Spontaneous Optic Nerve Compression in the Osteopetrotic (*op/op*) Mouse: A Novel Model of Myelination Failure

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We report a focal disturbance in myelination of the optic nerve in the osteopetrotic (*op/op*) mouse, which results from a spontaneous compression of the nerve resulting from stenosis of the optic canal. The growth of the *op/op* optic nerve was significantly affected, being maximally suppressed at postnatal day 30 (P30; 33% of age matched control). Myelination of the nerve in the optic canal was significantly delayed at P15, and myelin was almost completely absent at P30. The size of nerves and myelination were conserved both in the intracranial and intraorbital segments at P30, suggesting that the axons in the compressed site are spared in all animals at P30. Interestingly, we observed recovery both in the nerve size and the density of myelinated axons at 7 months in almost half of the optic nerves examined, although some nerves lost axons and became atrophic. *In vivo* and *ex vivo* electrophysiological examinations of P30 *op/op* mice showed that nerve conduction was significantly delayed but not blocked with partial recovery in some mice by 7 months. Transcardial perfusion of FITC-labeled albumin suggested that local ischemia was at least in part the cause of this myelination failure. These results suggest that the primary abnormality is dysmyelination of the optic nerve in early development. This noninvasive model system will be a valuable tool to study the effects of nerve compression on the function and survival of oligodendrocyte progenitor cells/oligodendrocytes and axons and to explore the mechanism of redistribution of oligodendrocyte progenitor cells with compensatory myelination.

Introduction

Myelination of the rat optic nerve is preceded by the well-coordinated postnatal migration of oligodendrocyte progenitor cells (OPCs) from the chiasmal end of the nerve to the retina (Small et al., 1987). Ensheathment of axons begins at postnatal days 6 (P6) and 7 (P7) and is not complete until P110 (Foran and Peterson, 1992; Dangata and Kaufman, 1997). The simple alignment of axons, the lack of neurons, and the well-described spatial-temporal series of events that occur during development have led to the optic nerve being a key structure in studying OPC migration and the signals that trigger survival, differentiation, and myelination (Skoff et al., 1980; Barres and Raff, 1994; Colello et al., 1995). Here, we report a disruption in this series of events in the osteopetrotic mouse (*op/op*) where compression of the nerve during development leads to a focal disturbance of myelination in the optic canal.

Osteopetrosis is a disease of both humans and animals in which genetic dysfunction in osteoclasts leads to a failure in bone remodeling (Tolar et al., 2004). The *op/op* mouse has an inactivating mutation in the colony-stimulating factor-1 (CSF-1) gene (Wiktor-Jedrzejczak et al., 1990; Yoshida et al., 1990). Because osteoclasts as well as the cells of monocytic lineage require CSF-1 for their development (Kodama et al., 1991a,b), the mouse develops osteopetrosis. The osteopetrotic features of *op/op* mice include the absence of incisors, a domed skull, shorter extremities, etc (Marks and Lane, 1976). However, the narrowed optic canal and compression of the optic nerve have not been reported to date. A previous study of *op/op* mice reported abnormalities in the visual-evoked potentials (VEP), but this was ascribed to neuronal abnormalities in the brain and the optic nerves were not examined (Michaelson et al., 1996).

Although blindness is one of the major symptoms in human osteopetrosis (Steward, 2003), the origin and cause of the visual disturbance are not well understood (Curé et al., 2000; Kerr et al., 2000; Cummings and Proia, 2004) perhaps because of the lack of an appropriate animal model. We describe here the first evidence that compression of the nerve in the optic canal results in a myelin defect that could account for visual disturbance in osteopetrosis in humans. To determine whether the focal compression of the optic nerve in *op/op* mice results from a developmental defect in myelination or demyelination and to help shed light on the pathophysiology of blindness in human osteopetrosis, we studied the temporal series of glial and myelin development in the optic

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Figure 1. The optic nerve of *op/op* mice is compressed at the optic canal. **A**, The optic nerve of 7-day-old *op/op* mouse. The nerve is translucent, indicating that myelination has not begun or is incomplete and compression of the nerve is not seen at the optic canal (arrowhead). **B**, Optic nerves on the cranial base of the *op/op* mouse at P30. The nerve compression coincided with the optic canal (arrows). The left compressed nerve was exposed by removing the sphenoid bone, showing that the compression was confined to the intracanalicular segment. OC, Optic chiasm; IC, intracranial nerve; IO, intraorbital nerve. **C**, Bilateral compression of the optic nerve of *op/op* mouse at P30, removed from the cranium. The compressed areas were translucent, indicating lack of myelin. **D**, Chronologically aligned optic nerves showed that the compression in *op/op* mice was present from P10 to P60, whereas the noncompressed wild-type (wt) nerve is not compressed and is normally myelinated at the optic canal. Scale bar: **D**, 1 mm.

nerve. We explored the OPC, glial cell, and myelin distribution in the nerve with time, the effect the myelin defect has on conduction in the nerve, and the potential ischemic basis for the lack of myelin in the optic canal. In addition, we determined whether spontaneous recovery could occur with time if the canal enlarged.

Materials and Methods

Animals. Breeding mouse pairs heterozygous for the *op* mutation (*csf1*^{+/*op*}) were purchased from The Jackson Laboratory. The colony was maintained at University of Wisconsin-Madison. Methods for animal husbandry, experimental procedures, and death in this study were approved by the University's Animal Care and Use Committee. Genotypes of their offspring were identified using PCR of genomic DNA from tail biopsies at P4–P10 (Lieschke et al., 1994). Absence of incisors in the *op/op* mutant causes problems with feeding and nutritional intake, resulting in their fatality before weaning (Ramnaraine and Clohisy, 1998). Therefore, to ensure sufficient nutrition for *op/op* pups, we adjusted the number of *op/op* pups to 4–6 per litter after the PCR genotyping. At P16, *op/op* mice were weaned and fed with liquid diet (D10012, Research Diets) twice a day in a 35 mm plastic dish lid placed on the cage floor. Powdered chow (Teklad, Harlan) and bottled water were also accessible *ad libitum*. This weaning protocol completely resolved the problem of early death of *op/op* mice (Kondo and Duncan, 2009). Wild-type control mice were weaned at P21 and fed only with pellet food.

Toluidine blue staining and electron microscopy

Wild-type mice and *op/op* littermates at 7, 10, 12, 15, 30, and 60 days and 7 months of age were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and then perfused transcardially with 10 mM PBS (PBS, pH 7.2) followed by Karnovsky fixative. The optic nerves were postfixed in the same fixative and 1% osmium tetroxide sequentially, and embedded in Epon; 1 μ m semithin sections were stained for myelin with 1% toluidine blue/1% sodium borate. For ultrastructural study, the plastic-embedded optic nerve was cut with a diamond knife at 80 nm, and sections mounted on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7600 electron microscope.

Immunohistochemistry. Mice were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The optic nerves were re-

moved, postfixed in the same fixative for 10 h, cryoprotected in 15% sucrose/PB, and cut on a cryostat horizontally at 10 μ m thickness. The following primary antibodies were used: rat anti-myelin basic protein (MBP, Millipore, 1:200), rabbit anti-neurofilament-200 (Millipore, 1:5000), rabbit anti- π form of glutathione S-transferase (GST- π , MBL, 1:10,000), rabbit anti-NG2 chondroitin sulfate proteoglycan (Millipore, 1:500), rabbit anti-glial fibrillary acidic protein (GFAP, Dako North America, 1:2000), and rat anti-mouse CD45 (Serotec, 1:500).

TUNEL assay and Ki67 immunostaining

At 8, 10, 12, and 15 days of age, the mice were perfusion-fixed with 4% paraformaldehyde ($n = 6$ nerves from three mice each group). The optic nerves were cut horizontally at 10 μ m and mounted on glass slides. TUNEL was performed using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions, except that the tissue was permeabilized by incubating slides with 0.3% Triton X-100 in PBS for 30 min instead of proteinase K. To identify mitotic cells, the slides were incubated in 10 mM citrate buffer, pH 6.0, for 30 min at 80°C, followed by reaction with rat anti-mouse Ki67 antigen antibody (1:20, Dako North America) for 1 h and AlexaFluor-488-conjugated donkey anti-rat IgG (Invitrogen) for 1 h. TUNEL-positive cells or Ki-67-positive cells were counted on photomicrographs in the intracanalicular, intracranial, and intraorbital segments. Cells were counted as positive only when DAPI nuclear stain overlapped. Subpial cells were excluded from counting. Using the MetaVue image analysis software (Molecular Devices), ratios of the number of TUNEL- or Ki67-positive cells to integrated signal intensities of DAPI nuclear counterstaining in the photomicrographs of immunostained optic nerve area were obtained and defined as TUNEL- or Ki67-index, respectively, assuming a positive correlation between the DAPI signals and the cell number.

Double immunofluorescent staining

The animals were perfusion-fixed with 2% paraformaldehyde in PB at P30. The optic nerves were removed immediately, postfixed in the same fixative for 30 min, cryoprotected, frozen with dry ice-cooled isopentane, cut horizontally on a cryostat at 10 μ m, and mounted on glass slides. The slides were incubated either with anti-Nav1.6 antibody (rabbit polyclonal, diluted 1:200, kind gift from Dr. Matthew Rasband) for 1 h, then with anti-caspr mouse monoclonal antibody (NeuroMab) for 1 h to label

Figure 2. Myelination is inhibited in the intracanalicular optic nerve of *op/op* mice. Toluidine blue-stained cross sections from the optic nerves of *op/op* mice at 7, 10, 15, 30, and 60 days, and 7 months of age. Whereas myelin develops with time both in the intraorbital (left panels) and intracranial (right panels) segments, the compressed intracanalicular nerve segment (middle panels) had only sporadic myelination of axons at P15 and P30, suggesting a failure in myelination. The appearances in the intraorbital and intracranial segments were comparable with those of wild-type control (data not shown). Note that only a few cell nuclei were identified in the intracanalicular nerve at P30. Scale bars: 20 μm ; insets, 200 μm .

Figure 3. Nerve area, axon myelination, and glial cell number are reduced in the compressed portion of the *op/op* optic nerve. **A**, Chronological changes in the cross-sectional area of *op/op* optic nerves. The areas of optic nerve cross section increase with time in areas of wild-type mice and the intraorbital and intracranial segments of *op/op* mice. However, the growth of optic nerve was suppressed in the intracanalicular segment of *op/op* mice from 7 through 60 d and showed partial recovery at 7 months. Data are mean $\pm$ SEM of 6 nerves from 3 animals. **B**, Chronological changes in the density of myelinated fibers in *op/op* optic nerves. The lack of myelin was most pronounced at P30 in the intracanalicular segment of *op/op* optic nerve, and myelination slowly proceeds thereafter. Data are mean $\pm$ SEM of 6 nerves from 3 animals. Significance was measured by one-way ANOVA followed by Scheffé's *F* post hoc test. Chronological changes in the number of glial cells in the intraorbital (**C**), intracanalicular (**D**), and intracranial (**E**) segments of the optic nerve. A significant decrease in glial number was seen only in the intracanalicular segment of *op/op* mice at P10, P15, P30, and P60, reaching the maximum reduction at P30. * $p < 0.05$, wild-type and *op/op* mice at the same age (one-way ANOVA followed by Bonferroni's post hoc test). ** $p < 0.01$, wild-type and *op/op* mice at the same age (one-way ANOVA followed by Bonferroni's post hoc test). *** $p < 0.001$, wild-type and *op/op* mice at the same age (one-way ANOVA followed by Bonferroni's post hoc test).

nodal and paranodal components of the node of Ranvier. Alexa Fluor-488-conjugated anti-rabbit IgG (1:100) and Alexa-Fluor-594-conjugated anti-mouse IgG (1:100) secondary antibodies were applied for 1 h. The antibodies described above were diluted in PBS containing 5% donkey serum. The slides were coverslipped using SlowFade (Invitrogen) and observed and photographed using the SPOT CCD camera (Diagnostic Instruments) on the Nikon Eclipse E800M light microscope. Negative control staining by omitting primary antibodies showed fluorescent signals in some vessels and leptomeninges, which originated from the anti-mouse secondary antibody. However, this nonspecific staining did not interfere with observation of axonal staining.

Morphometric analyses of myelination

The whole nerve was embedded noting the retinal end of the nerve and then the nerve cut in cross section from that end sequentially through the intraorbital portion, then the compression site in the optic canal, and then the intracranial nerve. Toluidine blue-stained cross sections from the three levels of the optic nerves were used in all morphometric analyses of myelin. At least 6 nerves (left and right) from 3 mice were analyzed from each group. The most severely constricted portion of nerve was selected to be representative of the intracanalicular segment of the *op/op* mice. Representative sections were taken from the mid portion between the intracanalicular segment and lamina cribrosa and between the intracanalicular segment and optic chiasm for the intraorbital and intracranial optic nerve, respectively. The area of the cross section of the optic

nerve was measured using the Bioquant image analysis software. All quantitation was done blinded.

To compare myelination in wild-type and *op/op* mice, the number of myelinated axons in the optic nerve was determined as previously described (Kondo et al., 2005; Kondo et al., 2011). Briefly, one rectangular area (1600×1200 pixels, $1246 \mu m^2$) was recorded from the central region of the optic nerve at each site of interest (intraorbital, canalicular, and cranial segments).

To evaluate the changes in the number of glia in the optic nerve, the number of cell nuclei was also counted in the toluidine blue-stained $1 \mu m$ cross sections. Subpial cells were excluded from the count.

FITC-BSA perfusion

To determine possible alternations in blood flow in the optic nerve of *op/op* mice, the vascular area within the nerve was measured. Wild-type and *op/op* mice ($n = 3$ each, P30) were transcardially perfused with PBS containing 10 U/ml heparin followed by 10 ml of 0.75 mg/ml FITC-BSA conjugate (Sigma-Aldrich) and 2% gelatin (Sigma-Aldrich) in 10 mM PBS and 20 ml of 0.75 mg/ml FITC-BSA conjugate and 4% gelatin in 10 mM PBS sequentially. All the reagents were injected at ~ 0.3 ml/s and kept at $\sim 37^\circ C$ during perfusion by maintaining the reagents at $43^\circ C$ before use. Immediately after perfusion, the animals were chilled on crushed ice for 10 min, and the optic nerves were removed and immersion-fixed in 4% paraformaldehyde/PB overnight at $4^\circ C$. The nerves were kept straight and embedded in 10% gelatin, so that cross sections could be cut precisely perpendicular to the axis through the entire optic nerve. All the sections were cut on the cryostat at $50 \mu m$ and collected and stored in order of sectioning in PBS containing 0.1% sodium azide at 10 sections per container. Six randomly selected sections per container were mounted on glass slides and coverslipped using SlowFade Gold (Invitrogen). Photographs were taken using the constant exposure time at 488 nm excitation for all slides examined, and the areas of FITC-containing vessels that had fluorescent intensities exceeding a fixed threshold value were determined using the MetaVue software.

Electroretinogram (ERG) and VEP

Two *op/op* and two wild-type mice were tested longitudinally at P30 and 7 months. Six *op/op* mice and six additional controls were tested at either P30 ($n = 3$) or 7 months of age ($n = 3$). Mice were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively, i.p.), and pupils dilated with 1.5% tropicamide. *In vivo* electrophysiologic recordings were made using a UTAS EMWIN system (LKC Technologies). ERGs were recorded using a shortened DTL corneal fiber electrode placed across the cornea wetted with 1.5% methylcellulose solution. A stainless steel subdermal reference electrode was inserted in cheek and rump for the reference and ground, respectively. Amplifiers gain for ERG was 1 K with a bandpass of DC–500 Hz. The VEP was recorded with the active subdermal electrode inserted along the midline of the visual cortex (Ren et al., 2000; Yu et al., 2011). The reference and ground was as for the ERG. The VEP amplifier gain was 20 K with bandpass from 1 to 100 Hz. The stimulus was a $3 \text{ cd} \cdot \text{s} \cdot \text{m}^{-2}$ xenon flash presented at a rate of 3/s presented on a dark ganzfeld background of $15 \text{ cd} \cdot \text{m}^{-2}$. Each trace is the averaged response to 80 flashes for both VEP and ERG recordings.

Ex vivo electrophysiology

Excised optic nerve preparation. Optic nerves (between the eye and the optic chiasm) were quickly excised from decapitated control and *op/op* mice. The nerves were mounted in a recording chamber for simultaneous electrophysiological and calcium imaging studies. The nerve was bathed with oxygenated (95% O₂ and 5% CO₂) Ringer's solution containing the following (in mM): NaCl 129, KCl 3, NaH₂PO₄ 1.2, CaCl₂ 2.4, MgSO₄ 1.3, HEPES 3, NaHCO₃ 20, and glucose 10 (Zhang et al., 2006). The pH of the solution was adjusted to 7.4 with NaOH or HCl as necessary.

Compound action potentials were recorded for analyzing conduction along optic nerves. Compound action potentials were evoked by a 125% supramaximal stimulus applied via the bipolar electrode to the cut end and recorded from a second suction electrode at the other cut end. The amplitude of the compound action potential data were analyzed using Pclamp 6.0 software (Molecular Devices). Nerve conduction was calculated by measuring the time between the stimulation artifact and the peak of compound action potentials and the distance of the nerve between the stimulating and recording electrodes.

For calcium imaging, we used a technique developed in Chiu's laboratory (Zhang et al., 2006) that selectively stains axons of rat optic nerves with calcium indicators. We used the cell-impermeant, high-affinity Oregon Green 488 BAPTA-1 (OGB-1, $K_d = 170$ nM, molecular weight = 1114) conjugated to the high molecular weight Dextran (molecular weight = 10,000; Invitrogen). Experiments were performed in axons of mice at P30. For dye loading, the normal saline solution in the recording pipette (attached to one cut end of the nerve) was replaced either with a high-K (140 mM) or a high-Na (140 mM), low calcium (0 calcium with or without 1 mM EGTA) solution containing 4–5 μ l of the cell-impermeant form of calcium indicators. Axons were stained by diffusion and/or axonal transport of the dyes from the cut end into the axonal cylinders. The final concentration of calcium dye in axons is estimated to be $<100 \mu$ M (Zhang et al., 2006). We typically performed calcium imaging at a site $\sim 1000 \mu$ m away from the dye-loading pipette. The dyes were allowed to remain in the loading pipette (also serve as the recording pipette) during the entire experiment. Calcium levels were not reported in absolute levels but as $\Delta F/F_0$, where F_0 is the baseline fluorescence signal before action potential stimulation.

Calcium imaging

Calcium images were viewed with a 40 $\times$ (Olympus) objective lens coupled to a Yokogawa spinning disk system (Zhang et al., 2006). The dyes were excited by an argon laser at 488 nm and confocal fluorescence signals collected through a 500-nm long-pass emission filter. Images of single axons were selected, and calcium images were streamed at a rate of 40 ms between frames. Image acquisition and on-line calculations were controlled through the MetaMorph software (Universal Imaging). All action potential and calcium imaging experiments were performed at a room temperature of $\sim 25^\circ\text{C}$.

Statistical analyses

Data are presented as mean $\pm$ SEM. Differences were assessed by one-way ANOVA followed by Bonferroni's *post hoc* test, except that an unpaired *t* test was used in comparisons for the nerve conduction velocity (see Fig. 10B) and calcium response (see Fig. 10D). Values of $p < 0.05$ were considered to be statistically significant.

Figure 4. The compressed nerve of the *op/op* mouse lacks glia at P60, although axons are unaffected. **A**, At P60 the intracanalicular segment of the optic nerve lacks glial cell nuclei in a majority of the nerve. A portion of the nerve in which glial cells are present coincides with a small number of adjacent myelinated fibers (*; enlarged in **B**). **C**, By contrast, the intracranial portion of the same nerve has a normal density of myelinated fibers, confirming the presence of a normal axon density along the entire nerve (*; enlarged in **D**). Scale bars: **A**, **C**, 50 μ m; **B**, **D**, 5 μ m.

Figure 5. Electron micrographs of the compressed optic nerve of *op/op* mice at P30. Most axons were myelinated in the intraorbital (**A**) and intracranial (data not shown) segments. However, the majority of axons were unmyelinated in the intracanalicular segment, and in this nerve only one myelinated axon (arrow) is present (**B**). Myelin debris or degenerating axons were not seen. Scale bars: **A**, 2 μ m; **B**, 1 μ m.

Results

Constriction in the optic nerve of *op/op* mice is associated with a focal reduction in number of myelinated axons and glia cells

On gross inspection, all *op/op* mice examined at P30 had bilateral constriction of the optic nerve in the optic canal (Fig. 1B, arrows). The compressed nerve was pale or transparent in contrast to the rest of the nerve, which was white because of myelination. At P7, myelination was not yet grossly obvious throughout the nerve, nor was the nerve constricted (Fig. 1A, arrowhead). The constriction, which was variable in degree between nerves, was consistently noted from P10 to P60 (Fig. 1D).

To determine whether the transparency of the optic nerve in the canal was the result of failure in myelination (dysmyelina-

Figure 6. At 7 months, the optic nerves in *op* mice show variable recovery. In the first mouse (**A**, **D**, **H**), the right optic nerve cannot be seen *in situ* (**A**), yet when trimmed and embedded it can be seen that the nerve is atrophic (**D**) and degenerates with only scattered myelinated axons present (**H**). In the second mouse (**B**, **E**, **F**, **I**, **J**), the left nerve has recovery of nerve area and axon myelination, whereas the right nerve is noticeably smaller (**B**, **F**), though well myelinated (**J**), possibly through the loss of some axons. In the third mouse (**C**, **G**, **K**), both the left and right optic nerves remain compressed at the canal, and there were only scattered myelinated fibers, yet axons appear to remain intact (**G**, **K**). Scale bars: **D–G**, 100 μ m; **H–K**, 10 μ m.

tion) or loss of myelin (demyelination), we chronicled myelin development of the *op/op* optic nerves at the intraorbital, intracranial, and intracranial segments from P7 through 7 months of age (Figs. 2 and 3). Myelination in the optic nerves of wild-type mice begins at P6–P7 and is complete by $\sim$ P110 (Foran and Peterson, 1992; Dangata and Kaufman, 1997). Toluidine blue myelin staining of cross sections of *op/op* optic nerves at P7 through 7 months of age revealed that myelination proceeds normally in the *op/op* intraorbital and intracranial segments (Figs. 2 and 3B). However, the intracranial/constricted segment of *op/op* nerves was strikingly different to the proximal and distal nerve in terms of the absence of myelin and glial cells (Fig. 3B,D). In particular, the number of myelinated axons was significantly decreased in the intracranial segment of mutant animals from P15–P60 and in some cases beyond. The reduction in myelinated axons corresponded with a reduction in the number of glial cells in this segment of the nerve, a reduction that became evident by P10, and was maintained through P60 (Fig. 3). From P15 to P30, there was a notable reduction in glial cells within the constricted nerve in the canal. The correlation between reduced glia number and myelinated axons in the constricted portion of the optic nerve was most apparent in one *op/op* mouse at P60 where the majority of the nerve lacked glia (Fig. 4). This region of the nerve noticeably lacked myelinated axons, whereas the portion of the nerve that contained a few glial cell nuclei also had some scattered myelinated axons (Fig. 4A,B). The intracranial portion of the same nerve, however, was normal in size and had a normal density of glial cell nuclei and myelinated fibers (Fig. 4C,D), suggesting that the glial-free area at the intracranial region was not an area of axon loss and gliosis but contained intact axons.

Electron microscopy confirmed that most axons were myelinated in the intraorbital (Fig. 5A) and intracranial (data not shown) segments of the *op/op* optic nerve at P30. In contrast, the intracranial segment contained predominantly non-myelinated axons, with some nerves having only rare myelinated axons (Fig. 5B).

To assess the degree of constriction on the optic nerves of *op/op* mice throughout development, we measured the cross-sectional area of the optic nerve at the intraorbital, intracranial, and intracranial segments of optic nerves at ages P7 through 7 months. The measurement of cross-sectional areas of the optic nerves confirmed that the intracranial segment of the nerve in *op/op* mice was significantly compressed from P10 through P60 (Fig. 3A). At 7 months, although the area of intracranial optic nerve in *op/op* mice was still significantly smaller compared with that in the wild-type, there was a significant recovery in the nerve area compared with P60 ($p = 0.0177$; no significant change was found in the intracranial segment at any other times). Although myelination in the intraorbital and intracranial segments of *op/op* nerves proceeded similar to the wild-type mice (Fig. 3B), the densities of myelinated axons in the intracranial segment of *op/op* mice were significantly lower after P15 compared with the wild-type control (Fig. 3B) and the difference reached a maximum at P30. Although the differences were not statistically significant, the density slightly increased from P10 to P15, then decreased at P30 in the intracranial segment of *op/op* mice, suggesting some loss of myelin. However, it appeared that the majority of axons were never myelinated rather than demyelinated (Figs. 2 and 3B).

Figure 7. Glial cells are reduced in the compressed optic nerve of *op/op* mice at P30, but axons are preserved. Immunohistochemistry on the longitudinal sections (**A–D, G, H**) and cross sections (**E, F**) of optic nerves from wild-type and *op/op* mice at P30 (**E, F** are from *op/op* mice only). **A**, MBP immunolabeling showed the absence of myelin in the compressed lesion of *op/op* mice. **B**, Neurofilament (200 kDa) is positive in the entire length of optic nerves and highly immunoreactive in the compressed lesion because the nerve fibers converge in the area and probably because the lack of myelin favored reaction of the antibody. Mature oligodendrocytes (**C, E**) and OPCs (**D, F**) were immunolabeled by anti-GST- π antibody and anti-NG2 antibody, respectively. GST- π revealed diffuse staining of myelin as well as strong immunoreactivity in the oligodendrocyte cell bodies, both of which were absent in the intracanalicular segment of the *op/op* optic nerve (**C–F**). GFAP immunolabeling shows relative absence of processes and cell bodies of astrocytes in the core lesion of the compressed optic nerve. **G**, In the periphery, however, GFAP immunoreactivity is dense with abundant astrocytic processes. **H**, The number of microglia and macrophages was highly reduced in the *op/op* optic nerve, although the compressed lesion contained some activated microglia (or macrophages).

Figure 8. TUNEL and Ki67 antigen detection did not reveal the mechanism of cell reduction in the intracanalicular optic nerve of *op/op* mice, but the compressed optic nerve of *op/op* mice is potentially ischemic. **A**, The number of TUNEL-positive apoptotic cells in the optic nerve of *op/op* mice was higher than that of wild-type mice in all regions and at all time points examined. **B**, There was no difference in the number of Ki67-positive proliferating cells between wild-type and *op/op* mice. Data are presented as an index (number of TUNEL- or Ki67-positive cells divided by integrated DAPI signals in each region). $n = 6$ nerves from 3 animals per group. **C**, The optic nerve blood flow was estimated as the total FITC-positive pixel areas in 50 μm cross sections of the optic nerve of 30-day-old wild-type and *op/op* mice transcardially perfused with FITC-conjugated BSA in gelatin. **D**, Percentage vascular area is shown by dividing the FITC-positive pixels (as in **C**) over the pixel area of the cross section. Both graphs indicated significant reductions of the estimated blood flow in *op/op* mice at the intracanalicular segment and 0.5 mm distal to the site. Data are expressed as mean ± SEM ($n = 6$ nerves per group) at every 0.5 mm from the intracanalicular segment (canal) toward the intraorbital segment (positive values) and the intracranial segment (negative values). * $p < 0.05$, compared with wild-type control in the same region (one-way ANOVA followed by Bonferroni's *post hoc* test). ** $p < 0.01$, compared with wild-type control in the same region (one-way ANOVA followed by Bonferroni's *post hoc* test).

Compared with the compressed optic nerve as described at P15–P60, by 7 months of age the myelin defects had improved in many of the nerves, commensurate with an increase in size of the nerve. Indeed, in the earliest group of 7-month-old mice examined, the density was significantly increased from P30 to 7 months in the intracanalicular segment of *op/op* mice (Fig. 3B), although there was no difference in wild-type, suggesting that a number of axons were myelinated as the nerve size expanded. However, in mice studied subsequently, it was noted that this was very variable (Fig. 6). Of the 15 7-month-old *op/op* mice studied (30 optic nerves), the nerves were graded as follows (1) complete recovery, 14; (2) normal nerve with only small areas of subpial dysmyelination, 5; (3) poor myelination throughout nerve but intact axons, 4; and (4) total atrophy of the nerve, 7. In no case were both optic nerves atrophic and degenerate, and in only one mouse did both optic nerves appear similar to that seen at P30 (i.e., both nerves remaining translucent at the optic canal; Fig. 6C,G,K).

In the recovered intracanalicular nerves, many of the myelinated axons appeared to have thinner myelin sheaths than at

the same site in wild-type mice. The G-ratio (the ratio of the inner axonal diameter to the total outer diameter of myelinated fiber) of intracanalicular optic nerve in the 7-month-old *op/op* mice was 0.661 ± 0.0844 (mean ± SD, $n = 264$ myelinated axons from 6 mice), which was slightly but significantly larger than that of wild-type control (0.635 ± 0.0942 , 374 myelinated axons from 6 mice; $p < 0.001$, Mann–Whitney *U* test (see Kondo et al., 2005 for methods)).

Glia are reduced but axons are preserved at P30 throughout the compression site of *op/op* mice

To investigate how the nerve compression influences the cellular components in the optic nerve of wild-type and *op/op* mice at P30, we studied the changes in the expression of different neural cell markers immunohistochemically. Myelin as revealed by MBP immunolabeling was almost absent from the compressed lesion (Fig. 7A). In contrast, there was no loss of neurofilament immunoreactivity in the entire length of *op/op* optic nerves, indicating that the axons were preserved (Fig. 7B). Mature oligodendrocytes and OPCs as labeled by anti-GST- π antibody and anti-NG2 antibody, respectively, were absent or scarce in the compressed nerve of *op/op* mice (Fig. 7C–F). GFAP immunolabeling showed a relative absence of processes and cell bodies of astrocytes in the core lesion of compressed optic nerve (Fig. 7G). However, GFAP immunoreactivity was dense in the nerve adjacent to the compression site, with abundant astrocytic processes. Because of the CSF-1 deficiency, the number of microglia and macrophages was highly reduced in *op/op* mice (Fig. 7H). However, the compressed lesion contained some activated microglia or macrophages with stout, less fibrous processes and high CD45 immunoreactivity.

To explore the decreased number of glial cells in the intracanalicular optic nerve in *op/op* mice at P30 (Figs. 2, 3, and 4), we hypothesized that the nerve compression may cause glial cell death in the intracanalicular segment. Thus, we performed TUNEL in the longitudinal sections of mice ranging from P8 to P15 of age. We did not include P30 as the majority of cells were already lost by this time in the intracanalicular segment. Interestingly in *op/op* mice, there was no significant increase in the number of TUNEL-positive cells in the intracanalicular segments compared with that in intracranial and intraorbital segments (Fig. 8A). The number of TUNEL-positive cells in *op/op* mice was higher than that in wild-type mice at all times in all three regions examined with statistically significant differences at P8 in the intracranial and intraorbital segments (Fig. 8A). Finally, no evidence of dying cells or tissue debris was seen in 1 μm sections or EM.

This unexpected result led us to determine whether the compression of the optic nerve may cause suppressed proliferation of cells of

oligodendrocyte lineage rather than an increase in cell death. Therefore, we investigated the number of Ki67-immunoreactive proliferating cells in the optic nerve. Ki67-immunoreactive cells were seen throughout the nerve at all time points examined, with decreasing trend with time (Fig. 8B). However, there was no significant difference in the numbers between *op/op* and wild-type mice or among the regions in the same genotype.

Compressed optic nerve is ischemic

Although it is obvious that the stenotic optic canal is responsible for the compression of optic nerve in *op/op* mice, the direct cause for myelination failure is not clear at this time. One of possible explanations for the mechanism by which the cells in the compressed nerve die or do not remain within the nerve at the compression site is an ischemic change in the intracranial optic nerve. To determine whether this occurs, we estimated the relative blood flow in the optic nerve of *op/op* and wild-type mice by perfusing the animals with the fluorescent dye, FITC, and measured the local blood volume in the optic nerve. The vascular volume in a 50 μ m section was significantly decreased in the intracranial segment and the neighboring intracranial segment ($p = 0.018$) of *op/op* mice (Fig. 8C). The vascular volume per nerve area was also significantly decreased in the intracranial segment and the neighboring intracranial segment of *op/op* mice, indicative of local ischemia in the intracranial segment of *op/op* optic nerve (Fig. 8D).

Local myelination failure disturbs conduction in the *op/op* optic nerve

The histopathological and immunohistochemical demonstration of preserved axons in the intracranial segment and intact myelin/axon integrity in the intracranial segment (distal nerve projection to the compression site) in the *op/op* optic nerve led us to investigate functional alterations of the optic nerve in this mouse. Thus, we examined the electrophysiological function of the nerve *in vivo* and *ex vivo*, as well as immunohistochemical localization of voltage-gated ion channels.

We measured flash ERGs and VEPs to examine whether the retinal response is indeed normal in mutants and whether the constricted optic canal would affect the VEP during development. The VEP is sensitive to optic nerve compression (Holder, 2004) and delayed in children with osteopetrosis (Thompson et al., 1998). Figure 9A–H shows an example of the ERGs and VEP evoked by flash stimulation from two mice tested at P30 and again at 7 months. The control mouse had a well-developed photopic ERG B-wave (Fig. 9A, arrow) and oscillatory potentials (bandpass filtered ERGs not shown) at P30 and 7 months (Fig. 9A,C). VEP in the control undergoes considerable increase in amplitude during this period (Fig. 9B,D). The *op/op* mouse in this example also had normal appearing ERG, at both ages (Fig. 9E,G) with normal latency for the B-wave and oscillatory potentials. The VEP in the *op/op* mouse was barely recordable at P30 (Fig. 9F), yet a robust cortical response was elicited at 7 months (Fig. 9H). However, the VEP N1 wave at 7 months (Fig. 9D, arrow) was delayed in the *op/op* mouse (Fig. 9H). All control mice showed VEP responses at P30 (Fig. 9B) with markedly increased amplitudes at 7 months (Fig. 9D) consistent with previous studies of murine flash VEP development (Yu et al., 2011; 2012). The remaining *op/op* mice showed some measurable, generally low-amplitude VEP at P30 and increased VEP amplitude at 7 months. These data support the idea that optic nerve constriction disrupts retinocortical transmission in the *op/op* mouse early

Figure 9. Compressed optic nerve of *op/op* mice is functionally impaired *in vivo*. **A–H**, Representative ERG and VEP traces recorded simultaneously in response to a medium intensity flash for a wild-type (**A–D**) and an *op/op* mouse (**E–H**). At 30 d, the b-wave of the photopic ERG (arrow) is robust in both the control (**A**) and *op/op* mouse at 30 d (**E**). At 7 months, ERGs remained stable in the control (**C**) and *op/op* mouse (**G**). In the control, the N1 wave of the flash VEP is very low amplitude but clearly present at 30 d (**B**) and increased markedly in amplitude with development (arrow) (**D**). In contrast, the flash VEPs in the *op/op* mouse were very low amplitude at 30 d (**F**), yet a VEP was clearly present at 7 months (**H**), albeit with reduced amplitude and increase peak times. Calibration: 100 mV, 50 ms.

in development and may be followed by significant recovery of function.

To explore whether nerve constriction leads to any change in conduction properties of the nerves, we measured compound action potentials excised (*ex vivo*) in the *op/op* and the wild-type nerve at P30 and 7 months (Fig. 10). At P30, the amplitude and velocity of the *op/op* action potential were significantly reduced to ~20% and ~50% of the wild-type, respectively (Fig. 10A). Consistent with the reduction in velocity, there was also an increase in the refractory period measured using standard paired-pulse studies; the refractory curve for *op/op* (○) is shifted to the right of the wild-type curve (●) by ~2 s at the 50% recovery level (Fig. 10C). In contrast to P30, the action potential in four optic nerves from three 7-month-old mutants exhibited a recovery in all three categories (amplitude, conduction velocity, and refractory period) to near wild-type values ($n = 4$) in age-match analysis (Fig. 10B). Both right and left nerves were examined microscopically in these mice. In the optic nerves that showed improved conduction velocity, microscopy confirmed an increase in myelination. In two nerves that were noted to be atrophic, an action potential could not be elicited; and on microscopy, no myelinated axons were seen.

Given that there was a nerve constriction, we explored whether abnormality occurs uniformly along the optic nerve. We addressed this using calcium imaging at P30 at the site of nerve constriction and nonconstriction. We found that, at the site of constriction, the stimulation-induced intracellular Ca elevation had a smaller amplitude (Fig. 10D) and a slower falling phase (Fig. 10E) than the wild-type. The slowing of the calcium transient suggests that it takes abnormally longer time at the constriction site to restore the calcium level to resting level after nerve stimulation.

Figure 10. Compressed optic nerve of *op/op* mice is functionally impaired in vitro. **A**, Compound action potential of wild-type ($n = 4$ nerves, P30; $n = 4$ nerves, 7 months) and *op/op* ($n = 4$ nerves, P30; $n = 4$ nerves, 7 months) mice, measured with suction electrodes and brief bipolar stimulation at P30 and 7 months. Notice a longer time to peak from the stimulation artifact for *op/op* at P30 but not 7 months of age, reflecting the reduced conduction velocity at P30 compared with the wild-type. Also note a smaller amplitude in the P30 *op/op* mice, reflecting that many axons are not conducting action potentials (room temperature 21°C). **B**, Velocity of compound action potentials in wild-type and *op/op* mice at P30 and 7 months of age. At P30, the wild-type velocity was 1.7 ± 0.16 m/s ($n = 4$) and *op/op* velocity was slower, at 0.75 ± 0.2 m/s ($n = 4$). **C**, Refractory period analysis of compound action potentials at P30 and 7 months. Refractory period measures how fast a second action potential recovers from a first stimulation. This recovery of the second action potential was assayed using standard twin-pulse stimulations with increasing interpulse intervals. Note that at P30 the curve for *op/op* mice (open symbol) was right shifted to the curve for wild-type mice (closed symbols). The average 50% recovery was 24 and 29 s for wild-type and *op/op* mice, respectively. The slight increase in refractory period (from 24 to 29 s) for *op/op* mice was consistent with a slower conduction velocity (**B**). **D**, Axonal calcium response at P30 evoked by repetitive action potentials (2 s at 80 Hz). The Ca response was expressed as changes relative to the resting level ($\Delta F/F$). Response of the P30 *op/op* nerve was measured at two locations for each nerve: at the unconstricted and the constricted region. Within the same *op/op* nerve, the Ca response was smaller at the constricted region ($\Delta F/F = 0.017 \pm 0.002$, $n = 4$) compared with the unconstricted region ($\Delta F/F = 0.038 \pm 0.005$, $n = 4$). The wild-type response was $\Delta F/F = 0.062 \pm 0.006$ ($n = 4$). Because the Ca response was measured from a large population of axons, the smaller response at the constricted region suggests either conduction block or reduced conduction at the constriction site. **E**, Actual axonal Ca response traces in the wild-type and *op/op* nerves. In the same *op/op* nerve, the amplitude of the Ca response is smaller at the constricted site compared with the unconstricted site. Further, it is interesting to note that the poststimulation Ca decline is slower at the constricted site (see inset; Ca responses normalized to the same amplitude). Because poststimulation Ca decline is determined by Ca buffering (Ca extrusion and/or Ca sequestration by intracellular organelles), the slower Ca decline at the constriction site (inset) suggests a selective abnormality in local Ca buffering at the constriction site of the *op/op* nerve. ** $p < 0.01$, compared with wild-type control. *** $p < 0.001$, compared with wild-type control.

Functional impairment in the compressed optic nerve was also demonstrated by immunohistochemistry for the fast sodium channel Nav1.6 and Caspr, both of which are necessary for effective saltatory conduction. The paranodal expression of Caspr and Nav1.6 expression, sequestered at the node of Ranvier, was maintained throughout the optic nerve of wild-type mice at P30 (data not shown). They were absent in the intracanalicular optic nerve of *op/op* mice at P30 (Fig. 11A, C) but were preserved both in the intraorbital and intracranial segments (Fig. 11A, B).

Discussion

Osteopetrosis is a heterogeneous group of heritable conditions in which a defect in bone resorption by osteoclasts causes abnormal bone remodeling and increased bone density (for review, see Tolar et al., 2004). Partial or complete blindness is one of the major neurological complications found in osteopetrosis, especially in the so-called malignant form with autosomal recessive traits (for review, see Steward, 2003). MRI identifies optic canal stenosis in most cases of the malignant osteopetrosis (Curé et al., 2000). However, the efficacy of optic nerve decompression as a treatment is yet unclear (Steward, 2003). The naturally occurring optic nerve compression in *op/op* mice studied here provides an excellent model to study pathophysiology of compression-induced nerve injury and to develop therapeutic approaches to visual loss in osteopetrosis and other similar conditions, such as fibrous dysplasia (Michael et al., 2000).

In a previous study on the *op/op* mice, Michaelson et al. (1996) reported an abnormal VEP, which they ascribed to an abnormal development of the cortical neural circuit. In this study, we have identified the reduced action potential and the delayed conduction in the *op/op* optic nerve (Fig. 9). However, our electrophysiological examinations of the excised *op/op* optic nerve (Fig. 10A–E) demonstrate that the abnormal VEP is largely the result of the compression-induced myelination failure in the optic nerve. In the previous study, the abnormal electrophysiology in *op/op* mice was partially improved after daily injections of recombinant CSF-1 protein starting from P2 (Michael et al., 2000). It would be interesting to know whether this treatment prevented the stenosis of optic canal as well as the development of cerebral neuronal circuit. Michaelson et al. (1996) also reported abnormal brainstem auditory-evoked potentials. It may be of great interest to investigate whether the VIII nerve is also compressed in *op/op* mice. However, the auditory system needs to be carefully examined because impaired vibration of auditory ossicles has recently been reported in another mouse model of osteopetrosis lacking either cytokine RANKL or transcription factor c-Fos (Kanzaki et al., 2011).

The lack of myelination of the compressed optic nerve is clearly the key question to be solved in this model. The data support this as primarily a failure of development of myelin as opposed to being a result of demyelination, although there may be a minor component of myelin breakdown. The apparent loss of glial cells at the compression site is the key to the myelination

failure. Despite careful investigation, cell death does not appear to be the primary reason for this glial cell paucity. An increase in TUNEL-positive cells was seen at all levels of the *op/op* optic nerve, although glial cell number was only reduced within the intracanalicular region, suggesting that cell death alone does not account for the glial cell reductions observed in the compressed region of the nerve. The lack of phagocytes in *op/op* mice may delay the clearance of apoptotic cells, which results in the sustained number of cells undergoing DNA fragmentation. It could also be ascribed to the lack of any trophic factors that are secreted by microglia and necessary for the maintenance of OPCs/oligodendrocytes in the developing optic nerve. For example, it has been shown that multiple trophic factors are required for the survival of developing oligodendrocytes in the rat optic nerve (Barres et al., 1993), and microglia are directly or indirectly responsible for the production of those factors (Czeh et al., 2011). In the normal development of the optic nerve (and likely all white matter), OPCs compete for the limited growth factors and nutrients within the neuropil, and ~50% die as a result (Barres et al., 1992a,b). Therefore, even small changes of blood flow to the nerve may be detrimental to their survival. As cell death was not greater in the intracanalicular segment of the *op/op* optic nerves, it may be that migrating OPCs and astrocytes “sense” a reduced oxygen tension within the nerve and migrate away from the intercanalicular segment. If this occurs, they apparently become evenly distributed throughout the intracranial and intraorbital segments, as there is no evidence of accumulation of cells at either side of the compressed nerve. Alternatively, the glial cells may simply sense pressure gradients within the compressed nerve, and, as the only component of the nerve that is migratory, move away from the compressed neuropil. Certainly, the nerve in the optic canal is at greater risk for disruption of myelination as it is already smaller in area than the rest of the nerve in wild-type mice, and myelination during development is delayed in the nerve within the canal in a number of species (Skoff, 1978, 1980). In addition, the nerve within the canal is less well vascularized than the intraorbital nerve (Skoff et al., 1980); hence, compression and subsequent ischemia would compound the differences seen during development. If ischemia is not the primary abnormality, the increase in pressure within the neuropil could have other effects on OPCs as it has been shown that the membrane of myelinating Schwann cells can be damaged by hydrostatic pressure without the influence of hypoxia *in vitro* (Frieboes and Gupta, 2009).

From the combined electrophysiological and calcium imaging analysis, several conclusions can be drawn regarding possible dysfunctions so prominent at P30. First, nerve constriction reduces the conduction velocity and slightly prolongs the refractory period. This is consistent with the measured ERG and VEP in whole animal. Second, calcium imaging suggests abnormal cal-

cium homeostasis at the nerve constriction site, reflected by a prolongation of the falling phase of the calcium transient (Fig. 10D,E). In the nonconstricted portion of the *op/op* nerve, the falling phase of the calcium transient is normal (compared with the wild-type nerve). Abnormal calcium homeostasis might make the constriction site vulnerable to axon degeneration.

The long-term outcome of compression of the optic nerve is mixed, based on the observations of the nerves in *op/op* mice at 7 months of age. We examined 15 7-month-old *op/op* mice (i.e., 30 optic nerves) and found a variable degree of recovery. As we have shown, that all *op/op* optic nerves are compressed at P30, although to a variable degree, it is likely that the most severely compressed nerves at this time are most susceptible to future degeneration, as was seen in five of 30 nerves examined (Fig. 6). In these cases, severe compression at the canal at P30 may lead to ischemia along the entire nerve, or anterograde and retrograde degeneration resulting from compression in the optic canal that leads to loss of all or the majority of axons. In contrast to this severe outcome, 14 nerves appeared to have recovered in terms of size of the nerve at the canal and in myelination, both as noted grossly and in the toluidine blue-stained 1- μ m sections. The mechanism of recovery in these nerves can only be speculated on. It seems likely, however, given the original paucity of cells of the oligodendrocyte lineage within the compressed/dysmyelinated nerve, that OPCs in the adjacent segments must have migrated at a later time point into the nerve within the canal, where they differentiated and myelinated axons. Measurement of the G ratio of myelinated axons showed a small but significant increase, suggesting that late-onset myelination resulted in axons with thin myelin sheaths resembling remyelination (Franklin and ffrench-Constant, 2008).

These observations have strong significance for osteopetrosis in children and the outcome of the disorder. Visual disturbance and blindness are a major part of this disorder; and at early ages, abnormalities in vision and VEPs can be found

Figure 11. Lack of model apparatus in the *op/op* mouse at the canal. Representative images of double immunofluorescent staining for voltage-gated sodium ion channel Nav1.6 (green) and contactin-associated protein (Caspr; red) (**A**) in the intracranial (**B**) and intracanalicular (**C**) segments of optic nerve in *op/op* mice at P30. Note that the normal nodal expression of Nav1.6 and paranodal expression of Caspr was lost in the intracanalicular segment, whereas they were maintained in other regions, suggesting that normal saltatory conduction was disturbed in the intracanalicular segment. Scale bars: **A**, 500 μ m; **B**, **C**, 10 μ m.

(Steward, 2003; Stark and Savarirayan, 2009). Although there is not total agreement on the value of surgical therapy to expand the optic canal, in one report this was shown to be successful (Hwang et al., 2000). Our observations in the *op/op* mouse make two important points with relevance to the human disease. First, this is the first report directly linking optic nerve deficits in osteopetrosis to disturbances in myelination. From our study on the *op/op* optic nerve, it can be surmised that a similar myelin deficit occurs in patients. Second, the current study suggests that severe compression of a portion of the nerve can lead to degeneration of the entire nerve; hence, human cases in which CT scans show a severe compression of nerve in the canal in early development should be targets for prompt neurosurgical opening of the canal.

In conclusion, we report the spontaneous local dysmyelination in the intracanalicular optic nerve segment of *op/op* mice. The compression appeared to cause local ischemia and focal disappearance of glial cells in the lesion. This glial cell loss and myelination failure reached a maximum at P30 and showed variable recovery thereafter. Such delayed myelination may not be called “myelin repair” because oligodendrocytes were eliminated before initiating myelination. However, this model will be useful in investigating how OPCs migrate from the adjacent intact areas of white matter into lesions, and differentiate into late-onset, myelinating oligodendrocytes.

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