Expression patterns and functional evaluation of the UNC5b receptor during the early phase of peripheral nerve regeneration using the mouse median nerve model.

UNIVERSITÀ DEGLI STUDI DI TORINO

EXPRESSION PATTERNS AND FUNCTIONAL EVALUATION OF THE UNC5b RECEPTOR DURING THE EARLY PHASE OF PERIPHERAL NERVE REGENERATION USING THE MOUSE MEDIAN NERVE MODEL

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Introduction: In this study, we evaluated the role of the Netrin-1 receptor UNC5b (Uncoordinated), a neuronal guidance molecule, during peripheral nerve regeneration using the mouse median nerve model. Materials and methods: Using Western blot analysis, we examined the expression changes of UNC5b after transection and microsurgical repair of the mouse median nerve distal to the transection site. We evaluated the histomorphometrical changes and functional recovery of the grasping force after median nerve transection and repair in wild-type (WT) mice and UNC5b$^{b/2}$ heterozygous mice. Results: In Western blot analysis, we could show a high increase of UNC5b in the nerve segment distal to the injury site at day 14. Histomorphometrical analysis did not show any significant differences between WT animals and heterozygous animals. Using the functional grasping test, we could demonstrate that peripheral nerve regeneration is signifi-cantly diminished in heterozygous UNC5b$^{b/2}$ mice. Conclusion: By using the mouse median nerve model in transgenic animals, we dem-onstrate that the Netrin-1 receptor UNC5b plays an important role during peripheral nerve regeneration.

Peripheral nerve injury is a common casualty. Although peripheral nerve fibers retain a considerable regeneration potential, recovery is usually rather poor, especially in case of large nerve defects. In these cases, nerve grafts and conduits are often used to bridge the defect.[1–3] Recently, biological tubulization with muscle-vein-com-bined grafts have been showed to restore the continuity of the nerve with good clinical and functional outcomes.[4] Before their use in patients, the muscle-vein grafts have been extensively studied in the rat median nerve model.[5,6] To gain further insight in the biology of nerve injury and repair, we focused our work on the role of the neuronal guidance molecule UNC5b.

The UNC5 (Uncoordinated) homolog family of Netrin receptors are single-pass transmembrane proteins. Four receptors have been found in mammals: UNC5a, b, c, and d.[7–11] UNC5s are composed of two extracellular Ig domains and two extracellular thrombospondin domains.[12] The intracellular sequence contains a ZU5 domain, a death domain,[13] and a DCC-binding domain.[14] Neuronal growth cones are guided to their targets by attractive or repulsive guidance molecules. Netrins, semaphorins, eph-rins, and slits are part of these families. Classical Netrin receptors are DCC (Deleted in Colorectal Cancer) and the UNC5 family. Depending on the receptor, Netrin-1 functions as a chemotropic or repulsive factor that medi-ates axonal outgrowth. Neurons expressing the DCC receptor[15] are attracted by Netrin-1, whereas the expres-sion of UNC5 converts attraction to repulsion.[14] Loss of function of DCC results in misrouting of axons that are normally attracted by Netrin-1.[16] Numerous studies sug-gest that long-range repulsion to Netrin-1 requires a ligand-gated association between the cytoplasmatic domains of UNC5 and DCC, whereas UNC5 without DCC is sufficient for short-range repulsion.[14,17,18]

Less is known about the role of the UNC5b receptor in the peripheral nervous system (PNS). In this study, we analyze the expression patterns of UNC5b after peripheral nerve transection and further evaluate the functional role of UNC5b using the mouse median nerve model for the assessment of peripheral nerve regeneration.[19]

MATERIALS AND METHODS

Animals and Surgical Procedure

All surgeries were performed using an anesthetic combination of fentanyl (0.05 mg/kg), midazolam (5 mg/kg), and medetomidin (0.5 mg/kg). The national guide-lines for laboratory animal care and safety were followed. A total number of 72 mice were used in this study.

To assess in vivo responses to peripheral nerve injury using Western blot analysis, 24 adult wild-type (WT) C57BL/6 mice weighing _30 g underwent transection of the left median nerve[19] directly after the branching of a constant sensible nerve running exactly parallel to the pectoralis muscle border (Fig. 1). The nerve was subse-quently repaired at 403 magnification using two epineu-ral stitches of 12–0 monofilament nylon. Similar to the procedure used for rats,[20] in order to prevent interfer-ences with the grasping test device during testing due to the use of the contralateral forepaw, the contralateral median nerve was transected at the middle third of the brachium and its proximal stump was sutured in the pec-toralis major muscle to avoid spontaneous reinnervation. Six mice were killed after 0, 7, 14, and 21 days postle-sion for Western blot analysis.

To assess the functional recovery after nerve transec-tion and quantitative morphology of nerve fiber regenera-tion, a total of 48 mice were used: 24 WT mice and 24 UNC5b$^{b/2}$ heterozygous mice (both on genetic back-ground C57BL/6). The mice were divided into four groups. Twelve WT animals and 12 UNC5b$^{b/2}$ heterozy-gous animals underwent transection and repair of the median nerve as described above. Twelve WT animals and 12 UNC5b$^{b/2}$ heterozygous animals only underwent operative exposure of the median nerve without transec-tion. Starting from day 5 postoperation, the grasping test was carried out every 5 days until day 50 when animals were euthanized for harvest of nerve samples.

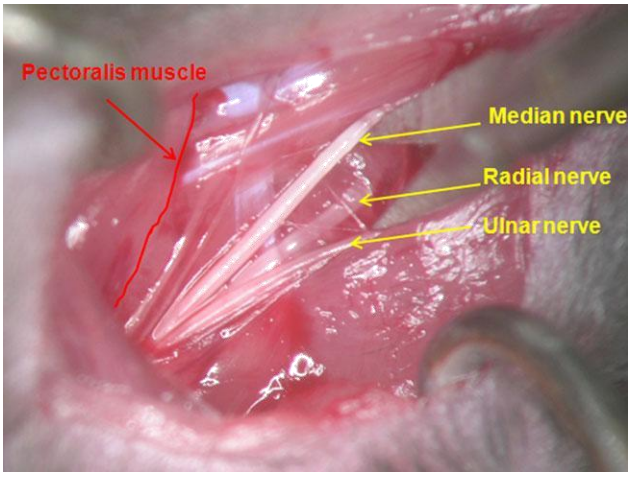

Figure 1. Intraoperative view of the murine axilla with the three main upper extremity nerves and the pectoralis muscle before transection of the median nerve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Western Blot Analysis

The explanted median nerves distal to the transection site (n 5 6) were lysed and homogenized. The lysed nerves were pooled and eluted in radio immunoprecipita-tion assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM so-dium orthovanadate, and 13 protease inhibitor mixture; Roche Molecular Biochemicals]. An equal volume of pro-teins was loaded on 10% sodium dodecyl sulfate (SDS) containing polyacrylamide gels for electrophoresis and transferred to a polyvinylidene fluoride (PVDF) mem-brane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat milk and Tris-buffered saline containing 0.05% Tween 20 for 1 hour, and then the membrane was immunoprobed with anti-UNC5b antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 48C. After three washes, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. After three more washes, the reaction was

visualized with the enhanced chemiluminescence reaction (ECL Detection system, Fluka-Luminol 09253, p-Coumaric acid; Sigma-Aldrich, Munich, Germany). Normalized relative intensity was determined using ImageJ V 1.43 Software.

Functional Grasping Test

The grasping test was performed using a precision balance connected to a rod. The test was performed as described by Tos et al.19 Each mouse was tested three times, and then the average value was recorded. Operated WT mice (n 5 12) were compared with operated UNC5bþ/2 mice (genetic background C57BL/6; n 5 12). Nonoperated WT mice (sham; n 5 12) and nonoperated UNC5bþ/2 mice (sham; n 5 12) served as controls.

Resin Embedding for Quantitative Morphology of Nerve Fiber Regeneration

For quantitative morphology of nerve fiber regenera-tion, an 8-mm-long segment of the median nerve distal to the site of lesion was removed. A 4/0 stitch was used to mark the proximal stump of the nerve segment. An 8-mm-long segment of uninjured median nerve, taken from a corresponding level, was withdrawn from the non-operated control animals and used as control. The nerve samples were fixed and prepared for design-based quanti-tative morphology of myelinated nerve fibers. Specimens were fixed by immediate immersion in a fixation solution, containing 2.5% purified glutaraldehyde and 0.5% saccha-rose in 0.1M Sorensen phosphate buffer for 3–4 hours. They were then washed in a solution containing 1.5% saccharose in 0.1M Sorensen phosphate buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Glauerts' embedding mixture of resins consisting equal parts of Araldite M and the Araldite Ha¨rter, HY 964 (Merck, Darmstatd, Germany), to which was added 1–2% of the accelerator 964, DY 064 (Merck), and 0.5% of the plasticizer dibutyl phthalate. A series of 2-/m-thick semi-thin transverse sections were cut starting from the distal stump of the median nerve segment, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany). They were stained by toluidine blue for high-resolution light microscopy examination, and design-based quantitative morphology for each nerve specimen
was carried out according to the stereological method described by Geuna et al.[21–24]

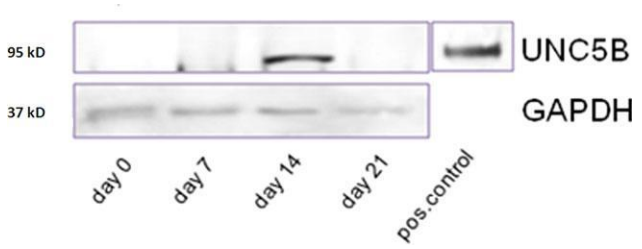

Figure 2. Western blot: High UNC5b protein expression at day 14 distal to the transection site. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Analysis

All data are presented as mean 6 SD. Statistical anal-ysis was performed using one-way analysis of variance test for histomorphometrical data. The different time-point assessments of the grasping test were compared by using analysis of covariance. A probability value of less than 5% was considered to be statistically significant.

RESULTS

UNC5b Protein Expression Using Western Blot

Analysis

We examined the protein expression changes of UNC5b after median nerve injury to further elicit the role of

UNC5b. In Western blot analysis, we could show a highly specific increase of UNC5b in the nerve segment distal to the injury site in WT animals at day 14 after pe-ripheral nerve transection and repair ($P > 0.001$). Before and after day 14, UNC5b was expressed at lower levels (Fig. 2). The normalized relative intensity of protein expression is presented in Figure 3.

Functional Grasping Test

Using the grasping test, we could show that peripheral nerve regeneration was significantly diminished in hetero-zygous UNC5b$^{b/2}$ mice. Although there was no difference in grip strength in both control groups (sham WT and sham UNC5b$^{b/2}$ mice), we could demonstrate a tremen-dous drop in total grip strength in operated UNC5b$^{b/2}$ animals when compared with operated WT animals ($P < 0.001$). On one hand, the grip strength of heterozygous mice only reached 60% of the strength of WT animals 50 days following nerve division and repair. Furthermore, they never got back their preoperative original strength. On the other hand, the functional recovery of operated UNC5b$^{b/2}$ mice moved on by far more slowly when compared with WT animals ($P < 0.001$; Fig. 4).

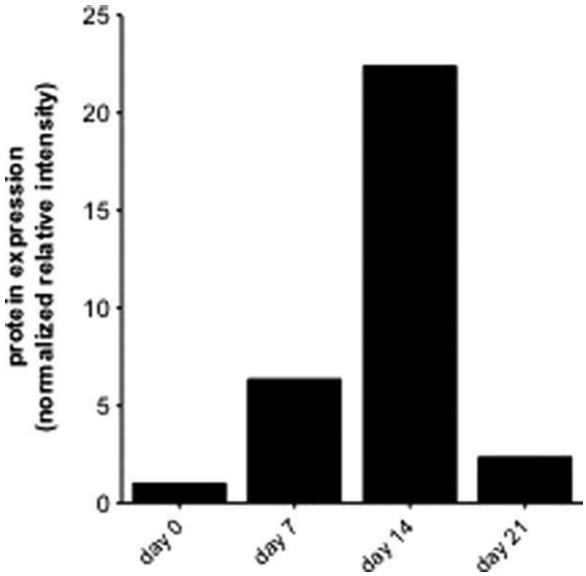

Figure 3. Normalized relative intensity of protein expression.

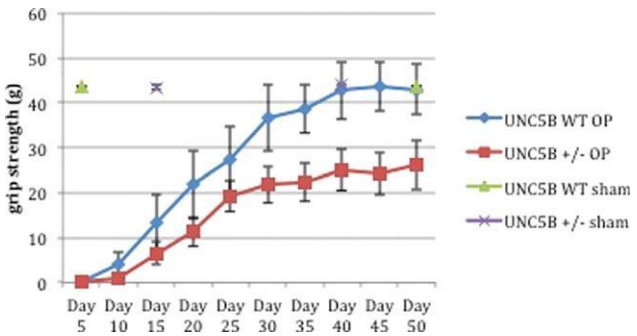

Figure 4. Functional grasping test: Grip strength of heterozygous mice only reaches 60% of the strength of WT animals ($P < 0.001$). Functional recovery of operated UNC5b$^{b/2}$ mice moves on by far more slowly when compared with WT animals, never reaching back the original force. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Histomorphology of Nerve Fiber Regeneration

Various morphological predictors of nerve regenera-tion were assessed by stereology at day 50 after nerve transection. Regarding total number of myelinated fibers after nerve transection and repair, we could show an

increased number of fibers in heterozygous animals when compared with WT animals; however, the difference was not significant ($P > 0.05$). Furthermore, the cross-sectional areas of transected and repaired nerves were larger in transgenic animals when compared with WT animals, without any significant difference ($P > 0.05$).

Axon diameter, fiber diameter, and myelin thickness were significantly diminished in WT animals and transgenic animals after transection and repair when compared with animals without nerve division ($P < 0.001$; $P < 0.001$; $P < 0.01$). Regarding the last three parameters, there was no significant difference between transected and repaired nerves of WT animals when compared with transected and repaired nerves of heterozygous animals ($P > 0.05$; Figs. 5 and 6).

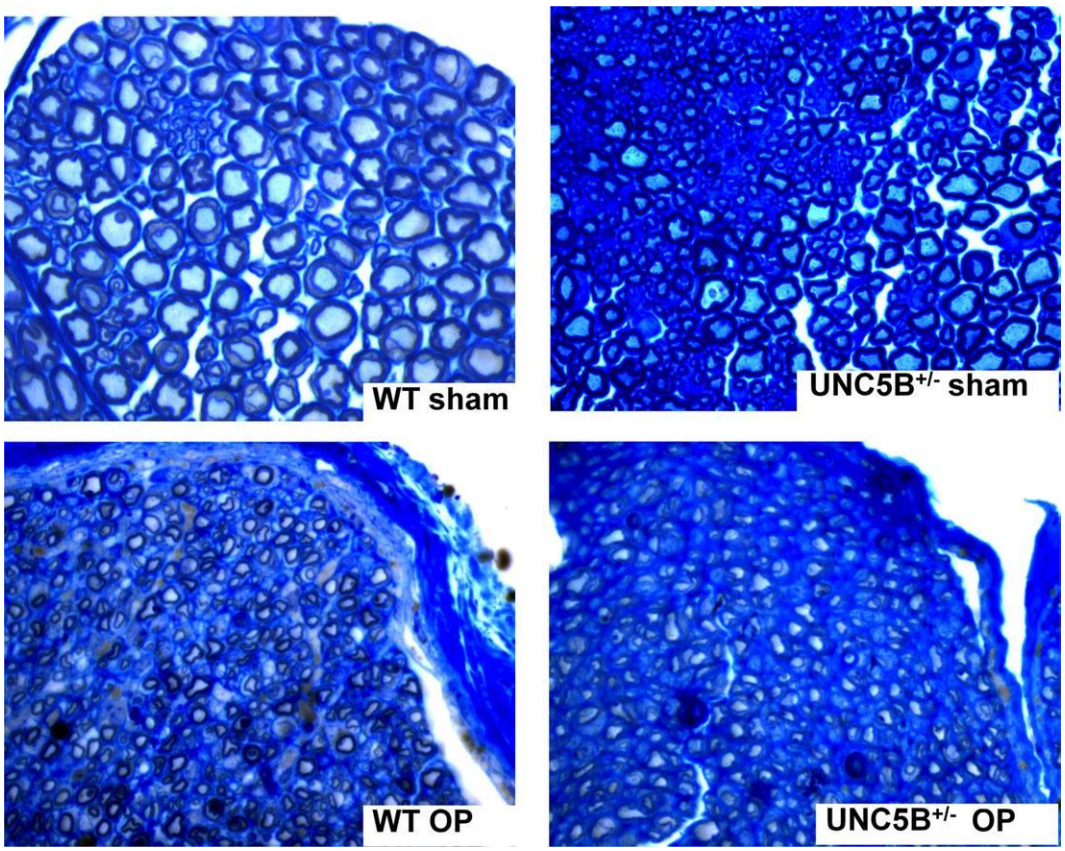

Figure 5. Histological cuts (toluidine blue): no significant differences in total number of fibers, cross-sectional area, and fiber density. Axon diameter, fiber diameter, and myelin thickness were significantly diminished in WT animals and transgenic animals after transection and repair when compared with animals without nerve division ($P < 0.001$; $P < 0.001$; $P < 0.01$). When comparing operated WT mice and operated UNC5bþ/2 mice, these parameters were without significant difference (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

In this article, we showed that UNC5b plays an important role during peripheral nerve regeneration. An important ligand of the UNC5b receptor is Netrin-1, which is known to direct cell and axonal migration during development. Another receptor of Netrin-1 is DCC. DCC mediates chemoattraction, whereas UNC5b mediates repulsion of outgrowing axons.11

The findings of our study identify UNC5b as an important factor for axonal regeneration after peripheral nerve injury. We clearly demonstrated a peak of UNC5b protein expression in Western blot analysis in the nerve segment distal to the injury site at day 14. After this time point, UNC5b protein expression drops down to the level of the uninjured nerve.

Using the grasping test,19 we showed that UNC5bþ/2 mice recover their grasping force more slowly than WT mice and never get back their initial preoperative force.

This is the first study to use the mouse median nerve model to evaluate peripheral nerve regeneration in transgenic animals. The histomorphometric results of regenerated nerve fibers did not reveal any differences

between WT and heterozygous animals. Recently, Muratori et al.25 showed that the regenerated nerve fibers did not return to the pretrauma size.

During development of the mammalian spinal cord, DCC expression is downregulated, whereas UNC5 homolog expression increases, indicating that UNC5 repellent signaling is the dominant response to Netrin-1 in the adult spinal cord.[11,26] After spinal cord injury, the expression of DCC and UNC5 proteins is reduced and corre-lates with poor axonal regeneration in these lesions.[27,28]

According to these results, we demonstrated poor func-tional recovery in UNC5b$^{b/2}$ mice after peripheral nerve transection when compared with WT animals.

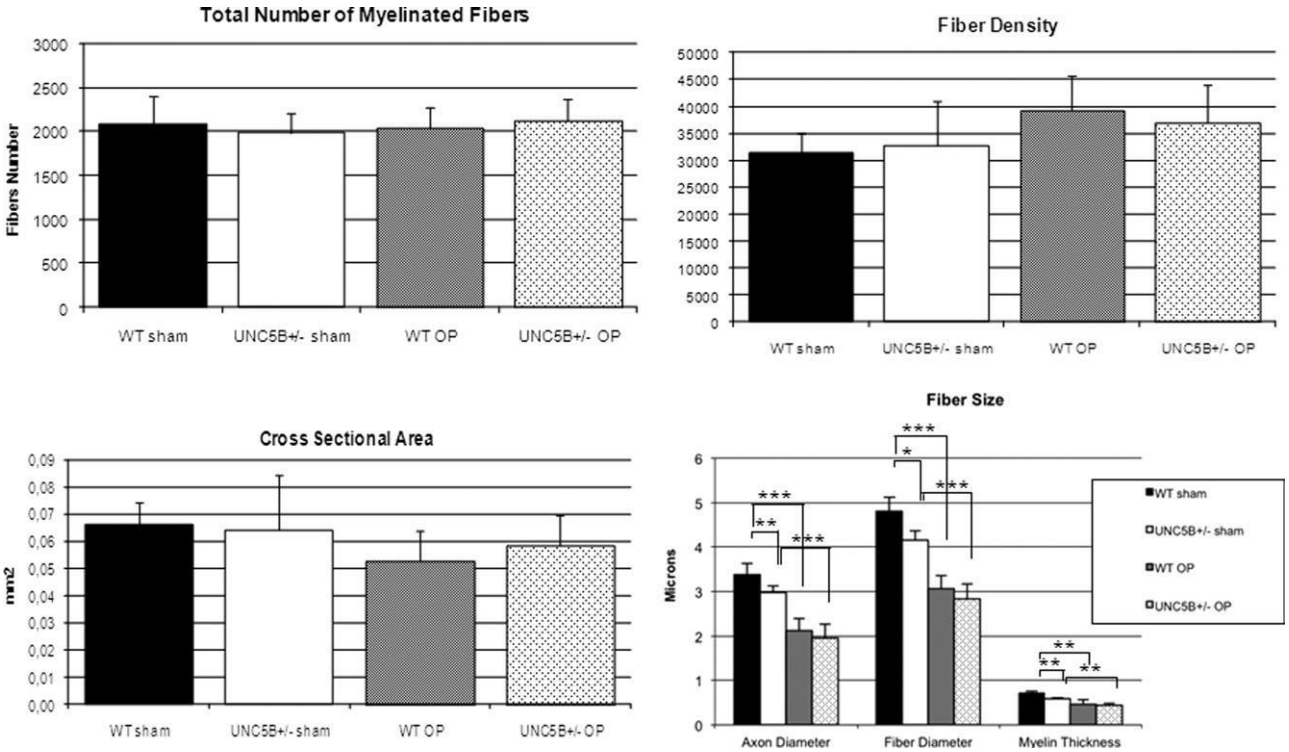

Figure 6. Histomorphometrical analysis: no significant differences in total number of fibers, cross-sectional area, and fiber density. Axon di-ameter, fiber diameter, and myelin thickness were significantly diminished in WT animals and transgenic animals after transection and repair when compared with animals without nerve division ($P < 0.001$; $P < 0.001$; $P < 0.01$). When comparing operated WT mice and operated UNC5b$^{b/2}$ mice, these parameters were without significant difference (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Furthermore, the UNC5b receptor and its ligand Netrin-1 are expressed in the adult vertebrate nervous system by oligodendrocytes in the central nervous sys-tem[29,30] and by Schwann cells in the PNS.[31] Webber et al.[30] demonstrated that Schwann cells direct peripheral nerve regeneration through DCC and UNC5b receptors. Lee et al.[32] showed that Netrin-1 induces proliferation of Schwann cells through the UNC5b receptor. Interestingly, UNC5b is specifically expressed during neoangiogenesis, as demonstrated by Larrivee et al.[33] and Lu et al.[34]

Hong et al.[4] demonstrated that during axonal naviga-tion, growth cones change their responsiveness to guid-ance cues as they progress through a ligand-gated associ-ation between cytoplasmatic domains of UNC5 and DCC family receptors. Once a growth cone has reached a par-ticular intermediate target, it must change its priority to move on to the next target.

We interpret our findings of high UNC5b protein expression at day 14 and massive drop of protein expres-sion after day 14 as follows: around this time point, the regenerating axonal growth cone passes through the ana-lyzed nerve segment distal to the lesion site (Fig. 7). It is ''surrounded'' by UNC5b expressed by Schwann cells in the immediate vicinity. A subtle balance between its ligand Netrin-1 and coreceptor DCC directs the

growth cone distally to its target organ. The finding that functional nerve recovery in UNC5b$^{b/2}$ mice is slower than that in WT animals is consistent with this model and partly explains why these animals never reach their initial grasping force.

Further studies are required to clarify the exact mech-anism of action of UNC5b during peripheral nerve regen-eration. It is slightly unclear why functional tests show large differences between the two groups but histomor-phometric data do not. At day 14, when the growth cone passes our analyzed nerve segment, UNC5b is highly expressed, showing the action of UNC5b at the tip of the growth cone. Electromicroscopical findings could be of special interest; however, these aspects were not addressed in this work. Future research has also to be focused on the distal neuromuscular junction site not analyzed in this study.

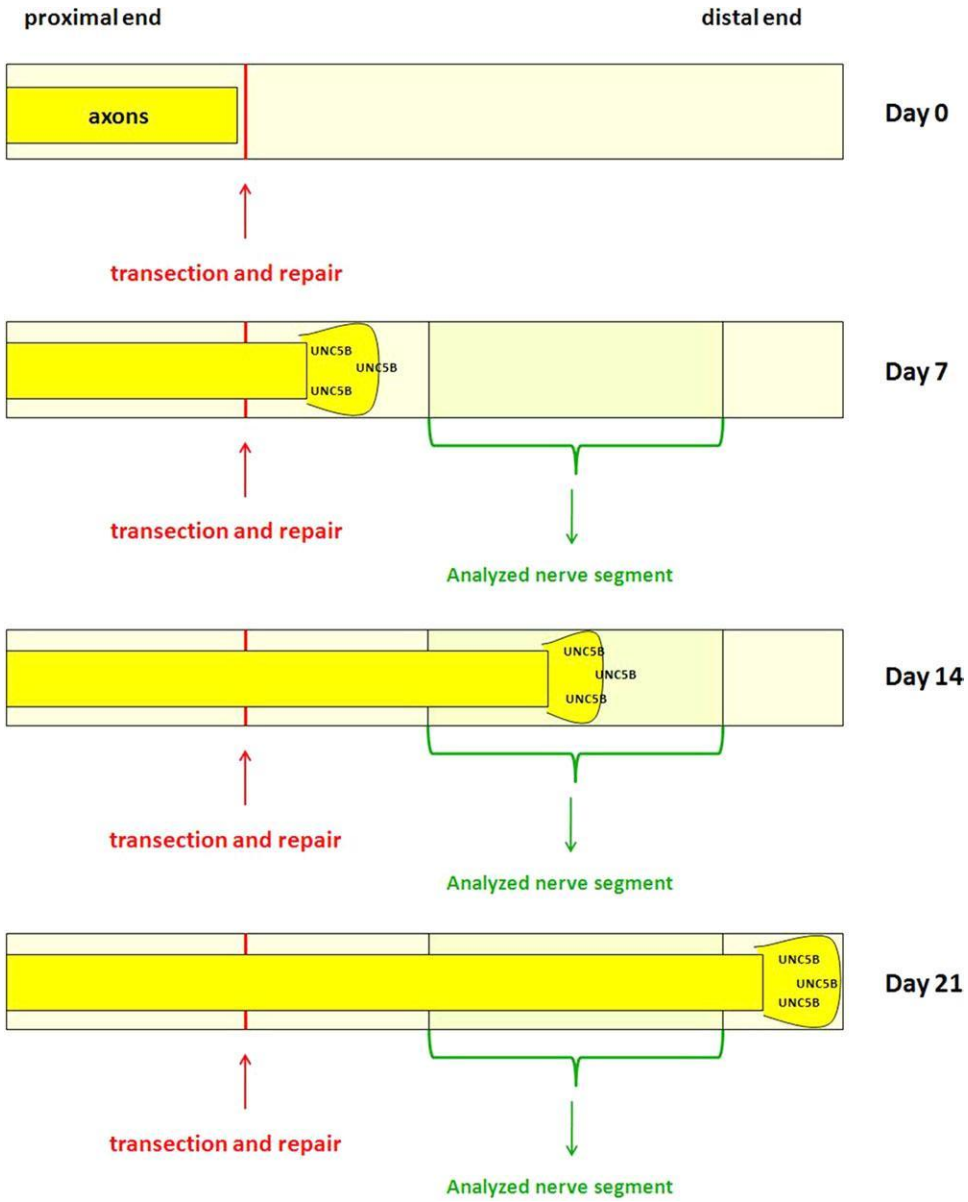

Figure 7. Interpretation of the results: Around day 14, axonal growth cone passes through the analyzed nerve segment distal to the lesion site. It is ‘‘surrounded’’ by UNC5b expressed by Schwann cells in the immediate vicinity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSION

In this work, we clearly demonstrated that the median nerve mouse model is a valuable tool to evaluate peripheral nerve regeneration in transgenic animals. We could show poor functional recovery in transgenic UNC5b$^{b/2}$ mice when compared with WT mice after median nerve transection and repair. Furthermore, we demonstrated high expression of UNC5b at day 14 after nerve division and repair. The main limitation is the very small nerve size requiring advanced microsurgical skills for performing epineural suturing. The long-term objec tive of these findings should be the local enhancement or addition of proteins supporting peripheral nerve regenera-tion or the specific block of molecules inhibiting nerve recovery.

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