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Oligodendrocytes regulate formation of nodes of Ranvier via the recognition molecule OMgp

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Abstract

The molecular mechanisms underlying the involvement of oligodendrocytes in formation of the nodes of Ranvier (NORs) remain poorly understood. Here we show that oligodendrocyte-myelin glycoprotein (OMgp) aggregates specifically at NORs. Nodal location of OMgp does not occur along demyelinated axons of either Shiverer or proteolipid protein (PLP) transgenic mice. Over-expression of OMgp in OLN-93 cells facilitates process outgrowth. In transgenic mice in which expression of OMgp is down-regulated, myelin thickness declines, and lateral oligodendrocyte loops at the node-paranode junction are less compacted and even join together with the opposite loops, which leads to shortened nodal gaps. Notably, each of these structural abnormalities plus modest down-regulation of expression of Na⁺ channel α subunit result in reduced conduction velocity in the spinal cords of the mutant mice. Thus, OMgp that is derived from glia has distinct roles in regulating nodal formation and function during CNS myelination.

Keywords

OMgp; myelination; Node of Ranvier; oligodendrocyte; Na⁺ channel

INTRODUCTION

Nodes of Ranvier (NORs) are specialized axonal domains where a constellation of cell-adhesion molecules, extracellular matrix (ECM) molecules and voltage-dependent Na^+ channels cluster. This unique architecture ensures the saltatory conduction of action potentials along myelinated fibers (Hodgkin and Huxley, 1952; Salzer, 2003). Notably, several oligodendrocyte-derived molecules, such as Tenascin-R (TN-R) (Bartsch *et al.*, 1993), NG2 (Butt *et al.*, 1999), myelin-associated glycoprotein (MAG) (Bartsch *et al.*, 1989), Nogo-A (Nie *et al.*, 2003) and oligodendrocyte myelin glycoprotein (OMgp) (Huang *et al.*, 2005) are located at distinct axonal domains. Some of these molecules mediate axoglial interactions during myelination. At NORs, clustered TN-R modulates the current amplitude of the voltage-gated Na^+ channels (Xiao *et al.*, 1999; Weber *et al.*, 1999). Both MAG and Nogo-A accumulate in CNS paranodes (Bartsch *et al.*, 1989; Nie *et al.*, 2003). Like Nogo-A and MAG, OMgp is expressed predominantly by oligodendrocytes, and binding of each of these three ligands to the Nogo-66 receptor (NgR) inhibits neurite outgrowth (Wang *et al.*, 2002; Kottis *et al.*, 2002). In OMgp-null mice, an increase of collateral sprouting at the NORs is observed (Huang *et al.*, 2005). However, the possible role of OMgp in the generation and maintenance of these specialized domains has not been elucidated clearly.

OMgp expression is detected in both CNS and PNS (Apostolski *et al.*, 1994; Huang *et al.*, 2005). OMgp comprises a series of tandem leucine-rich repeats (LRRs), a short cysteine-rich motif (CR) that has homology with an epidermal growth factor (EGF) motif, and the HNK-1 epitope, which makes it suited for cell recognition (Mikol *et al.*, 1990). Immunohistochemical observations with antibodies against either Gal (beta 1–3) GalNAc antigen or the molecule itself demonstrate that OMgp is associated with NORs (Apostolski *et al.*, 1994; Sheikh *et al.*, 1999; Vourc'h and Andres, 2004; Huang *et al.*, 2005). The temporo-spatial profile of expression indicates a possible role for OMgp in axoglial interaction and myelination (Mikol and Stefansson, 1988; Vourc'h *et al.*, 2003).

OBJECTIVE

This study aims to further explore the cellular location, physiological distribution and function along myelinated axons of OMgp, in addition to its role in inhibiting neurite outgrowth. Through analyzing *shiverer* mice, and transgenic mice in which proteolipid protein (PLP) is over-expressed and OMgp is down-regulated, potential roles for OMgp in the development of oligodendrocytes and myelin development and nodal formation are addressed using cell biological and electrophysiological approaches.

MATERIALS AND METHODS

Production of recombinant proteins

Full-length OMgp, and Na^+ channel $\beta 1$ and $\beta 2$ extracellular portions were cloned into the pGEX-KG vector for expression in *E. coli* and purified as GST-fusion proteins following standard procedures (Xiao *et al.*, 1996). Recombinant proteins and GST were visualized by Coomassie brilliant blue staining. Protein concentrations were determined using the BCA protein assay kit (BioRad).

Antibodies and plasmid constructs

Rabbit polyclonal antibodies against OMgp (Habib *et al.*, 1998), Caspr (Nie *et al.*, 2003); mouse monoclonal antibody against Caspr (K65/35) (Yang *et al.*, 2004) have been described previously. Goat polyclonal anti-OMgp (R&D system), rabbit polyclonal anti- Na^+ channel α subunit (pan-Nav), $\beta 2$ subunit (Nav $\beta 2$) (Alomone laboratories, Israel), mouse monoclonal antibody against 2',3'-cyclic nucleotide phosphorylase (CNPase) (11–5B; Sigma), pan-Nav

(K58/35; Sigma), K⁺ channels subunits Kv1.2 (K14/16) and Kvβ2 (K17/70; Upstate, USA), γ-tubulin (GTU88; Sigma), βIII-tubulin (Fluka) and O1 (Chemicon) were obtained commercially. Polyclonal anti-Navβ1 and anti-Navβ2 were raised in rabbits by injection of Navβ1-GST and Navβ2-GST, respectively, and affinity-purified. Full-length and ~1 kb antisense fragments of OMgp were cloned into GFP-encoding pTracer-CMV2 vector at *KpnI*-*XbaI* sites and *EcoRI*-*NotI*, respectively.

Cell culture and transfection

OMgp expressing CHO cells (OMgp-CHO) (kindly provided by Dr. Z.G. He, Harvard Medical School, USA) and OLN-93 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). OMgp-CHO cells were maintained in the presence of G418 (400 μg ml⁻¹). OLN-93 and OMgp-CHO cells were transiently transfected with full-length OMgp, antisense expressing constructs and the empty vector. All transfection experiments were carried out using Lipofectamine™ 2000 (Invitrogen) as instructed.

Primary cultivation of neurons, oligodendrocytes, astrocytes and oligodendrocyte precursor cells (OPCs) has been described previously (Hu *et al.*, 2003; Cui *et al.*, 2004). Oligodendrocyte-conditioned media were collected and concentrated with centricon-10 (Millipore).

OMgp-antisense transgenic mice

A ~1 kb fragment of OMgp was amplified from mouse brain cDNA and cloned into the pIRES vector in an antisense direction to produce pIRES-αOMgp, and a 1.8 kb fragment comprising the truncated OMgp and SV40 poly-A tail released by *KpnI*-*XbaI* digestion. This latter fragment was sub-cloned into the pSP72 vector to generate pSP72-αOMgp. The 1.4 kb myelin basic protein (MBP) promoter was amplified from mouse gDNA and sub-cloned into pSP72-αOMgp to generate pMBP-αOMgp. The construct was verified by DNA sequencing. A 3.2 kb transgene fragment was released from pMBP-αOMgp by *SphI*-*KpnI* digestion, isolated and microinjected into fertilized mouse oocytes. Routine genotyping of tail gDNA was performed with the PCR primer sequences 5'-acagctagtccaagaccca-3' (forward) and 5'-aagcccatgtgataggact-3' (reverse) to amplify a product of ~600 bp. At least three, independent, transgenic mouse lines were used in each experiment. All experiments involving animal application were approved by the Institutional Animal Care and Use Committee (IACUC) of Singapore General Hospital.

Other genetically modified mouse models

Transgenic mice that over-express PLP have been described (Rasband *et al.*, 2003), and *Shiverer* and wild-type mice were purchased from Jackson Laboratories (Bar Harbor).

Immuno-blotting analyses

Frozen tissues were homogenized in a lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% Triton X-100) with protease inhibitors (Roche Diagnostics). Following ultracentrifugation (150 000 *g* at 4° C for 45 min), the supernatants were collected and stored at -80°C. Alternatively, membrane fractions were prepared as described previously (Nie *et al.*, 2003). All samples were subjected to Bradford protein assay.

Equal amounts of protein samples were gel-separated and transferred onto nitrocellulose membranes. Immuno-blotting was performed using polyclonal antibodies against OMgp (1:1000), Navβ1 (1:500) and Navβ2 (1:200), and mouse monoclonal antibodies against CNPase (1:1000), pan-Nav (1:1000) and γ-tubulin (1:1000). Peroxidase-conjugated secondary antibodies were applied and blots visualized with an ECL™ detection kit (Amersham). The protein bands were measured using a Molecular Analyzer/Densitometer (Bio-Rad).

Confocal immunofluorescence staining

Animals were perfused sequentially with 0.1 M Ringer's solution and 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). For cryosection, spinal cords were removed immediately and immersed sequentially in 15% and 30% sucrose in PB. Sections (10 μ m) were mounted onto slides coated with poly-L-lysine.

For immunofluorescence staining, tissue slices were immersed in absolute acetone at -20°C for 20 min. Slices were then washed thoroughly in 0.1 M phosphate buffered saline (PBS, pH 7.6) containing 0.3% Triton X-100. After blocking with 10% normal goat serum (NGS) diluted in PB for 1 hr, tissue slices were incubated with primary antibodies at their optimum dilutions, either overnight or for 2 hrs at room temperature (RT). Cy2- and Cy3-conjugated secondary antibodies (Amersham) were used for 1 hr at RT. An additional cycle of labeling was also conducted for second primary antibodies. Images were acquired using a Carl Zeiss LSM5 confocal system and quantitated with the software Zeiss LSM Image Browser.

Electron and immuno-electron microscopy

Animals were transcardiacally perfusion-fixed with 2% paraformaldehyde plus 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Spinal cords were removed and post-fixed in the same solution for 2 hrs. The trimmed samples were immersion-washed overnight and further post-fixed in 1% osmium tetroxide (OsO_4) containing 1.5% potassium ferro-cyanide for 2 hrs, followed by dehydration in an ascending concentrations of alcohol and absolute acetone. After gradually infiltrated with Araldite 502 (EMS), the blocks were embedded and polymerized overnight at 60°C . Ultrathin sections (~ 90 nm) were counterstained with uranyl acetate and lead citrate.

For immuno-electron microscopy (IEM), animals were perfusion-fixed in 4% paraformaldehyde plus 0.1% glutaraldehyde. To start the pre-embedding staining, tissue blocks were cut into 100- μ m-thick slices with a vibratome before staining according to the ABC (avidin-biotin complex) method. Briefly, spinal cord slices were incubated with OMgp antibody (1:1000 at 4°C overnight) after 10% NGS blocking. Biotinylated anti-rabbit secondary antibody (Vector Lab) was applied for 1 hr. Tissue samples were incubated further in ABC for 1 hr, followed by reaction with DAB solution. Areas of interest were selected for standard dehydration and infiltration. All samples were visualized and photographed under a Jeol 1220 electron microscope.

In vitro Na^+ current recording

Whole-cell voltage clamp recordings were made in CHL cells that express Nav1.2 β 1 β 2 (Xiao *et al.*, 1999). The pipette solution contained (in mM): 140 CsCl, 1 CaCl_2 , 10 EGTA, 2 MgCl_2 and 10 HEPES, pH 7.2 with CsOH. The external recording solution contained (in mM): 126 NaCl, 10 CsCl, 10 HEPES, 1 MgCl_2 , 2 CaCl_2 and 10 D-Glucose (pH 7.3 with CsOH; 300–305 mOsm L^{-1}). All experiments were performed at RT (21 – 25°C). Cells were held at -110 mV and step-depolarized to the range -40 to $+30$ mV for 50 ms. Either OMgp-GST (50 ng ml^{-1} and 50 μg ml^{-1}) or GST were added into the bath and currents recorded 5, 10 and 20 min after application of OMgp.

Whole-cell, perforated-patch recordings of voltage-gated Na^+ currents were performed on cultured hippocampal neurons (12–14 days old) from embryonic day 18 (E18) Sprague Dawley rats. To isolate Na^+ currents the external recording solution contained (in mM): 140 NaCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 D-Glucose, 10 TEA-Cl, 5 4-AP, 0.2 CdCl_2 , pH 7.4 (310 mOsm/L); the patch pipette solution was (in mM): 130 CsCl, 10 TEA-Cl, 2 MgCl_2 , 1 CaCl_2 , 11 EGTA, 10 HEPES, pH 7.2. Pipettes were tip-filled with internal solution and then back-filled with internal solution containing amphotericin B (400 ng ml^{-1} , Sigma). Neurons were voltage-

clamped at -80 mV, followed by 50 msec depolarization to -20 mV, at which time the Na^+ current reached its maximum. The currents were recorded continuously with an interval of 30 secs between stimulations. OMgp-GST and GST were added directly to the recording bath to a final concentration of 50 ng ml^{-1} and then washed by perfusion with a standard external solution. Data were analyzed by Clampfit 9.0 (Axon Instruments).

Conduction velocity recording

Compound action potentials (CAPs) were recorded in the ventral funiculus of the spinal cord using a suction electrode as described earlier (Weber *et al.*, 1999). Briefly, spinal cords were removed quickly from the animals and hemisected. The ventral funiculus was carefully dissected in Krebs's solution (in mM; 126 NaCl, 26 NaHCO_3 , 3 KCl, 1.3 NaH_2PO_4 , 2 MgCl_2 , 10 dextrose and 2 CaCl_2 ; pH ~ 7.4). The funiculus was held straight in a custom-made recording chamber with a thread tied to one end and mild suction from the recording electrode at the other, and perfused at $3\text{--}5 \text{ ml min}^{-1}$ in the oxygenated (95% O_2 , 5% CO_2) medium at 25°C . As a stimulus, a $0.1 \text{ }\mu\text{s}$, square-wave-current pulse was applied at one end of the funiculus via a bipolar Pt/Ir electrode (FHC). The voltage signal from the other end was recorded with a suction electrode, high-pass filtered at 50 Hz, and digitized at 20 kHz, using Clampex software and GeneClamp 500B amplifier (Axon Instruments). Conduction velocity was defined as l/t , where l is the length of the funiculus and t is the time from stimulus onset to the response peak.

Quantitative and statistic analyses

Raw data were collected from either at least three independent experiments or different animals and are presented as mean $\pm$ SEM. OMgp labeling at NORs is quantified relative to respective markers. The relative intensities of OMgp staining in transfected OLN-93 cells have been compared using Student's *t*-test. In OMgp-transgenic and wild-type mice, the concentrations OMgp and CNPase, G ratio, axon diameter and conduction velocity have been compared using the independent-samples Student's *t*-test. Nodal distance, nodal index, paranodal length, G ratio, number of detached paranodal loops in axons of different size have been compared in OMgp-transgenic and wild-type mice in accordance with one-way ANOVA. Statistic analyses utilized softwares SPSS11.5 for windows and Origin 7.0. *P* values of <0.05 are considered significantly different.

RESULTS

Oligodendroglial OMgp clusters at the CNS NORs

To explore roles of OMgp in axoglial interactions, we first investigated the distribution of OMgp along the CNS myelinated axons and discovered independently that OMgp accumulates specifically at NORs. As evidenced by double-immunofluorescence staining in spinal cord (Fig. 1Aa,b), OMgp labeling (green) was flanked by either juxtaparanodal Kv1.2 (red; Fig. 1Aa,a') or paranodal Caspr/paranodin labeling (red; Fig. 1Ab,b'), which indicates the nodal location of OMgp. Nodal location of OMgp was also detected in the brain and optic nerve (not shown). On longitudinal sections of adult rat spinal cord, immuno-reactivity was undetectable after the antibody was pre-absorbed with OMgp-GST proteins (Fig. 1Ac), which confirms the specificity of OMgp labeling at NORs.

We observed a link between OMgp clustering and axon diameter. To determine the portion of OMgp in all NORs in spinal cords from adult rats, we quantitated the nodal deposition of OMgp relative to the axonal markers Kv1.2 and Caspr. Values of $83.4 \pm 10.3\%$ and $74.7 \pm 3\%$, respectively (Fig. 1Ad), indicate that not all NORs in the spinal cord contain OMgp. In support of this, OMgp is detected in all NORs in axons of diameter $\geq 1 \text{ }\mu\text{m}$, but only in 8.9%, 34.0% and 72.7% of NORs in axons with smaller diameters ($<0.3 \text{ }\mu\text{m}$, $0.3\text{--}<0.5 \text{ }\mu\text{m}$ and $0.5\text{--}<1 \text{ }\mu\text{m}$,

respectively) (Fig. 1Ae). This pattern indicates that clustering of OMgp at NORs is related proportionally to axon diameter. Given that myelin thickness is proportional to axon diameter (Smith *et al.*, 1982; Salzer, 2003), our results indicate that OMgp might originate from myelin/oligodendrocytes.

Immunofluorescence label of OMgp was detected over a wider area than Caspr (Fig. 1Ab,b', yellow), which indicates that it might be located in a cell process that abuts the node. For more details, we performed IEM in accordance with the pre-embedding ABC method. Results show that axonal domains are devoid of OMgp immuno-reactive particles. Rather, particles are derived from the adjacent cells that have been shown positive for NG2 staining (Fig. 1Af,g) (Butt *et al.*, 1999).

To further determine the identities of OMgp-expressing cells, different cell types in the mammalian CNS were isolated, cultured *in vitro*, and immuno-stained using antibodies against OMgp in combination with their specific molecular markers. The soma and processes of both NG2-positive, bipolar, OPCs and O1-positive mature oligodendrocytes were labeled strongly by anti-OMgp antibodies (Fig. 1Ba,b). By contrast, β -III-tubulin-positive neurons and GFAP-reactive astrocytes were not labeled (Fig. 1Bc,d).

OMgp possesses a glycosylphosphatidylinositol (GPI) tail through which the protein is embedded on the cell surface (Mikol and Stefansson, 1988). The GPI linkage, however, is susceptible to phospholipase cleavage; akin to contactin/F3 and OMgp might, therefore, exist in a released form, even in natural conditions (Durbec *et al.*, 1992). To verify this, the medium from primary cultures of rat oligodendrocytes was concentrated. In addition, biochemical soluble and membrane fractions were prepared from immature and mature brains. An OMgp protein of ~120 kDa was identified in the membrane tissue fraction, with a faster migrating OMgp (~100 kDa) in the oligodendrocyte medium and the soluble fraction (Fig. 1Be). Thus, nodal OMgp might have both membrane-bound and soluble forms.

Together, our results demonstrate that OMgp that accumulates specifically at NORs in the CNS is derived from oligodendrocytes, although it might also be expressed by a subpopulation of neurons (Habib *et al.*, 1998). Deposition of OMgp at the border between nodal and paranodal regions, which might act as the pivotal site that maintains the integrity of both domains (Zhang *et al.*, 2005), is closely related to axon size. This leads us to speculate that OMgp might play significant roles in myelination and nodal formation.

OMgp clustering depends on myelination

We were therefore interested to explore the relationship between OMgp clustering and myelin development. As illustrated by immuno-blotting analysis (Fig. 2A), OMgp protein is present in brain at birth, is up-regulated at postnatal day 5 (P5) and remains relatively high until P30. Thus high concentrations of OMgp are present during active myelination. Next, we performed fluorescent immuno-staining to identify clustering of OMgp with Na⁺ and K⁺ channel when the NORs specialize gradually. OMgp clustering at the NORs commenced at ~P6 and increasingly occurred later on (Fig. 2B), indicating that OMgp follows clustering of Na⁺ channels in matured NORs. This notion was confirmed by analyses in which OMgp was stained in spinal cord sections of MBP-deficient (*Shiverer*) mice and transgenic mice that over-express PLP, two animal models in which there is severe demyelination. As described previously (Nie *et al.*, 2003; Rasband *et al.*, 2003), Caspr and Kv1.2 (Fig. 2C) are distributed diffusely along myelinated axons in *Shiverer* and PLP-mutant mice. Notably, OMgp is undetectable at nodes in demyelinated axons but distributed rather diffusely in both mutants (Fig. 2C) compared with wild-type mice. These observations indicate that clustering of OMgp relies on the integrity of subcellular axonal domains.

OMgp over-expression is associated with mature morphology in an oligodendrocyte cell line

The OLN-93 cell line resembles immature oligodendrocytes because it has a bipolar morphology under routine culture conditions (Hu *et al.*, 2003). Immuno-blotting detects endogenous OMgp in OLN-93 cells, albeit at a very low level, and in CHO cells transfected with OMgp (Fig. 3Aa). To observe effects on the biology of OLN-93 cells, OMgp expression was either increased or repressed by transient transfection with full-length and antisense-encoding constructs, respectively. Over-expression of OMgp by >3 fold, determined by immunofluorescence intensity, (Fig. 3Ac) promoted cellular arborization from OLN-93 cells, whereas blocking endogenous expression of OMgp had no significant effects on the cells, which displayed typical bi-polar morphology. These results implicate a potential role for OMgp in oligodendrocyte maturation.

Hypo-myelination in transgenic mice in which OMgp is down-regulated

The antisense construct used in the transfection study repressed OMgp expression significantly *in vitro*, as shown by comparing the labeling intensity in transfected cells with surrounding untransfected cells (Fig. 3Ab,c). Using the antisense strategy to study indispensable functions of OMgp during development we generated OMgp-transgenic mice by transducing the antisense construct under control of the MBP promoter (Fig. 3Ba). Significant down-regulation of OMgp in these transgenic mice was confirmed by immuno-blotting for OMgp and other myelin components in protein extracts from spinal cords of OMgp transgenic and wild-type mice. By comparison, the level of CNPase was not altered significantly. All three transgenic lines showed consistent results in the parameters tested (Fig. 3Bb).

We then analyzed myelination in OMgp-transgenic mice by electron microscopy. First, the axon diameters in mutant mice did not differ significantly from those in wild-type mice (Fig. 4Aa–c). Because myelin sheaths appeared to be thinner in the mutants, we measured G ratios (the numeric ratio between the diameter of the axon proper and the outer diameter of the myelinated fiber) (Michailov *et al.*, 2004). G ratios were significantly higher in large axons (diameter $\geq 1 \mu\text{m}$) of transgenic mice than wild-type mice (Fig. 4Ad–f). These observations indicate hypo-myelination of thicker axons in OMgp-transgenic mice, which confirms a role for OMgp in oligodendrocyte maturation and myelination.

Nodal architecture is disorganized in OMgp-transgenic mice

The mature structure of NORs is complete during the late phase of myelination (Hodgkin and Huxley, 1952; Pedraza *et al.*, 2001). To determine whether OMgp affects nodal formation, we performed further EM analyses on longitudinal sections of OMgp-transgenic and wild-type mice to scrutinize the nodal architecture. The OMgp mutants have a major abnormality in the node–paranode junction, because lateral oligodendrocyte loops are less compacted and have either a looser or inverted assembly compared with wild-type mice (Fig. 4Ba–c). Furthermore, statistical analyses indicate that although the abnormality occurs significantly in axons of diameter 0.5–1 μm , it is more pronounced in axons of $\geq 1 \mu\text{m}$ (Fig. 4Ca). As a result, the nodal length in larger axons ($\geq 1 \mu\text{m}$) is significantly reduced in transgenic mice compared with their wild-type littermates. This is corroborated by comparing the nodal index (Fig. 4Cb), which is defined by $(N1+N2)/D$, where N1 and N2 are the upper and lower nodal distances, respectively, and D is axon diameter, measured from electron micrographs (Fig. 4Ba). The paranodal length is also increased in axons $\geq 1 \mu\text{m}$ in the OMgp mutant (Fig. 4Cc). These observations indicate that OMgp stabilizes lateral oligodendrocyte loops that anchor to the axon surface to demarcate the nodal and paranodal domains.

During myelination the immunoglobulin recognition molecule F3/contactin co-localizes and interacts with Caspr to form transverse bands, the electron-dense, ladder-like partitions or septa in the paranodal region, where myelin loops are glued to axons (Rosenbluth, 1995; Salzer,

2003). Paranodal architectures, particularly the transverse bands, were further compared in transgenic and wild-type mice. However, we saw no misaligned transverse bands or malformation in paranodal regions except that most lateral loops were reversed (Fig. 4D). These observations underscore the specific role of OMgp at NORs.

Impaired expression and function of Na⁺ channels in OMgp-transgenic mice

Na⁺-channel clustering at NORs makes the non-myelinated segments functional structures for the proper conduction of action potentials. Because OMgp localizes in NORs and functions to stabilize nodal formation, we analyzed Na⁺ channel clustering and expression at NORs in OMgp-transgenic mice. As shown by double immuno-staining with antibodies against Na⁺ channel α subunits and Caspr, clustering of Na⁺ channels appears normal in the OMgp mutants (Fig. 5Ac,e), but the physical gap between each pair of Caspr labeling is narrowed, significantly in axons of diameter $\geq 1 \mu\text{m}$ (Fig. 5Ad,f,g). This confocal microscopy analysis, thus, confirms the nodal shortening detected previously by EM. However, it also indicates that OMgp is not essential for Na⁺ channel clustering at NORs, which is consistent with the previous finding that OMgp clusters behind Na⁺ channels (Fig. 2B).

TN-R, another oligodendrocyte-associated protein, modulates Na⁺ currents *in vitro* and action potential conduction *in vivo* (Xiao *et al.*, 1999; Weber *et al.*, 1999). To determine whether OMgp has similar roles in modulating voltage-gated Na⁺ channels, hippocampal neurons and Chinese Hamster Lung (CHL) cells co-transfected with Na⁺ channel α , $\beta 1$ and $\beta 2$ subunits (Xiao *et al.*, 1999) (not shown) were recorded using whole-cell voltage clamp and perforated-patch recordings, respectively. The amplitude of Na⁺ currents was not significantly altered after treatment with either OMgp-GST or GST control protein (Supplemental Fig. 1). These results indicate that OMgp is unlikely to have a direct influence on Na⁺ currents. However, immuno-blotting analyses identified a significant reduction in the concentration of Na⁺ channel α subunits, but not $\beta 1$ and $\beta 2$ subunits in OMgp-transgenic mice compared with wild-type mice. Quantitatively, expression of Na⁺ channel α subunits is reduced by ~40% compared to wild-type samples (Fig. 5B), which indicates that OMgp has a role in regulating the expression of Na⁺ channel α subunits *in vivo*.

We wanted to see how action potential conduction is altered by the disorganization in nodal architecture and reduction in Na⁺-channel expression in the OMgp mutants. Therefore, we recorded compound action potentials from the ventral funiculus of spinal cords in OMgp-transgenic and wild-type mice. The conduction velocity in OMgp-transgenic mice ($4.4 \pm 0.3 \text{ m sec}^{-1}$) is significantly lower than in wild-type mice ($5.5 \pm 0.4 \text{ m sec}^{-1}$) (Fig. 5C).

CONCLUSIONS

- Clustering of OMgp at NORs is related closely to axonal size in the CNS.
- Nodal OMgp derives from either myelinating oligo dendrocytes or oligodendrocyte-like cells that abut NORs, in either membrane-bound or soluble forms.
- OMgp plays a specific role in myelin development and in axoglial interaction to regulate nodal formation via an unidentified mechanism.
- OMgp is required for proper expression of Na⁺ channels and axonal conduction.

DISCUSSION

Independently, we have identified that OMgp clusters at NORs, which confirms a recent observation of another group (Huang *et al.*, 2005). We have also demonstrated *in vitro* and *in vivo* that nodal OMgp is derived from oligodendrocytes and/or oligodendrocyte-like cells.

Clustering of OMgp during postnatal development is associated with and depends on myelination. *In vitro* over-expression of OMgp promotes cellular arborization in OLN-93, an immature oligodendrocyte line. In mice in which OMgp is down-regulated via the antisense strategy there is hypo-myelination and disorganization in nodal architectures in the CNS. Although lacking direct effects on Na⁺ currents, OMgp appears to regulate Na⁺-channel expression and electrophysiological functions of axons. Together, our results demonstrate a novel role for OMgp in regulating myelination and nodal formation at postnatal stages.

OMgp is a novel component of nodal complex

Huang *et al.* propose that OMgp is a nodal molecule (Huang *et al.*, 2005) on the basis of the proteomic identification of OMgp in presumed nodal membranes. Although the nodal membranes described by this group are rich in nodal and paranodal markers, they encompass juxtaparanodal Kv1.2 and the presence of internodal axolemma was not excluded unequivocally. Moreover, their proteomic analysis did not detect several known nodal membrane proteins, including Na⁺-channel subunits, neuronal cell adhesion molecule (NrCAM) and NG2 (Dawe *et al.*, 2006). Here, we provide evidence that OMgp accumulates in NORs of myelinated axons in the CNS, which supports the notion that OMgp is a nodal molecule. During early stages of axoglial interaction, OMgp expression and clustering is related closely to the process of myelination. Whereas in demyelinating animal models, such as MBP-deficient (*Shiverer*) mice and transgenic mice that over-express PLP (Rasband *et al.*, 2003), OMgp distribution is ectopic and dispersed rather than clustered into the NORs. These observations indicate that accumulation of OMgp in the NORs might depend on myelin integrity and subcellular axonal polarization. Notably, OMgp carries the Thomsen-Friedenreich glycan that has been localized specifically to NORs (Apostolski *et al.*, 1994; Kleene *et al.*, 2004). Autoantibodies against this glycan are associated with motor neuron disease and sensorimotor pathology (Thomas, 1996). Thus, the Gal (beta 1–3) Gal NAc-bearing moieties might be targets of immune-mediated neuropathies (Sheikh *et al.*, 1999). These observations lend support to the functional significance of OMgp at the CNS nodes.

OMgp is expressed by oligodendrocytes (Mikol and Stefansson, 1988) and a subset of projection neurons (Habib *et al.*, 1998). In the present study, we have further affirmed that OMgp is expressed by OPCs, myelinating oligodendrocytes and oligodendrocyte-like cells abutting to the NORs. Huang *et al.* present immunohistochemical evidence for the localization of OMgp to NG2-positive processes at NORs (Huang *et al.*, 2005). However, we can not rule out the possibility that NG2 and, most probably, OMgp, which bear glycosylphosphatidylinositol (GPI) tails that are homologous to F3/contactin, can be produced as a soluble protein (Durbec *et al.*, 1992; Martin *et al.*, 2001). Indeed, our biochemical evidence validates the existence of both soluble and membrane-bound forms of OMgp. We therefore believe that nodal OMgp is functionally associated with oligodendrocytes and myelin.

OMgp regulates CNS nodal formation

A crucial feature of axon–oligodendrocyte interaction and myelination is the formation of NORs, the unmyelinated, regularly placed axonal segments. It is known that the myelin-sheath thickness or number of myelin lamellae is proportional to axon caliber (Smith *et al.*, 1982). During myelin development, the adhesion of lateral oligodendrocyte loops to axolemma via recognition molecules is considered to be a key step in the formation and maintenance of nodal and paranodal architectures (Pedraza *et al.*, 2001; Hildebrand *et al.*, 1993; Berthold, 1996), by which internodal extension generates an increasing compression against the nodes (Pedraza *et al.*, 2001). Notably, Huang *et al.* observe prolonged paranodes and nodal lengths in OMgp-null mice, via Caspr immunolabeling. This result indicates a role for OMgp in axoglial interaction. Unfortunately, they provide no additional analyses, for example electron microscopy, to determine how axoglial junctions at the paranodes and nodal structures are

altered in this mutant. We have performed comprehensive analyses in OMgp-transgenic mice in which OMgp is reduced by 50–70%, and observed a significant abnormality in the node–paranode junction. Disorganized lateral glial loops at the node–paranode junctions and shortening of nodal span in the OMgp mutants strongly indicate that OMgp has a role in attaching lateral glial loops and demarcation of node–paranode junctions. Given its role in maintaining nodal architecture, however, how OMgp interacts with axonal partners, particularly the components of NrCAM/neurofascin/Na⁺ channels, awaits further studies.

Huang *et al.* have observed that collateral axonal sprouting increasingly arises at the NORs where OMgp is lost. Many inhibitory proteins are associated with NORs, such as TN-R, TN-C, NG2, versican and phosphocan (Weber *et al.*, 1999; Evers *et al.*, 2002; Martin *et al.*, 2001; Oohashi *et al.*, 2002). We have confirmed co-localization of OMgp, TN-R and NG2 at NORs in the spinal cord (not shown). Generally, the inhibition of neurite outgrowth and sprouting by Nogo-A, MAG and OMgp varies depending largely on neuronal types, ages and species (Johnson *et al.*, 1989; Turnley and Bartlett, 1998; Woolf *et al.*, 2003). Hence, a question remains as to how significantly OMgp arrests the collateral sprouting during development and in adulthood.

The role of OMgp in myelin development

As an oligodendrocyte-myelin associated molecule, OMgp is postulated to have a role in myelination. Of our particular note is that OMgp is not detected in all NORs and is undetectable in a subset of smaller axons (Fig. 1Ae). There appears to be a proportional relationship between the accumulation of OMgp and axon diameter, which indicates that a change in the concentration of OMgp might have a significant impact on a subpopulation of axons, at least.

In addition, we have confirmed that the antisense strategy used *in vitro* represses endogenous expression of OMgp. To ensure that neuronal expression of OMgp is not affected (Habib *et al.*, 1998), we used the myelin-specific MBP promoter to generate transgenic mice in which expression of OMgp is down-regulated. Hypo-myelination occurs in the OMgp mutants. First, although OMgp is a minor component of myelin in amount, loss of OMgp might contribute to inadequate myelin production. Second, OMgp might have an active role in the development of myelin because over-expression of OMgp *in vitro* promotes cell arborization, which is a morphological feature of oligodendrocyte maturation. It will be interesting to further analyze the role of OMgp in myelination in OMgp-null mice. LINGO-1, which has been identified recently as a co-receptor for NgR, is also implicated in the regulation of CNS myelination (Mi *et al.*, 2005). Given that OMgp interacts with NgR (Wang *et al.*, 2002), it remains to be explored whether OMgp interacts with NgR/LINGO-1 to interfere with the oligodendrocyte maturation and myelination.

Regulation of Na⁺-channel expression

A soluble oligodendrocyte protein is thought to induce Na⁺-channel clustering in NORs (Kaplan *et al.*, 1997; Kaplan *et al.*, 2001), which indicates that Na⁺ channels on axons might be regulated by mechanisms that are triggered by glial cells. Similarly, loss of dystroglycan in Schwann cell microvilli results in a decrease of Na⁺-channel density and slows nerve conduction in the PNS (Saito *et al.*, 2003). In TN-R-deficient mice, conduction velocity is reduced in optic nerves (Weber *et al.*, 1999) because TN-R modulates Na⁺ currents (Xiao *et al.*, 1999) but not Na⁺-channel expression (Weber *et al.*, 1999; Kaplan *et al.*, 2001). Nevertheless, Na⁺-channel clustering is unaltered in OMgp-transgenic mice, and the Na⁺ flux across the membrane via the α subunit-forming Na⁺-channel pore is not affected directly by treatment with OMgp protein. However, the concentration of Na⁺ channel α subunit in mutant mice is lower than in wild-type littermates. Although it is not clear whether OMgp regulates Na⁺-channel expression directly, at NORs OMgp might form part of a complex that, via the

β subunits, changes the number of functional Na^+ channels at the membrane (Qu *et al.*, 1995; Kazarinova-Noyes *et al.*, 2001; McEwen *et al.*, 2004).

Axoglial interaction is involved in the onset of myelination and axonal polarization (Girault and Peles, 2002). Our previous studies have demonstrated that activation of Notch receptor by axonal F3/contactin promotes oligodendrocyte maturation (Hu *et al.*, 2003). The present study provides evidence that oligodendrocytes regulate myelin development and nodal formation via the oligodendrocyte-derived protein OMgp.

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Fig. 1. Oligodendrocyte-derived OMgp locates at NORs

(A) OMgp is detected in NORs in the spinal cord. a,b, Longitudinal sections of normal adult rat spinal cord white matter subjected to double immunofluorescence using polyclonal antibodies to OMgp (green) and monoclonal antibodies against Kv1.2 (red; a) and Caspr (red; b), respectively. a',b', higher magnification images from a and b, respectively. c, Similar staining of adult rat spinal cord, using OMgp antibodies that were pre-incubated with quantitatively >500-fold excess OMgp-GST proteins. No specific labeling is observed. d, Nodal OMgp labeling was counted relative to two markers Kv1.2 and Caspr in spinal cords from at least three animals ($n = 200$). Relative ratios are $83.4 \pm 10.3\%$ and $74.7 \pm 3\%$, respectively. e, Quantification of OMgp immunolabeling, relative to Caspr on different sized

axons. f,g, IEM studies in adult rat spinal cords using OMgp antibodies. DAB-immuno-reactive particles are associated with the perinodal cell bodies and accumulate at NORs. Abbreviations: N, node of Ranvier; PN, paranode. Scale bars: a–c, 20 μ m; a',b', 5 μ m; f,g, 1 μ m. (B) Isolated OPCs, mature oligodendrocytes, neurons and astrocytes were double stained by antibodies against their respective markers NG2 (a), O1 (b), β -III-tubulin (c) and GFAP (d) (all green), as well as OMgp antibody (red). e, Soluble (S) and membranous fractions (M) of homogenized brains were prepared from rats at P15 and 4 months (4 Mon), respectively. Concentrated culture medium was collected from rat primary oligodendrocytes (OLs-cm) and subjected to OMgp immunoblotting (IB). Arrowheads indicate bands corresponding to OMgp. Note that a lower size of OMgp (~100 kD) is identified in both oligodendrocyte medium soluble tissue fraction.

Fig. 2. OMgp expression and clustering during CNS myelination

(A) Protein extracts from rat whole brains at P0, P5, P7, P8, P10, P15, P21, P30 and adult were subjected to immuno-blotting analyses using antibodies to OMgp and γ -tubulin (as loading control). (B) Immuno-staining of longitudinal sections of spinal cord from rats at P6, P10, P15 and P21 using antibodies against pan- Na^+ channels (Nav, red), K^+ channel $\beta 2$ subunits (Kv $\beta 2$, red) and anti-OMgp (green). Arrowheads indicate NORs. Insets are higher magnification images. OMgp clustering commences at P6 and becomes prevalent by P15. Scale bars: a–g, 10 μm . (C) longitudinal sections of cervical spinal cord of (b) MBP-deficient and (a) wild-type mice (age 2 months), and (c,d) of PLP over-expressing transgenic mice (age 7 months) were immuno-stained using antibodies against OMgp (green), in combination with

either anti-Caspr or anti-Kv1.2 (all red) as indicated. Arrowheads denote the retained OMgp clusters at some NORs. Scale bars: 10 μ m.

Fig. 3. Antisense constructs down-regulate OMgp expression *in vitro* and *in vivo*

(A) a, Immuno-blotting assay confirms the presence of OMgp in OLN-93 cells, in OMgp-transfected CHO cells and brain. b, OLN-93 cells transfected with control (Con.), OMgp full-length (FL) or antisense (AS) encoding constructs. The transfected cells, which are GFP fluorescent, were then stained with OMgp antibodies. Enhanced expression of OMgp is associated with cellular arborization of OLN-93 cells, whereas cells in which OMgp is down-regulated retain bipolar morphology. c, Quantification of OMgp fluorescence intensity in transfected cells. The average value of surrounding, non-transfected cells is set at 100. OMgp intensity in the Con., FL and AS transfected cells are 100 ± 5.36 , 364 ± 29.87 and 11 ± 11.81 , respectively. $**P < 0.01$. (B) a, A transgenic construct was generated by cloning murine cDNA that encodes OMgp in an anti-sense direction downstream of the MBP promoter. b, Immuno-

blotting of extracts of spinal cord from OMgp-transgenic mice and wild-type mice using antibodies against OMgp, CNPase and γ -tubulin (the last as the loading control). The protein bands were quantified and compared. Relative to wild-type littermates, the normalized intensities (mean $\pm$ SEM) in three transgenic mice (#1, 2 and 3) are $31.49 \pm 10.42\%$, $36.25 \pm 11.06\%$, $49.08 \pm 7.67\%$ for OMgp; and $84.6 \pm 9.65\%$, $72.70 \pm 12.60\%$, $97.3 \pm 2.67\%$ for CNPase bands, respectively. $**P<0.01$.

Fig. 4. CNS hypo-myelination and disorganized nodal architecture in mice in which OMgp is down-regulated

(A) Representative EM photos showing the ultrastructure of myelin sheaths in spinal cords of OMgp-transgenic mice (b) and wild-type littermates (a) (all aged 2 months). Axon diameters (c) and G ratios, which represent myelin thickness (d) were compared between groups. e, G ratio plotted against different axon diameters. Note that increment of the G ratio in transgenic mice is more pronounced in larger axons. f, The G ratios of different sized axons. There is a significant reduction of myelin thickness in axons of diameter $\geq 1 \mu\text{m}$ in OMgp mutant mice compared with wild-type mice. Scale bars: a, b, $2 \mu\text{m}$. ** $P < 0.01$. (B) EM analyses of longitudinal spinal cord sections of OMgp-transgenic (b, c) and wild-type mice (a) (all aged 2

months). Arrows indicate either inverted or detached oligodendrocytic loops. The parentheses mark the spans of nodal gap and linear segments indicate axonal diameter. Scale bars: 500 nm. (C) Morphometric analyses reveal significant nodal shortening and disorganization of lateral loops in OMgp-transgenic mice in axons of diameter $\geq 1 \mu\text{m}$. a, Detached loops in OMgp-transgenic mice are increased significantly in axons of diameter $\geq 0.5 \mu\text{m}$. b, To evaluate the nodal span in relation to axonal diameter, we calculated the nodal index, defined by $(N1+N2)/D$ as indicated in (A). Nodal index is decreased significantly in axons of diameter $\geq 1 \mu\text{m}$ in OMgp tg mice compared with wild-type mice ($n = 40$). c, Paranodal lengths in OMgp-transgenic mice are also significantly increased in axons of diameter $\geq 1 \mu\text{m}$. $*P < 0.05$, $**P < 0.01$. (D) EM of longitudinal sections of the spinal cord of OMgp-transgenic (b) and wild-type (a) mice (both 2-months old) showing paranodal architectures. Black arrows indicate transverse bands and white arrows the inverted glial loops. There appears to be a normal array of paranodal transverse bands in OMgp-transgenic mice. Scale bars: 200 nm.

Fig. 5. Impaired Na⁺ channel expression and slowed conduction velocity in OMgp-transgenic mice (A) a,b, Confocal micrographs of spinal cord sections of OMgp-transgenic mice and wild-type littermates stained using antibodies against Na⁺ channel α subunit (pan-Nav; red) and Caspr (green). Arrows indicate NORs. c-f, Higher magnification images from a and b. g, Nodal length quantified from confocal micrographs of different sized axons. There is a significant reduction in nodal length in axons of diameter ≥ 1 μm in transgenic mice compared wt mice. **P* < 0.05. Scale bars: a,b, 10 μm; c-f, 5 μm. (B) Immuno-blotting analyses of spinal cord extracts from three OMgp-transgenic mice and their wild-type littermates, using antibodies to Na⁺ channel α, β1 and β2 subunits (γ-tubulin used as the internal control). Protein bands were measured with a densitometer and quantified. Normalized to that of their wild-type littermates, staining intensity (mean ± SEM) in three transgenic mice (#1, 2, 3) is $64.80 \pm 3.81\%$, $60.87 \pm 5.62\%$ and $56.50 \pm 4.13\%$ for the Na⁺ channel α subunit, $86.30 \pm 6.64\%$, $81.41 \pm 5.39\%$ and $80.53 \pm 4.45\%$

for the $\beta 1$ subunit, and $84.33 \pm 7.41\%$, $86.14 \pm 6.93\%$ and $91.91 \pm 6.50\%$ for the $\beta 2$ subunit, respectively. $*P < 0.05$. (C) Conduction velocity in spinal cords of OMgp-transgenic mice is significantly lower than that in wild-type littermates. a, Representative recordings (average of 20 traces) of compound action potentials from equal lengths of the ventral funiculus of spinal cords. Stimulus artifact is removed for clarity. Arrow marks onset of the stimulus (100 μ sec). b, Conduction velocity in OMgp-transgenic mice ($n = 10$) is 20% lower than in wild-type mice ($n = 8$). $*P < 0.05$.

Supplemental Figure 1. OMgp has no direct effects on the voltage-gated Na⁺ currents

a, In the cultured neurons, the Na⁺ currents were recorded continuously with an interval of 30 s between two stimuli. TTX (1 μ M) could completely abolish the Na⁺ currents. b, A representative recording showing the Na⁺ currents recorded in the absence or presence of OMgp-GST or GST. c, Na⁺ currents during OMgp application and after washout of OMgp were normalized to the control (for each group, $n = 8$). The relative values were 0.999 ± 0.018 after 50 ng/ml OMgp and 0.988 ± 0.044 compared with the control.