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Maintenance of the relative proportion of oligodendrocytes to axons even in the absence of BAX and BAK

Kumi Kawai^{1,2,6}, Takayuki Itoh^{1,2,6}, Aki Itoh^{1,2}, Makoto Horiuchi^{1,2}, Kouji Wakayama^{1,2}, Peter Bannerman^{2,3}, James Y. Garbern⁴, David Pleasure^{1,2}, and Tullia Lindsten⁵

¹ Department of Neurology, University of California Davis, School of Medicine, Sacramento, California 95817, USA

² Institute for Pediatric Regenerative Medicine, Shriners Hospitals for Children Northern California, Sacramento, California 95817, USA

³ Department of Cell Biology and Human Anatomy, University of California Davis, Davis, California 95616, USA

⁴ Department of Neurology and Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan 48201, USA

⁵ Department of Pathology and Laboratory Medicine, and Abramson Family Cancer Research Institute, The University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

Abstract

Highly purified oligodendroglial lineage cells from mice lacking functional *bax* and *bak* genes were resistant to apoptosis after *in vitro* differentiation, indicating an essential role of the intrinsic apoptotic pathway in apoptosis of oligodendrocytes in the absence of neurons (axons) and other glial cells. These mice therefore provide a valuable tool with which to evaluate the significance of the intrinsic apoptotic pathway in regulating the population sizes of oligodendrocytes and oligodendroglial progenitor cells. Quantitative analysis of the optic nerves and the dorsal columns of the spinal cord revealed that the absolute numbers of mature oligodendrocytes immunolabeled for aspartoacylase, and adult glial progenitor cells expressing NG2 chondroitin sulfate proteoglycan, were increased in both white matter tracts of adult *bax/bak*-deficient mice, and, to a lesser extent, *bax*-deficient mice, except for no increase in NG2-positive progenitor cells in the dorsal columns of these strains of mutant mice. These increases in mature oligodendrocytes and progenitor cells in *bax/bak*-deficient mice were unexpectedly proportional to increases in numbers of axons in these white matter tracts, thus retaining the oligodendroglial lineage to axon ratios at most 1.3-fold of the physiological numbers. This contrasts to the prominent expansion in numbers of neural precursor cells in the subventricular zones of these adult mutant mice. Our study indicates that quantitative homeostatic control of the oligodendroglial lineage is distinct from that of neural precursor cells, and that other regulatory mechanism(s), in addition to apoptotic elimination through the intrinsic pathway, prevent the overproduction of highly mitotic oligodendroglial progenitor cells.

Keywords

apoptosis; Bcl-2-related protein family; differentiation; aspartoacylase; NG2

Address for communications: Takayuki Itoh MD, PhD, 601A Shriners Hospitals for Children Northern California, 2425 Stockton Boulevard, Sacramento, California 95817, USA, takito@ucdavis.edu.

⁶The authors contributed equally to this study.

Introduction

Apoptotic elimination of superfluous cells has been considered a critical process to fine-tune the population size of a cell lineage. In this respect, the oligodendroglial lineage has been intensively studied (Barres and Raff 1999; Miller 2002). Oligodendroglial progenitor cells (OPCs) are generated in excess compared with the axons to be myelinated, and these redundant cells become susceptible to apoptosis when they begin to differentiate. In optic nerves, 50% of newly generated oligodendrocytes are eliminated by apoptosis (Barres et al. 1992), while apoptosis appears to play a lesser role in determining oligodendroglial population in other portions of the central nervous system (CNS) (Trapp et al. 1997). This process is experimentally enhanced in mice overexpressing a platelet derived growth factor A subunit (PDGFA) transgene in neurons (Calver et al. 1998). A mechanism underlying this homeostatic phenomenon is that, once OPCs exit the cell cycle and commence terminal differentiation, they become dependent on axon-derived signals, such as Neuregulin-1 and Laminin-2, for their survival (Colognato et al. 2002; Corley et al. 2001; Fernandez et al. 2000; Frost et al. 1999). Even in adults, myelinating oligodendrocytes are likely to continue to receive trophic support from axons, as evidenced by apoptosis of mature oligodendrocytes in association with Wallerian degeneration of distal axons following axotomy (Dong et al. 2003).

Some OPCs persist in adult CNS tissues. This population, comprising 5 to 10% of total adult CNS cells, is surface positive for NG2 chondroitin sulfate proteoglycan, and is believed to contribute to CNS repair by generating oligodendrocytes and astrocytes (Nishiyama et al. 2009; Polito and Reynolds 2005). The mechanisms regulating these adult OPC remain elusive.

The core apoptotic machinery consists of the extrinsic (or death receptor-mediated) and the intrinsic (or mitochondrion-mediated) pathway (Green 2000; Puthalakath and Strasser 2002). The intrinsic pathway is regulated by proteins of the Bcl-2 family of genes. The multidomain pro-apoptotic members of this family, BAX and BAK, act as a checkpoint through which death signals elicit the mitochondrial outer membrane permeabilization and subsequent release of apoptogenic proteins from mitochondria, depending on their functional balance to the anti-apoptotic Bcl-2 family members BCL-2 and BCL-X_L (Cheng et al. 2001; Wei et al. 2001). Knockout mouse strains of *bax* and *bak* genes provide valuable tools to analyze roles for the intrinsic pathway in apoptosis of various cell lineages (Knudson et al. 1995; Lindsten et al. 2000; 2003). In this study, we found that myelin-producing oligodendrocytes from mice deficient in both BAX and BAK were viable *in vitro* for a prolonged period even in the absence of axons and other glial cells. We therefore hypothesized that *bax/bak* double knockout (DKO) oligodendrocytes are resistant to apoptotic elimination even in the absence of axon-derived survival cues, and that this resistance would result in excess numbers of myelinating oligodendrocytes in the CNS of DKO mice. Surprisingly, our data show that, in contrast to the prominent accumulation of neural precursor cells in the periventricular zone of DKO mice in the previous report (Lindsten et al. 2003), the quantitative balance of myelinating oligodendrocytes and NG2-positive adult glial progenitor cells to axons was relatively well maintained in adult DKO mice.

Materials and Methods

Mouse strains with targeted disruption of *bax* and/or *bak*

The parental strains with targeted null mutation of *bax* and *bak* genes used in this study have been reported previously in detail by Knudson et al. (1995), and Lindsten et al. (2000), respectively. *bax*^{+/-} *bak*^{+/-} and/or *bax*^{+/-} *bak*^{-/-} mice were mated to generate all genotypes used in this study. Genotyping of offspring was performed by PCR of genomic DNA samples from tail snips or brain tissues with the following sets of primers modified from the previous report (Lindsten et al. 2000).

Sense primer for bax wild-type allele: 5' -CACTAAAGTGCCCGAGCTGAT

Sense primer for bax mutant allele: 5' -ACTTCCATTTGTCACGTCCTG

Antisense common primer for bax: 5' -TGACCAGAGTGCGTAGGAGTC

Sense primer for bak wild-type allele: 5' -CTCTTCACCCCTTACATCAGT

Sense primer for bak mutant allele: 5' -CCTTCTTGACGAGTTCTTCTG

Antisense common primer for bak: 5' -GAGAGCCATGAGATGTTTAGC

Animals were housed in standard laboratory cages with an unrestricted access to food and water, and maintained under 12-hour light/dark cycles. All experiments using the animals were performed in conformity with the procedure approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Reagents and Culture media

The reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. The culture medium used for mixed glial cells and panned oligodendroglial cells (designated as GM in this study) was composed of a 3:7 mixture (v/v) of B104 neuroblastoma-conditioned medium and the N1 medium (high glucose Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 6 mM glutamine, 10 ng/ml biotin, 5 µg/ml bovine insulin (SIGMA I6634), 50 µg/ml human transferrin (SIGMA T2036), 30 nM sodium selenite, 20 nM progesterone, 100 µM putrescine, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) as final concentrations). To induce differentiation of oligodendrocytes, the culture medium was switched to the differentiation medium (DM; a 1:1 mixture (v/v) of high glucose DMEM and Ham's F-12 medium supplemented with 4.5 mM glutamine, 10 ng/ml biotin, 12.5 µg/ml bovine insulin, 50 µg/ml human transferrin, 24 nM sodium selenite, 10 nM progesterone, 67 µM putrescine, 0.4 µg/ml 3, 5, 3', 5'-tetraiodothyronine (SIGMA T0397), 100 units/ml penicillin, and 100 µg/ml streptomycin as final concentrations).

Mouse oligodendroglial culture

Primary oligodendroglial cultures from single mouse pups were prepared by a procedure modified from that reported for rats (Horiuchi et al. 2006; Itoh et al. 2002). Whole brain was dissected from a single 0 to 2 day-old mouse pup, and submerged into ice-cold Leibovitz's L-15 medium. Under a dissecting microscope, olfactory bulbs, cerebral cortex and the hindbrain were removed. A small piece of the removed tissue was saved for genotyping. After removing meninges and vessels including choroidal plexus, the rest of the brain tissue was cut into small chunks with a 21-gauge needle, and digested by 0.0625% (w/v) trypsin in Ca²⁺ and Mg²⁺-free Hank's Balanced Salt Solution (HBSS) for 20 min. Enzymatic digestion was terminated by adding soybean trypsin inhibitor (SIGMA T6522, 0.058 mg/ml), DNase I (SIGMA D5025, 0.0044 mg/ml) and fraction V bovine serum albumin (SIGMA A2058, 0.33 mg/ml). Dissociated cells were obtained by passing the softened chunks through a 1 ml hand pipette tip several times, and collected by centrifugation at 365× g for 5 min. The cells were resuspended in the minimum essential medium alpha medium (Invitrogen, Cat# 12571) containing 5% (v/v) fetal bovine serum and 5% (v/v) calf serum, and plated onto a 10 cm culture dish. One day after plating, attached cells (designated as passage 0) were washed with HBSS to remove serum, and thereafter maintained in the GM medium for 5 to 7 days when the proliferating cells were approximately 80% confluent. Cultures were fed with fresh GM medium every two days. The mixed glial cells were collected after a brief trypsinization, transferred to a cryoprotection medium composed of 93% (v/v) GM medium and 7% (v/v) dimethyl sulfoxide, and kept frozen in liquid nitrogen until enough cells of each genotype were

obtained and pooled for an experiment. We confirmed that the survival and differentiation of the mixed glial cells from wild-type mice were not significantly altered by this single freezing-thawing cycle. When enough cells had been collected, the frozen cells were thawed, and plated in the GM medium onto a 10 cm culture dish (passage 1). At 2 days after replating, cells of the oligodendroglial lineage were selected by immunopanning procedures modified from the initial report by Wang et al. (2001). The mixed glial cells were washed with Ca^{2+} and Mg^{2+} -free HBSS, suspended in the N1 medium containing 0.1% (w/v) factor V bovine serum albumin, and plated on the negative panning plate coated with anti-Thy 1.2 antibody (clone 30H12 from American Type Culture Collection, Manassas, VA) for 30 min at 37 °C. The nonadherent cells were then transferred to the O4 positive panning dish. After the serial immunopanning, approximately 2 to 4×10^5 purified O4-positive cells were obtained from a single brain. These cells (passage 2) were plated onto poly-D-lysine coated 12 mm round coverslips or multi-well plates in the GM medium supplemented with bovine basic FGF (R&D systems Inc., Minneapolis, MN, 5 ng/ml), and human recombinant PDGFA homodimer (R&D systems Inc., 2 ng/ml). The purified O4-positive cells were maintained in the GM medium for 24 h to ensure their attachment to the coverslips, and then switched to the DM medium to induce terminal differentiation. Half of the DM medium was exchanged every two days.

Quantitative RT-PCR

Total RNA was isolated by RNeasy RNA extraction kit (Qiagen, Valencia, CA) from O4-positive passage 2 cells at 1 day after immunopanning. RT-reaction was done as reported previously (Itoh et al., 2003). Quantitative PCR was performed by MX3005P (Stratagene, La Jolla, Ca) using TaqMan Assay-on-Demand assay kits (assay Nos.: Mm00437783_m1, Mm00477631_m1, Mm00432050_m1, and Mm00432045_m1 for Bcl-2, Bcl-x, Bax, and Bak, respectively). Plasmids containing PCR-amplified cDNA of the target genes were serially diluted and used for the concentration standards. For standardization, GAPDH cDNA levels were quantified with TaqMan Rodent GAPDH Control Reagents according to manufacturer's instruction, and the absolute cDNA amounts were expressed as ratios to GAPDH cDNA.

Immunocytochemistry

For O4 staining, cells cultured on glass coverslips were incubated for 30 min with O4 hybridoma supernatant at a 1:2 dilution at room temperature (23-25 °C), rinsed with PBS three times, then incubated with tetramethylrhodamine-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 30 min, and post-fixed in ice-cold 5% acetic acid ethanol for 10 min. For myelin basic protein (MBP) staining, cells were fixed in 4% paraformaldehyde for 25 min at 4 °C, rinsed with PBS three times, permeabilized in 0.1% Triton-X and 10% normal goat serum in PBS for 30 min at room temperature, rinsed with PBS three times, incubated with the 3B12 hybridoma (generous gift from V. Lee, The University of Pennsylvania) supernatant for 60 min at room temperature, rinsed with PBS three times, and then incubated with the fluorescein isothiocyanate-conjugated anti-rat IgG antibody. Nuclear staining with Hoechst 33258 (SIGMA B2883) was performed before mounting with Vectashield (Vector Laboratories, Burlingame, CA).

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling) assay

DNA fragmentation was detected by the TUNEL method (Gavrieli et al. 1992) with minor modifications. The 3'-OH ends of fragmented DNA were directly labeled with tetramethylrhodamine-6-dUTP (Molecular probes, Eugene, OR) by terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) or Hoechst 33258, and TUNEL-positive and TUNEL-negative nuclei were counted under a fluorescence microscope. Approximately 500 nuclei were counted for each culture condition.

Immunohistochemistry and *in situ* TUNEL

Adult mice (150 to 250 day-old) were deeply anesthetized with a ketamine/xylazine cocktail and perfused transcardially by PBS followed by 4% (w/v) paraformaldehyde in PBS. The optic nerve and the spinal cord were removed under a dissecting microscope, and post-fixed in 4% (w/v) paraformaldehyde in PBS for additional 1 h. The tissues were rinsed in PBS overnight at 4 °C, soaked in 15% (w/v) sucrose overnight and then in 30% (w/v) sucrose overnight at 4 °C, and embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound. Six μ m-thick tissue sections were prepared by a cryostat (Leica CM3050S, Leica Microsystems, Wetzlar, Germany).

The tissue sections were rinsed in PBS, and preincubated in a blocking solution (2% (w/v) BSA, 2% (v/v) normal goat serum and 0.05% (v/v) Triton X-100) for 1 h. Then, primary antibodies were applied overnight at 4 °C. The primary antibodies used were anti-NG2 antibody (1:100, rabbit polyclonal, AB5320, Millipore, Temecula, CA), anti-MBP antibody (1:20, rat monoclonal, Cat# NB600-717, Novus Biologicals, Littleton, CA), anti-aspartoacylase (ASPA) antibody (1:2000, rabbit polyclonal from Dr. James Garbern) (Hershfield et al. 2006), anti-neurofilament M (NF-M) antibody (1:200, rabbit polyclonal, Cat# AB1987, Millipore), anti-glutathione *S*-transferase antibody (1:125, mouse monoclonal, Cat# 610718, BD Biosciences, San Jose, CA), and anti-adenomatous polyposis coli (APC) antibody (1:200, mouse monoclonal CC-1, EMD Chemicals, Gibbstown, NJ). The slides were washed in PBS, followed by a 1 h-incubation in tetramethylrhodamine or fluorescein isothiocyanate-conjugated anti-rabbit, anti-mouse or anti-rat secondary antiserum (1:250, Jackson ImmunoResearch). Nuclei were counterstained with DAPI, and the labeled tissues were mounted with Vectashield.

Apoptotic cells were detected *in situ* using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, Madison, WI) according to the manufacturer's instruction. Briefly, the frozen tissue sections were post-fixed in ice-cold acetone for 10 min, rinsed in PBS and subjected to the terminal deoxynucleotidyl transferase reaction. All nuclei were colabeled with DAPI. Due to a low incidence of apoptotic cells in a cross-section of the optic nerve, multiple cross-sections were randomly counted to obtain the averaged TUNEL-positive cell number per section in each case.

Quantitative histological analysis

Digitized fluorescent images of each cross section were acquired with a fluorescence microscope (Axioplan 2, Carl Zeiss MicroImaging, Thornwood, NY) equipped with a digital camera (AxioCam, Carl Zeiss MicroImaging) and imaging software (AxioVision Release 4.5, Carl Zeiss MicroImaging). Numbers of DAPI-positive nuclei, ASPA-positive and NG2-positive cells per cross section were counted manually in no less than 3 sections per tissue. Pericytes/smooth muscle cells in developing vasculature are also known to express NG2 (Stallcup, 2002), but they could be excluded easily by their distinct morphology associated with vessels in the quantitative analysis. At least three mice were analyzed in each genotype.

To quantify axon numbers, sections double stained with anti-MBP and anti-NF-M antibodies were examined using a confocal microscope (Nikon Eclipse TE2000-E, Nikon Instruments, Kawasaki, Japan). Optical sections of confocal fluorescence images were sequentially acquired using a 100 $\times$ oil objective (NA=1.30) and EZ-C1 3.40 software, and montages were constructed. Nine hundred μ m² square areas were randomly selected for optic nerves, while, for spinal cords, a specified 900 μ m² area was selected at the most dorsal part of the dorsal column just adjacent to the meninges and along the midline. Almost all axons in this region consisted of relatively large diameter ascending axons (>1 μ m in diameter) from dorsal ganglion neurons. All the axons positive for NF-M in a 900 μ m² area were counted manually. In both tissues, 9 areas per mice, and at least 3 mice per genotype were analyzed.

Cross sectional areas were measured with the stereology software, SlideBook (Nippon Roper, Tokyo, Japan) using the digital images obtained by a fluorescent microscope (Olympus BX51, Olympus, Tokyo, Japan) equipped with a digital camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan).

Statistics

For the quantitative analyses, mean values of at least three independent counting results were calculated for each animal, accumulated and used for the further statistical comparison. P values were calculated by either Welch's unpaired *t*-test for unequal variances (for the comparison between wild-type and DKO mice) or one-way ANOVA followed by the Bonferroni/Dunn post-hoc test (for the data sets from more than three genotypes). Results were considered significant, when $p < 0.05$ (one-tailed).

Results

Mouse oligodendroglial cultures

In this study, we used O4 as a reliable surface marker for positive selection of mouse cells committed to the oligodendroglial lineage, because a large proportion of mouse oligodendroglial progenitors are negative for A2B5, a typical surface marker for rat bipotential glial progenitors of oligodendrocytes and type-2 astrocytes (O2A progenitors), until they acquire O4 antigen (Fanarraga et al. 1995; our unpublished data). Figure 1a shows purified O4-positive cells of the oligodendroglial lineage from wild-type mice at 24 hr after immunopanning. In contrast to the relatively uniform morphology of rat A2B5-positive progenitors with 2 to 3 slender processes, mouse O4-positive cells were heterogeneous in morphology, consisting of small round cells without processes, bipolar cells and multipolar cells with branched processes. At 24 h after plating, the cultures were transferred to DM to induce *in vitro* terminal differentiation. Despite the initial heterogeneous morphology, most cells turned into multiprocess-bearing cells at 2 days in DM, more than 90% of which were O4-positive (Figs. 1b and c). During the same period, however, differentiating purified O4-positive cells from wild-type mice rapidly died in DM (Figs. 1b and c), although a majority of rat oligodendrocytes can be maintained for a week (data not shown). We confirmed that the mouse cells died by apoptosis using TUNEL assay (Fig. 3a).

Roles of Bax and Bak in apoptosis of differentiated oligodendrocytes

The multidomain proapoptotic members of the Bcl-2 family, BAX and BAK, are expressed at relatively high levels throughout *in vitro* differentiation from progenitor cells to mature oligodendrocytes (Itoh et al. 2003), and function as key regulatory molecules in activation of the intrinsic apoptotic pathway (Wei et al. 2001). We determined steady state mRNA levels of the four major multi-domain Bcl-2-related protein genes, *bcl-2*, *bcl-x*, *bax* and *bak* in the O4-positive cells purified from *bax*- and/or *bak*-deficient mice. The qPCR results demonstrated that *bax* mRNA was 10 times more abundant than *bak* mRNA, and, more importantly, that there were no significant compensatory changes in transcriptional regulation of the two major antiapoptotic members *bcl-2* and *bcl-x* in *bax*- and/or *bak*-deficient O4-positive cells (Fig. 2).

To examine whether loss of BAX and/or BAK protects differentiating purified O4-positive cells from apoptosis in DM, wild-type, *bax*^{+/-} *bak*^{-/-}, *bax*^{-/-} *bak*^{+/-}, and *bax*^{-/-} *bak*^{-/-} purified O4-positive cells were cultured in DM. As demonstrated by the TUNEL assays (Fig. 3a-d), virtually no apoptotic cells were seen in the *bax*^{-/-} *bak*^{-/-} (DKO) oligodendrocyte cultures after 5 days in DM, compared to the large numbers of apoptotic cells in the wild-type and *bax*^{+/-} *bak*^{-/-} oligodendroglial cultures. An intermediate viability was obtained in the *bax*^{-/-} *bak*^{+/-} cultures, where approximately half of the cells survived. Quantitative analysis of viability at multiple time points is shown in Fig. 3e. More than 90% of DKO oligodendrocytes

were still viable even after 14 days in DM. The presence of a single *bax* allele was sufficient for oligodendrocytes to restore 'normal' susceptibility to apoptosis following *in vitro* differentiation, whereas a single copy of *bak* only partially restored susceptibility. These results suggest that, whereas BAX is the predominant determinant in oligodendroglial apoptosis induced by loss of trophic supports, long-term high viability is only obtained in the DKO oligodendrocytes.

Terminal differentiation progresses independently of apoptosis

As more than 90% of DKO oligodendrocytes remained viable for as long as 14 days in DM (Fig. 3e), these cultures provide a convenient system to study the progress of terminal oligodendroglial differentiation induced by loss of trophic supports, in the virtual absence of apoptosis. The proportion of MBP-positive cells was immunocytochemically determined in the cultures from wild-type and DKO mice. In the DKO cultures, there were 65% MBP-positive cells at 5 days, and 85% at 9 days in the DM medium (Table 1). These results clearly demonstrated that, when the intrinsic apoptotic pathway was blocked, most of the purified O4-positive cells could complete terminal differentiation, and thus differentiation is independent of the BAX/BAK checkpoint. On the other hand, in the wild-type cultures at 5 days in the DM medium, more than 80% of the whole population had died by apoptosis. Most of the dead cells in the wild-type cultures did not show any MBP immunoreactivity, whereas residual MBP immunoreactivity usually remained detectable for at least a few days after the death of MBP-positive cells in the cultures (Fig. 4a–d). These results suggest that, when both differentiation and apoptosis are initiated simultaneously, as may be the case in this *in vitro* system, apoptosis was completed before MBP expression, as measured by immunocytochemistry, was first seen.

As demonstrated in Fig. 4e–h, approximately 50% of *bax*^{-/-} *bak*^{+/-} oligodendrocytes and more than 90% of DKO oligodendrocytes were alive at 14 days in the DM medium, as judged by their nuclear morphology, TUNEL- and MBP-staining. Despite the prolonged survival of DKO oligodendrocytes without evidence of apoptosis even under our stringent culture condition, the processes became irregular and sometimes fragmented with punctuated MBP-staining at 14 days, compared with MBP-positive finely branched processes with membranous sheets at 5 days (Compare Figs. 4d and 4f, h).

Apoptotic cells were significantly reduced in the optic nerve during CNS myelination of DKO mice

We next tested these *in vitro* results *in vivo* during developmental CNS myelination. We confirmed that numbers of TUNEL-positive cells in the optic nerves of wild-type postnatal mice reached a peak around 13 to 14 days after birth. Thus, TUNEL-positive cells were counted in multiple 6 μm-thick transverse sections of the optic nerves of 13 to 14 day-old wild-type mice and DKO mice. In the wild-type optic nerve, the averaged incidence of TUNEL-positive cells per cross-section was 2.03 ± 0.56 (n=3), whereas there were 0.17 ± 0.29 (n=3) TUNEL-positive cells in the DKO optic nerve ($t_{3,0}=5.15$, $P=0.014$, Welch's unpaired *t*-test), confirming that developmental apoptotic pruning is also largely dependent on the intrinsic apoptotic pathway.

Total cell number and mature oligodendrocytes are increased in the *bax*- and *bax*, *bak*-double deficient white matter

These *in vitro* and *in vivo* results further prompted us to test the prediction that, in *bax*-single knockout (SKO) or DKO mice, more cells of the oligodendroglial lineage would persist *in vivo*. Optic nerves and spinal cords from 150 to 250 day-old mice of the four genotypes, wild-type, *bax*^{+/-} *bak*^{-/-}, *bax*^{-/-} *bak*^{+/-}, and *bax*^{-/-} *bak*^{-/-}, were subjected to quantitative histological analyses. Sections were stained with DAPI to count the number of nucleated cells present (Fig. 5), and mature oligodendrocytes were identified by their immunoreactivity for

N-acetyl-L-aspartate amidohydrolase (ASPA, aspartoacylase, EC 3.5.1.15) (Madhavarao et al. 2004) and quantified in 6 μm -cross sections of the optic nerve and the dorsal column of the spinal cord at the first thoracic spinal segment (T1). This ASPA-positive population overlapped with almost 100% of the cells immunoreactive to glutathione *S*-transferase π isoform (Tansey and Cammer 1991), and $93 \pm 4\%$ of the cells labeled with CC1 monoclonal antibody (Bhat et al., 1996) (from three adult mice; Supplemental Fig. 1), which confirmed the report by Madhavarao et al. (2004) that ASPA is a cytoplasmic marker of mature oligodendrocytes.

The cross sectional area of the optic nerve was increased in *bax*-SKO and more significantly in DKO (Figs 5a-d and Table 2), and this was accompanied by significant increases in the number of nucleated cells as measured by DAPI staining ($F_{3,13}=29.9$, *bax*-SKO: $P<0.0001$; DKO: $P=0.0002$; Fig. 5e). The number of ASPA-positive cells was also significantly increased ($F_{3,13}=18.5$, *bax*-SKO: $P=0.0006$; DKO: $P=0.0004$; Fig. 5f and Table 2), in proportion to the increase in all nucleated cells. The analysis of the dorsal column of the T1 spinal cord showed that the increase in the cross-sectional area was not as apparent as that of the optic nerve in *bax*-SKO and DKO mice. However, a significant increase in both number of nucleated cells and number of ASPA-positive cells was seen in the DKO mice (DKO (nucleated cells): $F_{3,11}=4.27$, $P=0.014$; DKO (ASPA-positive cells): $F_{3,11}=6.04$, $P=0.003$; Figs. 5e and 5f, respectively, and Supplemental Fig. 2).

NG2 chondroitin sulfate proteoglycan-positive adult OPCs are increased in the *bax*- and *bax*, *bak*-double deficient optic nerve but not in the dorsal column

We have previously shown that the size of the neural stem cell pool in the subventricular zone is significantly increased by loss of the BAX/BAK checkpoint (Lindsten et al. 2003). Therefore, we next examined whether the BAX/BAK checkpoint affected the size of the NG2-positive adult OPC pool as well. The optic nerve and spinal cord sections of the four genotypes, adjacent to those used for ASPA staining, were immunohistologically examined for NG2-positive cells. In optic nerves, NG2-positive cells were increased significantly in *bax*-SKO and DKO mice ($F_{3,11}=5.74$, *bax*-SKO: $P=0.012$; DKO: $P=0.034$; Fig. 6). The increase was proportional to that of all nucleated cells, as observed in mature oligodendrocytes. In dorsal columns, however, NG2-positive cells were not significantly increased in *bax*-SKO and DKO mice (Fig. 6e and Supplemental Fig. 2).

Cells of the oligodendroglial lineage in the white matter were not increased far beyond the normal proportion to myelinated axons by loss of the BAX/BAK checkpoint

Axons are believed to be a principal regulator of the population size of oligodendrocytes. We hypothesized, therefore, that if the intrinsic apoptotic pathway is involved in this regulatory process, oligodendrocytes per unit of axons should be increased more in DKO compared to wild-type mice. To test this hypothesis, we compared myelinated axon numbers per 100 μm^2 area in the optic nerve and in the dorsal column, and the cross sectional areas of these tissues from DKO mice and wild-type mice, because the highest increase of mature oligodendrocytes in both tissues was observed in DKO mice. Myelinated axons in the cross-sections were immunohistochemically identified by their immunoreactivity to NF-M surrounded by the MBP-positive myelin using a confocal microscope (Fig. 7). Interestingly, the myelinated axon densities in the optic nerve and the dorsal column of DKO mice were nearly equal to those of wild-type mice (Fig. 7, and Tables 2 and 3). Therefore, the estimated total number of myelinated axons in either the optic nerve or the dorsal column was increased in DKO mice with a simple positive correlation with the transverse sectional area of the tissue. As demonstrated in Table 2, the transverse sectional area of the DKO optic nerve was increased by 1.8-fold compared with that of the wild-type optic nerve, whereas the number of ASPA-positive cells in the DKO optic nerve was increased by 2.2-fold. As a consequence, the averaged number of ASPA-positive mature oligodendrocytes per 10,000 myelinated axons was increased by 20% in the

DKO optic nerve compared to the wild-type optic nerve. Based on the same calculation, approximately 30% more ASPA-positive mature oligodendrocytes per 10,000 myelinated axons were present in the DKO dorsal columns compared to the wild-type dorsal columns (Table 3). On the other hand, NG2-positive OPCs per unit number of axons in DKO mice increased at most 10% in the optic nerve but not in the dorsal column compared with those in wild type mice (Tables 2 and 3).

Discussion

In vitro differentiation of highly purified OPCs to myelin-producing oligodendrocytes can be initiated by removal of mitotic factors, and is completed as a default process in the absence of other CNS populations. Indeed, availability of PDGF is a key extrinsic factor to determine the timing at which proliferating OPCs exit the cell cycle and differentiate *in vivo* (Calver et al. 1998; McKinnon et al. 2005; van Heyningen et al. 2001). However, *in vivo* differentiation of OPCs is likely to be a process which is more actively regulated by various extrinsic signals (Birchmeier 2009; Bozzali and Wrabetz 2004; Laursen and French-Constant 2007; See and Grinspan 2009; Vartanian et al. 1999), suggesting that *in vitro* differentiation is not identical to *in vivo* differentiation. We observed apoptosis of ‘differentiating’ oligodendrocytes prior to full maturation after *in vitro* differentiation. This apoptosis might be a consequence of removal of mitotic factors rather than lack of axon-derived survival signals, as shown in other cell types (Wei et al. 2001). Even if this is the case, our *in vitro* finding of long-term survival of MBP-positive oligodendrocytes from *bax*^{-/-} *bak*^{+/-} and DKO mice still supports our conclusion that loss of the BAX/BAK checkpoint is sufficient to make mature oligodendrocytes resistant to apoptosis in the absence of axonal support.

In most lineages, either *bax*- or *bak*-deficient cells are as susceptible to apoptosis by various insults as wild-type cells (Cheng et al. 2001; Lindsten et al. 2000; 2003; 2005; Wei et al. 2001). In oligodendrocytes, however, a single *bax* allele was sufficient to restore normal susceptibility to *in vitro* differentiation-induced apoptosis, whereas *bax*^{-/-} *bak*^{+/-} oligodendrocytes demonstrated partial resistance. This predominance of BAX over BAK in proapoptotic function is consistent with prior studies of oligodendrocytes (Dong et al. 2003) and of subsets of peripheral and CNS neurons during development (Deckwerth et al. 1996; White et al. 1998). Although these results are partly explained by gene dosage, as demonstrated by 10-fold higher *bax* mRNA levels compared with *bak* mRNA levels in O4-positive cells (Fig. 2), it still remains to be clarified why BAX is the predominant determinant in oligodendroglial apoptosis induced by *in vitro* differentiation.

In general, known survival promoting-factors for oligodendrocytes, such as insulin-like growth factor-1, ciliary neurotrophic factor, neurotrophin-3, and neuregulin-1, bind to cognate receptor tyrosine kinases (RTKs), and subsequently activate intracellular signaling cascades including the mitogen-activated protein kinases, and phosphatidylinositol 3-kinase-Akt pathways. Both pathways are directly and indirectly involved in transcriptional control and posttranslational modification of several Bcl-2 family proteins, particularly BAD and BIM, members of “BH3 (BCL-2 homology 3) domain-only” protein (BOP) subclass (Brunet et al. 2001). In a model of the BAX/BAK checkpoint, allosteric conformational changes of BAX and BAK, directly or indirectly triggered by active BOPs, elicit the mitochondrial outer membrane permeability (Cheng et al. 2001). Therefore, it is reasonable that the post-mitochondrial apoptotic events cannot take place in oligodendrocytes lacking both BAX and BAK, even though some BOPs are activated by loss of RTK-mediated survival signals.

The number of axons is believed to determine the number of oligodendrocytes (Barres and Raff 1999; Miller 2002). This notion was supported by evidence that, when the axon number of the optic nerve was experimentally increased by overexpressing human BCL-2 under the

neuron-specific enolase (NSE) promoter, the number of oligodendrocytes was increased in proportion to the increase in axons by means of a decrease in normal oligodendrocyte death and an increase in OPC proliferation (Burne et al. 1996). However, oligodendrocytes in the NSE-Bcl-2 transgenic mouse were susceptible to apoptosis by loss of axonal contact, and not rescued from cell death when the nerve was transected (Burne et al. 1996). In contrast, we show that *bax/bak* DKO oligodendrocytes are resistant to apoptosis in the absence of axons, at least *in vitro*. The DKO mouse model thus provides a more stringent model system to directly evaluate the contribution of apoptotic elimination mediated by the intrinsic pathway to homeostatic control of oligodendrocyte numbers *in vivo*.

White et al. (1998) reported that many of the extra axons were atrophic in the *bax*-deficient optic nerve resulting in a 44% increase in the axon number per unit area of the optic nerve. Nevertheless, we observed no significant difference in axon numbers when we compared wild-type, *bax* single deficient (data not shown) and DKO optic nerves (Fig. 7 and Table 2). Moreover, the axonal density and total axon number per unit area results were quite comparable to those previously reported for wild type mice (Burne et al. 1996; Inman et al. 2006). Therefore, we concluded that a relatively physiological oligodendrocyte to axon ratio (at most 1.2-fold increase) can be established in the adult optic nerve even in the absence of apoptotic elimination through the intrinsic pathway in oligodendrocytes and in retinal ganglion neurons. Recent studies on demyelinating diseases have revealed an additional role of myelinating oligodendrocytes in supporting long-term axonal function and survival, thus resulting in axonal loss after a prolonged period of demyelination (Nave and Trapp 2008). This mutual dependence might be attributable to homeostatic balance between myelinating oligodendrocytes and myelinated axons as well.

We obtained similar results in the dorsal columns, although the extent of the increase in oligodendrocytes was much milder than that of the optic nerve. This is presumably due to the heterogeneity of dependence on programmed cell death among the subsets of neurons whose axons make up the dorsal column. In addition, the number of oligodendrocytes that undergo apoptosis during development might be different amongst CNS regions. De Louw et al. (2002) reported that only 32% of all the TUNEL-positive apoptotic cells in the spinal cord white matter of neonatal rats were positive for an oligodendrocyte marker. This percentage was far lower than the number shown by Barres's group, where a maximum of 91% of the dead cells were identified as oligodendrocytes in the developing optic nerve. Despite the methodological difference between the two studies, the incidence of developmental apoptosis of oligodendrocytes is likely to be lower in the spinal cord than in the optic nerve.

On the other hand, little is known about the homeostatic quantitative control of adult oligodendroglial progenitor cells. Our results indicate that the increase in adult NG2-positive cells due to inactivation of the intrinsic apoptotic pathway is smaller than the increase in ASPA-positive oligodendrocytes. Logically, the following four factors determine the number of adult NG2-positive cells; 1) their proliferation rate, 2) number of these cells migrating in or out of the tract, 3) number of these cells differentiating into other cell types, and 4) number of these cells undergoing cell death. Since very few progenitor cells undergo apoptosis (McBride et al. 2003), it is quite unlikely that this death mechanism has a principal role in determining the physiological number, which is in agreement with our result. In contrast, neural stem-like cells accumulate in the subventricular zone of the cerebral hemispheres in the DKO brain, suggesting that the BAX/BAK checkpoint negatively regulates the size of the neural stem cell pool (Lindsten et al. 2003). Our observation suggests that there is a substantial difference in the contribution of the intrinsic apoptotic pathway to quantitative regulation of the two distinct cellular pools for regeneration in the adult CNS.

In summary, our results suggest that, although loss of the BAX/BAK check point substantially increases both oligodendrocytes and axons, other mechanisms such as axonal influence on proliferation and migration of OPCs could maintain numbers of the oligodendroglial lineage relatively proportional to axonal numbers. However, conditional inactivation of the BAX/BAK checkpoint in the oligodendroglial lineage will be required to further clarify this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Immunopanning-purified mouse oligodendroglial cells. (a) Phase contrast microscopy of O4-positive cells at 24 hr in the GM medium after immunopanning. These cells were heterogeneous in morphology at this time point, when the cells were switched to the DM medium to induce terminal differentiation. Phase contrast (b) and immunofluorescence (c) pictures of oligodendroglial cells from a single wild-type neonatal mouse at 2 days in the DM medium. The cells were immunocytochemically labeled for O4 antigen (red) and the nuclei were stained with Hoechst 33258 (blue) (c). Concomitant O4-negative cells (arrows) accounted for less than 5% but could not be eliminated completely in the primary cultures in this study. Approximately 30% of the cells were already dead with fragmented or shrunk nuclei as shown by Hoechst staining (arrow heads). These dead cells are easily identified in the phase contrast picture (b). Scale bar, 100 μ m.

Figure 2.

Steady state mRNA levels of the four major multi-domain Bcl-2-related protein genes, *Bcl-2*, *Bcl-x*, *Bax* and *Bak* in O4-positive mouse cells of the oligodendroglial lineage from *bax*- and/or *bak*-deficient mice. The reverse transcribed cDNA levels were quantified by quantitative PCR (see the Materials and Methods). Each plot represents the calculated result from the cells from each single pup of the indicated genotype. Null mutation of *bax*, *bak* or both caused no remarkable compensatory transcriptional changes in the antiapoptotic genes. Abbreviations for the genotypes are WT; wild-type, *bak*-SKO; *bax*^{+/+} *bak*^{-/-}, *bax*-SKO; *bax*^{-/-} *bak*^{+/+} and DKO; *bax*^{-/-} *bak*^{-/-}. Note that the data are plotted as ratios to copy numbers of *GAPDH* cDNA on a logarithmic scale.

Figure 3.

TUNEL assay of oligodendrocyte cultures following *in vitro* differentiation. Cultures from wild-type (a), *bax*^{+/−} *bak*^{−/−} (b), *bax*^{−/−} *bak*^{+/−} (c), and *bax*^{−/−} *bak*^{−/−} (d) mice at 5 days in the DM medium are shown. Phase contrast pictures are superimposed on the fluorescent pictures to demonstrate the morphology of dead and alive cells. TUNEL-positive cells were labeled with tetramethylrhodamine (pink, arrowheads) in addition to the nuclear staining with Hoechst (blue). Scattered DNA remnants stained with tetramethylrhodamine are also present in the pictures, because removal of the cells from the positive panning plates at the final step of the immunopanning causes cell damage. However, these are easily distinguished from TUNEL-positive nuclei. Scale bar, 100 μm. E. Incidence of TUNEL-positive oligodendrocytes in the cultures from single wild-type (open circles), *bax*^{+/−} *bak*^{−/−} (closed triangles), *bax*^{−/−} *bak*^{+/−} (closed squares), and *bax*^{−/−} *bak*^{−/−} (closed circles) mice at various time points after transfer to the DM medium. Data are presented as mean ±1SD.

Figure 4.

In vitro differentiation and viability of purified O4-panned oligodendrocytes.

Oligodendrocytes were stained for MBP (green) along with nuclear labeling (blue). Panel a to d demonstrate representative phase contrast (a, c) and immunofluorescence (b, d) pictures of wild type and $bax^{-/-} bak^{-/-}$ oligodendrocytes at 5 days in the DM medium. By this time point, most of the cells were dead (arrowheads in b) without expression of MBP in the wild-type culture (a, b), whereas both MBP-positive and MBP-negative (asterisks) cells were almost all viable in the DKO culture (c, d). Note that MBP-positive mature oligodendrocytes were more robust in the DKO culture than in the wild-type culture. Panel e to h demonstrate representative phase contrast (e, g) and immunofluorescence (f, h) pictures of $bax^{-/-} bak^{+/-}$ and $bax^{-/-} bak^{-/-}$ oligodendrocytes at 14 days in the DM medium. Despite the presence of some dead cells (arrowheads in e and f), many cells still remained alive and MBP-positive in the $bax^{-/-} bak^{+/-}$ culture (e, f). Almost all DKO oligodendrocytes were alive and MBP-positive (g, h) even at 14 days after induction of *in vitro* differentiation. Note that MBP-immunoreactivity shows irregular punctate staining in both cultures at this time point (compare with d). Arrows indicate concomitant fibroblast-like cells. Scale bar, 100 μ m.

Figure 5.

ASPA-positive mature oligodendrocytes in the optic nerve and in the dorsal column of the T1 spinal cord. Six μm -thick tissue cross-sections were stained for ASPA (red) followed by DAPI staining (blue), and the fluorescent images were superimposed. Panel a-d demonstrate representative images of ASPA-positive cells in the optic nerve of adult wild-type (a), *bax*^{+/+} *bak*^{-/-} (b), *bax*^{-/-} *bak*^{+/+} (c) and *bax*^{-/-} *bak*^{-/-} (d) mice (representative ASPA-positive cells are enlarged in the inset). Scale bar, 100 μm . Total nucleated (DAPI-positive) cells and ASPA-positive cells were counted in each 6 μm -thick cross-section of the optic nerve and of the dorsal column. The summarized results of nucleated cell numbers and ASPA-positive cell numbers per cross-section from at least three animals of each genotype are shown in graph e and graph f, respectively. Abbreviations for the genotypes are wt/wt; wild-type, wt/Ho; *bax*^{+/+} *bak*^{-/-}, Ho/wt; *bax*^{-/-} *bak*^{+/+} and Ho/Ho; *bax*^{-/-} *bak*^{-/-}. In the optic nerve, ASPA-positive cell numbers were significantly increased in *bax*-SKO (150 ± 31 , $n=4$) and DKO (186 ± 41 , $n=3$) compared to the wild type control mice (76 ± 7 , $n=5$). In the dorsal column, ASPA-positive cell numbers were also significantly increased in DKO (206 ± 37 , $n=3$) compared to the wild type control mice (125 ± 25 , $n=5$), but not in *bax*-SKO (134 ± 32 , $n=4$) mice. n indicates number of the animals examined. Data are presented as mean $\pm$ 1SD. ** $p < 0.01$, * $p < 0.05$.

Figure 6.

NG2-positive cells in the optic nerve and in the dorsal column of the T1 spinal cord. Six μm -thick tissue cross-sections were stained for NG2 (red) followed by DAPI staining (blue). Panel a-d demonstrate representative images of NG2-positive cells (an enlarged representative NG2-positive cell is shown in the boxed inset) in the optic nerve of adult wild-type (a), *bax*^{+/+} *bak*^{-/-} (b), *bax*^{-/-} *bak*^{+/+} (c) and *bax*^{-/-} *bak*^{-/-} (d) mice. Scale bar, 100 μm . NG2-positive cells were counted in each 6 μm -thick cross-section of the optic nerve and of the dorsal column.

Graph e demonstrates the summarized results of NG2-positive cell numbers per cross-section of the optic nerve and the dorsal column from at least three mice of each genotype.

Abbreviations for the genotypes are wt/wt; wild-type, wt/Ho; *bax*^{+/+} *bak*^{-/-}, Ho/wt; *bax*^{-/-} *bak*^{+/+} and Ho/Ho; *bax*^{-/-} *bak*^{-/-}. NG2-positive cell numbers in the optic nerve were significantly increased in *bax*-SKO (17 ± 3.1 , $n=5$) and DKO (16 ± 6.6 , $n=3$) mice compared to the wild type control mice (9.0 ± 3.9 , $n=4$), whereas there was no significant difference in NG2-positive cell numbers in the dorsal column among genotypes (n indicates number of the animals examined). Data are presented as mean $\pm$ 1SD. ** $p < 0.01$, * $p < 0.05$.

Figure 7.

High-magnification photomicrographs of the optic nerve and the dorsal column of the T1 spinal cord. The optic nerve (a, c) and the dorsal column (e, g) cross sections from wild-type (a, e) and *bax*^{-/-} *bak*^{-/-} (c, g) mice were double labeled for NF-M (red) and MBP (green). All the axons immunopositive for NF-M were counted in the single channel images for NF-M as shown in b, d, f, and h. There was a clear difference in axonal diameters and densities between the optic nerve and the dorsal column, with smaller and more numbers of axons in the cross sections of the optic nerve. However, there was no apparent difference in axonal diameters and densities between the wild-type optic nerve (a, b) and the DKO optic nerve (c, d). The findings were similar between the wild-type dorsal column (e, f) and the DKO dorsal column (g, h). Abbreviations used are wt; wild-type, ON; optic nerve, T1; dorsal column of the T1 spinal cord. Scale bar, 10 μ m.

Table 1

Incidence of myelin basic protein-positive oligodendrocytes from wild-type and *bax*^{-/-} *bak*^{-/-} (DKO) mice after *in vitro* differentiation. Approximately 300 cells on average in at least four image fields were examined in each independent experiment.

Days in the DM medium	Wild-type	<i>bax</i> ^{-/-} <i>bak</i> ^{-/-} (DKO)
5	60 ± 24% (n=3)	65 ± 8% (n=4)
9	N.D.	85 ± 7% (n=4)

Mean ± 1 SD. N.D.; not determined, because the majority of the cells were dead.

Table 2

Comparison of axons, ASPA-positive and NG2-positive cells in the optic nerve of wild-type and DKO mice.

	Wild type mice	DKO mice	DKO/Wild type (fold change)
Cross sectional area of the optic nerve ($\times 10^3 \mu\text{m}^2$)	95 $\pm$ 15 (n=8)	168 $\pm$ 27 (n=8) ($t_{11,2}=6.67$, $P=0.00003$)	1.8
Axon number per 100 μm^2 area of the optic nerve	46 $\pm$ 8 (n=8)	49 $\pm$ 9 (n=8) ($t_{13,6}=0.732$, $P=0.477$)	1.1
Estimated axon number in the optic nerve ($\times 10^3$)	44 $\pm$ 9 (n=8)	82 $\pm$ 20 (n=8) ($t_{9,95}=4.88$, $P=0.0007$)	1.9
ASPA ⁺ cell number in a 6 μm -thick cross-section of the optic nerve	90 $\pm$ 20 (n=8)	194 $\pm$ 29 (n=8) ($t_{12,5}=8.31$, $P=0.000002$)	2.2
NG2 ⁺ cell number in a 6 μm -thick cross-section of the optic nerve	9 $\pm$ 3 (n=5)	18 $\pm$ 5 (n=6) ($t_{8,08}=4.03$, $P=0.004$)	2.0
Calculated number of ASPA ⁺ cells per 10,000 axons per cross-section	21 $\pm$ 6 (n=8)	26 $\pm$ 9 (n=8) ($t_{12,3}=1.07$, $P=0.307$)	1.2
Calculated number of NG2 ⁺ cells per 10,000 axons per cross-section	2.3 $\pm$ 0.9 (n=5)	2.5 $\pm$ 0.9 (n=6) ($t_{8,62}=0.342$, $P=0.741$)	1.1

n indicates the number of animals examined. Statistical values (Welch's unpaired *t*-test) in comparison between DKO and wild-type mice are shown.

Table 3

Comparison of axons, ASPA-positive and NG2-positive cells in the dorsal column of the T1 spinal cord of wild-type and DKO mice.

	Wild type mice	DKO mice	DKO/Wild type (fold change)
Cross sectional area of the dorsal column ($\times 10^3 \mu\text{m}^2$)	188 $\pm$ 22 (n=4)	224 $\pm$ 19 (n=6) ($t_{5,82}=2.65$, $P=0.039$)	1.2
Axon number per specified 100 μm^2 area of the dorsal column #	24 $\pm$ 4 (n=4)	24 $\pm$ 4 (n=6) ($t_{6,62}=0.079$, $P=0.939$)	1.0
Estimated axon number in the dorsal column ($\times 10^3$)	44 $\pm$ 5 (n=4)	53 $\pm$ 7 (n=6) ($t_{7,71}=2.29$, $P=0.053$)	1.2
ASPA ⁺ cell number in a 6 μm -thick cross-section of the dorsal column	128 $\pm$ 19 (n=4)	206 $\pm$ 29 (n=6) ($t_{7,98}=5.14$, $P=0.0009$)	1.6
NG2 ⁺ cell number in a 6 μm -thick cross-section of the dorsal column	13 $\pm$ 2 (n=4)	13 $\pm$ 2 (n=4) ($t_{5,28}=0.244$, $P=0.817$)	1.0
Calculated number of ASPA ⁺ cells per 10,000 axons per cross-section	30 $\pm$ 7 (n=4)	39 $\pm$ 6 (n=6) ($t_{5,70}=2.18$, $P=0.075$)	1.3
Calculated number of NG2 ⁺ cells per 10,000 axons per cross-section	2.9 $\pm$ 0.6 (n=4)	2.5 $\pm$ 0.2 (n=4) ($t_{3,65}=-1.10$, $P=0.338$)	0.9

n indicates the number of animals examined. Statistical values (Welch's unpaired *t*-test) in comparison between DKO and wild-type mice are shown.

see the Materials and Methods for the specification.