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Bex1 is involved in the regeneration of axons after injury

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Abstract

Successful axonal regeneration is a complex process determined by both axonal environment and endogenous neural capability of the regenerating axons in the central and the peripheral nervous systems. Numerous external inhibitory factors inhibit axonal regeneration after injury. In response, neurons express various regeneration-associated genes to overcome this inhibition and increase the intrinsic growth capacity. In the present study we show that the brain expressed X-linked (Bex1) protein was over-expressed as a result of peripheral axonal damage. Bex1 antagonized the axon outgrowth inhibitory effect of myelin-associated glycoprotein (MAG). The involvement of Bex1 in axon regeneration was further confirmed *in vivo*. We have demonstrated that Bex1 knock-out mice showed lower capability for regeneration after peripheral nerve injury than wild-type animals. Wild type mice could recover from sciatic nerve injury much faster than Bex1 knock-out mice. Our findings suggest that Bex1 could be considered as regeneration associated gene.

Keywords

Bex1; axon injury; regeneration

Introduction

The signalling molecules activated after axonal injury transduce information to the cell body leading to the expression of regeneration-associated genes (RAGs) and increased intrinsic growth capacity of the neurons (Abe and Cavalli 2008). Lesioned axons in the peripheral nervous system (PNS) have the capacity to undergo successful regeneration but lesioned axons of the central nervous system (CNS) do not achieve any functionally significant regeneration. This arises not only from the limited endogenous ability of CNS neurons to regrow, but from numerous inhibitory factors in the CNS environment such as myelin associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), Nogo and chondroitin sulfate proteoglycans (CSPGs) (Yiu and He 2006). OMgp and MAG are well-characterized components of myelin in both the CNS and PNS (Yamashita et al. 2002, Chen et al. 2007), while Nogo is only expressed in the CNS (Yiu and He 2006). MAG, OMgp and Nogo exert their inhibitory effect on axonal growth and regeneration through binding to tripartite receptor complex $p75^{NTR}$/NgR/LINGO-1 and activation of RhoA (Fournier et al. 2001, Wang, and Kim 2002, Mi et al. 2004, Yiu and He 2006). Recently, it has been shown

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that MAG, OMgp and Nogo can also bind to paired immunoglobulin-like receptor B (PirB) and transduce signals that inhibit axon regeneration in vitro (Atwal et al. 2008). Successful regeneration requires that axons synthesize new proteins to overcome this inhibition (Verma et al. 2005).

In a previous study, we reported that Bex1 is upregulated along with a number of RAGs like GAP43, ATF3 and SPRRA1 in Pmp22 mutant mice which exhibit a demyelinating neurodegeneration in PNS (Nattkämper et al. 2009). Enhanced Bex1 expression was detected in spinal motor neurons (MNs) of Pmp22 mutants (Nattkämper et al. 2009) as well as in two other mouse mutants that develop MN degeneration, the pmn and the wobbler mice (Perrin et al. 2006).

Bex1 is an adapter molecule involved in $p75^{NTR}$ signalling and is presumably involved in neuroprotection. Association of Bex1 with $p75^{NTR}$ can provide a switch for growth inhibition or regeneration decisions mediated by the $p75^{NTR}$ (Vilar et al. 2006). This could modulate the signals propagated from $p75^{NTR}$ and trigger axonal regeneration.

In the present study we showed that the expression of Bex1 was upregulated in spinal cord MNs after axonal injury. Furthermore, our data from Bex1-KO mice illustrated that Bex1 was required for neurons to be able to overcome the inhibitory effect of MAG and recover faster from injury.

Materials and Methods

Animals

Wild-type (WT) mice (C57BL/6 or 129/SvEv) were obtained from Charles River in Sulzfeld, Germany. Two mouse lines with altered gene dosage for Pmp22 obtained from our breeding colonies were Pmp22 knock-out mice (Pmp22-KO) (Adlkofer et al. 1995) and mice overexpressing Pmp22 (Pmp22-Tg) (Magyar et al. 1996). Bex1-deficient mice (Bex1-KO) were described recently (Koo et al. 2007) and mice deficient for myelin protein zero (MPZ-KO) were originally provided by Prof. R. Martini, University of Würzburg (Giese et al. 1992). Tissues from mice deficient in MAG (MAG-KO) were kindly provided by Prof. R. Schnaar, Johns Hopkins University (Pan et al. 2005). All animal experimental procedures were in agreement with legal ethical requirements in strict accordance to the guidelines of the German law for protection of animals. All efforts were made to minimize animal suffering and to reduce the number of animals required.

Plasmids

The mammalian expression vector for GFP-Bex1 fusion was kindly provided by Prof. C. Ibáñez (Vilar et al. 2006).

Neuronal cultures and transfection

Primary cultures of MNs from spinal cords of E13-14 mouse embryos were prepared as described before with some modifications (Azzouz et al. 2000). Briefly, spinal cords were dissected from mouse embryos. After removing meninges and dorsal root ganglia (DRG), the ventral half of the lumbar part of spinal cords were incubated for 15 min in 0.025% trypsin at 37°C. The tissue was then triturated with a fire-polished Pasteur pipette. The cell suspension was gently layered on a 6.8% HistoDenz cushion (Sigma, Deisenhofen, Germany) (w/v in Neurobasal medium) and to enrich the low buoyant density cells (MNs), it was centrifuged at 800 g for 15 min at 4°C. The interface layer enriched in MNs was collected, diluted in complete Neurobasal medium (see below), and centrifuged again through a 4% bovine serum albumin (BSA) cushion at 450 g for 10 min. The cells were then

re-suspended in complete Neurobasal medium (Invitrogen, Leiden, Netherlands) and were plated at a density of 10,000 cells/well in 24-well-plates containing coverslips previously coated with poly-L-ornithine (1.5 μg/mL; 1h at 37°C) and laminin (3 μg/mL; 1h at 37°C) and incubated at 37°C and 5% CO_2. Complete Neurobasal medium containing 2% B27-supplement, 2% horse serum, 1% penicillin, 1% streptomycin and 1% glutamine (all from Invitrogen), was supplemented with 10 ng/ml CT-1, 10 ng/ml human neurotrophin-3 (NT-3), 10 ng/ml human brain-derived neurotrophic factor (BDNF), and 10 ng/ml rat ciliary neurotrophic factor (CNTF; all from Sigma). Neurons were fixed on day 3 *in vitro* with 4% paraformaldehyde and 20% sucrose in phosphate-buffered saline (PBS) for 20 min at 4°C. To analyze the localization of Bex1 in neurons, they were stained with rabbit polyclonal anti-Bex1 (ab69032; 1:500; Abcam, Cambridge, UK), mouse monoclonal Tau1 (MAB3420; 1: 500; Chemicon, CA), as a marker for axons, chicken polyclonal anti-Map1 (ab5392 1:500) as a marker for minor neurites (Schwamborn et al., 2007) and Alexa-conjugated antibodies (Alexa Fluor 594, 488 or 350) (Molecular Probes; Breda, Netherlands, 1:1000) as secondary antibody.

Axon length measurement was performed with the NIH program ImageJ (Bethesda, MD) and the plug-in NeuronJ. On around 30 random images from each condition, the primary axon of each cell was measured with the tracing algorithm contained in NeuronJ and statistical analysis was performed.

Cryosection preparation and immunolabelling

Mice were anesthetized and perfused with 4% PFA followed by cryoprotection in 30% sucrose for 6 h at 4°C. The spinal cords were prepared from the fixed embryos and embedded at −20°C in Tissue Tek. Cryosections (16 μm) were cut using a Leica cryostat and mounted onto glass slides. The sections were rinsed in PBS, post-fixed in 4% PFA for 20 min at 20°C, permeabilized in 0.1% Triton X-100/0.1% Na-Citrate in PBS for 5 min and blocked with 5% fetal calf serum in PBS for 1 h at 20°C. Sections were incubated with the primary antibody diluted in the blocking solution overnight at 4°C and then rinsed three times in PBS. Primary antibodies used were rabbit polyclonal anti-Bex1 (ab69032; 1:500; Abcam), mouse monoclonal SMI32 (ab73273; 1:500; Abcam). Sections were then incubated with Alexa-conjugated (Alexa-Fluor 594 or 488) secondary antibodies (Molecular Probes; 1:1000) for 60 min at 20°C. Slides were then mounted in mounting medium containing 4', 6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI).

Sciatic nerve crush and demyelination

Mice were anesthetized intraperitoneally with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and the sciatic nerve was exposed. The left sciatic nerve was crushed at midthigh level with a flattened forceps for 30 s. In the sham-operated controls, the sciatic nerve was exposed but not injured.

For sciatic nerve demyelination experiments, after exposure of sciatic nerves, 5 μl lysolecithin solution (1% in sterile PBS, pH 7.4; L-alpha–lysophosphatidylcholine; Sigma) was applied topically using a 34-gauge needle over a length of 5-6 mm, ensuring that the nerve remained coated with the solution for 10 min after which the reagent was washed off with sterile saline (Wallace et al. 2003). Sham control animals were prepared by treating the nerve as above with sterile PBS lacking lysolecithin.

Muscles and skin were sutured above the crush or injection site and the mice were allowed to recover.

Analysis of the sciatic nerve semi-thin sections

Seven days after crush injury or induced demyelination, mice were euthanized and sciatic nerves were quickly dissected and fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4) for 12 h, then washed in cacodylate buffer, post-fixed for 2 h in 1% OsO_4/0.1 M cacodylate buffer (pH 7.4), dehydrated in a graded alcohol series followed by acetone, and finally embedded in epoxy resin. Semi-thin cross-sections (1 μm thick) were cut from injured nerve specimens located 1 mm proximal to the lesion epicenter and stained with toluidine blue (1%) for 20 min; the corresponding sections from intact nerve specimens were also examined. The stained sections were then examined under a light microscope (AxioCam 2; Carl Zeiss, Jena, Germany).

Retrograde labelling of spinal cord MNs in vivo

Retrograde labelling was performed for the quantification of spinal MN perikarya within the ventral horn to quantify the number of MNs whose axons extended beyond the injury site (Nattkamper et al. 2009). Seven and 14 d after sciatic nerve injury, mice were anaesthetized and incisions were made into the gluteal muscle at the level of the right hip and femur exposing the injured sciatic nerve. Small crystals of the lipophilic fluorescent-tracer DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethyl carbocyanine, Molecular Probes) were placed 7 mm distal to the crush lesion site and fixed with an absorbable gelatine sponge after applying an appropriate amount of oily liquid (Freund's adjuvant incomplete, Sigma) to dissolve DiI. The dye was then transported in the retrograde direction to the corresponding MN perikarya. The skin was sutured and the mice were kept for another 48 h in their cages. After 2d, mice were anesthetized again and perfused with 4% PFA. Spinal cords were removed and processed for cryosectioning. Cryostat-serial sections (25 μm) were viewed under fluorescence illumination, and labelled MNs were counted in every section. The total number of labeled MNs per section was quantified from cryosections. Counting was performed blindly with respect to treatment. Summarized counts were taken by averaging across several sections from multiple mice.

Behavioural analyses

Three different tests were used to assess the recovery of hindlimb motor and sensory function after sciatic-nerve injury. Ten mice were used for each functional evaluation; *Rota-rod test* was used to assess the motor coordination and balance of the animals on days 7, 14, 21 and 28 after operation. The Rota-rod apparatus (Rotarod 3375-5; TSE Systems; Bad Homburg, Germany) consisted of a plastic rod (7 cm in diameter) with a gritted surface, flanked by two large discs to prevent interference from other animals, at a height of 20 cm from the floor. Mice were trained for 5 days to become acquainted with the apparatus. Before the daily training, mice were allowed to adapt to the rod for 5 min. For each session, mice were subjected to 10 rotarod trials. The experimental mice were placed on the rod, and the rotation was started at a speed of 4 rpm and accelerated to 40 rpm in 270 sec. The latency period until the mice fell off the apparatus was monitored for 300 sec.

Gait analysis was used to measure Sciatic Functional Index (SFI). The SFI evaluates crucial aspects of locomotion involving recovery of hindlimb sensory and motor function (Contreras et al. 1995). The hind feet of mice were painted with ink and the mice allowed to walk freely on a blank strip of paper 15 cm wide and 60 cm long. Distance to Opposite Foot (TOF), Print Length (PL), Total Toes Spreading (TS) and the distance between Intermediary Toes (IT) were measured as described before (de Medinaceli et al. 1982). The results from three sets of footprints were determined for 10 mice of each experimental group. The tracks were analyzed according to the empirical equation determined by de Medinaceli et al. (1982).

Toe-pinch reflex was used to assess the recovery of sensory function as described previously (Siconolfi and Seeds 2001) by pinching the most distal portion of the last three toes (third, fourth and fifth) of the injured hindlimb with a flattened forceps. Foot withdrawal was recorded as positive responses indicative of recovery. The percentage of mice that respond to toe pinch for the first time on days 7, 14, and 28 after the lesion was recorded. Digits 1 and 2 were not tested since they are partially innervated by the saphenous nerve (Navarro et al. 1995). Only the three lateral digits were tested before and after sciatic nerve crush for up to 28 days.

Quantitative real-time PCR

MN perikarya from the spinal cord of five mice from each mouse strain were dissected by Laser-capture microdissection as described before (Nattkämper et al. 2009). The total RNA was extracted from dissected MNs (~ 2000 MNs from each animal) with a total RNA isolation kit (RNeasy Micro Kit; Qiagen) according to the manufacturer's protocol and used as a template in RT-PCR. Three animals were used for each analysis. Quantitative Real-time PCR using QuantiTect® Syber Green PCR Master Mix, QuantiTect® Primer assay primers designed for mouse Bex1 (QT02278843; Qiagen) was done according to the manufacturer's protocol with Mastercycler®ep realplex (Eppendorf, Hamburg, Germany). All samples were analyzed in triplicate and the values normalized to the reference housekeeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The comparative Ct method was used for quantification of transcripts. Measurement of Ct was performed in triplicates. Following real-time PCR, the generated real-time PCR data were automatically processed based on the respective standard curve data by realplex software. Following export of these processed data into MS Excel, RNA levels were calculated relative to a baseline sample based on Schmittgen and Livak (2008).

Statistical analysis

Data are presented as average ± SEM. Repeated-measures ANOVA with Bonferroni-Holm post hoc analysis was completed to assess statistical differences between genotypes. Subsequent ANOVA analysis was completed to assess differences between genotypes at each time point after lesion.

Results

Bex1 is expressed in cultured MNs

We first analyzed the expression of Bex1 in cultured MNs prepared from E13-14 mice. MNs were fixed at 3 DIV and analyzed by immunofluorescence to determine the subcellular localization of Bex1 (Fig. 1). Anti-Tau-1 and anti-MAP2 antibodies were used as markers for axons and minor neurites, respectively (Schwamborn et al., 2007). Bex1 was present in the soma and was detectable in all neurites in a punctate staining pattern. No selective enrichment in axons or minor neurites was apparent.

Bex1 expression is upregulated in MNs after axonal damage

We previously demonstrated that Bex1 expression was upregulated in MNs of the spinal cord of Pmp22 mutant mice (Nattkämper et al. 2009) which undergo axonal degeneration as a secondary consequence of demyelination (Sancho et al. 1999).

To determine whether induced expression of Bex1 is a general response of MNs to peripheral axonal damage, changes in Bex1-mRNA expression were analyzed in MNs of MPZ-KO mice and MAG-KO mice, and also in wild-type mice following axonal demyelination or axonal-crush injury. MAG-KO and MPZ-KO mice are known to show substantial peripheral axonal damage as a consequence of myelin deficiency (Fruttiger et al.

1995, Carenini et al. 1996, Yin et al. 1998, Frei et al. 1999, Samsam et al. 2003, Pan et al. 2005). Seven days after crush injury, mice were euthanized and single MNs from spinal cord were dissected using LCM to study overall changes in Bex1 mRNA expression using quantitative real-time PCR (qRT-PCR).

In MAG-KO mice, Bex1 mRNA was slightly elevated (2.30 ± 0.37 fold). In comparison, the qRT-PCR analysis of MNs from MPZ-KO mice revealed an increase in Bex1 mRNA levels of 5.75 ± 0.40 fold. MNs harvested from the spinal cord of wild type mice after sciatic nerve lesion displayed a 12.06 ± 0.60 fold increase in Bex1-mRNA content (Fig. 2A) compared to controls with sham injury. In contrast, no significant Bex1-mRNA upregulation was observed in MNs of mice subjected to experimental demyelination by topical treatment with lysolecithin (1.60 ± 0.69 fold). Lysolecithin focally dissolves myelin sheaths around axons and causes reversible demyelination without overt axonal damage (Wallace et al. 2003). The extent of demyelination in lysolecithin treated mice was analyzed by toluidine blue staining of semi-thin sections of sciatic nerves. A maximum of 55% of fibres were completely demyelinated, and the remaining myelinated fibres exhibited myelin of reduced thickness (Suppl. Fig. 1).

To elucidate the expression of Bex1 protein, tissue sections from lumbar spinal-cord preparations were immunostained with anti-Bex1 antibody and anti-nonphosphorylated neurofilament (SMI32). While Bex1 was hardly detectable in MNs of control WT mice (naive) and sham-injured mice (Fig. 2B), an unambiguous increase of Bex1 staining in MNs of MPZ-KO mice and mice subjected to sciatic nerve crush was observed. Bex1 was found rather uniformly distributed in the cytoplasm of MNs with an occasional nuclear localization. In MAG-KO, a slight increase in Bex1 expression was detectable. No significant increase in Bex1 protein was detected in WT mice after demyelination by lysolecithin or in sham experiments (Fig. 2B). These observations are consistent with the quantitative data seen in Figure 2A.

Bex1 can promote axonal outgrowth on non-permissive substrate

The finding that Bex1 expression is upregulated in the MNs after different types of axonal injury suggests that Bex1 might be involved in the regeneration of damaged axons. To further study the function of Bex1 in axonal growth, we assessed its influence on axon outgrowth of mouse MNs in culture. No clear difference in the axonal length of MNs cultured on growth permissive substrate (PLL) was ascertained between MNs of Bex1-KO mice (transfected with GFP), MNs of wild type mice (transfected with GFP) and MNs which are over-expressing Bex1 (transfected with GFP-Bex1) (Fig. 3). The axonal length of wild-type MNs was affected by Bex1 expression when they were cultured on growth-non-permissive (PLL + MAG (4μg/ml)) substrate. In concordance with previous reports, the axonal length of MNs cultured on non-permissive substrate was decreased (132 ± 18; compared to 193 ± 12 in PLL; $p < 0.01$, ANOVA). However, MNs over-expressing Bex1 could partially overcome the inhibitory effect of MAG and grew longer axons (164 ± 15; compared to 190 ± 24 in PLL; $p < 0.01$, ANOVA) on non-pemissive substrate (Fig. 3). The axonal length of neurons cultured from Bex1-KO mice were also affected by MAG (113 ± 25; compared to 188 ± 22 on PLL; $p < 0.01$, ANOVA). But they did not show significant difference with the axon length on WT neurons cultured in PLL+MAG.

Bex1-KO mice showed a normal phenotype

To obtain insight into the significance of Bex1 on axonal growth and regeneration *in vivo*, we used Bex1-KO mice (Koo et al. 2007). These Bex1-KO mice were viable and fertile and indistinguishable from wild type littermates and developed normally. However, it has been reported that their exercise performance is compromised compared to WT mice and they

have a slower rate of skeletal muscle regeneration following injury (Koo et al. 2007). The axons and myelin of Bex1-KO peripheral nerves appeared normal (Suppl. Fig. 2). The gait was normal in Bex1-KO, as indicated by footprint analysis, and motor coordination and balance were tested on the rotarod at 8 week of age and no deficits were recorded in the knock-out mice (however, see next section; Fig 5A, B and C).

Bex1-KO mice showed reduced axonal regeneration in vivo

To test the influence of Bex1 on axonal regeneration in the PNS *in vivo*, sciatic nerve lesions in 8 week-old mice were performed. Most of the motor axons were injured as demonstrated by a significant reduction of 61% in the number of retrogradely labelled MNs in the spinal cords of SN-injured mice compared with sham operated mice 7 days after the nerve crush (Fig. 4B; $n = 3$; $p < 0.01$, ANOVA). The number of MNs that were labelled by the DiI was significantly higher in WT mice compared to Bex1-KO mice (261 ± 35 in WT compared to 145 ± 29 in KO; $p < 0.01$, ANOVA). The difference in the number of retrogradely labelled MNs in WT compared with Bex1-KO mice was larger two weeks after crush injury (Fig. 4B; 685 ± 51 in WT compared to 316 ± 47 in KO; $p < 0.01$, ANOVA). These results indicate that the WT mice exhibited more rapid axonal regeneration than the KO mice.

Bex1-KO were compromised in their ability to recover from sciatic nerve injury

The recovery of motor/sensory function was compared between WT (n=5) and Bex1- KO (n=5) mice on 7, 14, 21 and 28 d post injury, using the Rotarod test to measure the motor coordination and balance, gait analysis with Sciatic Functional Index (SFI) (de Medinaceli et al. 1982), and the toe- pinch reflex-analysis (Siconolfi and Seeds 2001).

Before sciatic-nerve injury no functional deficits were observed in Rotarod performance between WT and Bex1-KO groups (Fig. 5A). However, acute functional deficits compared with baseline performance were observed for both WT and Bex1-KO groups at day 7 (WT = 3.6%, KO = 4%; percentage (%) of mean duration compared with the presurgery control value) and day 14 (WT = 17%, KO = 16%) after injury although there was no significant difference in Rotarod results between Bex1-KO mice and wild-type littermates at these time points (Fig. 5A). However, WT performance recovered more rapidly and up to 49 % of baseline at day 21 and 72 % of baseline at day 28 whereas Bex1-KO mice showed significantly lower recovery (29% of baseline at day 21 and 59 % of baseline at day 21; $p < 0.01$, ANOVA). Although both groups had similar baseline-performance ability before injury and exhibited similar deficits on days 7 and 14 after injury, their performance abilities differed significantly between WT and KO animals on day 21 ($p < 0.01$, ANOVA) and day 28 ($p < 0.01$, ANOVA). In control groups with sham injury, the Bex1-KO mice displayed reduced performance and earlier exhaustion compared to WT mice at 7 and 14 days post-injury (Suppl. Fig. 3), which could reflect the lower rate of muscle regeneration in Bex1-KO mice (Koo et al. 2007). The muscle injury recovered within two weeks and it seems that the major contribution to the observed difference between WT and Bex1-KO mice after sciatic-nerve injury was related to delayed axonal regeneration rather than muscle regeneration.

Sciatic functional index (SFI) characterizes hindlimb use and foot and toe positions. Unlesioned animals have an SFI of zero (±10) (Pot et al. 2002). Both Bex1-KO and WT mice showed normal SFI values before the lesion. Sciatic-nerve crush induced extensive functional loss and resulted in a negative SFI at day 7. At 14 days post-injury, the recovery of Bex1-KO mice was significantly less complete than in the wild-type littermates ($p < 0.01$, ANOVA) (Fig. 5B). Both WT and Bex1-KO mice recovered to the same level after 28 days (Fig. 5B). Although, SFI index at day 21 displayed a trend of recovery but it did not reach

statistical significance (P = 0.118, ANOVA). No significant difference was observed between WT and Bex1-KO mice in control groups with sham injury.

We also examined the recovery of sensory function by testing the reflex of the hindlimb (lateral extension accompanied by a foot flexure) after toe pinching. Before sciatic nerve crush, all mice showed equal sensitivity in the toe pinch reflex, a pinch of toe 3, 4, or 5 (tested separately) reliably induced a rapid retraction of the leg. After sciatic nerve injury, the pinch reflex response of the third, fourth, and fifth toe was abolished in all animals. The recovery was monitored over 28 days, and the percentage of animals responding to toe pinch for the first time on days 7, 14, and 28 after injury was calculated (Fig. 5C). While sensory function is recovered in wild type mice by 28 days, the rate of the return of the toe reflex was delayed for Bex1-KO mice compared to WT ($n = 5$; $p < 0.01$, ANOVA). No significant difference was observed between WT and Bex1-KO mice in control groups with sham injury.

Discussion

In addition to its importance in nerve impulse conduction, the myelin sheath is necessary for stability and physical maintenance of axons. It also provides a track along which axonal growth can occur. However, some myelin proteins pose an obstacle to axon growth and regeneration. Axons in PNS have the intrinsic capability to express proteins which help them to overcome this inhibition whereas neurons in CNS cannot activate the same intrinsic growth capacity (Chen et al. 2007).

In our previous study (Nattkämper et al. 2009) using Pmp22-mutant mice, which undergo peripheral axonal damage as a result of myelin deficiency (Sancho et al. 1999, Nattkämper et al. 2009), we found that RAGs like c-Jun, ATF3, SPRR1A, and GAP43 were upregulated in spinal cord MNs raising the possibility of activation of the regeneration process in these mutants after injury. Along with these RAGs, Bex1 was also highly upregulated in these mouse models highlighting a potential role of Bex1 in peripheral axon regeneration. It was striking that Bex1 was recognized previously as playing a role in skeletal muscle regeneration (Koo et al. 2007). Additionally, the interaction of Bex1 and $p75^{NTR}$ was particularly intriguing (Vilar et al. 2006).

Bex1 belongs to a family of small proteins of unknown function, but with several features suggesting roles as adaptors or modulators of intracellular signaling pathways. Bex1 is primarily expressed in nerve cells (Alvarez et al. 2005). Bex1 is involved in the $p75^{NTR}$/TrkA/B pathway (Vilar et al. 2006). Currently, five highly homologous members have been found, namely Bex1 (Rex3/NADE4), Bex2 (NADE5), Bex3, Bex4 and Bex5.Bex1 and Bex2 show 87% homology in their amino acid sequences (Mukai et al. 2000). Bex1 and Bex2 were shown to be present at high levels in rat and mouse retinal-ganglion cells (RGC) in both the soma and the axons (Bernstein et al. 2006) and in olfactory-sensory neurons as well as in several populations of CNS neurons (Koo et al. 2005). This is in agreement with our results showing that Bex1 is expressed in MNs of the spinal cord and is localized in the soma and the axons. In humans, Bex1 and Bex2 show similar expression patterns within the CNS. While Bex2 show a strong expression only in the CNS, Bex1 reveals a high expression level also in peripheral tissues (Koo et al. 2005, Alvarez et al. 2005). Interestingly, it has been shown that expression of Bex1 and Bex2 increases in RGCs after axonal damage resulting from stroke (Bernstein et al. 2006). Bex1 and Bex2 are also expressed in the hematopoietic system and in various tumor cells (Mukai et al. 2000, Chien et al. 2005, Alvarez et al. 2005, Fischer et al. 2007, Naderi et al. 2007). A recent study suggested that Bex1 functions as a tumor suppressor gene (Ding et al. 2009). Bex1 is a signal transduction mediator whose function is not yet fully understood. Bex1 interacts

directly with the intracellular domain of $p75^{NTR}$(Vilar et al. 2006) as well as with calmodulin (Koo et al. 2007), and this interaction could have a potential influence in triggering regeneration.

Bex1 up-regulation is a general response of injured axons to myelin-induced inhibition of axonal regeneration

Induced expression of Bex1 is not only restricted to axonal damage in Pmp22 mutants (Nattkämper et al. 2009) but it was also observed as a result of peripheral axonal damage in MPZ-KO mice. This excludes the possibility that the expression of Bex1 is a consequence of altered Pmp22 expression in the MN of Pmp22-mutant strains. Pmp22-mutant mice and MPZ-KO mice suffer from myelin deficiency while lysolecithin, which induced only myelin damage without axonal damage, could not induce Bex1-upregulation. On the other hand the expression of Bex1 is highly upregulated after axonal crush injury without myelin deficiency (Fig. 2). The upregulation of Bex1 is not restricted to MNs, but our experiments showed that the expression of Bex1 was also upregulated in sensory neurons in DRGs after sciatic nerve injury (data not shown).

These data show that Bex1 expression may result from axonal damage rather than from demyelination. MAG-KO mice also suffer from myelin deficiency and, as a secondary effect, from axonal damage in a later phase of the disease (Pan et al. 2005). Interestingly, in MAG-KO mice the Bex1 was only slightly elevated.

Bex1 counteracts the inhibitory effect of MAG on axonal growth

As shown here, the expression of Bex1 in lesioned axons was up-regulated in the presence of MAG. Bex1 expression enhances outgrowth when the neurons are cultured on substrates, such as MAG, that normally inhibit neurite outgrowth. MAG is known to signal to the axons via interaction with $p75^{NTR}$/NgR/LINGO-1 and inhibits axonal growth and regeneration (Mukhopadhyay et al. 1994, Domeniconi et al. 2002, Dubreuil et al. 2003, Robak et al. 2009). MNs cultured on non-permissive substrate, containing MAG, grew shorter axons (Fig. 3). However, MNs over-expressing Bex1 could overcome the inhibitory effect of MAG and grew longer axons in the presence of MAG. Thus, Bex1 expression is advantageous for axonal outgrowth only when neurons are cultured on a myelin-inhibitory component like MAG substrate. It seems that the basal level of Bex1 expression *in vitro* is not sufficient to overcome myelin inhibition. That is why the axon length of Bex1-KO neurons cultured on PLL+MAG is not significantly different with the axon length in WT neurons cultured on PLL+MAG. However, *in vivo* conditions are different from those *in vitro*. There are mechanisms *in vivo* that sense injury and induce gene expression (Abe and Cavalli 2008) which potentially can also up-regulate Bex1 expression after injury (Fiure 2A). Furthermore, *in vivo* there are additional factors to MAG, like OMgp and Nogo, which bind to $p75^{NTR}$ receptor and are affected by Bex1. Thus the lack of Bex1 in Bex1-KO mice in vivo is expected to be more pronounced than the in vitro condition with MAG as the sole inhibitory molecule.

Bex1 is required for proper functional recovery after sciatic nerve injury

The analysis of Bex1-KO mice demonstrated that they have a reduced capability for regeneration after peripheral nerve injury than WT animals. The most supportive evidence was provided by crush-injury experiments of sciatic nerves. Sciatic-nerve crush is a well-established model for studying nerve regeneration and functional alterations associated with peripheral-nerve injury. WT mice recover from sciatic-nerve injury much faster than Bex1-KO mice. Our studies demonstrated that mice deficient in Bex1 protein possess a reduced capability to regenerate injured axons than do WT littermates. We have evidences that this effect maybe partialy arises from the the function of Bex1 on modulation of RhoA activation

after injury, which would ultimately result in better axonal growth. On the other hand, Bex1 could be also involved in regeneration via another mechanism. Bex1 can interact with calmodulin (Koo et al. 2007), and it may be involved in the signaling pathway by which receptor-mediated calcium- fluxes regulate growth-cone activity.

In conclusion, we have demonstrated here that Bex1 is an intracellular mediator which may antagonize the inhibitory effect of MAG. Bex1 should be considered to be a RAG since our results suggest that Bex1 may play a role in promoting nerve repair after injury in the PNS. These findings open interesting possibilities for exploring Bex1 function in the control of neuronal regeneration in the CNS, an important point for future consideration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Bex1	brain expressed x-linked 1 protein
CNS	central nervous system
DAPI	4’, 6-diamidino-2-phenylindole dihydrochloride hydrate
DIV	days *in vitro*
KO	knock out
LCM	laser capture microscopy
MAG	myelin associated glycoprotein
MN	motor neuron

MPZ	myelin protein zero
OMgp	oligodendrocyte myelin glycoprotein
pmn	progressive motor neuronopathy
PLL	Poly-L-Lysine
PNS	peripheral nervous system
qRT-PCR	quantitative real-time polymerase chain reaction
RAG	regeneration associated gene
RGC	retinal ganglion cells
SFI	sciatic functional index
WT	wild type

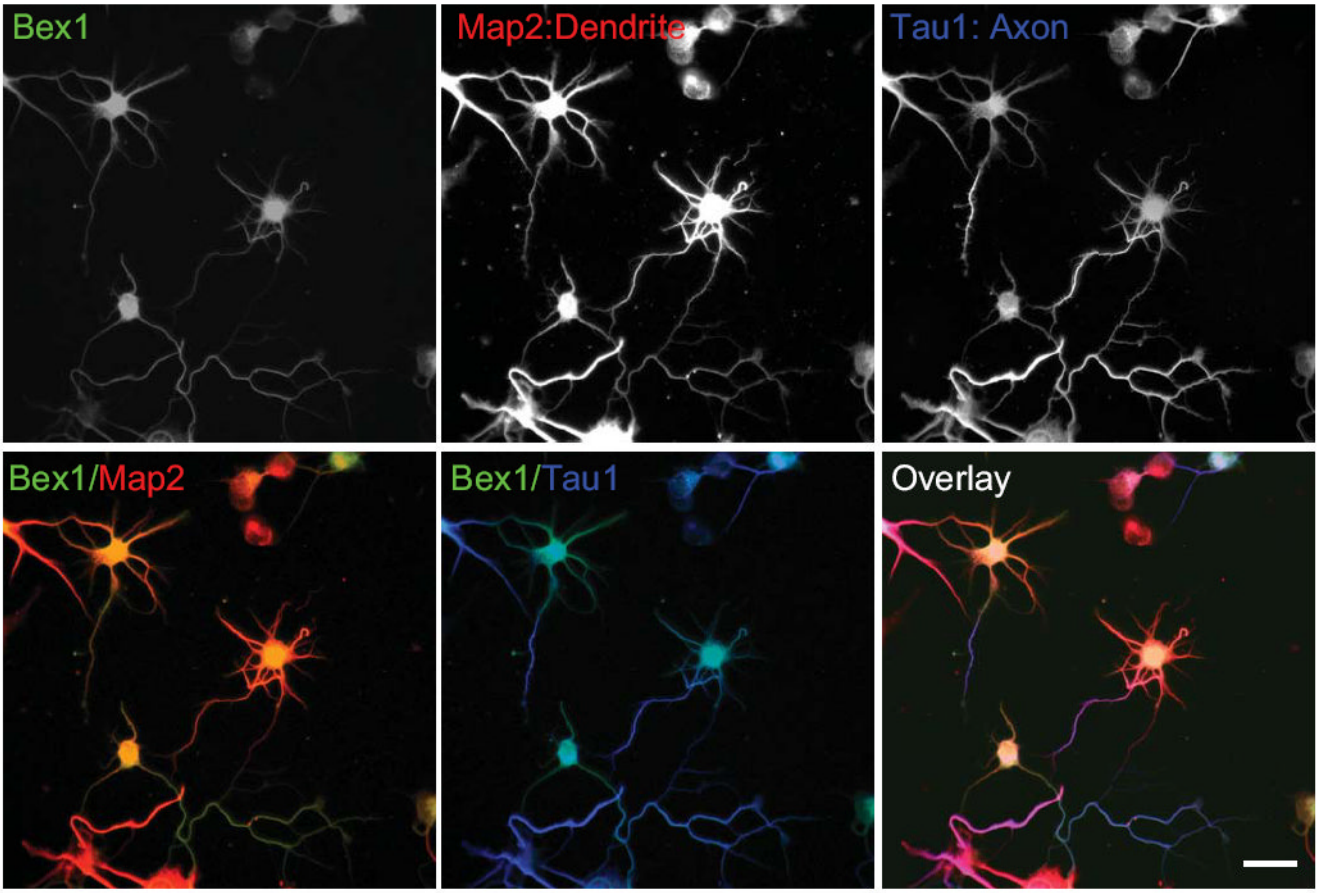
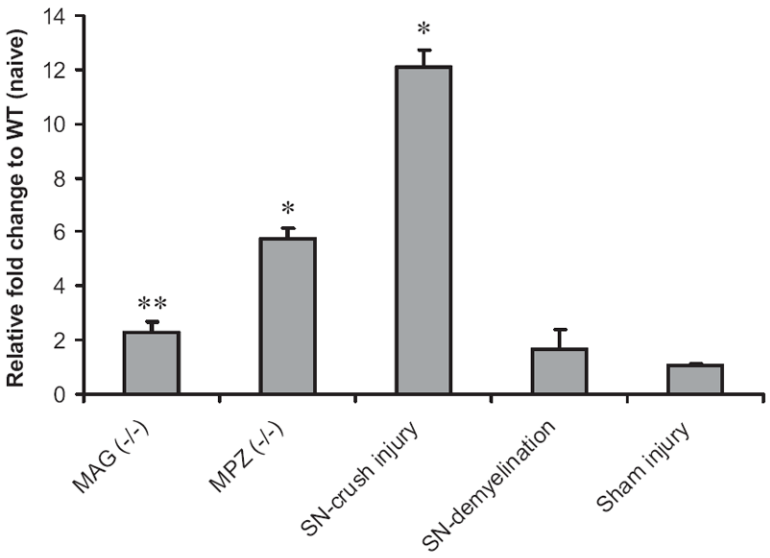


Fig. 1. Bex1 expression in axons

MNs from E13-14 mouse embryos were fixed at 3 DIV and stained with antibodies specific for Bex1 (green), MAP2 (red) and the Tau-1 antibody (blue). The scale bar is 40μm.

A



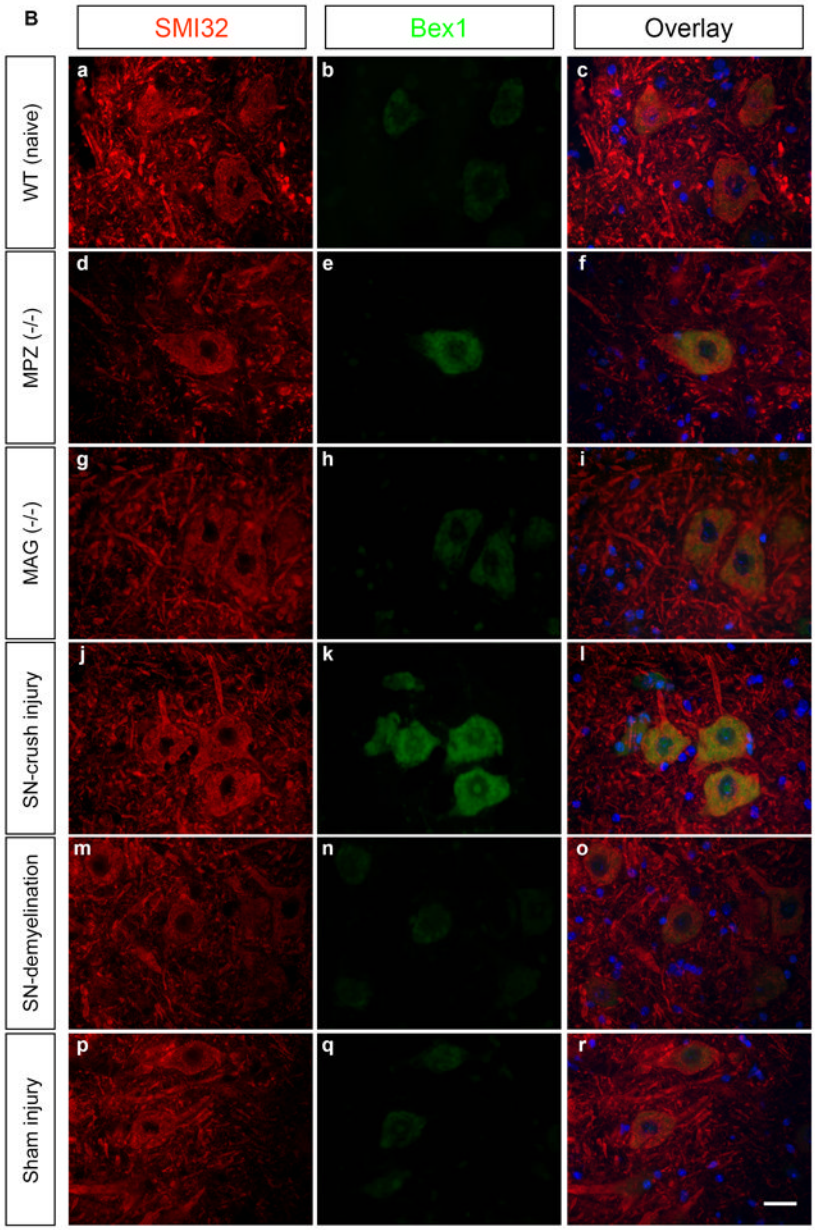


Fig. 2. Bex1 upregulation in MNs after axonal injury

a) MNs from the spinal cord of MPZ-KO and MAG-KO mice and mice subjected to SN-crush injury or demyelination were dissected by Laser capture microdissection and their total RNA was used for quantitative real-time PCR. n =3, error bars represent SEM, * $p < 0.001$, ** $p < 0.005$, ANOVA compared to MNs derived from un-injured WT mice **b)** To monitor the expression of Bex1 protein, the tissue sections from lumbar-spinal cord of MPZ-KO and MAG-KO mice and mice subjected to sciatic-nerve crush-injury or demyelination were stained with anti Bex1 (green) and anti-nonphosphorylated neurofilament (SMI32; a marker of neurons) (red) antibodies. DAPI (blue) was used for nuclear staining. Scale bar is 50 μm. Note elevation of Bex immunostaining following crush injury.

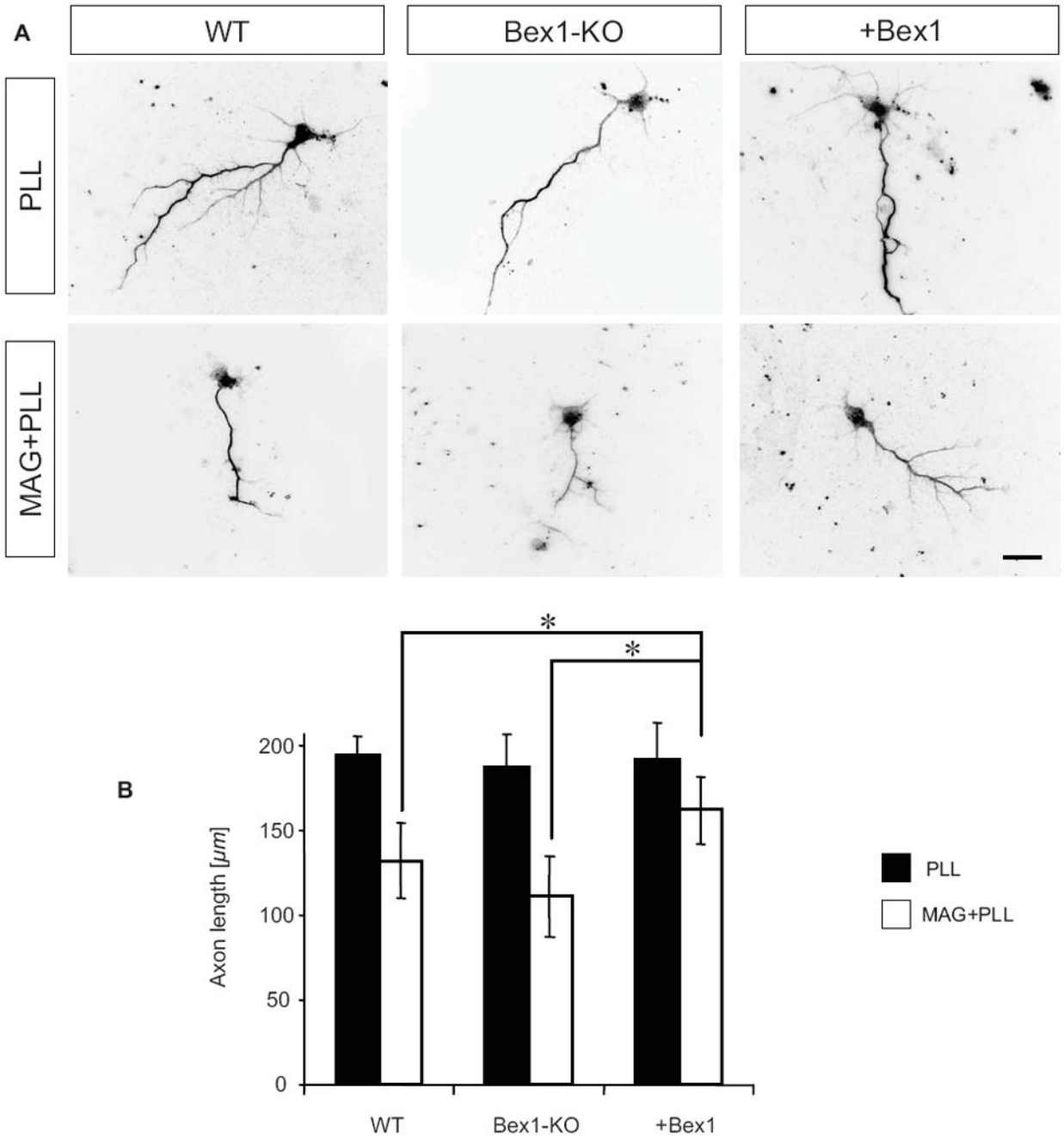


Fig. 3. The function of Bex1 under MAG signalling on axonal growth

a) MNs prepared from E13-14 WT or Bex-KO mice embryos were cultured on PLL substrate or PLL + MAG (4μg/ml). WT- MNs were transfected 2 h after plating with expression vectors for GFP, or GFP-Bex1, and Bex-KO- MNs were transfected 2 h after plating with expression vectors for GFP. Transfected cells (GFP-positive) were analyzed at 3 DIV. The scale bar is 40 μm. **b)** The average length of the axons are measured from the soma along the process to its tip by using ImageJ software (released by NIH) (n=3, means ± SEM; * $p < 0.01$, ANOVA compared to neurons cultured on just PLL).

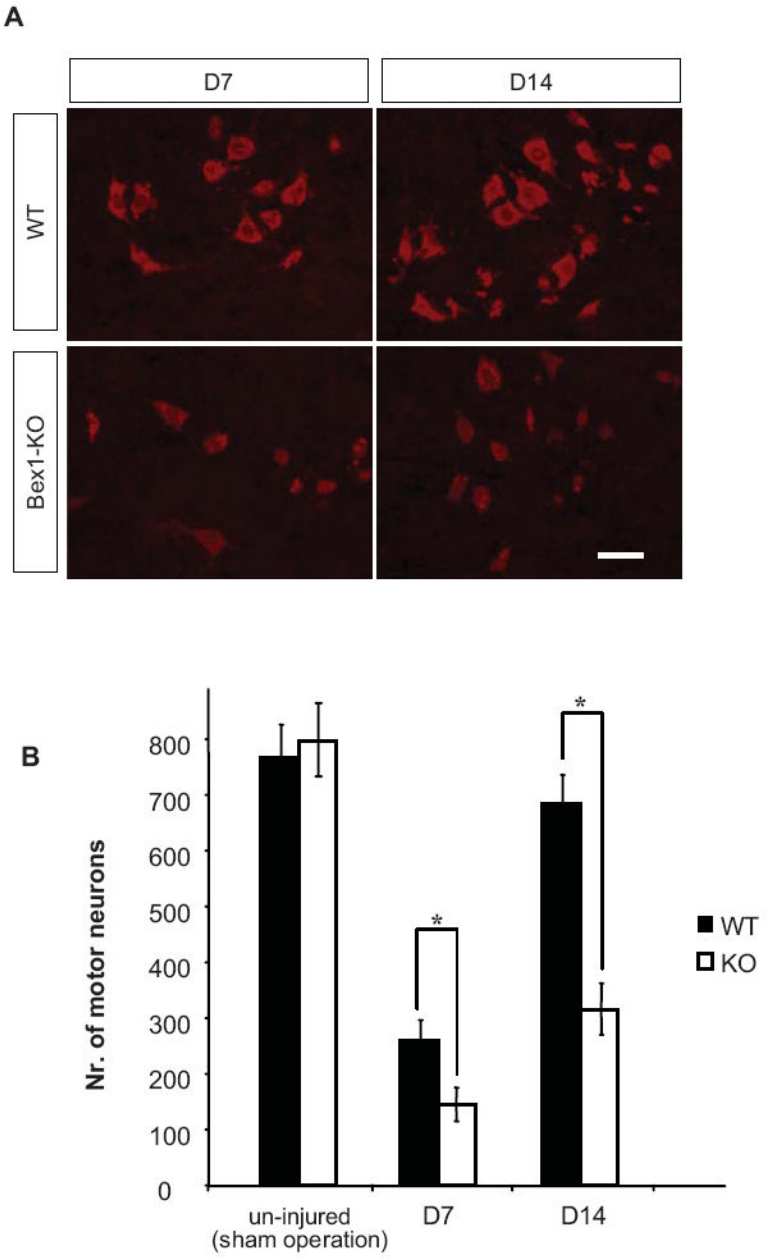


Fig. 4. Retrograde labelling of spinal cord MNs *in vivo*

Retrograde labelling was performed for the quantification of spinal-MN perikarya within the ventral horn that projects to the sciatic nerve. 7 and 14 days after sciatic-nerve injury, the sciatic nerve was exposed again and a small crystal of DiI was placed 7 mm distal to the crush lesion site. The dye was transported retrogradely to the MN perikarya. In the sham-operated controls, the sciatic nerve was exposed but not injured. After 2 days, spinal cords were removed and sectioned. The serial sections were analyzed by microscope (a) and the number of labelled MNs was counted on each section (b), data are presented as average ± SEM. Repeated-measures ANOVA with Bonferroni-Holm post hoc analysis was completed to assess statistical differences.

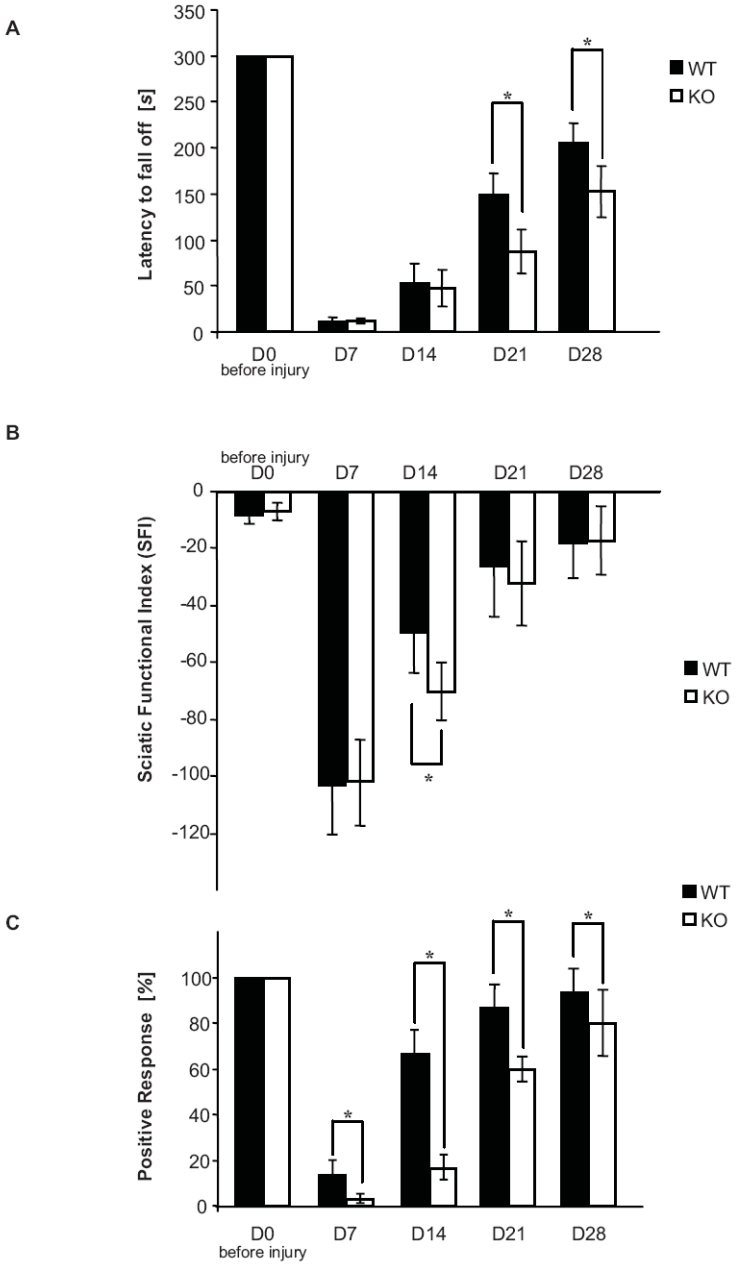


Fig. 5. Behavioral analysis illustrates functional recovery following SN injury

a) Rotarod test was used to assess the motor coordination and balance of the animals on days 7, 14, 21 and 28 after operation. Rotation was started at a speed of 4 rpm and accelerated to 40 rpm in 270 sec. The latency period until the mice fell off the apparatus was monitored for 300 sec. **b)** Gait analysis was used to measure Sciatic Functional Index (SFI). The hind feet of mice were painted and the mice allowed to walk freely on a blank strip of paper. The SFI was calculated after measuring the distance to Opposite Foot (TOF), Print Length (PL), Total Toes Spreading (TS) and distance between Intermediary Toes (IT). **c)** Toe-pinch reflex was used to assess the recovery of sensory function by pinching the most distal portion of the last three toes of the injured hindlimb with a flattened forceps. Foot withdrawal was recorded as positive responses indicative of recovery. n = 3, data are

presented as average ± SEM. Repeated-measures ANOVA with Bonferroni-Holm post hoc analysis was completed to assess statistical differences.