,C). Quantification of the percentage of GFP⁺ SCs expressing P0 in the lesion showed that the relative amount of remyelination achieved by exogenously transplanted SCs over that of endogenous SCs was significantly higher and increased by twofold in the Inh X-SC group compared with CON-SC group (Inh X-SCs: $40 \pm 10\%$; CON-SCs: $21.1 \pm 9\%$; Fig. 10E). The enhanced remyelination by exogenous SCs may be explained by improved SC migration/survival upon Inh X treatment. Moreover, exogenous SCs expressed the paranodal protein Caspr, indicating that these cells have the ability to reassemble and establish nodes of Ranvier (Fig. 10F,G).

Discussion

During development SCs migrate along developing nerves modulating their growth and guidance. They recapitulate similar functions in bands of Büngner in response to nerve injury. Their ability to promote axonal growth during PNS development and repair has made them ideal candidates for CNS repair. Even though animal models have proven that SCs possess axon regeneration and remyelination properties in the CNS, a major impediment in developing SC-based therapeutic strategies for CNS repair is their poor migration and survival within the adult CNS. The reasons for their poor migration and survival within the adult CNS are not well understood. It is believed that astrocytes play a role in restricting SC migration within the CNS (Lakatos et al., 2003a, 2003b). We have, however, previously reported that transplanted SCs do not migrate into the white matter but migrate preferentially along the meninges and blood vessels (Baron-Van Evercooren et al., 1993; Bachelin et al., 2010). This led us to investigate the possibility that myelin, a major component of white matter, plays a role in poor SC migration and survival in the adult CNS. Our results demonstrate that when SCs interact with MAG, an inhibitor present in myelin, or myelin in general, p75 undergoes γ -secretase-dependent cleavage, and this event is necessary to inhibit SC migration and induce their death both *in vitro* and *in vivo*.

It is well known that MAG and other molecules present in CNS myelin are inhibitory to neurite outgrowth from neurons (Filbin, 2003). MAG inhibits neurite outgrowth via cleavage of p75. p75 cleavage is necessary for the action MAG on neurons. Blocking p75 cleavage using a specific γ -secretase inhibitor, Inh X, overcomes MAG-mediated neurite outgrowth inhibition (Domeniconi et al., 2005). Taking a lead from this study, we conducted a series of experiments to understand the mechanisms of MAG/myelin interaction with SCs. First, we provide evidence that MAG/myelin inhibits SC migration via p75 cleavage. We then demonstrate that the blockade of p75 cleavage using Inh X

Figure 10. Blockade of p75 cleavage enhances SC remyelination at the lesion site. **A–D, F, G**, Photomicrographs of the longitudinal spinal cord sections showing the cellular organization at the lesion site, 4 weeks after transplantation of control (**A, B, F**) and InhX-treated (**C, D, G**) SCs. **A, C**, General view of GFP⁺ SCs in the lesion is visualized by GFAP (red) immunostaining. Scale bar, 200 μ m. **B, D**, Visualization of P0⁺ myelin-like sheaths corresponding to GFP⁺ profiles; insets are enlarged views of dotted squares illustrating colabeling of GFP⁺/P0⁺ myelin-like structures. Scale bar, 25 μ m. **E**, Quantification of GFP staining associated with P0 staining as an index of remyelination by the grafted SCs. Bars represent the mean $\pm$ SEM of 12 sections/animal and 6–12 animals/group. * $p < 0.032$ by Mann–Whitney t test. **F, G**, Illustration of Caspr⁺ paranodes associated with GFP⁺ profiles at the level of nodes of Ranvier (arrows). Scale bar, 20 μ m.

blocks MAG/myelin-mediated inhibition of SC migration. Therefore, blocking p75 cleavage is a potential therapeutic strategy to enhance SC migration *in vivo*. Additionally, while both full-length MAG and MAG (1–3) bind to SCs with high affinity and specificity, MAG (1–3) does not induce p75 cleavage and consequently does not inhibit SC migration, suggesting that Ig domains four and five are necessary for MAG-induced effects in both neurons and SCs. Furthermore, we demonstrate that cul-

tured SCs express high levels of NgR1 and LRP1, the two known receptors for MAG in neurons. Our hypothesis is that upon binding to SCs via NgR1 or LRP1, MAG induces p75 cleavage, resulting in the inhibition of migration. Other investigations, however, have shown that LRP1 promotes SC migration (Mantuano et al., 2008, 2010). This can be explained by the fact that LRP1 exerts different effects with different ligands by inducing the assembly of different coreceptor systems. For example, when MAG binds

to LRP1, p75 is recruited into LRP1 complex and RhoA is activated (Stiles et al., 2013). This is a key pathway by which myelin inhibitors inhibit neurite outgrowth in neurons (Yamashita et al., 2002). Whereas when tissue-type plasminogen activator and $\alpha 2$ macroglobulin bind to LRP1, ERK1/2, and AKT are activated (Mantuano et al., 2010). It is possible that while the binding of matrix metalloproteinase-9 to LRP1 promotes SC migration via ERK1/2 and AKT activation, the binding of MAG inhibits migration via p75 cleavage. Further experiments are necessary to test whether MAG binding results in the assembly of LRP1/p75 and/or NgR1/p75ntr in SCs.

In addition to its role in nerve growth and differentiation, p75 is involved in cell death. For example, when BDNF or pro-BDNF is added to sympathetic neurons in culture, p75 undergoes cleavage, releasing p75ICD. The release of ICD via a series of downstream events involving the activation of p75 interacting factors, TRAF6 and NRIF, results in apoptosis (Kenchappa et al., 2006). Our results demonstrate that prolonged incubation of SCs (≥ 24 h) with MAG or myelin induces their death. Interestingly, MAG/myelin-induced SC death also requires p75 cleavage, and blockade of p75 cleavage using Inh X improves SC survival. Then the question is how can a single ligand (MAG) induce two different effects in SCs? It is possible that upon long-term exposure to MAG, via p75 cleavage, a death pathway similar to pro-neurotrophins is activated, whereas a short-term exposure to MAG, also via p75 cleavage, activates a distinct pathway, perhaps involving a small GTPase, RhoA. Indeed, MAG has been shown to activate RhoA, an event downstream of p75 cleavage. RhoA activation disrupts the cytoskeleton to induce growth cone collapse in neurons (Yamashita et al., 2002; Mimura et al., 2006). Blocking p75 cleavage blocks the activation of RhoA (Domeniconi et al., 2005). In other words, the MAG/myelin signaling pathway bifurcates after p75ICD formation to one that induces the inhibition of migration and, second, that induces death. Further experiments are necessary to validate this idea.

The *in vitro* results clearly demonstrate that blockade of p75 cleavage is sufficient to block the MAG/myelin-mediated effect on SC migration and survival. Our logical next step was to investigate whether blockade of p75 cleavage improves SC migration and SC survival *in vivo* in the demyelinated adult CNS. To test this, we grafted SCs pretreated with Inh X or not into the adult mouse spinal cord 2 mm away from a demyelinating lesion. In this paradigm, SCs migrate toward the lesion in which they encounter MAG and myelin debris. Our results demonstrate that Inh X-pretreated SCs migrate more extensively from the graft site to the lesion site, leading to enhanced recruitment by the lesion. Inh X treatment also reduced the number of dying cells in the lesion site. The observed increase in SC recruitment at the lesion site may result from a combined effect on the increase in migration and the survival of the Inh X-pretreated SCs. A previous study (Su et al., 2007) reported that anti-NgR antibodies facilitate the migration of olfactory ensheathing cells through white matter tracts. In this study, anti-NgR antibodies were infused into the injured spinal cord. Since several cell types, including microglia (Yan et al., 2012), macrophages (McDonald et al., 2011), neural precursor cells (Wang et al., 2008; Mathis et al., 2010), astrocytes (Sato et al., 2005), and oligodendrocyte precursor cells (Huang et al., 2012), involved in lesion repair also express NgR, the infused anti-NgR antibody could bind to NgRs expressed in any of these cells, thereby blocking NgR signaling. To avoid any nonspecific effect of Inh X on other cell types, instead of infusing Inh X into the animals we pretreated SCs with Inh X before transplantation. Our results demonstrate that transient blockade of p75

cleavage is sufficient to promote SC migration, survival, and remyelination *in vivo*.

Furthermore, Inh X-pretreated SCs expressed processes reminiscent of normal SC morphology, whereas untreated SCs appeared round *in vivo*. Similar morphological changes were observed *in vitro* when SCs were cocultured on CHO-MAG cells, suggesting that MAG inhibits SC process extension, a step necessary for migration. Previous studies have shown that MAG causes growth cone collapse/repulsion in neurons via the activation of RhoA (Yamashita et al., 2002; Mimura et al., 2006). It is likely that SC interaction with MAG also activates RhoA, which then causes cytoskeletal disruption leading to reduced process length. Indeed, blocking p75 cleavage using Inh X reverses the effect of MAG on SC morphology. Moreover, Inh X-SCs expressed longer processes *in vivo*. Treatment with Inh X may therefore induce SCs to recapitulate their behavior during nerve development and repair (Feltri et al., 2016).

Next, we assessed whether transplanted SCs were capable of remyelinating the demyelinated axons. We allowed mice to survive for 28 d after transplantation, by which time remyelination of LPC-induced spinal cord lesions is expected to be complete (Bachelin et al., 2010). We double immunostained the spinal cord sections for the myelin marker P0 and GFP. The number of myelinating GFP⁺ cells was higher in Inh X-treated group compared with control. These cells also expressed the nodal protein Caspr, which is indicative of node formation and myelination, a process known to correlate with improved neural conduction (Black et al., 2006; Sasaki et al., 2006).

Together, our results demonstrate that MAG/myelin inhibits SC migration and reduces their survival via p75 cleavage. Blockade of p75 cleavage significantly improves SC migration and survival in the presence of MAG/myelin. Moreover, Inh X-SCs migrate extensively and survive better in the demyelinating adult CNS environment *in vivo*. The transplanted SCs exhibit signs of remyelination and node formation. These data suggest that MAG and myelin play a role in restricting SC migration and survival, resulting in inefficient SC transplantation *in vivo*. Blockade of p75 cleavage is therefore a putative therapeutic strategy to achieve successful SC-mediated repair of the adult CNS after an injury or a disease.

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