

N-Propionylmannosamine stimulates axonal elongation in a murine model of sciatic nerve injury

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Abstract

Increasing evidence indicates that sialic acid plays an important role during nerve regeneration. Sialic acids can be modified *in vitro* as well as *in vivo* using metabolic oligosaccharide engineering of the N-acyl side chain. N-Propionylmannosamine (ManNProp) increases neurite outgrowth and accelerates the reestablishment of functional synapses *in vitro*. We investigated the influence of systemic ManNProp application using a specific *in vivo* mouse model. Using mice expressing axonal fluorescent proteins, we quantified the extension of regenerating axons, the number of regenerating axons, the number of arborising axons and the number of branches per axon 5 days after injury. Sciatic nerves from non-expressing mice were grafted into those expressing yellow fluorescent protein. We began a twice-daily intraperitoneal application of either peracetylated ManNProp (200 mg/kg) or saline solution 5 days before injury, and continued it until nerve harvest (5 days after transection). ManNProp significantly increased the mean distance of axonal regeneration (2.49 mm vs. 1.53 mm; $P < 0.005$) and the number of arborizing axons (21% vs. 16%; $P = 0.008$) 5 days after sciatic nerve grafting. ManNProp did not affect the number of regenerating axons or the number of branches per arborizing axon. The biochemical glycoengineering of the N-acyl side chain of sialic acid might be a promising approach for improving peripheral nerve regeneration.

Key Words: N-propionylmannosamine; sialic acid; glycoengineering; sciatic nerve; peripheral nerve; branching; arborisation; Thy1-YFP mouse; nerve regeneration

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Introduction

N-Acetylneuraminic acid (Neu5Ac), or sialic acid (Sia), is expressed as terminal sugar in most glycoproteins and glycolipids. Sia is intimately connected with intermolecular and intercellular interactions. Within the nervous system, Sia is part of the polysialic acid (PSA) that prevails in the neural cell adhesion molecule (NCAM), which is a prototypic member of the immunoglobulin family of adhesion molecules (Hildebrandt and Dityatev, 2013). Polysialylation of NCAM plays an important role in brain development, nerve regeneration and neural plasticity (Daniloff et al., 1986; Maier et al., 1986; Brodkey et al., 1993; Zhou and Zhou, 1996; Franz et al., 2005; Hildebrandt et al., 2007; Jungnickel et al., 2009). PSA is a major regulator of cellular plasticity in brain development (Rutishauser, 2008; Schnaar et al., 2014). PSA modulates cell interactions *via* multiple mechanisms, including the attenuation of homophilic and heterophilic cell-cell and cell-matrix adhesion (Hildebrandt and Dityatev, 2013). During the development of the peripheral nervous system, the polysialylated form of NCAM is highly expressed in Schwann cells and axons. Its expression decreases in adulthood, but increases again after injury and during regeneration (Covault et al., 1986; Daniloff et al., 1986; Maier et al., 1986; Brodkey et al., 1993; Carratu et al., 1996; Zhou and

Zhou, 1996; Fu and Gordon, 1997; Rutishauser, 1998; Franz et al., 2005; Hildebrandt et al., 2007; Jungnickel et al., 2009). Polysialylation of NCAM during regeneration seems to be crucial for regenerating motor axons to find the appropriate motor Schwann cell tubes – a process known as preferential motor reinnervation (Brushart, 1993; Brushart et al., 1998; Franz et al., 2005, 2008). Brief electrical stimulation of the traumatized nerve enhances peripheral nerve regeneration (Gordon et al., 2008, 2009), and increasing evidence indicates that this phenomenon can be explained at least partially by up-regulation of PSA expression (Franz et al., 2008; Vogt et al., 2012).

Sialylation can be modified biochemically *in vitro* and *in vivo* using non-physiological precursors such as N-propionylmannosamine (ManNProp) and other N-acylmannosamines (Buttner et al., 2002; Gagiannis et al., 2007; Pon et al., 2007). The promiscuity of the enzymes of the Sia biosynthesis allows ManNProp to be metabolised to the respective N-propionylneuraminic acid (Neu5Prop). This process is very similar to the physiological metabolism of N-acetylmannosamine to Neu5Ac (Kayser et al., 1992; Kepler et al., 1995; Schmidt et al., 1998, 2000; Wolf et al., 2012). Posttranslational modification of glycoconjugates by Neu5Prop significantly replaces natural Sia by up to 85%

without increasing overall sialylation, as shown *in vitro* (Butner et al., 2002) and *in vivo* (Gagiannis et al., 2007).

Biochemical glycoengineering of sialic acid has revealed unexpected new biological characteristics of Sia, e.g., influencing the expression of genes involved in the neuronal differentiation (Kepler et al., 2001; Kontou et al., 2008).

In this paper, we use a mouse sciatic axotomy and transplantation model to show, for the first time, the impact that systemic application of ManNProp has on early peripheral nerve regeneration *in vivo*.

Materials and Methods

Animals

Twelve thy-1-YFP mice (YFP⁺) (Jackson Laboratory, Bar Harbor, Maine, USA) with mixed gender and a weight of 25–30 g, which express yellow fluorescent protein (YFP) in a subset of their axons (B6.Cg-Tg (thy1-YFP) 16Jrs/J; Jackson Laboratory, Bar Harbor, Maine, USA), were divided into two experimental groups (Feng et al., 2000; Witzel et al., 2005). C57Bl/6 mice ($n = 12$) with mixed gender and a weight of 25–30 g served as YFP⁻ donors for peripheral nerve grafting. The State Animal Protection Committee of Berlin (Germany; Application Number G0041/07) approved all procedures.

Surgical procedure

All surgical procedures were performed on YFP⁺ mice at the age of 12–16 weeks. Anesthesia was induced with isoflurane (Forene®, Wiesbaden, Germany) and continued by intraperitoneal injection of a mixture of xylazine (16 mg/kg) and ketamine (100 mg/kg). A dorsal approach was used for the bilateral sciatic nerve exposure in YFP⁺ mice. After

transection of the sciatic nerve proximal to the trifurcation, the distal nerve section was completely removed and partially replaced by a 1-cm sciatic nerve graft harvested from YFP⁻ mice. Proximal coaptation of the nerve graft to the transected sciatic nerve of the YFP⁺ mice was performed after careful alignment, and a semicircular rubber splint was used to hold the two ends in place (Dow Corning; 0.025 inch ID) (Witzel et al., 2005). This resulted in a tension-free splint repair and avoided the irritation that would have been caused by end-to-end nerve suture. A single stitch secured the epineurium to the rubber at each end of the splint (11-0 nylon; **Figure 1**). No distal coaptation of the nerve graft was performed. The mice received a daily subcutaneous injection of 0.1 mg/kg buprenorphine as an analgesic.

Experimental groups

The first experimental group (ManNProp, $n = 6$) received a twice-daily intraperitoneal application of peracetylated ManNProp (200 mg/kg; produced in our biochemical lab) diluted in physiologic saline solution. Peracetylation renders sugars lipophilic, which means they can be transported into the cell without the need for a limited, specific monosaccharide transport system. Inside the cell, non-specific esterases quickly split off the acetyl groups. As a result, much less sugar is needed to induce biological effects. The strength and timing of the dosage of ManNProp in its peracetylated form were based on published data (Kayser et al., 1992; Gagiannis et al., 2007). The second experimental group (saline, $n = 6$) served as control and received a twice-daily intraperitoneal application of physiologic saline solution (10 mL/kg) only. Treatment started 5 days

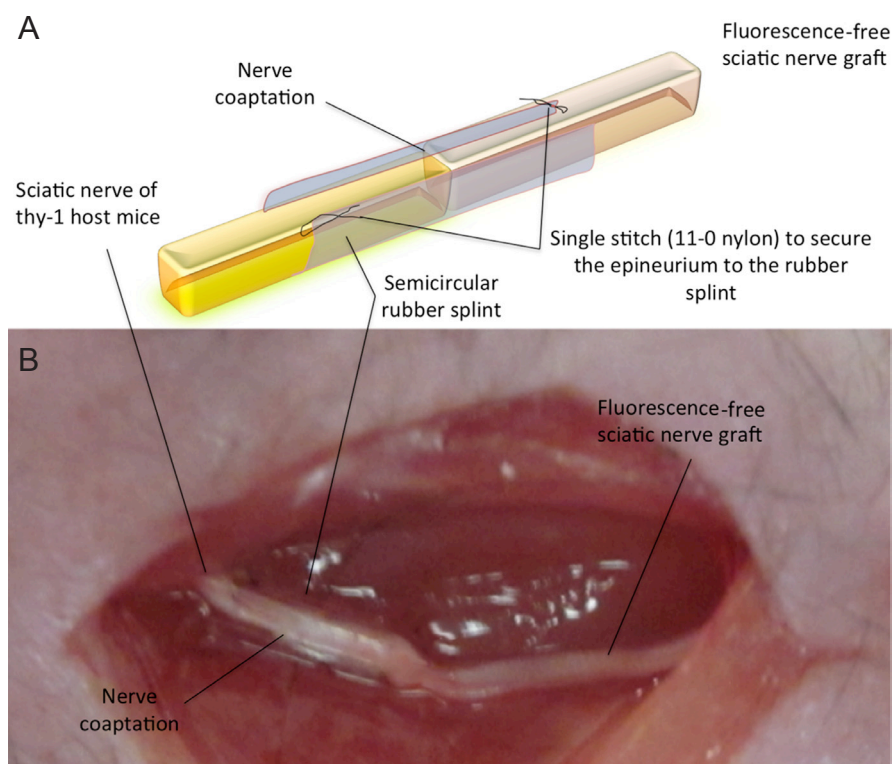


Figure 1 Visualization of the mouse model. (A) Schematic image of the semicircular silicon rubber splint that surrounds the repair site and is sutured to each nerve stump, far away from the fragile repair site. The proximal fluorescent sciatic nerve stump (left side, light yellow) is attached to the non-fluorescent nerve graft (right side, brown). (B) *In situ* aspect of the right sciatic nerve region after nerve grafting.

Table 1 Description of the outcome values

Group	25%	50%	75%	Mean	Difference	P value
ManNProp	3 mm	3 mm	2 mm	2.49 mm	0.96 mm	> 0.005
Saline	2 mm	1 mm	1 mm	1.53 mm		

ManNProp: N-Propionylmannosamine. 25%, 50%, and 75% represent 25th, 50th, and 75th percentiles, respectively.

before surgery and was continued for additional 5 days after surgery, until nerve harvest. Donor mice were not treated.

We used thy-1 mice as host animals. Their partial fluorescent axons made it easy for us to quantify regeneration distance and morphology. To exclude interference from fluorescent debris caused by Wallerian degeneration and thus difficulties in quantifying the results, we used fluorescence-free nerve grafts for the distal nerve section. The distal end of the nerve graft was not coapted, which ruled out any influence from the target organ (Frey et al., 1996). The follow-up period was limited by the length of the nerve graft (1 cm). Five to seven days after nerve repair, the fastest axons had exceeded the distal end of the graft and therefore could not be used for evaluation.

Nerve harvesting and fixation

Five days after surgery, both sciatic nerves, including the grafts, were harvested after sacrificing the mice by CO₂ inhalation. After the semicircular rubber splint had been removed, the nerve was carefully stretched out on a piece of wood and sutured with 9-0 nylon at both ends. The nerves were fixed for 24 hours in 4% paraformaldehyde. We produced 100- μ m longitudinal sections on a freezing microtome. For the imaging of Schwann cell tubes, we used antibodies against mouse laminin produced in rabbit in a dilution of 1:250 and an incubation time of 12 hours (Catalogue-No: L9393, Sigma, Munich, Germany) and Alexa Fluor 568 as a secondary antibody produced in a goat at a dilution of 1:300 and an incubation time of 2 hours (Catalogue-No: A-21069, Invitrogen, Darmstadt, Germany).

Evaluation of tissue sections

Regeneration distance

Sections were analyzed with standard fluorescence microscopy (Carl Zeiss Microscopy GmbH, Berlin, Germany) at a wavelength of 568 nm and 466 nm for the yellow auto-fluorescence of the axons. All counts performed were done manually during fluorescent microscopy without using professional software. The coaptation site was set as the zero reference point. We counted the number of fluorescent axons proximal to the coaptation site. The number of axons that were present exactly 1 mm proximal to the coaptation was defined as the maximum number of axons able to regenerate. We also counted the regenerating axons at intervals of exactly 1 mm distal to the coaptation site. Retrograde regenerating axons were excluded from the count. Each axon branch was counted as one axon at each millimetre point (Figure 2).

The numbers of regenerating axons present at each millimetre distal to the coaptation site were set against the maximum number of axons able to regenerate and displayed as Kaplan-Meier curves. The mean and standard deviation of the distance, as well as the distance of the 25th, 50th and 75th percentiles (representing the fastest, middle and slowest 25% of regenerating axons), could be determined for each experimental group.

Percentage of regenerating axons

The fluorescent axons 1 mm proximal and distal to the coaptation site were counted in order to calculate the percentage of regenerating axons for each group (mean \pm SD).

Branches and arborization

The total number of regenerating axons (RA), arborizing axons (AA) and branches (B) was counted in the zone running from the transection to 2 mm inside the nerve graft. We used these results to calculate the ratio of AA to RA. We also calculated the mean count and standard deviation of branches per regenerating axon as the ratio of B to RA, and per arborizing axon as the ratio of B to AA for each experimental group.

Statistical analysis

Statistical significance of the compared Kaplan-Meier curves was determined by the log-rank test. Statistical analysis of the percentage of regenerating axons and branching was performed by Student's *t*-test after testing for normal distribution. The significance level was set at $P < 0.05$. Statistical analysis was performed using IBM SPSS Statistics 22 software (Ehningen, Germany).

Results

After 5 days, the regeneration distance in the ManNProp group increased significantly compared to the saline group ($P < 0.005$; Figure 2). The mean regeneration distance achieved was 2.49 ± 0.05 mm for the ManNProp group and 1.53 ± 0.04 mm for the saline group. Regeneration distances of the 25th, 50th and 75th percentiles were 3, 3, and 2 mm for the ManNProp group, and 2, 1, and 1 mm for the saline group (Figure 3, Table 1). The mean percentage of arborizing regenerating axons after 5 days was higher between the ManNProp group compared with the saline group ($P < 0.05$; Figure 4). The difference of the number of regenerating axons between the ManNProp group and saline group was not found ($P > 0.05$; Figure 5). The mean number of branches per regenerating axon in the ManNProp group was similar to the saline group ($P > 0.05$; Figure 6). There were no differences found in the number of branches per arborizing axon between the ManNProp group and the saline group ($P > 0.05$; Figure 7).

Discussion

Systemic treatment of mice with ManNProp significantly increased the distance of axonal elongation and the number of arborizing axons 5 days after nerve axotomy. This supports

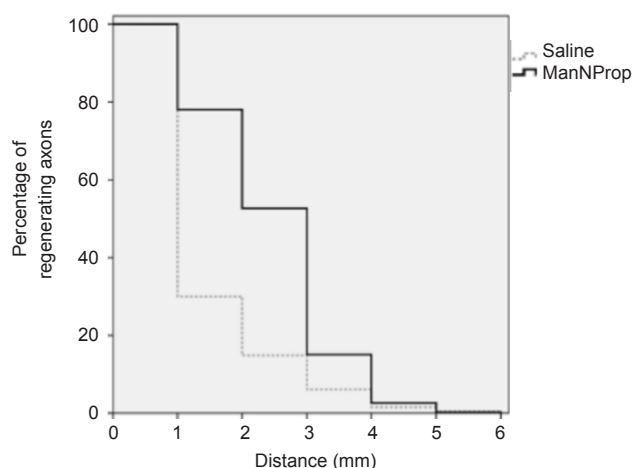


Figure 3 N-Propionylmannosamine (ManNProp) increases distance of axonal regeneration.

Treatment with ManNProp significantly increased axonal elongation at 5 days after sciatic nerve grafting ($P > 0.05$; log-rank test).

previous *in vitro* results, which showed that treating PC12 cells with ManNProp increased neurite outgrowth and accelerated significantly the reestablishment of the perforant pathway in brain slices *in vitro* (Schmidt et al., 1998, 2000; Buttner et al., 2002). Recent findings indicate that ManNProp influences signal transduction, as shown by the increased expression of phosphorylated Erk 1/2 (Kontou et al., 2008). ManNProp also stimulates the secretion of thioredoxin, a small redox protein that promotes neurite outgrowth (Kontou et al., 2008; Horstkorte et al., 2010). These phenomena might be due to ManNProp itself or to the biochemical modification of Sia in receptor glycoproteins on the cell surface enhancing the recognition and transmission of growth factor signals.

In the first few days after nerve axotomy, the biggest obstacle facing regenerating axons is crossing the repair site. The morphology of regenerating axons is mainly characterized by lateral movement, arborization and pruning (Ramón y Cajal and May, 1928). Although increased arborization is presumed to be associated with nerve malcoaptation or scarring, recent data suggest that it could also be a pathway selection mechanism that benefits regeneration specificity (Brushart, 1990, 1993; Brushart et al., 1998; Franz et al., 2005, 2008; Witzel et al., 2005). Already in 1972, it was found that transected axons produce multiple sprouts that advance distally in the so called “regenerating unit” (Morris et al., 1972; Mackinnon et al., 1991). During embryonic development of the motor nervous system and central nervous system, arborization and collateral pruning are recognized as the way that the projections fine-tune themselves (Redfern, 1970; Brown and Booth, 1983; Stelzner and Strauss, 1986). Branching is undoubtedly a substrate and energy consumptive process. Since evolution has preserved this mechanism means that it must offer some kind of benefit. Biochemical glycoengineering of sialic acid might, in future, be a promising way of beneficially influencing peripheral nerve regeneration.

Taken together, systemic treatment of mice with Man-

NProp increases the regeneration distance, the number of axons that arborize and the degree of axonal branching in mouse models of sciatic axotomy and transplantation.

Author contributions: CW and WR designed the study. CW and GK performed the research. WR and GBS interpreted the data and performed the critical revision of the paper for important intellectual content. GK was also responsible for data collection and statistical analysis and wrote the paper. All authors approved the final version of this paper.

Conflicts of interest: None declared.

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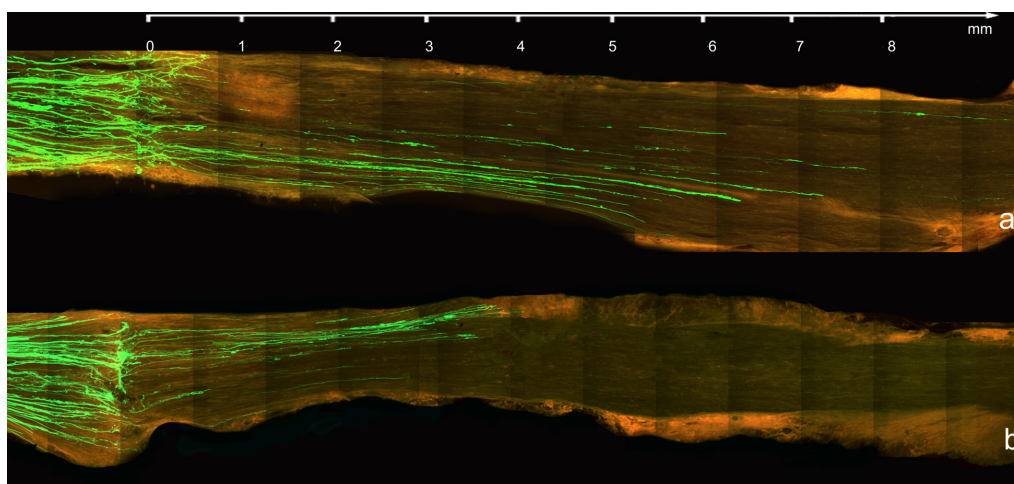


Figure 2 Overview of nerve repairs in 100 μm sections assessed by fluorescence microscopy (wavelength: 568 nm and 466 nm). The proximal fluorescent stump is on the left, the repair site runs vertically at 0 mm and the recipient non-fluorescent graft is on the right. Sensory and motor axons that express yellow fluorescent protein are greenish-yellow, while basement membrane that contains laminin is light red. The samples are harvested 5 days after nerve repair. Panel a: A nerve section sample from the *N*-propionylmannosamine (ManNProp) group showing a significantly increased regeneration distance. Panel b: A nerve section sample from the saline group showing a shorter regeneration distance than the ManNProp group.

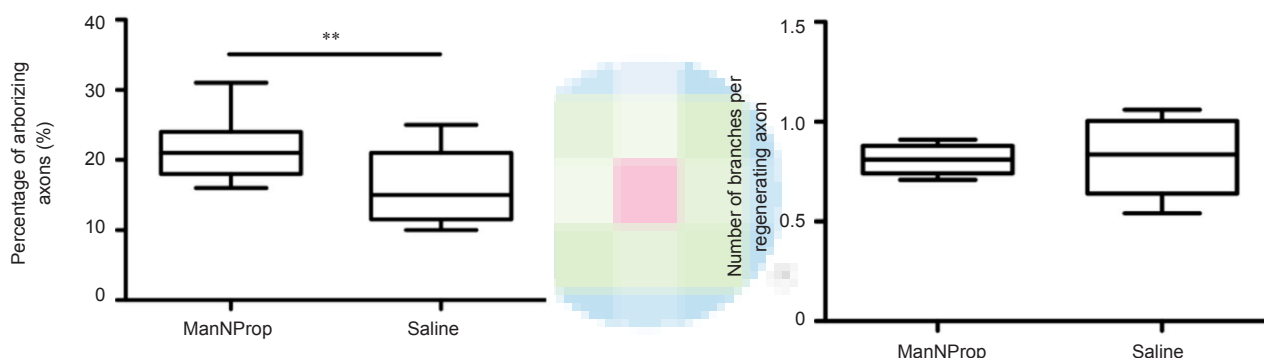


Figure 4 *N*-Propionylmannosamine (ManNProp) increases the number of arborizing axons. We counted the number of arborizing axons in the zone running from the coaptation site to 2 mm inside the distal stump. The results were set against the total number of regenerating axons. ManNProp ($21 \pm 0.05\%$) increased the number of arborizing axons compared to saline treatment ($16 \pm 0.05\%$; $**P < 0.05$; mean \pm SD; Student's *t*-test; $n = 6$ for each group).

Figure 6 Treatment with *N*-propionylmannosamine (ManNProp) did not affect the number of branches per regenerating axon. The mean value of branches per regenerating axon in the ManNProp group was 0.80 ± 0.07 (saline group: 0.82 ± 0.18 ; $P > 0.05$; mean \pm SD; Student's *t*-test; $n = 6$ for each group). The number was calculated as the ratio of the number of arborizing axons to the total number of regenerating axons.

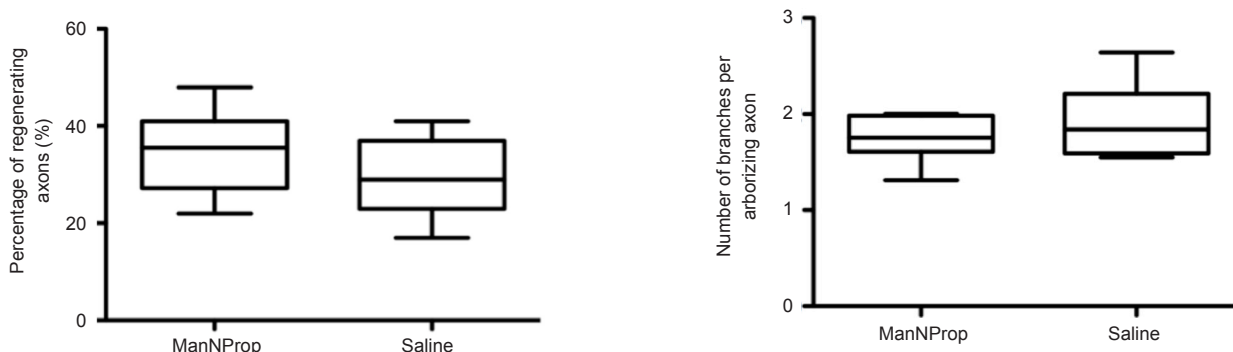


Figure 5 Treatment with *N*-propionylmannosamine (ManNProp) did not affect the number of regenerating axons. The number of regenerating axons was $35 \pm 0.09\%$ in the ManNProp group and $30 \pm 0.09\%$ in the saline group ($P > 0.05$; mean \pm SD; Student's *t*-test; $n = 6$ for each group).

Figure 7 The number of branches per arborizing axon was comparable between the groups. No significant difference was seen in the number of branches per arborizing axon after *N*-propionylmannosamine (ManNProp) treatment (1.75 ± 0.23) (saline group: 1.91 ± 0.37 ; $P > 0.05$; mean \pm SD; Student's *t*-test; $n = 6$ for each group). The number was calculated as the ratio of the number of branches to the total number of regenerating axons.

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