RESEARCH ARTICLE

Specific Inhibition of Secreted NRG1 Types I–II by Heparin Enhances Schwann Cell Myelination

Yael Eshed-Eisenbach, Aaron Gordon, Natalya Sukhanov, and Elior Peles

Primary cultures of mixed neuron and Schwann cells prepared from dorsal root ganglia (DRG) are extensively used as a model to study myelination. These dissociated DRG cultures have the particular advantage of bypassing the difficulty in purifying mouse Schwann cells, which is often required when using mutant mice. However, the drawback of this experimental system is that it yields low amounts of myelin. Here we report a simple and efficient method to enhance myelination *in vitro*. We show that the addition of heparin or low molecular weight heparin to mixed DRG cultures markedly increases Schwann cells myelination. The myelin promoting activity of heparin results from specific inhibition of the soluble immunoglobulin (Ig)-containing isoforms of neuregulin 1 (i.e., NRG1 types I and II) that negatively regulates myelination. Heparin supplement provides a robust and reproducible method to increase myelination in a simple and commonly used culture system.

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Key words: PNS, Schwann cells, myelin, neuregulin, heparan sulfate proteoglycans

Introduction

Myelin, produced by Schwann cells in the peripheral nervous system (PNS), allows fast and efficient nerve conduction that is necessary for normal function and survival. During the formation of peripheral nerves, Schwann cells undergo a series of distinct developmental stages, starting from migrating neural crest cells to myelinating and nonmyelinating Schwann cells of peripheral nerves (Kidd et al., 2013). Moreover, fully differentiated Schwann cells can trans-differentiate to form specialized repair cells in response to injury (Jessen et al., 2015). These complex developmental and postdevelopmental programs are controlled by both axonal and extracellular matrix-derived signals (Monk et al., 2015; Salzer, 2015). A central mediator of axon–glia communication in the PNS is Neuregulin-1 (NRG1) and its ErbB2/ErbB3 receptor heterodimer, which regulate nearly all developmental stages of the Schwann cell lineage (Grigoryan and Birchmeier, 2015). These include cell proliferation, survival and migration at early stages, progression from precursor cells to immature Schwann cells at later stages, and then axonal sorting and myelination (Jessen et al., 2015).

NRG1 is comprised of a number of alternatively spliced isoforms (types I–VI), which all contain an EGF-like domain that is necessary and sufficient for receptor activation (Birchmeier and Nave, 2008). In peripheral nerves, NRG1 I–II are mostly expressed by SCs, whereas NRG1 III is expressed by neurons (Stassart et al., 2013). NRG1 I–II are secreted proteins containing an immunoglobulin (Ig)-like domain in their N-terminal region, while NRG1 III is a transmembrane protein containing a cysteine-rich domain instead of the Ig fold (Falls, 2003). NRG1 III requires Schwann cell–axon contact (juxtacrine signaling) and is necessary for Schwann cell differentiation and myelination (Michailov et al., 2004; Taveggia et al., 2005). In contrast, NRG1 I–II act in a paracrine/autocrine fashion, and although they are necessary for efficient remyelination (Jessen et al., 2015; Stassart et al., 2013), they are dispensable for developmental myelination (Michailov et al., 2004; Stassart et al., 2013). Studies using Schwann cell/dorsal root ganglion (DRG) neurons culture revealed that soluble NRG1 could both promote and inhibit myelination depending on the concentrations used (Syed et al., 2010; Zanazzi et al., 2001). A unique feature of NRG1 I–II compared to NRG1 III is their ability to

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bind heparan sulfate proteoglycan (HSPGs) through an heparin-binding domain (HBD) present in their N-terminal Ig-like domain (Falls, 2003). Binding to HSPGs may increase the local concentration of NRG1 I–II, resulting in sustained ErbB receptor activation in Schwann cells (Li and Loeb, 2001; Ma et al., 2009; Sudhalter et al., 1996).

Here, we sought to explore whether the interaction between NRG1 and HSPGs regulates ErbB2/3 activation and myelination. We report that the addition of heparin to dissociated mouse DRG culture dramatically increases myelin production. Heparin supplement provides a robust and reproducible method to increase myelination in a simple and commonly used culture system that circumvents the use of purified mouse Schwann cells, which are poorly grown *in vitro* (Paivalainen et al., 2008). The effect of heparin resulted from specific inhibition of ErbB2/3 receptor activation by soluble NRG1-II, but not by the axonal transmembrane NRG1 III. Our results also suggest that the interaction of NRG1 I–II with HSPG facilitate continuous ErbB2/3 receptor activation, which negatively regulates Schwann cell myelination.

Materials and Methods

DRG Cultures and Purified SC Cultures

Dissociated mouse and rat DRG cultures were prepared from embryos at day 13.5 and 15.5 of gestation, respectively. DRGs were dissociated with trypsin and plated at a density of 4×10^4 per 13 mm coverslip, coated with Matrigel (BD Biosciences) and poly-D-lysine (Sigma). Cultures were grown for 2 days in Neurobasal medium supplemented with B-27, glutamax, penicillin/streptomycin (all from Gibco Life Technologies) and 50 ng/mL NGF (Alomone Labs). Cultures were then grown for eight additional days in BN medium containing Basal medium–Eagle (Sigma) ITS supplement (Gibco), glutamax (Gibco), 0.2% BSA (Sigma), 4 mg/mL D-glucose (Sigma), 50 ng/mL NGF and antibiotics. To induce myelination, cultures were grown in BNC medium (BN supplemented with 15% heat inactivated fetal calf serum, and 50 μg/ml L-ascorbic acid). Cultures were fixed for analysis after 10–12 additional days. In some experiments the following reagents were added to the BNC medium: heparin (Sigma 3393), NRG1(EGF) (Peprotech #100-3); NRG1-II (NDFβ1 ECD purified using heparin columns) (Peles et al., 1992); Clexane (Sanofi); Fragmin (Pharmacia). Rat Schwann cells were purified from dissociated DRG cultures based on their fast adhesion to Primaria plates (BD Falcon). Purified Schwann cells were grown in proliferation medium (DMEM, 3% FBS, 10% NDFβ conditioned medium, 2 μM forskolin, 2 mM glutamine, and 1 mM pyruvate). In order to test for soluble NRG1 activity, Schwann cells were grown for 18–24 h with medium that contained 0.5% FBS and lacked NDFβ, before being treated with different neuregulins or conditioned media for 30 min at 37°C. Cells were then lysed in 2× Laemmli sample buffer and analyzed by Western Blot using antibodies for P-Erb3, ErbB3, and P-AKT (Cell Signaling), P-ERK (Santa Cruz), and tubulin (Sigma) as a loading control.

Neuronal Membrane Preparation

In order to purify DRG neuron membranes, DRG neurons grown for 10–12 days in culture were collected in ice cold PBS and homogenized extensively using Dounce homogenizer. After an initial 20*g* centrifugation for 20 min, supernatant was centrifuged at 20,000*g* for 1 h at 4°C. Pellets were resuspended in DMEM 0.1% FBS and seeded on starved Schwann cells (i.e., incubated overnight with DMEM/0.5% FBS) by 200*g* 10 min centrifugation at 4°C. After a 25 min incubation at 37°C cells were lysed and analyzed by Western blot as described above.

Immunofluorescence Labeling and Proliferation Assays

For immunofluorescence labeling of cell cultures, cells were fixed with 4% PFA for 10 min at RT. Myelinating DRG cultures were then permeabilized in methanol for 5 min at −20°C. Following three washes in PBS, cultures were incubated in blocking solution (PBS, 1% glycine, 5% normal goat serum, 0.1% TritonX-100) for 45 min at RT. Cultures were subsequently incubated overnight at 4°C with primary antibodies rat anti-MBP (Chemicon); mouse anti-MAG (clone 513, Roche); diluted in blocking solution. After extensive washing with PBS, cultures were incubated with fluorophore-conjugated secondary antibodies (Jackson Laboratories) for 45 min at RT, and then washed in PBS and mounted in elvanol. For BRDU proliferation assay, BRDU (10 μM, sigma) was added to the culture medium for 2 h at 37°C before fixation with 4% PFA. Coverslips were then washed with PBS and incubated with 2N HCL for 20 min at 37°C. After further washing with PBS, slides were incubated for 45 min at RT with a blocking solution as described above and then incubated for 1h at RT with mouse anti BRDU antibody (Sigma), washed and incubated with donkey anti mouse Cy3 (Jackson) and DAPI (Sigma). Fluorescence images were obtained using an Axioskop2 microscope equipped with an ApoTom imaging system (Carl Zeiss) fitted with a Hamamatsu ORCA-ER CCD camera, or with Eclipse 90i Nikon microscope equipped with an ORCA-ER camera. Images were acquired and processed using the Zen2012 (Carl Zeiss) or Volocity (PerkinElmer) and Photoshop software (Adobe). Image analysis was done using Volocity and ImageJ.

Results

Heparin Increases Myelination in Dissociated DRG Cultures

Mouse and rat embryonic DRGs are commonly used as a tool to study myelin formation by Schwann cells in culture. They are often used at two configurations: dissociated cultures containing a mixture of Schwann cells and neurons (Fex Svenningsen et al., 2003), or as coculture (Johnson et al., 2001), in which purified Schwann cells are added to a bed of isolated DRG neurons. Although myelination is much less robust in dissociated DRG cultures than in coculture, the former has the advantage that it bypasses the need of using mouse Schwann cells that are poorly grown in isolation (Paivalainen et al., 2008). In an effort to promote myelination *in vitro*, we examined whether heparin, which regulates the activity of several growth factors (Billings and Pacifici, 2015), could enhance myelination of dissociated mouse DRG cultures. Cultures were

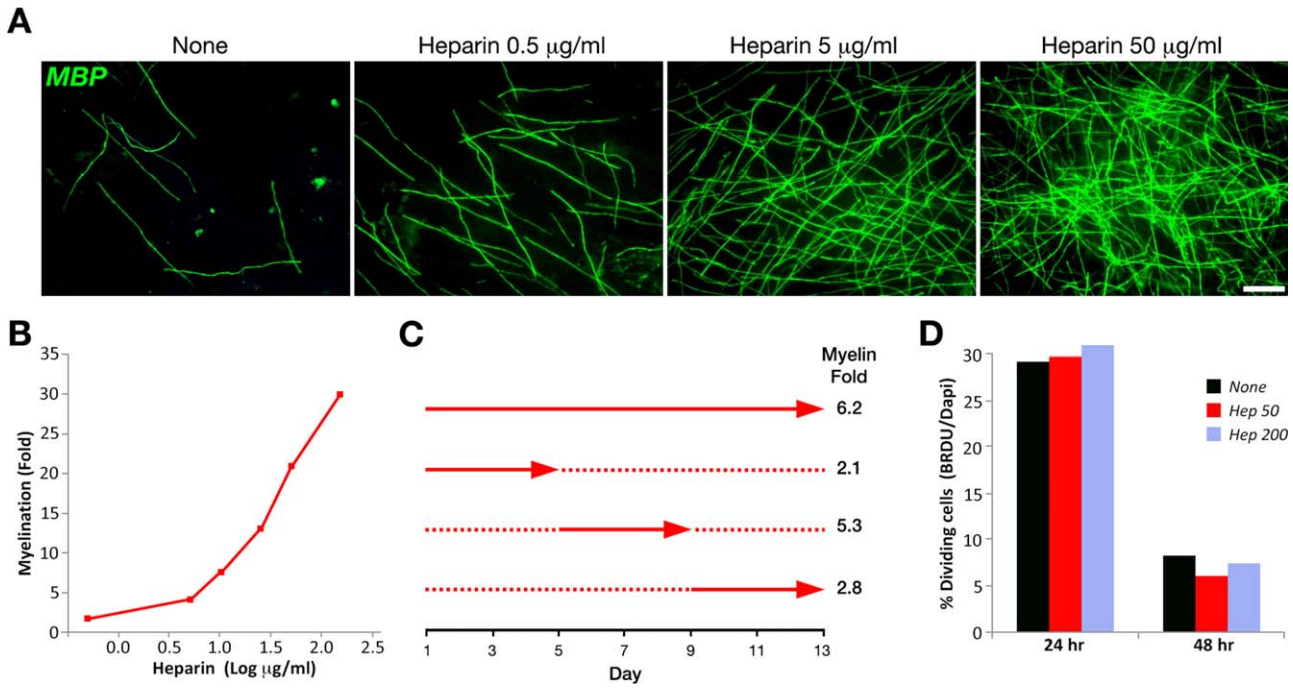

FIGURE 1: Heparin increases myelination by Schwann cells *in vitro*. A. Immunolabeling of dissociated mouse DRG cultures grown in the absence (None) or presence of the indicated concentration of heparin, using an antibody to MBP. B. Quantification of the effect of heparin. The fluorescence intensity of MBP positive myelin internodes is plotted as a function of the log heparin concentration. C. Heparin affects myelination during different time windows. Heparin was added to the myelination medium of dissociated mouse DRG cultures for the entire myelination period (days 1–13) or to three separate 4-day segments indicated by red arrows. Myelination was assessed by MBP staining and is registered as fold increase compared to untreated cultures. Dotted red line represents time of culture in the absence of heparin. D. Heparin did not affect Schwann cell proliferation. Dissociated mouse DRG cultures were grown in myelination medium (None) or myelination medium supplemented with the indicated concentration (μg/mL) of heparin for 24 or 48 h. BRDU labeling was performed for 2 h before fixation and the percentage of dividing cells in each culture was calculated. Scale bar: A. 60 μm.

prepared from 13.5 day embryos and grown for 8 days in a medium that supports axonal extension and Schwann cell proliferation before switching to myelination inducing medium. As depicted in Fig. 1A, we found that the addition of 0.5–50 μg/mL heparin to the myelination medium produced a dramatic increase in the number of Schwann cells that myelinate their associated axons. This effect was dose dependent ranging from a 1.7-fold increase at 0.5 μg/mL to a 30-fold increase at 150 μg/mL (Fig. 1B). The myelination enhancing effect of heparin did not depend on the substrate as it was observed when the cultures were grown on different matrices such as Matrigel and laminin-coated coverslips (data not shown).

When grown in the presence of 5 μg/mL heparin, the maximal (6.2-fold) enhancement of myelination was observed when the heparin was present during the entire 12 days myelination period (Fig. 1C). To determine whether shorter incubation times with heparin are sufficient to enhance myelination, we added heparin at three separate 4-day segments (Fig. 1C). Supplementing the myelination medium with 5 μg/mL heparin during the first (i.e., days 1–4) or the last (i.e., days 8–12) 4 days resulted in only a 2–3-fold increase myelination (as measured by MBP fluorescence intensity) while the addition of heparin to days 5–9 resulted in a substantial increase in myelination corresponding to 80% of the effect observed at the maximal exposure time. The addition of heparin to the myelinating medium did not affect cell proliferation as determine by bromodeoxyuridine (BrdU) incorporation (Fig. 1D). This finding is in line with previous studies showing that the majority of cell proliferation in these cultures occurs in the first few days after plating before the induction of myelination (Salzer and Bunge, 1980). Since the heparin we used was unfractionated crude heparin isolated from porcine tissues, we wanted to exclude the possibility of an active contaminating material and thus tested the effect of two synthetic low molecular weight heparins Enoxaparin (Clexane) and Deltaparin (Fragmin). Both had a similar effect that was comparable to the unfractionated heparin (Fig. 2).

To further examine whether the myelinogenic activity of heparin is due to its effect on Schwann cell differentiation or on myelin membrane wrapping, we immunolabeled dissociated mouse DRG cultures with antibodies to MAG and MBP, which mark early (promyelinating) and late (myelinating) differentiation stages of myelinating Schwann cells, respectively. We found that after 6 days in myelination medium, only heparin-treated cultures still contained numerous promyelinating MAG positive Schwann cells that did not express MBP (Fig. 3). During the next 4 days, these cells continued to

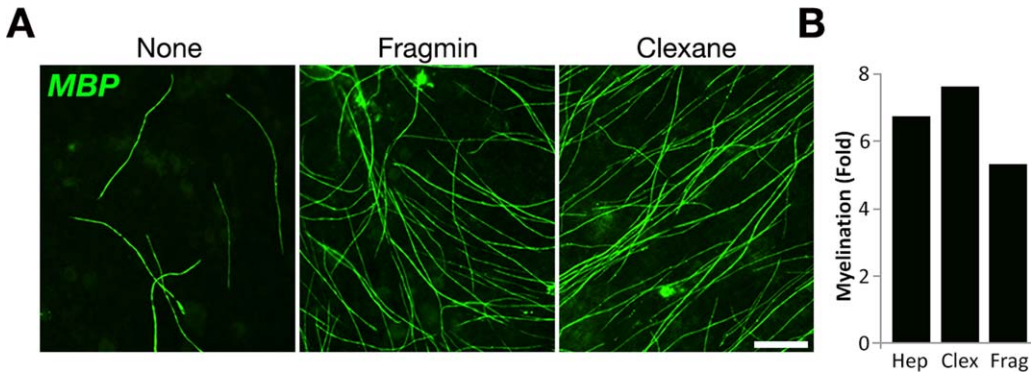

FIGURE 2: Low-molecular-weight heparins enhance Schwann cell myelination. A. Immunolabeling of dissociated mouse DRG cultures grown in the absence (None) or presence of 10 IU/mL low molecular weight synthetic heparins Clexane and Fragmin. B. Quantification of the effect of LMW heparins compared to unfractionated heparin. All heparins were added at 10 UI/mL. The fluorescence intensity of MBP positive myelin internodes was measured and is presented as folds increase compared to nontreated cultures (Hep—unfractionated heparin; Clex—clexane; Frag—fragmin). Scale bar: A, 40 μm.

differentiate and by 10DIV almost all MAG positive segments also expressed MBP. Although we cannot exclude a direct additional effect of heparin on myelin membrane wrapping, our results indicate that heparin promotes the early differentiation of Schwann cells. They also indicate that in the presence of heparin, Schwann cells cultured with neurons continue to differentiate from immature to promyelinating/early myelinating cells for a longer period of time. This notion is in line with

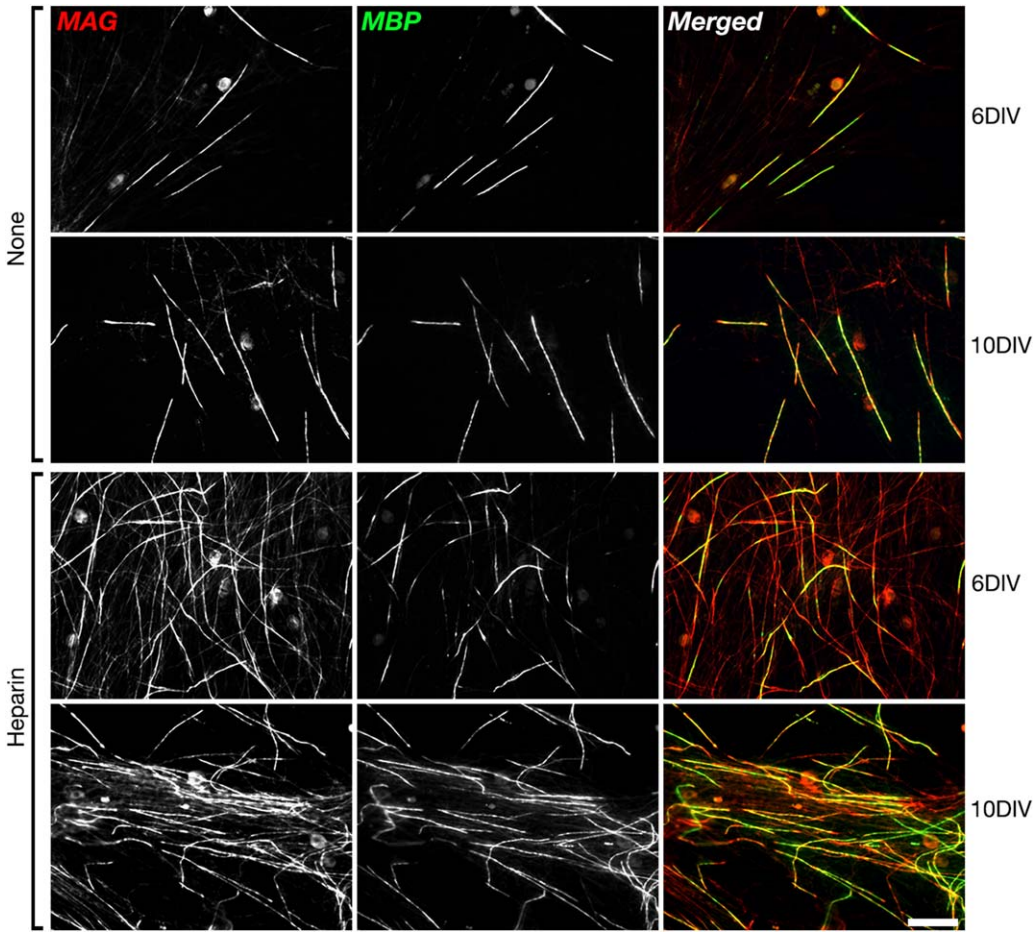

FIGURE 3: Heparin increases the number of promyelinating Schwann cells. Dissociated mouse DRG cultures were supplemented with 10 μg/mL heparin 2 days after plating, fixed at 6DIV or 10DIV, and immunolabeled using antibodies to MAG (red) and MBP (green) antibodies. Note the increase in MAG positive segments at 6DIV. Scale bar: 80 μm.

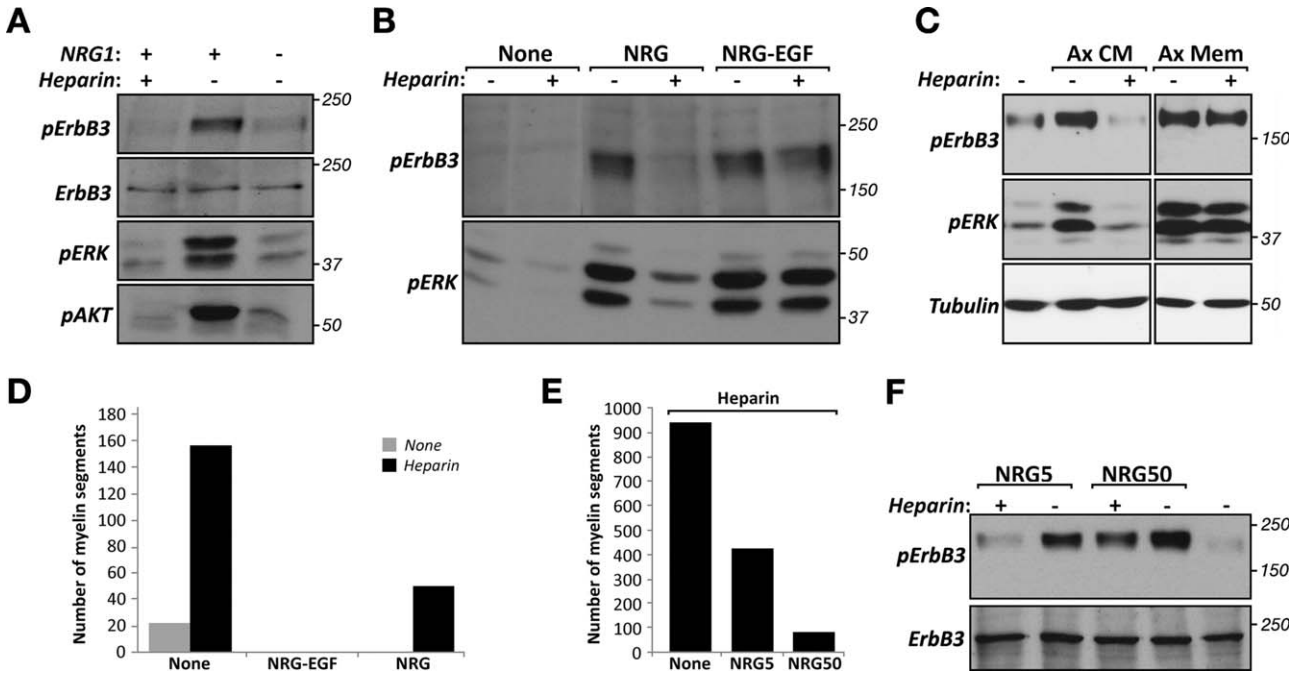

FIGURE 4: Heparin specifically inhibits signaling by soluble Ig containing NRG1 I–II. A. Heparin inhibits NRG1 I–II induced phosphorylation of ErbB3 and its downstream effectors ERK and AKT. Rat Schwann cells were incubated with soluble NRG1 I–II for 30 min in the absence or presence of 30 μg/mL heparin as indicated. Western blot analysis was done using antibodies to phosphorylated ErbB3 (*pErbB3*), ErbB3 (*ErbB3*), or phosphorylated ERK (*pERK*) or AKT (*pAKT*). **B.** The effect of heparin depends on the Ig domain of NRG1. Western blot analysis of rat Schwann cells treated with recombinant NRG1 type II (NRG) or only the EGF domain of NRG1 (NRG-EGF) using antibodies to phosphorylated ErbB3 (*pErbB3*) or ERK (*pERK*). Heparin specifically inhibited ErbB3 and ERK phosphorylation induced by NRG1 I–II and not by its EGF domain. **C.** Heparin inhibits ErbB3 signaling induced by soluble, but not by membrane bound forms of NRG1. Western blot analysis of rat Schwann cells treated with DRG neurons conditioned medium (Ax CM) or with neuronal membrane preparation (Ax Mem), in the absence or presence of heparin, using the indicated antibodies on the left of each panel. Note that heparin could only block signaling by secreted NRG1. **D.** Overcoming the inhibitory effect of NRG1 by heparin requires its Ig domain. Dissociated rat DRG cultures were grown in the absence (None) or presence of inhibitory concentration of NRG1 I–II (NRG) or the EGF domain of NRG1 (EGF), either in the presence (gray bars), or absence (black bars) of heparin. Fixed cultures were labeled with anti-MBP antibodies and the number of MBP positive segment was counted. Note that heparin is able to override the inhibitory effect of NRG1 I–II but not that of NRG1 EGF domain. **E,F.** The ability of heparin to rescue myelination in NRG1-inhibited cultures correlates with inhibition of ErbB3 activation in Schwann cells. Myelinating mouse DRG cultures were grown with 30 μg/mL of heparin in the absence (None) or presence of two inhibitory concentrations of NRG1 I–II. The number of myelin segments is shown (E). Western blot analysis of isolated rat Schwann cells that were left untreated or treated for 30 min with the same factor combination (F).

the observation that treatment of isolated rat Schwann cells with heparin resulted in downregulation of c-jun mRNA which is a negative regulator of myelination (Parkinson et al., 2008) (Supporting Information Fig. 2).

Heparin Blocks the Activity of NRG1 I–II

Schwann cell differentiation and myelination is controlled by the combinatorial action of several signaling systems of which NRG1 is central. NRG1 I–II isoforms secreted by Schwann cells and sensory neurons contain an immunoglobulin domain that binds heparan sulfate proteoglycans and heparin. To test whether the myelination enhancement capacity of heparin involves NRG1 signaling, we treated primary rat Schwann cells for 30 min with NRG1 Iβ in the presence or absence of heparin. Western blot analysis revealed that heparin blocked ErbB3 phosphorylation and consequently the activation of its downstream effectors ERK and Akt (Fig. 4A). Inhibition of ErbB3 and ERK phosphorylation by heparin was only observed when Schwann cells were treated with NRG1 Iβ and not with a recombinant protein harboring only the EGF domain (Fig. 4B), demonstrating that the action of heparin depends on the presence of the Ig domain of NRG1. Previous studies have shown that axon–glial NRG1 signaling depends on the transmembrane isoform NRG1 III that lacks the Ig-like heparin-binding domain (Michailov et al., 2004; Taveggia et al., 2005). To test whether the axo-glial contact-dependent NRG1 III signal is affected by heparin, we stimulated Schwann cells with either axolemmal preparation of DRG neurons, or with DRG neurons conditioned medium (both derived from the same cultures), in the presence or absence of heparin (Fig. 4C). While both conditioned medium and the axonal membrane preparation induced ErbB3 and ERK phosphorylation, heparin only blocked ErbB2/3 activation induced by the medium. These

results demonstrate that heparin inhibits the signaling elicited by secreted, but not by the membrane-associated, isoforms of NRG1.

Inhibition of Ig-EGF NRG1 Signaling by Heparin Promotes Myelination

It was previously reported that when added as soluble recombinant proteins, both NRG1-II and NRG1 III increase myelination at low concentration but inhibit it at high concentrations (Syed et al., 2010). Interestingly, it was found that soluble NRG1-II becomes inhibitory to myelination at much lower concentrations than soluble NRG1 III. When we added NRG1-II and NRG1-EGF at inhibitory concentrations to mixed DRG cultures, we found that heparin could override the inhibitory effect of exogenous NRG1-II but not that of NRG1-EGF (Fig. 4D). This suggests that the positive effect of heparin on myelin formation in culture is a result of inhibiting the negative effect of soluble NRG1.

We next examined whether the capacity of heparin to override the inhibitory effect of NRG1-II on myelination correlated with its ability to block ErbB2/3 signaling. To this end, we added heparin to myelinating mouse DRG cultures grown in the presence of two inhibitory concentrations of NRG1-II (Fig. 4E), and treated starved rat Schwann cells for 30 min with the same factor combination (Fig. 4F). We found that the ability of heparin to inhibit ErbB3 phosphorylation correlated with its ability to reverse the negative effect of recombinant NRG1-II on myelination. Notably, the addition of heparin did not affect cell proliferation, as determined by BRDU labeling (data not shown). Altogether these results suggest that heparin enhances myelination in mixed DRG cultures by inhibiting the negative effect of soluble NRG1-II secreted by the neurons, Schwann cells or both cell types.

Discussion

Mixed sensory neurons and Schwann cells cultures prepared from embryonic mouse DRGs, as well as DRG/Schwann cells coculture, are commonly used to study PNS myelination. The first is a relatively simple culture system, in which DRG neurons are populated and then myelinated by Schwann cells and dedifferentiated satellite Schwann cells that are present within the ganglia. In contrast, in the coculture configuration, Schwann cells purified from sciatic nerve or DRG ganglia are added to isolated sensory neurons. Although myelination in cocultures is more robust than in mixed dissociated DRG cultures, it requires the use of purified mouse Schwann cells, which are difficult to obtain. Given the increasing number of mutant mice strains, enhancing myelination of dissociated DRG cultures is of great interest. In this study, we show that the addition of heparin or low molecular weight heparin to mixed mouse DRG culture results in 5–30 fold increase in myelination. The optimal concentration of heparin we routinely use is 10 μg/mL, although higher concentrations could be used without significantly affecting neuronal and Schwann cells health. The maximal enhancement of myelination was observed when heparin was added to the cultures early on with the induction of myelination by ascorbic acid (i.e., myelination medium).

Myelination of the PNS is controlled by various paracrine, autocrine and juxtacrine signals (Grigoryan and Birchmeier, 2015; Monk et al., 2015). Among these, NRG1 signaling regulates all stages of Schwann cells development, differentiation and myelination. NRG1 signaling thus requires tight regulation that is provided by spatial and temporal control of its expression, alternative splicing, proteolytic processing of precursor proteins, and by interaction with HSPGs (Gambarotta et al., 2013). Furthermore, this signaling system requires a balance between the action of axolemma-bound (NRG1 III) and secreted (NRG1 I–II) forms of NRGI that could function in different manners. For example, while axonal NRG III is essential for myelination during development, NRG1 I–II is required for remyelination by dedifferentiated Schwann cells after injury (Fricker et al., 2009). This complex regulation of NRG signaling is likely disturbed in culture conditions, where Schwann cells that detach from neighboring axons during culture preparation are expected to upregulated NRG1 I–II (Stassart et al., 2013). We suggest that the dramatic effect of heparin on Schwann cells differentiation into myelinating cells in DRG cultures results from its inhibitory effect on the immunoglobulin-containing isoforms of NRG1. This conclusion is based on the following observations: (i) Heparin blocks phosphorylation of ErbB3 receptor and its downstream effectors ERK and AKT induced by NRG1 I–II, but not by NRG1 III which is present in axonal membrane preparations. (ii) Heparin does not affect signaling induced by recombinant NRG1 protein that only contains its EGF domain. (iii) Overcoming the inhibitory effect of NRG1 I–II on myelination by heparin depends on the presence of its immunoglobulin like domain. (iv) The ability of heparin to rescue myelination in NRG1 I–II inhibited cultures correlates with the inhibition of ErbB3 phosphorylation in Schwann cells. (v) In contrast to dissociated mouse DRG cultures, heparin has only a minor effect on rat cultures, which contain lower levels of soluble NRG1 I–II (Supporting Information Fig. 1). (vi) In isolated Schwann cells, heparin causes downregulation of c-jun (Supporting Information Fig. 2), a transcription factor that is induced by autocrine production of NRG1-II in Schwann cells that undergo dedifferentiation after peripheral nerve injury (Arthur-Farraj et al., 2012; Fricker et al., 2011; Stassart et al., 2013).

In agreement with previous studies (Syed et al., 2010; Zanazzi et al., 2001), our results suggest that the timing of

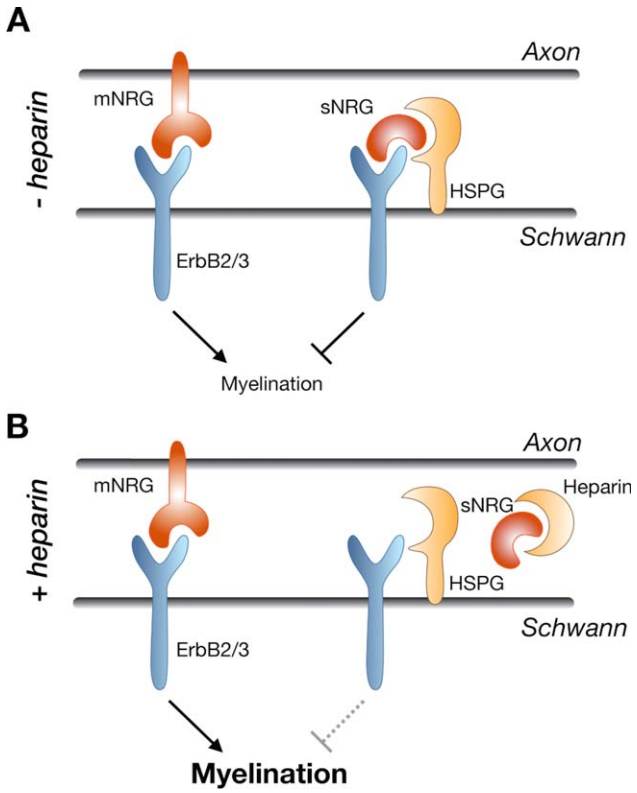

FIGURE 5: Proposed mechanism underlying the effect of heparin on myelination. Myelination is regulated by the balanced action of the stimulatory axonal membrane bound NRG1 III and the inhibitory soluble NRG1 I–II that are secreted by both Schwann cells and neurons. The addition of heparin to dissociated Schwann cell/DRG neuron cultures specifically inhibits NRG1 I–II (and not NRG1 III), thereby resulting in enhanced myelination.

Schwann cell myelination is controlled by an interplay between the enhancing action of axonal NRG1 III and the inhibitory activity of soluble NRG1 I–II (Fig. 5). Such a regulation may be of particular importance when Schwann cells are not in contact with high enough levels of axonal NRG1 III, as occurs in denervated nerves or when Schwann cells contact small caliber axons. Interestingly, once stable axonal contact is made, transmembrane NRG1 III negatively regulates the expression of NRG1 I–II in Schwann cells (Stassart et al., 2013), thereby facilitating further differentiation. Our results are consistent with previous studies, showing that soluble NRG1 II bind HSPGs, and that heparin inhibits its ability to stimulate ErbB receptor phosphorylation (Li and Loeb, 2001). In myotube cultures for example, NRG1 II binding to endogenous HSPG produced sustained signaling necessary for acetylcholine receptor gene expression (Li and Loeb, 2001). Signaling by the NRG1 II produced a 4-fold increase in receptor phosphorylation compared to the NRG1 II lacking the immunoglobulin domain. In agreement, in Schwann cells the concentration threshold beyond which soluble NRG II and III become inhibitory for myelination was much lower for heparin binding NRGII (Syed et al., 2010). Although NRG1 I–II secreted by Schwann cells is not necessary for myelination, i.e., when axonal NRG1 III is present, it is required for Schwann cells redifferentiation after injury (Stassart et al., 2013). However, as both neurons and Schwann cells secrete NRG1 I–II (Ma et al., 2011), the role of this specific isoform has not been tested in developmental PNS myelination. It would thus be interesting to study PNS myelination in isoform-specific mutants using conditional alleles, in addition to a timely overexpression of NRG1 I–II during postnatal development. Moreover, it may be of interest to test for myelin-related phenotypes in mice carrying mutations in enzymes that control HSPG biosynthesis as well as in extracellular sulfatases as such mutations have been shown to alter signaling by heparin binding growth factors (Phillips et al., 2012).

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