Supporting Online Material for

Glial Membranes at the Node of Ranvier Prevent Neurite Outgrowth

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This PDF file includes:

Materials and Methods

Other Supporting Online Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/1118313/DC1)

Movies S1 and S2
Supporting Online Material

Movie S1. OMgp$^+$ glial processes converge and ensheath the CNS node of Ranvier.

Please view movie (Movie S1.OMgp.mov) with Quicktime.

Movie S2. NG2$^+$ glial process (in green) encircles the node of Ranvier. Please view movie (Movie S2.NG2.mov) with Quicktime.
Materials and Methods

Antibodies and Reagents

Following primary antibodies were used for microscopy and immunoblots: Rabbit polyclonal anti-MAG, DM20/PLP, MBP, NG2 (Chemicon), Kv1.1 potassium channel (Alamone Labs), OMgp (D. Mikol), contactin (G. Gennarini), and neurofascin (P. Brophy); guinea pig polyclonal anti-Caspr; mouse monoclonal anti-sodium channel (Sigma), neurofilament (Sigma), GFAP (Dako) and Rip (Developmental Studies Hybridoma Bank). Reagents for electron microscopy and rabbit conjugated gold particles were obtained from Electron Microscopy Sciences.

Axogliasome Isolation

CNS white matter (WM) tissues were obtained from dissected human corpus callosum (H. Haroutunian), bovine corpus callosum (Max Insel Cohen), or mouse brainstem (Charles River). Axogliasomes isolation was modified from synaptosome preparation as described (5). Compact myelin was further enriched as described (6). Axogliasomes were further enriched by hypo-osmotic shock and refloated twice more.

Multidimensional Protein Identification Technology

Axogliasomes from human WM were extracted with 400µl MeOH and 100µl chloroform (4:1 v/v), vortexed, followed by addition of 300µl H2O. The extract was vortexed again and pelleted for 30 minutes at 14,000 rpm at 4º C. The supernatant was removed, and the pellet washed and pelleted in 500µl MeOH. The pellet was then resuspended in EtOH and incubated in –20º C overnight to make the extracted membrane water-soluble, then
pelleted at 14,000 rpm in 4°C for 30 minutes and resuspended in 200µl H2O, and sonicated 3 times 15 seconds on ice. 50µl 8M guanidine (Sigma) was then added to the suspension to a final concentration of 2M. The extract was mixed, and incubated (60°C, 15 minutes). The supernatant was then collected and subjected for MudPIT analysis as described (12).

**Electron Microscopy**

Adult rat optic nerves were dissected and prepared for electron microscopy as described(5). For preparation of axogliasomes, membranes were pelleted prior to fixation in 4% glutaraldehyde in 1X PBS, pH 7.5 for 2 hours on ice. For pre-embed immunoelectron microscopy, 50µl of bovine axogliasomes was diluted to 250µl with H2O, and extracted (v/v) with 250µl of 2X Extraction Buffer (2.0% Tx-100, 40 mM Tris, pH 6.5, 0.2 mM CaCl₂, 0.1 mM PMSF). The sample was then vortexed, incubated on ice for 20 minutes and pelleted. The supernatant was discarded, and the pellet resuspended in 500µl Incubation Buffer (0.5% Tx-100, 20 mM Tris, pH 6.5, 0.1 mM CaCl₂, 0.1 mM PMSF) by pipetting up and down until pellet is fragmented, followed by homogenization with a hand-held homogenizer. 10µl of rabbit anti-Caspr (1:50 dilution) was added and incubated with rotation for at least 2 hours in 4°C. The labeled axogliasome extract was overlaid on top of a 1M sucrose cushion (20 mM Tris, pH6.5, 0.1 mM CaCl₂, 0.1 mM PMSF), and pelleted in an ultracentrifuge SW55 rotor at 26,000 rpm for 20 minutes to remove unbound antibodies then resuspended in 250µl Incubation Buffer. 10µl of 6nm gold conjugated goat-anti-rabbit antibody (1:25 dilution) was added to the labeled extract and incubated with rotation for 1 hour, and pelleted through a sucrose cushion as
The pellet was resuspended in 250 µl Incubation Buffer, transferred to an Eppendorf tube and centrifuged at 15,000 rpm for 10 minutes to obtain a visible and dense pellet. The labeled sample was subsequently fixed in 4.0% glutaraldehyde and prepared for electron microscopy.

**Cell Cultures and Axon Outgrowth Assay**

Embryonic day 17 Cb tissues from rat pups were dissected, meninges stripped, and placed into L15 media (Gibco). The collected tissues were treated with 0.25% trypsin in L-15 media at 37°C and mechanically dissociated with a plastic Pasteur pipette. The trypsin was inactivated and with addition of 10% serum, and the cells washed and centrifuged twice in L15 media. The final pellet was resuspended in NB++ (Neurobasal media (Gibco), 2.0% B27, 0.3% L-glutamine (Gibco) and 100 ng/ml nerve growth factor (NGF) (Boehringer-Mannheim)). For the axon outgrowth assay, 50 µg/ml Poly-L-lysine was coated overnight on Lab-Tek Permanox chamber slides (Nalge Nunc International). The slides were then washed and dried. Mouse axogliasomes and myelin were diluted in H2O to the indicated concentrations per µl. 1 µl membrane spots at the indicated concentration were placed onto the slide wells and allowed to dry for 3 hours before plating. The dissociated cells were seeded at approximately 0.5-1.0 x 10⁵ cells/ml in NB++ into each well. After 36 hours, the slides were fixed, immunostained with Crmp-2 antibody followed by a peroxidase reaction with the Vectastain Elite ABC Kit (Vector Laboratories). The lengths of axons (50-100) were measured by NIH ImageJ software. For DRG explants, postnatal day 8 DRGs were dissected from rat spinal cords and placed in L-15 media. Each ganglion was transferred to a Poly-L-lysine treated glass coverslip.
in a 4 well Nunc dish that was coated with 20µl of diluted axogliasomes at indicated concentrations per µl. After 7 days in NB\textsuperscript{++} media, the ganglia were immunostained, and the lengths of axons, based on the radius of axon outgrowth from axon tips to the ganglion, were measured by NIH ImageJ software.

**Immunocytochemistry**

PNS tissues were obtained from post-natal day 18 rat sciatic nerves, fixed for 15 minutes in 4% paraformalddehyde, transferred to 1X PBS, and cut longitudinally into small 3-5 mm sections. A piece of sciatic nerve was then transferred to a drop of 1X PBS on a Superfrost/Plus glass slide, where the perineurium was stripped and removed, and the PNS nerves teased with No 5 superfine forceps (Roboz). The teased fibers were subsequently dried overnight in room temperature, and stored in –80°C until use. CNS tissues were obtained from 4% paraformaldehyde perfused post-natal day 6, 10 and 40 rat spinal cords and brains, and post-natal day 21 mouse spinal cords from OMgp null mice, and cryo-protected through a 12%, 15%, 20% sucrose series and embedded by rapid freezing in Tissue Tek OCT for cryosectioning. For sodium channel staining, the rats were perfused transcardially via 4% paraformaldehyde filled 30 cc syringes for no more than 5 minutes, and dissected tissues were post-fixed for another 5-10 minutes before cryo-protection. Longitudinal and transverse sections (15µm) were generated and placed on Superfrost/Plus glass slides (Fisher), and stored at –80°C until use. For immunofluorescence, the slides containing CNS or PNS tissue sections were rinsed in 1X PBS for 5 minutes and blocked for 1 hour in blocking solution (5% normal goat serum, 0.2 % Tx-100 in 1X PBS). Indicated primary antibodies in blocking solution were
incubated overnight at room temperature, washed briefly in 1X PBS, and species specific secondary antibodies conjugated to rhodamine, fluorescein, or cy5 in blocking solution were incubated for 60 minutes. The sections were washed 3 times in 1X PBS for 10 minutes, applied in mounting medium (50 mM Tris, pH 8.6, 2.5% DABCO, 90% glycerol), sealed, and examined with a Leica TCS 4D confocal scanning microscope.

**Generation of OMgp null mouse**

To create an OMgp- targeting construct, mouse genomic 129/ SvJ DNA was isolated from a lambda genomic library (Stratagene # 946313; Stratagene, LaJolla, CA); a 9.9 kb NotI- EcoRV fragment was subcloned into pBSK\(^+\), then targeted by homologous recombination in bacteria to insert eGFP reporter gene at the initiating ATG. The final construct deleted the entire 1-1299 nt single exon coding sequence of OMgp. This construct was used to target the OMgp locus in V6.5 embryonic stem (ES) cells (obtained from R. Jaenisch). Correctly targeted cells were identified by Southern blotting of XbaI digested ES cell DNAs and injected into C57Bl/6 blastocysts to generate chimeric mice. Chimeras were crossed to C57Bl/6 mice to generate heterozygous founder mice. Genotypes were determined by three-primer PCR of tail DNA. The forward primer 5'-'CGAATGCTAACTGACCCATGC and the two reverse primers 5'-GAACAGTCCACATGCCTGTGCC and 5'-GATGCCCTTCAGCTCGATGCG yielded 207 bp wild-type and 496 bp mutant allele products in a 35-cycle reaction (94°C for 20 sec., 65°C for 30 sec., 72°C for 30 sec). The OMgp null mice analyzed were of a mixed 129SvJ, C57Bl/6 background.
Nodal size and collateral sprouting determination

High resolution image stacks from 21 day old mice spinal cord cryosections were analyzed in double blind manner using the NIH ImageJ program (http://rsb.info.nih.gov/ij/). Nodal size was considered as the distance between two Caspr stained hemi-paranodes connected by neurofilaments, while axonal diameter corresponded to the highest value of paranode length perpendicular to neurofilament stain. Nodal length values were normalized by corresponding axonal diameter and statistical difference was analyzed with Mann-Whitney Rank Sum Test (SigmaStat 3.1 for Windows). Collateral sproutings were identified by detection of labelled axons in between 2 hemi-paranodes. 10 spinal cord sections from either OMgp null or wild type mice were examined.