Matrix Pathobiology

A Mouse Model of Schwartz-Jampel Syndrome Reveals Myelinating Schwann Cell Dysfunction with Persistent Axonal Depolarization *in Vitro* and Distal Peripheral Nerve Hyperexcitability When Perlecan Is Lacking

Marie Bangratz,^{*†‡} Nadège Sarrazin,^{*†‡}
Jérôme Devaux,[§] Désirée Zambroni,[¶]
Andoni Echaniz-Laguna,^{||**††} Frédérique René,^{||**}
Delphine Boërio,^{††} Claire-Sophie Davoine,^{*†‡}
Bertrand Fontaine,^{*†‡§§} Maria Laura Feltri,^{¶¶}
Evelyne Benoit,^{†‡} and Sophie Nicole^{*†‡§§}

From INSERM, U975, Research Center of the Brain and Spinal Cord Institute,* U975, Paris, France; the Université Pierre et Marie Curie-Paris 6,[†] UMR5 975, Paris, France; the Centre National de la Recherche Scientifique,[§] UMR 7225, Paris, France; the Research Center in Neurobiology and Neurophysiology of Marseille,[§] Centre National de la Recherche Scientifique, UMR 6231, Aix-Marseille University, Marseille, France; the Division of Genetics and Cell Biology,[¶] San Raffaele Scientific Institute, Milano, Italy; INSERM, U692,^{||} Strasbourg, France; the Université de Strasbourg,^{**} UMR5 692, Strasbourg, France; the Department of Neurology,^{††} Hôpitaux Universitaires de Strasbourg, Strasbourg, France; Centre National de la Recherche Scientifique, UPR 3294, the Neurobiology Institute of Alfred Fessard,^{††} Gif sur Yvette, France; Assistance Publique-Hôpitaux de Paris,^{§§} Groupe Hospitalier Pitié-Salpêtrière, Department of Neurology, Reference Center for Muscle Channelopathies, Paris, France; and the Hunter James Kelly Research Institute,^{¶¶} State University of New York at Buffalo, Buffalo, New York

Congenital peripheral nerve hyperexcitability (PNH) is usually associated with impaired function of voltage-gated K⁺ channels (VGKCs) in neuromyotonia and demyelination in peripheral neuropathies. Schwartz-Jampel syndrome (SJS) is a form of PNH that is due to hypomorphic mutations of perlecan, the major proteoglycan of basement membranes. Schwann cell basement membrane and its cell receptors are critical for the myelination and organization of the nodes of Ranvier. We therefore studied a mouse model of SJS to determine whether a role for perlecan in these functions could account for PNH when perlecan is lacking. We revealed a role for perlecan in the longitudinal elongation and organization of myelinating Schwann cells because perlecan-deficient mice had shorter internodes, more numerous Schmidt-Lanterman incisures, and increased amounts

of internodal fast VGKCs. Perlecan-deficient mice did not display demyelination events along the nerve trunk but developed dysmyelination of the preterminal segment associated with denervation processes at the neuromuscular junction. Investigating the excitability properties of the peripheral nerve suggested a persistent axonal depolarization during nerve firing *in vitro*, most likely due to defective K⁺ homeostasis, and excluded the nerve trunk as the original site for PNH. Altogether, our data shed light on perlecan function by revealing critical roles in Schwann cell physiology and suggest that PNH in SJS originates distally from synergistic actions of peripheral nerve and neuromuscular junction changes. (Am J Pathol 2012, 180: 2040–2055; DOI: 10.1016/j.ajpath.2012.01.035)

Peripheral nerve hyperexcitability (PNH) is clinically characterized by spontaneous and continuous muscle activity. This activity leads to impaired muscle relaxation, mus-

Supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Association Française Contre les Myopathies, Centre National de la Recherche Scientifique (CNRS) and Université Pierre et Marie Curie-Paris 6 (SN), Telethon Italia GGP08021, and the European Community's Seventh Framework Programme (FP7/2007-2013) HEALTH-F2-2008-201535, UE-FP7 (NGIDD) (M-LF). The UC Davis/National Institute of Neurological Disorders and Stroke/National Institute of Mental Health NeuroMab Facility is supported by NIH grant U24NS050606 and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, UC Davis. N.S. holds a postdoctoral fellowship from the Neuropôle de Recherche Francilien. M.B. holds a doctoral fellowship from the Ministry of High Education and Research and from the Association Française contre les Myopathies. S.N. holds a Contrat d'interface from Assistance Publique-Hôpitaux de Paris.

Accepted for publication January 10, 2012.

M.B. and N.S. contributed equally to this work.

Supplemental material for this article can be found on <http://ajp.amjpathol.org> or at doi: 10.1016/j.ajpath.2012.01.035.

Address reprint requests to Sophie Nicole, Ph.D., Centre de Recherche de l'Institut du Cerveau et de la Moelle Épinrière, ICM, Hôpital Pitié-Salpêtrière, 47, bld de l'Hôpital, 75651 Paris Cedex 13, France. E-mail: sophie.nicole@upmc.fr.

cle twitching at rest (visible myokymia), and cramps.¹ PNH is observed in a heterogeneous group of diseases with autoimmune-mediated and non-autoimmune-mediated forms, including genetic disorders. PNH is usually associated with loss of function of voltage-gated K⁺ channels (VGKCs), which regulate neuronal firing by setting the membrane potential in the subthreshold range.² Accordingly, two inherited forms of PNH are due to dominant mutations in VGKCs: episodic ataxia type 1 with myokymia (Online Mendelian Inheritance of Man no. 160120) is associated with mutations of the *KCNA1* gene impairing functions of Kv1.1,^{3,4} and benign familial neonatal convulsions (Online Mendelian Inheritance of Man no. OMIM 121200) are due to dominant-negative mutations in *KCNQ2* encoding Kv7.2, respectively.^{5,6}

Schwartz-Jampel syndrome (SJS), also known as chondrodystrophic myotonia, is a rare autosomal recessive human disorder characterized by permanent muscle stiffness associated with chondrodysplasia (reduced size, hip dysplasia, kyphoscoliosis, and bowing of the long bones) that develops during childhood.⁷ Electroneuromyography (ENMG) investigations show spontaneous bursts of repetitive discharges with constant shape, amplitude (100 to 400 μ V), and frequency (7 to 80 Hz). These discharges are sensitive to blockade of neuromuscular transmission with curare but not to proximal axotomy, indicating that they originate from motor nerve fibers or neuromuscular junction (NMJ).^{8,9} Other ENMG parameters, such as compound muscle action potentials (CMAPs) and nerve conduction velocities, are normal, making SJS a pure form of PNH.^{9,10} SJS results from hypomorphic mutations of the gene encoding perlecan, the major heparan sulfate proteoglycan (HSPG) of basement membranes (BMs).^{11–13} Perlecan is not crucial for the initial formation of BM but provides critical stability to the scaffold formed by laminins and collagen IV networks. This stability enables BM to withstand mechanical stress as shown by the late (E12–E14) embryonic lethality of perlecan-null mice.^{14,15} In addition to this structural role, perlecan participates in cell signaling through direct binding interactions with α -dystroglycan, integrins β 1 and β 3, and several growth factors (fibroblast growth factors, platelet-derived growth factor, and vascular endothelial growth factor).¹⁶ Despite its widespread expression and functions, a partial lack of perlecan in mammals results in PNH and chondrodysplasia only, suggesting critical neuromuscular functions for perlecan.^{11,12,17} One of these functions is to anchor the collagen-tail form of acetylcholinesterase (AChE) to the synaptic BM at the NMJ.¹⁸ Accordingly, reduced levels of perlecan lead to a partial endplate AChE deficiency in mouse models of SJS.¹⁷ However, we showed that this partial endplate AChE deficiency potentiates muscle force but does not itself induce PNH.

PNH is observed in congenital peripheral neuropathies, where focal demyelination may promote spontaneous activity by generating ectopic impulse and ephaptic transmission (ie, abnormal communication where an action potential in one axon can cause depolarization of an adjacent axon).¹⁹ Accordingly, spontaneous activity at ENMG has been recorded in various mouse models with demyelinating

neuropathy, such as *P0*^{−/−}, *Pmp22*^{−/−}, *Trembler*, *Trembler-J*, and *Pmp22*-overexpressing mice.²⁰ The efficient propagation of action potential along the axon depends on the precise distribution of ion channels into distinct domains at the node of Ranvier. This distribution results from specialized and complex interactions between axons and myelinating Schwann cells.²¹ Voltage-gated Na⁺ channels are highly concentrated at the node where they are essential for the generation of action potential. KCNQ2 is also located at the node, whereas Kv1.1 and Kv1.2 are clustered in the juxtaparanodes concealed by the myelin sheath.^{22,23} The Schwann cell BM is critical for myelination by promoting Schwann cell survival, proliferation, maintenance, and regeneration.²⁴ Laminin-211 and its cell receptor dystroglycan are also crucial for the formation of nodes of Ranvier by promoting Na⁺ channel clustering and organizing Schwann cell microvilli.^{25,26}

These observations prompted us to examine the peripheral nervous system in a mouse model of SJS to determine whether myelination and the nodes of Ranvier are disorganized when the amount of perlecan is reduced in Schwann cell BM. We reveal a role for perlecan in Schwann cell physiology with polyaxonal myelination, short internodes, and numerous Schmidt-Lanterman incisures (SLIs) due to development defects and lengthening of nodes of Ranvier due to maintenance defects in perlecan-deficient mice. Moreover, we demonstrate dysmyelination of the preterminal axon associated with denervation-reinnervation processes at the NMJ. Finally, we suggest that persistent axonal depolarization may occur when perlecan is lacking and exclude the nerve trunk as being the generator site of spontaneous activities, thereby supporting the hypothesis that PNH in SJS originates close to the NMJ.

Materials and Methods

Mice

The hypomorphic perlecan (*Hspg2*^{C1532Yneo}) mutant line mimicking SJS has been previously described.¹⁷ This line contained the missense c.4595G>A mutation, which encodes the p.C1532Y amino acid substitution in exon 36 and the selection cassette *PGK-neo* in intron 36 of the endogenous *Hspg2* gene. It was backcrossed onto a DBA/2J background. Mice were genotyped by PCR amplification of genomic DNA. Homozygous *Hspg2*^{C1532Yneo/C1532Yneo} mutant mice were studied at the age of 2 and 8 months and age-matched wild-type (WT) littermates were used as controls. PLN refers to *Hspg2*^{C1532Yneo/C1532Yneo} homozygous mutant mice and WT refers to *Hspg2*^{+/+} homozygous WT mice. A minimum of three mice per genotype were studied for each analysis. Experiments complied with the guidelines established by the French Council on Animal Care Guide for the Care and Use of Laboratory Animals (EEC86/609 Council Directive - Decree 2001-131) and the experimental protocols were approved by the French Departmental Direction of Animal Protection (agreements 75-952, 91-453, and 3959).

RT-PCR and Quantitative PCR

Total RNA was extracted from sciatic nerves using Trizol reagent (Invitrogen, Cergy Pontoise, France) and was reverse transcribed with random hexamer primers, in accordance with the manufacturer's protocol (Thermoscript RT-PCR System; Invitrogen). Splicing events between *PGK-neo* and *Hspg2* were studied using primers binding within exon 35, exon 37, and *PGK-neo* as previously described.¹⁷ Quantitative real-time PCR analyses were performed using a TaqMan Real-Time PCR detection system (ABI7000; Applied Biosystems, Villebon sur Yvette, France). The reaction mix of the PCR was made up of 115 ng of first-strand cDNA from the reverse transcription reaction (QPCR Mastermix; Eurogentec, Angers, France) and the prevalidated TaqMan Gene expression assays (Applied Biosystems) Hs99999901_s1 (universal 18S RNA, used for the normalization of the results) and Mm00464544_g1 (*Hspg2*).

Western Blot Analyses

Sciatic nerves were homogenized in radioimmunoprecipitation assay lysis buffer containing PBS at pH 7.4, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, and protease cocktail inhibitor (Sigma-Aldrich, Lyon, France). Homogenates were centrifuged at 6000 × g for 10 minutes at 4°C and the supernatant was collected. Protein concentrations were determined using the Bradford method (Bio-Rad, Marnes-la-Coquette, France). A total of 25 µg of total protein extracts was fractionated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Molsheim, France) for Western blot analyses. Membranes were blocked with nonfat milk in PBS and incubated with a polyclonal antibody against Caspr (gift from Dr. Laurence Goutebroze), fibronectin (Sigma-Aldrich), and monoclonal antibodies against Kv1.1 [clone K36/15; University of California (UC) Davis/NIH NeuroMab Facility, Davis, CA] or neurofilament 200 kDa (NF200, clone NE14; Sigma-Aldrich). After intensive washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies (Thermo Scientific, Illkirch, France). Chemiluminescence was detected by autoradiography using the super signal pico ECL reagent (Thermo Scientific). Quantification of bands was performed using ImageJ software version 1.45i (NIH, Bethesda, MD) (<http://rsbweb.nih.gov/ij/>) in accordance with the instructions of the developers.

Tissue Specimens

Sciatic nerve fibers and tibialis anterior (TA) were immediately removed after mouse euthanasia and fixed with fresh 4% paraformaldehyde diluted in 0.1 mol/L phosphate buffer, pH 7.4, for 1 hour (sciatic nerves) or overnight (whole muscles) at 4°C. Nerves were included in OCT medium before freezing for transverse (8 to 10 µm) or longitudinal (30 µm) sections prepared on superfrost plus slides using a cryostat (Leica Microsystems SAS, Nanterre, France). Teased nerve fibers were prepared after the nerve was desheathed, transferred on superfrost

plus slides (CML, Nemours, France), dried for 30 minutes at room temperature, and stored at −80°C until further use. For staining of intramuscular nerve fibers and NMJ, whole TA was embedded in 3% agarose (Sigma-Aldrich) dissolved in PBS, and longitudinal floating sections (50 µm) were prepared by Vibratome sectioning (Leica Microsystems SAS).

Immunofluorescence Microscopy

Floating longitudinal sections of TA and slides were post-fixed if required by immersion in methanol or acetone at −20°C for 20 minutes, depending on the primary antibodies used, washed twice in PBS, and blocked at room temperature for 2 hours with PBS containing 3% bovine serum albumin, 5% normal goat serum, and 0.1% Triton X-100. Sections and slides were incubated at 4°C overnight in a wet chamber with primary antibodies diluted in the blocking solution. They were then washed twice in PBS and incubated at room temperature with Alexa fluor 488 or 555 (Invitrogen) or AMCA (Millipore) conjugated secondary antibodies. TA sections were then put on slides. Slides were mounted in Vectashield mounting medium without or with DAPI to stain nuclei (Vector Laboratories Inc., Burlingame, CA). The following primary antibodies were used: monoclonal antibodies against α-dystroglycan (clone VIA4-1; Millipore), laminin-α2 (clone 4H8-2; Sigma-Aldrich), Voltage-gated Na⁺ channel α-subunits (pan-Nav, clone S8809; Sigma-Aldrich), utrophin (clone DRP3/20C4; Novocastra Laboratories Ltd, Newcastle on Tyne, UK), Caspr (clone K65/35; UC Davis/NIH NeuroMab facility), NF200 (clone NE14; Sigma-Aldrich), synaptophysin (clone SVP-38; Sigma-Aldrich), and domain IV of perlecan (clone A7L6; Millipore); and polyclonal antibodies against Caspr/paranodin and pan-neurofascin (gift from Dr. Laurence Goutebroze), agrin (gift from Prof. Markus A. Rüegg), TAG-1 (gift from Dr. Domna Karagogeos), gliomedin (Abcam, Paris, France), Kv1.1 and Kv1.2 (Alomone Labs, Jerusalem, Israel), KCNQ2,²³ phospho-ERM (Cell Signaling Technology, Danvers, MA), NrCam (Abcam), S100 (Dako, Trappes, France), and myelin basic protein (MBP) (Millipore). Postsynaptic nicotinic acetylcholine receptors (AChRs) were stained with tetramethylrhodamine conjugated α-bungarotoxin (BTX; Molecular Probes, Life Technologies, Villebon sur Yvette, France). For FM1-43 [*N*-(3-triethylammoniumpropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide] staining, teased nerve fibers were incubated in a solution containing 2 µM FM1-43 (Molecular Probes, Cergy Pontoise, France) for 15 minutes and washed in PBS.

Images were acquired using an epifluorescence (Leica DMRA) or confocal laser scanning (Leica SP2) microscope (Leica Microsystems SAS). Measurements of nodal (pan-Nav immunostaining) and paranodal (Caspr/paranodin immunostaining) length and width, nodal area, SLI spacing, and internodal lengths (ILs) were performed on confocal flat projections using ImageJ software. The diameter of individual teased fibers was measured after immunostaining with the secondary goat anti-mouse antibodies that underlined the fiber sur-

face. Quantification of perlecan immunostaining was performed using confocal stack projection of immunostaining performed on teased nerve fibers. The threshold was adjusted and pixel intensity (formatted into constant rectangles) was measured in the nodal or internodal regions using ImageJ software. Quantification of terminal Schwann cell (tSC) number was performed by calculating the number of nuclei surrounded by S100 staining on whole muscle sections stained with anti-S100, BTX, and DAPI. The length of nonmyelinated preterminal segments and the MBP staining thickness were measured on confocal stack projection of whole muscle preparations stained with anti-MBP, BTX and DAPI. The axonal length was measured from the distal end of MBP staining to the first terminal arborization apposed to AChR using ImageJ software.

EM Analyses

The sciatic nerves of 8-month-old WT and PLN mutant mice were dissected and fixed by immersion in 2% glutaraldehyde in 0.12 mol/L phosphate buffer, pH 7.4, overnight at 4°C. Nerves were washed in phosphate buffer and osmicated in 1% OsO₄ for 2 hours at room temperature. They were divided into 5-mm segments, dehydrated in graded ethanols, infiltrated with propylene oxide/Epon mixture (1:1) and then Epon, and polymerized at 60°C. Semithin sections were stained with toluidine blue (1% in 0.12 mol/L phosphate buffer) and viewed with a Leica DMRA light microscope. Ultrathin sections were stained with lead–uranyl acetate and photographed with a LEO 912AB or a Philips CM120 transmission electron microscope (EM).

The diameters of myelinated axons were calculated on the total surface of the semithin sections of sciatic nerves using the g-ratio calculator plug-in developed for ImageJ software.²⁷ Axons and nerve fibers were grouped into increasing size categories separated by 1 μm to calculate their distribution. The g ratios were measured as ratios of axonal to total-fiber perimeters in equivalent 124 × 124-μm areas from each nerve. The f ratio was calculated as ratio between the length of the myelinated Schwann cell membrane corresponding to cytoplasmic regions and the length of the membrane corresponding to appositions. These lengths were measured using ImageJ software on random 17 × 17-μm EM micrographs of transverse nerve sections as previously described.²⁸

Electroneuromyography

Seven PLN mice and 5 WT mice were examined each month from the age of 2 to 8 months. Recordings were made with a standard ENMG apparatus (Dantec Dynamics S.A.S, Nozay, France) in accordance with the guidelines of the American Association of Electrodiagnostic Medicine. Mice were anesthetized with 1 mg/kg of ketamine chlorhydrate and 0.5 mg/kg of xylazine (Rompun; Bayer HealthCare, Loos, France). A monopolar needle electrode (diameter, 0.3 mm; 9013R0312; Medtronic, Boulogne-Billancourt, France) was inserted into the tail of the mouse to ground the system. Recordings were made in the muscle of interest with a concentric needle elec-

trode (diameter, 0.3 mm; 9013S0011; Medtronic). Spontaneous activity was considered intermittent when it was initiated by needle electrode movement or voluntary effort and continuous when it was recorded without any triggering factor.

Multiple Measures of Motor Nerve Excitability in Vivo

The excitability properties were assessed *in vivo* by means of minimally invasive electrophysiologic methods, using the Qtracw software version 10/25/2007 (Institute of Neurology, London, UK) as previously described.²⁹ Briefly, mice were anesthetized by isoflurane inhalation (AErrane; Baxter S.A. Maurepas, France) and placed on a heating pad to maintain body temperature between 35.2°C and 35.8°C (measured using a rectal probe). Electrical stimulations were delivered on the tibial branch of the sciatic nerve by surface electrodes, and CMAP was recorded using needle electrodes inserted into the plantar muscle. Five different excitability tests (stimulus-response and strength-duration curves, threshold electrotonus, current-threshold relationship, and recovery cycle) were performed. Overall, more than 30 parameters were determined from the tests and analyzed.

In Vitro Electrophysiology

The sciatic nerves were quickly dissected from mice after euthanasia and were transferred into artificial cerebrospinal fluid, which contained 126 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl₂, 2 mmol/L MgSO₄, 1.25 mmol/L NaH₂PO₄, 26 mmol/L NaHCO₃, and 10 mmol/L dextrose, at pH 7.4 to 7.5. The nerves were cut into 2-cm segments, placed into a three-compartment recording chamber, and perfused (1 to 2 mL/min) in artificial cerebrospinal fluid equilibrated with 95% O₂/5% CO₂. The bath temperature was kept constant at 35°C. In some experiments, 10 mmol/L of tetraethylammonium chloride (TEA; Sigma-Aldrich) was applied to block VGKCs. The distal end was stimulated supramaximally (duration of 40 μs) through two electrodes isolated with Vaseline, and recordings were performed at the proximal end. Signals were amplified, digitized at 500 kHz, and stored on a hard disk.

To test the effects of KCl, nerves were desheathed and solutions were applied in the central compartment of the chamber (1.4 cm in length). Measurements were made once the effects had reached a steady state, typically 30 to 45 minutes after application. Nerves were continuously stimulated at a frequency of 0.25 Hz. The delay and duration of compound nerve action potentials (CNAPs) were calculated at half-maximal amplitude; the maximal amplitude and area under the curve were also measured. For recruitment analysis, the amplitude of the CNAPs was measured and plotted as a function of the stimulation frequency. For refractory period analysis, two stimuli were applied at different intervals, and the amplitude of the second CNAP was measured and plotted as a function of the stimulus interval. To ensure that the amplitude of the second response was accurately assessed, the

first response was subtracted from all of the recordings. To measure CNAP attenuation after repetitive stimulation, trains of pulses were applied at frequencies ranging from 100 to 1000 Hz. The duration of the train was kept constant at 100 ms, and the amplitude of the last CNAP was measured and compared with the amplitude of the CNAP before repetitive stimulation.

Statistical Analyses

All data in the text, figures, and table legends are expressed as mean $\pm$ SEM except in box plots. Box plots summarized the following five standard values from bottom to top: sample minimum (lower vertical bar), lower quartile (lower side of the rectangle), median (middle bar in the rectangle), upper quartile (upper side of the rectangle), and sample maximum (upper vertical bar). The equality of variances between the mutant and the control groups was estimated using the F-test or Lilliefors's test. Differences between control and mutant values were tested using the parametric, unpaired, two-tailed Student's *t*-test, two-way analysis of variance, or the non-parametric Mann-Whitney *U*-tests, depending on the equality of variances (GraphPad Prism version 5.04; GraphPad Software, La Jolla, CA). Contingent tables were compared using Fisher's exact test or the χ^2 test, depending on the number of data. Distributions were compared using the Kolmogorov-Smirnov test (freely available at http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html). Differences among groups were considered significant when $P < 0.05$.

Results

Expression of Perlecan in Schwann Cell BM and at the Nodes of Ranvier

We first analyzed the expression of perlecan in WT mice and PLN mice with two mutations in the perlecan gene that exert a cumulative hypomorphic effect: one missense mutation (p.C1532Y) in exon 36 and the selection cassette *PGK-neo* in intron 36.¹⁷ Quantitative real-time PCR performed on reverse-transcription products showed a high expression of perlecan in sciatic nerves of 8-month-old WT mice that was lowered by 59% in PLN mice ($P < 0.05$; Student's *t*-test; data not shown). Qualitative PCR confirmed the presence of two mutant mRNA populations in sciatic nerve samples of PLN mice as previously observed in skeletal muscles (Figure 1A).¹⁷ One corresponded to the full-length perlecan mRNA containing the missense mutation, which induces a reduced secretion of full-length mutant perlecan by promoting its intracellular retention. The other mutant mRNA population resulted from alternative splicing with *PGK-neo*, which was predicted to introduce a premature stop codon and be degraded before translation.

Immunostaining analyses showed that perlecan was present within the perineural and Schwann cell BM and was more abundant around the nodes of Ranvier and in the synaptic BM in WT mice (Figure 1B). A reduced

amount of perlecan was observed in all BMs of adult (2- and 8-month-old) PLN mice, which was estimated to be 30% of the amount of perlecan in Schwann cell BM of WT mice (Figure 1C). This reduction level was similar to the perlecan deficiency observed previously in other tissues.¹⁷ The reduction was specific to perlecan because immunostaining of laminin- $\alpha 2$, α -dystroglycan, and utrophin, which have direct or indirect binding interactions with perlecan, was similar between PLN and WT mice (see Supplemental Figure S1, A–C at <http://ajp.amjpathol.org>). An apparent up-regulation of agrin—another HSPG present in Schwann cell BM—was observed in PLN mice. This up-regulation might partially compensate for the lack of perlecan as already reported in skeletal muscles.¹⁷ The high expression of perlecan in Schwann cell BM, especially around the nodes of Ranvier, and its reduction in adult PLN mice prompted us to pursue our investigations of the peripheral nervous system.

Development of PNH with Aging

PLN mice displayed neuromyotonic discharges, ie, bursts of motor unit action potential firing at high rates (120 to 300 Hz), at the age of 8 months, whereas all other neurophysiologic parameters were normal on ENMG investigations.⁸ We further investigated these mutants by performing an ENMG survey from the age of 2 to 8 months to determine whether PNH was present at younger ages. PLN mice aged 2 months already had PNH in paraspinal and facial (levator palpebrae) muscles where neuromyotonic discharges were recorded in response to stimulation (Table 1). However, neuromyotonic discharges were not recorded in the hind limb (gastrocnemius) at this age. PNH progressively worsened and neuromyotonic discharges were detected in the gastrocnemius in response to needle movement and were continuously recorded in paraspinal and facial muscles of older mice. We took advantage of the development of PNH with age to determine its relationship with any morphologic change by comparing PLN mice aged 2 and 8 months.

Polyaxonal Myelination and Increased g Ratio When Perlecan Is Lacking

We performed histologic and EM analyses of sciatic nerves of 8-month-old mice to determine whether changes occur when perlecan is lacking. Gross examination of semithin sections did not reveal dysmyelination in the sciatic nerves of mutant mice (Figure 2A). The nerve of 8-month-old PLN mice also appeared normal at the ultrastructural level (Figure 2B). The perineural sheath was well organized, and the Schwann cell BM was present and well compacted around all of the Schwann cells investigated (see Supplemental Figure S1D at <http://ajp.amjpathol.org>). Sensitive C-fibers in Remak bundles were normal in accordance with the normal sensitive nerve conduction velocities recorded *in vivo* in PLN mice,⁸ and their pain sensitivity behavior (hot plate and tail flick test) was not different from WT mice (data not

Table 1. Development of Peripheral Nerve Hyperexcitability with Aging in PLN Mutant Mice

Muscle	Spontaneous activity	
	2-month-old	8-month-old
Gastrocnemius	Absent	Intermittent
Paraspinal muscles	Intermittent	Continuous
Levator palpebrae	Intermittent	Continuous

Seven PLN mice were surveyed from age 2 to 8 months by ENMG. The term intermittent describes spontaneous activity initiated by needle electrode movement or voluntary effort. The term continuous describes spontaneous activity that was recorded spontaneously. Spontaneous activity was never recorded in WT mice ($n = 5$).

shown). Polyaxonal myelination, in which a single myelin sheath enclosed several axons, was more frequently seen in PLN mice than in WT mice [$1.9\% \pm 0.5\%$ in PLN mice ($n = 1748$); $0.3\% \pm 0.1\%$ in WT mice ($n = 1243$); $P < 0.001$; Fisher's exact test] (Figure 2B). No evidence for demyelination or remyelination was detected.

Morphometric analyses showed that the mean nerve fiber diameter was similar between PLN and WT mice [$5.7 \pm 0.1 \mu\text{m}$ in PLN mice ($n = 3890$) and WT mice ($n = 4307$)]. Furthermore, the distribution of axonal diameters was slightly shifted toward higher values in PLN mutants (Figure 2C). As a consequence, the mean g ratio was marginally but significantly increased (+5%) in PLN versus WT mice [0.64 ± 0.01 in PLN ($n = 837$); 0.61 ± 0.01 in WT ($n = 646$); $P < 0.001$; Mann-Whitney U -test]. Plotting g ratios as a function of the axon diameter showed that all axonal categories were affected (Figure 2D). Altogether, our data excluded major dysmyelination or demyelination when perlecan is reduced by 70% in Schwann cell BM, which was concordant with the normal neurophysiologic parameters recorded in PLN mice during ENMG investigations.⁸

Figure 1. Reduced amount of perlecan in Schwann cell BMs of PLN mutant mice. **A:** Schematic representation of the mutated *Hspg2*^{C1532Yneo} allele and analysis of the splicing events occurring among exons 35, 36, and 37 of the perlecan gene in sciatic nerve samples of 8-month-old PLN and WT mice. The c.4595G>A substitution encoding the p.C1532Y missense mutation is located in exon 36, and *PGK-neo* is located in intron 36. Agarose gel resolution of RT-PCR products showed normally spliced perlecan mRNAs containing the c.4595G>A mutation in PLN samples using primers binding within exons 35 and 37 (right). Hybrid mRNAs, resulting from splicing between exon 35 and *PGK-neo*, were also observed in PLN mutant samples using primers binding within exon 35 and *PGK-neo* (left). **B:** Representative perlecan immunostaining (green) of sciatic nerve sections (top; DAPI in blue), teased sciatic nerve fibers (middle; Caspr in red), and longitudinal TA sections (bottom; AChR in red) from 8-month-old mice. Perlecan is present in perineurial and Schwann cell BM in WT mice. Perlecan is more abundant in the BM around the nodes of Ranvier as demonstrated by immunostaining of teased WT sciatic nerve fibers with paranodes stained with an antibody directed against Caspr (middle) and in the synaptic BM as shown by fluorescent staining of postsynaptic AChR with BTX (bottom). A reduced amount of perlecan was observed in all BM of PLN mice with a punctuate staining that results from the intracellular retention of the mutated p.C1532Y perlecan. Scale bars: 50 μm (top), 40 μm (middle), and 20 μm (bottom). **C:** Quantitative analysis of perlecan immunostaining demonstrates that the amount of perlecan in the BM around the nodes of Ranvier and along internodes was reduced by 70% in PLN mice (white bars) compared with the amount observed in WT mice (black bar) ($***P < 0.001$, two-way analysis of variance). Results are expressed as mean $\pm$ SEM percentage of perlecan immunostaining along internodes or around nodes with the mean percentage of WT immunostaining set to 100. Numbers of values are indicated in parentheses on the graph (with $n = 64$ nodes and $n = 124$ internodes for WT mice).

Figure 2. Polyaxonal myelination and decrease of myelin thickness in PLN mice. **A:** No major differences were observed between transverse semithin sections (toluidine blue staining) of sciatic nerves from 8-month-old PLN mice and WT mice. Scale bar = 50 μm. **B:** Transverse EM analyses of sciatic nerves did not detect major defects in PLN mice compared with WT mice except for the increased amount of polyaxonal myelination events. Scale bar = 2 μm. **C:** Distribution of percentage of fibers in relation to the axon diameter showed a small but significant shift toward higher values in PLN mice compared with WT mice ($P < 0.001$, χ^2 test; $n = 8423$ and $n = 6860$ for WT and PLN mice, respectively). **D:** Distribution of the g-ratio in relation to the axon diameter showed a marginal but significant increase in all categories between PLN mice and WT mice (* $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$, Student's *t*-test for each axonal category).

Increased Nodal and Paranodal Lengths without Molecular Disorganization of the Nodes of Ranvier in Old PLN Mice

Because focal demyelination may induce ectopic impulses,¹⁹ we searched for such events in 8-month-old PLN mice on teased sciatic nerve fibers. We used the FM1-43 dye, which is a fluorescent lipophilic molecule with high affinity to lipid-rich structures, to stain myelin. Although this analysis did not detect demyelinated segments along nerve fibers, it revealed an increased mean length (+17%) of the unstained nodal gap in PLN mice versus WT mice (5.8 ± 0.2 μm in PLN; 4.9 ± 0.1 μm in WT mice; $P < 0.001$; Mann Whitney *U*-test) (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). This change was not seen in 2-month-old PLN mice in which the small increase (+4%) in mean nodal gap length was not statistically different from age-matched WT mice (6.6 ± 0.2 μm in PLN mice and 6.4 ± 0.1 μm in WT mice).

The organization of the nodes of Ranvier depends on complex axoglial interactions.²¹ Immunostaining of the main axonal and glial proteins specific to nodal, paran-

odal, and juxtaparanodal regions was performed on teased sciatic nerve fibers to check their organization. In concordance with FM1-43 staining, we observed a small but significant increase in the length of Na⁺ channel staining in 8- but not 2-month-old PLN mice versus age-matched WT mice (Figure 3, A and B). Nevertheless, the area of Na⁺ channel clusters remained proportional to the axonal diameters, suggesting a preserved nodal function (Figure 3C). Paranodes stained with Caspr were also found to be longer in 8- but not in 2-month-old PLN mice versus WT mice (Figure 3, D and E). Despite these modifications, paranodal and nodal immunostaining did not show any overlap (Figure 3F). Glial (gliomedin, P-ERM, and neurofascin-155 stained with a pan-neurofascin antibody) and axonal (NCAM and neurofascin-186 stained with a pan-neurofascin antibody) components, which are critical for the nodal organization, also displayed a normal immunostaining pattern in 8-month-old PLN mutants (see Supplemental Figure S3A at <http://ajp.amjpathol.org>). Perlecan is critical for the resistance of BM to mechanical stress. To exclude any effect of nerve fiber teasing, we analyzed nodes in longitudinal nerve

Figure 3. Increased length of nodal and paranodal regions in 8-month-old PLN mice. Immunostaining of nodal (**A**) and paranodal (**D**) regions in the nodes of Ranvier with Pan-Na⁺ and Caspr markers, respectively, shows apparently normal (**middle**) and longer (**bottom**) nodes and paranodes in PLN mice versus WT mice. Box plot representations of nodal (**B**) and paranodal (**E**) lengths showed the presence of longer nodes in 8-month-old (8m) but not in 2-month-old (2m) PLN mice versus WT mice (**P < 0.001, Mann-Whitney U-test). Box plot graphs depict minimal value, lower quartile, median, upper quartile, and maximal value from bottom to top with the number of values indicated in parentheses. **C:** The representation of the area filled by Na⁺ channels in relation to the axon diameter did not show a difference between 8-month-old PLN and WT mice. **F:** A representative co-immunostaining of teased nerve fibers showed a normal organization without any overlap of nodal (anti-Pan Na⁺ channel in green) and paranodal (anti-Caspr in red) regions at the nodes of Ranvier in 8-month-old PLN mice (**bottom**). Scale bar = 5 μm.

sections of 8-month-old mice. Nodal length was still significantly higher in PLN mice ($1.8 \pm 0.1 \mu\text{m}$, $n = 70$) than in WT mice ($1.4 \pm 0.1 \mu\text{m}$, $n = 129$) ($P < 0.001$, Mann-Whitney U-test), thereby excluding the effect of fiber teasing on the increased nodal length in mutant samples.

Ultrastructural analyses of nodes with a special attention on Schwann cell microvilli, which are disorganized when dystroglycan and laminin- $\alpha 2$ are lacking,^{25,26} did not detect major changes in 8-month-old PLN mice (see Supplemental Figure S3B at <http://ajp.amjpathol.org>). Calculation of nodal gap lengths on EM preparations confirmed the existence of higher values in PLN mice than in WT mice (see Supplemental Figure S3C at <http://ajp.amjpathol.org>). These results suggested that the overall organization of the nodes of Ranvier was preserved in 8-month-old mutants despite a mild increase in nodal and paranodal length.

Overexpression of Kv1.1 Channels in PLN Mice

Because impaired KCNQ2 and Kv1.1 activity leads to PNH both in humans and mice, we first checked whether their expression is modified when perlecan is lacking.^{3,5,30,31} KCNQ2, which mediates a slow K⁺ current, is located at the node, where it would prevent resting membrane potential fluctuations and aberrant repetitive firing.²³ Kv1.1 and Kv1.2, which contribute to a fast K⁺

conductance that may prevent reexcitation after action potential or participate in the generation of the internodal resting potential, are concentrated at the juxtaparanodes under the myelin sheath.²² Although KCNQ2 immunostaining was similar in PLN and WT mice, which further confirmed the structural integrity of nodes, juxtaparanodal staining of Kv1.1 and Kv1.2 was more intense in both 2- and 8-month-old PLN mice versus WT mice (Figure 4, A and B). The juxtaparanodal overexpression was specific to VGKC because immunostaining of TAG-1 and connexin 29—two other juxtaparanodal proteins present in the axoglial and glial membranes, respectively—were similar in PLN and WT mice (see Supplemental Figure S3A at <http://ajp.amjpathol.org>). Immunoblotting confirmed that the level of Kv1.1 was significantly increased (1.9-fold) in the sciatic nerve samples of 8-month-old PLN mice using fibronectin (a marker of extracellular matrix), NF200, or Caspr as controls of equal loading (Figure 4, C–E). These data excluded a loss of expression or abnormal location of VGKCs when perlecan is reduced, suggesting that more complex mechanisms account for PNH in SJS.

Changes in Internodal Organization of Nerve Fibers

When exploring the organization of nodes of Ranvier, we noticed a marked increase in the number of SLIs and

Figure 4. Overexpression of juxtaparanodal VGKCs in PLN mice. **A:** Representative immunostaining of Kv1.1 and Kv1.2 (red) with Na⁺ channels (green) showing more intense juxtaparanodal Kv1.1 and Kv1.2 stainings in 8-month-old PLN mice versus WT mice. Kv1.1 staining was also observed in the juxtaesaxon, a circumferential strip extending along the internode, and in the juxtainterspaces, which appeared to be more numerous in PLN than in WT mice (**bottom**). **B:** KCNQ2 immunostaining (red) was present at the nodes of PLN mice, stained with anti-Pan Na⁺ channels (green) and did not differ from that observed in WT mice. Scale bar = 15 μ m. **C:** Representative immunoblot analyses of Kv1.1, NF200, Caspr, and fibronectin expression in sciatic nerves from 8-month-old WT and PLN mutant mice, suggesting a higher amount of Kv1.1 in mutant samples than in WT samples. Quantification of immunoblot analyses using fibronectin (**D**) or Caspr (**E**) signal for normalization shows that the amount of Kv1.1 was significantly increased in PLN samples compared with WT samples. Results are reported as means $\pm$ SEM (* P < 0.05; Mann-Whitney U -test with number of studied samples indicated in parentheses).

adjacent juxtainterspaces in PLN mice. SLIs are cytoplasmic channels within compact myelin formed by gap junctions that contained glial components found at the node (neurofascin 155, ERM) and connexins 29 and 32.³² A tripartite axonal strand consisting of paranodal molecules (Caspr) flanked by juxtainterspaces (Kv1.1, Kv1.2, Caspr2, TAG1) extends below the SLIs in the inner mesaxon.³³ More numerous SLIs and associated juxtainterspaces were observed in PLN mice when we immunostained connexin 29, NrCAM, neurofascins, ERM, Caspr, and VGKCs (Figure 5A and data not shown). This finding suggests that the additional SLIs have a normal molecular composition and are fully functional. The higher density of SLIs along internodes was documented by the reduced distance (~40%) between adjacent SLIs in 8-month-old PLN mice versus WT mice (Figure 5B). This increase was not due to

the increased axonal diameter because the number of SLIs relative to the nerve fiber diameter was still higher in PLN mice (data not shown). Furthermore, we found that the difference between PLN and WT mice was less pronounced at 2 months of age compared with 8 months because the mean distance between adjacent SLIs was decreased by 27% at 2 months in PLN mice versus WT mice. This finding indicates development and maturation defects in the formation of SLIs when perlecan is lacking.

We then examined whether the increased number of SLIs was associated with an increased IL in PLN mice. Mean IL was unexpectedly 33% lower in 2- and 8-month-old PLN mice compared with WT mice and remained so after normalization to the fiber diameter (Figure 5C and data not shown). A concomitant increase in the number of myelinating Schwann cells evaluated by the number of

Figure 5. Higher number of SLIs and shorter length of internodes in PLN mice. **A:** Representative immunostaining of SLIs with NrCAM and connexin 29 (Cx29) proteins showing the increased number of SLIs along the teased sciatic nerve fibers of PLN mice versus WT mice. Scale bar = 30 μ m. Box plot representations of distances between adjacent SLIs (**B**) and ILs (**C**) showed the decreased values in 2- (2m) and 8- month-old (8m) PLN mice compared with age-matched WT mice ($***P < 0.001$, Mann-Whitney *U*-test). Box plot graphs depict minimal value, lower quartile, median, upper quartile, and maximal value from bottom to top with numbers of values indicated in parentheses. **D:** Visualization of internodes immunostained with pan-neurofascin (pan-NF in green) and Schwann cell nuclei (DAPI in blue) in teased sciatic nerves showed shorter internodes concomitant to an increased number of myelinating Schwann cells in 8-month-old PLN mice compared with WT mice. SLIs, nodes, and Schwann cell nuclei are labeled with asterisks, arrowheads, and n, respectively. Scale bar = 40 μ m. **E:** Calculation of abaxonal appositions per Schwann cell in WT and PLN mice did not show any significant difference in distributions between the two genotypes ($n = 121$ for PLN mice and $n = 135$ for WT mice).

nuclei beneath Schwann cell BM was observed (Figure 5D). Disruption of the dystroglycan complex impairs Schwann cell elongation, resulting in shorter IL in parallel with alteration of Cajal bands, which are the cytoplasmic channels located between the abaxonal myelin sheath and the Schwann cell cytoplasmic membrane.³⁴ We therefore determined whether lack of perlecan affects the Cajal bands. For this purpose, we calculated the number of appositions between the abaxonal surface of the myelin sheath and the cytoplasmic membrane on transversal EM analyses. The number of appositions was not significantly different between PLN and WT mice at the age of 8 months (Figure 5E). In addition, f ratio, a value that quantitatively assesses the compartmentalization degree of the Schwann cell cytoplasm, was similar between PLN mutants and WT mice [0.60 ± 0.06 in PLN mice ($n = 53$); 0.63 ± 0.06 in WT mice ($n = 82$)]. These data suggest that the reduced IL resulting from PLN deficiency involves a function independent of cytoplasmic compartmentalization of myelinating Schwann cell into Cajal bands.

Dysmyelination of the Preterminal Segment with Aging

Finally, we investigated the distal myelinated segment near the NMJ because it could be a more susceptible region to demyelination. As previously reported for extensor digitorum longus,¹⁷ we observed major NMJ remodeling in TA longitudinal sections of 2- and 8-month-old PLN mice with incomplete apposition of presynaptic (nerve terminal) and postsynaptic (AChR) components,

thin terminal nerves and short sprouting of tSCs suggestive of reinnervation processes by nerve terminal sprouting, and streaky pattern of AChRs (Figure 6A). The number of tSCs at the NMJ was similar between mutant and WT mice (3 ± 1). Focusing on S100 staining showed a clear limit between the thick myelinated preterminal nerve segment—located before the first axonal arborization apposed to AChRs—and the thin, nonmyelinated terminal segment in the NMJ of WT mice. In addition to this normal S100 immunostaining pattern, we saw thinner preterminal S100 staining in mutant mice. This pattern was related to processes of denervation and reinnervation by nerve sprouting because it was associated with NMJs composed of thinner preterminal axons, areas of AChRs devoid of terminal nerve, and tSC sprouting (Figure 6A).

Denervation-reinnervation processes are usually associated with demyelination and Schwann cell dedifferentiation to promote axon regrowth.³⁵ We immunostained the myelin sheaths in intramuscular nerves with MBP to determine whether preterminal demyelination occurs when perlecan is lacking. If we did not observe interrupted preterminal MBP staining in PLN mice, we saw longer (1.5-fold) nonmyelinated segments compared with WT mice at the age of 8 months even in apparently well-innervated NMJs (Figure 6, B and C). This change was not seen at the age of 2 months. Finally, measuring the thickness of distal MBP staining showed that the high values seen in WT mice were absent in PLN mice at the age of 8 months (Figure 6D), suggesting remyelination of the preterminal segment in PLN mice. Perlecan deficiency therefore leads to developmental changes at the NMJ with denervation-

Figure 6. Denervation-reinnervation processes at the NMJ with dysmyelination of the preterminal segment in PLN mice. **A:** Representative fluorescent staining of postsynaptic AChRs (BTX in blue), Schwann cells (anti-S100 in green), and motor nerve terminals [anti-NF200 and anti-synaptophysin (sy) in red] in longitudinal sections of TA from 8-month-old mice. NMJs of WT mice displayed AChRs with a pretzel-like organization that is recovered by nerve terminal and tSCs. S100 staining of the preterminal nerve segment is thick with well-defined positions of Schwann cell nuclei, which are suggestive of myelination (top). In PLN mice, AChRs displayed a streaky pattern with poor or no pretzel-like organization. Denervation processes were seen in mutant NMJs with areas of AChRs devoid of nerve terminal (arrowhead) and thin preterminal S100 staining (arrow). NMJs with highly fragmented AChRs were also seen in 8-month-old PLN mice (asterisk). Scale bar = 20 μm. **B:** Box plot representations of nonmyelinated preterminal segment lengths showed increased values in PLN mutant mice compared with WT mice. The difference was statistically significant at the age of 8 months ($***P < 0.001$, Mann-Whitney *U*-test). **C:** A representative fluorescent staining of AChRs (BTX in blue), myelin sheath (anti-MBP in green), motor axon, and nerve terminal (anti-NF200 and anti-synaptophysin (sy) in red) showed a longer nonmyelinated preterminal segment in a well-innervated NMJ of an 8-month-old PLN mouse compared with a WT mouse. Scale bar = 20 μm. **D:** Box plot representations of the thickness of preterminal MBP staining showed the absence of high values in 8-month-old PLN mice compared with WT mice. The absence of high values in PLN mice was less pronounced at the age of 2 months. All box plot graphs depict minimal value, lower quartile, median, upper quartile, and maximal value from bottom to top with number of values indicated in parentheses.

reinnervation processes that induce dysmyelination of the preterminal myelinated segment with aging.

No Major Changes in Neuromuscular Excitability in Vivo

Our previous ENMG investigations of 8-month-old PLN mice did not indicate major neurophysiologic changes.⁸ Nevertheless, increased amount of VGKCs and reduced ILs could induce subtle changes along the nerve trunk. To test this hypothesis, we performed a multimodal evaluation of nerve excitability based on threshold tracking techniques. These approaches specifically investigate ion conductance and axonal membrane properties

in vivo.^{29,36} Five different excitability tests (stimulus-response and strength-duration curves, threshold electrotonus, current-threshold relationship, and recovery cycle) were performed by stimulating the tibial branch of the sciatic nerve and recording CMAP from the plantar muscle in 2- and 8-month-old mice *in vivo*. Off-line treatment did not reveal differences between 2-month-old WT and PLN mice (data not shown). In particular, parameters sensitive to fast VGKC activity, such as threshold changes occurring in the early phase of depolarizing electrotonus and superexcitability in the recovery cycle, were normal (see Supplemental Figure S4 and Supplemental Table S1 at <http://ajp.amjpathol.org>). This excluded a physiologic effect of the overexpression of

Figure 7. Increased threshold undershoot in 8-month-old PLN mutant mice. **A:** Threshold changes (threshold reduction or increased excitability plotted upwards) induced by a 100-ms subthreshold depolarizing current determined *in vivo* from the plantar muscle of 8-month-old WT (filled symbols) and PLN (open symbols) mice. **B:** Threshold undershoot was characterized by a 1–6 fold threshold increase after the end of the 100 ms current in PLN mice compared with WT mice. Data are expressed as means $\pm$ SEMs with number of values in parentheses (** $P < 0.01$; Student's *t*-test).

Kv1.1. At 8 months, all parameters were still normal except for an increased threshold undershoot (Figure 7; see also Supplemental Table S1 at <http://ajp.amjpathol.org>).

Threshold undershoot corresponds to the reduction in excitability after the end of a long-lasting subthreshold depolarizing current and is associated with the gradual deactivation of slow VGKCs.³⁷ Although a greater undershoot might indicate a slower deactivation of the slow VGKCs, the normality of the other threshold electrotonus parameters that depend on slow VGKCs excluded changes in their activity. Nevertheless, the significant increase of threshold undershoot in parallel with the development of PNH prompted us to further examine the *in vitro* electrophysiologic properties of isolated sciatic nerves in 8-month-old mice. The amplitude, duration, and conduction velocity of CNAPs assessed *in vitro* were not significantly different in 8-month-old PLN and WT mice (Table 2). In addition, the recruitment and refractory period of sciatic nerve axons were unaffected (data not shown). We tested whether TEA, which blocks most VGKCs, had any effect on CNAPs in PLN mice. A solution of 10 mmol/L TEA did not alter the conduction velocity or the shape of the CNAPs in WT or PLN mice (see Supplemental Table S2 at <http://ajp.amjpathol.org>). This finding further demonstrates that overexpression of Kv1.1 channels did not participate in the nerve excitability of PLN mice.

Axonal Depolarization of PLN Nerves *in Vitro*

Slow VGKCs are activated in response to prolonged depolarization—as may occur during high-frequency activity—to prevent inappropriate after-discharge and are unlikely to be activated by a single action potential.³⁸ Motor units fire at mean frequencies of discharges ranging from 30 Hz (slow skeletal muscles) to 100 Hz (fast skeletal muscles) and are recruited with initial frequencies up to 500 Hz.³⁹ We therefore examined nerve excitability *in vitro* using frequencies of stimulation ranging from 100 to 500 Hz. If no differences were seen in these physiologic ranges, we observed greater attenuations of mutant CNAPs with higher frequencies. We therefore tested

higher frequencies of stimulation (600 to 1000 Hz) and observed a drastic attenuation of CNAPs in PLN nerves compared with WT nerves (Figure 8A).

Several phenomena may be responsible for these effects: i) increased intrinsic VGKC activity, ii) increased Na^+/K^+ ATPase pump activity, or iii) persistent axonal depolarization. The amplitude of the posttetanic hyperpolarization was not significantly increased in PLN nerves, excluding an implication of the Na^+/K^+ ATPase pump (data not shown). We then tested the hypothesis of a persistent axonal depolarization in response to high frequencies of stimulation by raising the extracellular K^+ concentration. When extracellular K^+ concentration was raised to 12 or 15 mmol/L, the amplitude of CNAPs in response to a single stimulus decreased to greater extent in PLN nerves than in WT nerves (Figure 8B). A significant difference in CNAP attenuation during trains of stimulation at physiologic frequencies (100 to 500 Hz) was seen between PLN nerves and WT nerves when extracellular K^+ concentration was raised to 12 mmol/L (Figure 8C). These data suggest that a persistent axonal depolarization may occur in PLN nerves *in vitro* during a train of action potentials. Because axonal depolarization may favor ectopic impulses, we recorded action potentials at high gain amplification to determine whether spontaneous activity occurs in mutant sciatic nerves. No spontaneous activity was recorded in isolated sciatic nerves of PLN mice under any previously tested conditions *in vitro*. This last result excludes the nerve trunk as a generator site of spontaneous activity when perlecan is lacking, which favors the hypothesis of an origin close to the NMJ for PNH in SJS.

Discussion

We provide important new clues to the neuromuscular functions of perlecan and their relationship with SJS, a rare disease providing a pure model for the understanding of congenital PNH. We show that perlecan plays a role in Schwann cell growth (myelin thickness and internodal elongation) and structure (SLI formation) during development. Moreover, we demonstrate a role for perlecan in the maintenance of the preterminal nerve segment with age. If we exclude the nerve trunk as the generator site of PNH, we reveal that a reduced amount of perlecan might induce changes in slow VGKCs functioning with persistent axonal depolarization under certain conditions *in vitro*. Our data strongly argue for a presynaptic origin of PNH in SJS, where

Table 2. *In Vitro* Electrophysiologic Characteristics of Sciatic Nerves at 35°C

Characteristic	WT mice	PLN mice
Amplitude (mV)	5.7 $\pm$ 0.7	4.5 $\pm$ 0.6
CV $V_{1/2}$ (m/s)	56.3 $\pm$ 1.8	53.2 $\pm$ 2.2
CV V_{max} (m/s)	38.9 $\pm$ 1.4	36.2 $\pm$ 1.3
Duration $V_{1/2}$ (ms)	0.30 $\pm$ 0.02	0.31 $\pm$ 0.01
<i>n</i>	23 (12)	28 (14)

V_{max} and $V_{1/2}$ are the maximal and the half-maximal amplitude of CNAP, respectively. Data are expressed as means $\pm$ SEMs. *n* is the number of nerves tested with the number of animals indicated in parentheses.

Figure 8. In vitro effects of depolarization induced by raising extracellular K⁺ on sciatic nerve activity of 8-month-old PLN mice. **A:** CNAP amplitude obtained in response to repetitive stimulations (100 ms, 100 to 1000 Hz) at 35°C represented as a function of the stimulation frequency at physiologic (3 mmol/L) extracellular K⁺ concentration. The amplitude of the last CNAP is expressed as a percentage of the amplitude of the CNAP before repetitive stimulation. The CNAPs from PLN mice were increasingly attenuated at high-frequency stimulation (700 to 1000 Hz) compared with WT mice (filled squares). **B:** Amplitude of CNAPs from PLN and WT mice was measured at increasing extracellular K⁺ concentrations. The attenuation of CNAP amplitude obtained in response to a single stimulus was higher in PLN nerves (white bars) than in WT nerves (black bars) when extracellular K⁺ was raised to 12 and 15 mmol/L. **C:** CNAP amplitude obtained in response to increasing train frequency (100 ms, 100 to 500 Hz) represented as a function of the stimulation frequency obtained at high (12 mmol/L) extracellular K⁺ concentration. A higher degree of CNAP attenuation was seen at all frequencies of stimulation in PLN nerves compared with WT nerves when extracellular KCl was increased to 12 mmol/L. The graphs depict means $\pm$ SEMs (* P < 0.05 and ** P < 0.01; Student's *t*-test with n > 10 from at least eight independent mice).

three changes may act in combination to generate spontaneous activity: synaptic AChE deficiency,⁸ axonal depolarization persisting focally during trains of nerve action potentials, and dysmyelination of the preterminal axonal segment.

According to its status of major HSPG of BM, perlecan is present in all Schwann cell BMs. We show that perlecan is dispensable for Schwann cell differentiation and during the promyelinating stages because only rare polyaxonal myelination events and no large bundles of unsheathed axons were observed when perlecan was reduced by 70%. In mice deficient for laminin-211, defective radial sorting is more severe in spinal roots than in distal nerves.²⁶ Major hypomyelination in the spinal roots of PLN mice is functionally excluded because the F-wave latency, which is the second CMAP resulting from the backfiring of a small portion of the motor neurons in response to the antidromic propagation of the initial stimulus, is within the normal range (A. Echaniz-Laguna, unpublished data). We also demonstrate that perlecan is concentrated in the BM around the nodes of Ranvier. At this location, perlecan has been hypothesized to bind the secreted gliomedin multimers to facilitate clustering of cell adhesion molecules and Na⁺ channels during formation of nodes of Ranvier.⁴⁰ However, the normal gliomedin staining and nodal organization that we observed in PLN mutant mice exclude such a critical role for perlecan. The only modification seen in the perlecan-deficient nodes was an increased length for some nodal and paranodal segments with age. A possible explanation for nodal lengthening is demyelination,⁴¹ but such events were not observed in PLN mutant mice. Age-related changes in the nodes of Ranvier with altered densities of Na⁺ channels and disorganized microvilli have been reported in mice with selective deletion of dystroglycan.²⁵ The small nodal lengthening observed in PLN mice might therefore result from the direct binding interaction of PLN with α -dystroglycan. The absence of major myelination or nodal alterations, such as those observed in laminin-211- and dystroglycan-deficient mice, may be due to a marginal role of perlecan in these functions, to the residual amount of

perlecan present in the mouse model of SJS, or to a functional compensation by other HSPG such as agrin. This phenotype is fully concordant with the human phenotype because clinical or neurophysiologic signs of peripheral neuropathy have never been reported in patients with SJS.^{9,10} Using a mouse model with a complete lack of perlecan restricted to Schwann cell BM to overcome the early lethality of perlecan knockout will be of interest for a follow-up of the questions raised by our studies.

We reveal a more important role for perlecan in the longitudinal elongation and organization of myelinating Schwann cells with shorter internodes, additional SLIs, and increased amount of fast VGKCs when perlecan is lacking. Despite the shorter ILs, nerve conduction velocities are normal in PLN mice, both *in vivo* and *in vitro*. There are at least two possible explanations for this. First, not all internodes showed a reduced length in PLN mice. Second, the small increase in axonal diameter may compensate for the reduced IL in PLN mice because the conduction velocity is proportional to both axon diameter and IL.⁴² During nerve growth, Schwann cells elongate longitudinally to match the extension of their associated axon concomitantly to the radial growth of myelin.⁴³ Critical to the process is the cytoplasmic compartmentalization of myelinating Schwann cells into Cajal bands, which depends on laminin-211, dystroglycan, utrophin, and periaxin-DRP2 axes.³⁴ We did not observe defective cytoplasmic compartmentalization in PLN mice by calculating both the number of appositions and the f ratio, suggesting that this mechanism is not responsible for shorter ILs when perlecan is lacking. Increased Schwann cell proliferation before the onset of myelination may also account for reduced ILs.⁴⁴ Schwann cell functions are regulated by numerous axolemma-based and nonaxonal membrane signaling pathways dependent or independent from the extracellular matrix.⁴⁵ Perlecan is able to bind growth factors critical for the regulation of Schwann cell functions, including neuregulin, fibroblast growth factors, and platelet-derived growth factors, and to modulate

their activity by regulating their fixation rate to their cell surface receptors.^{16,46} The changes in Schwann cell functions observed when perlecan is lacking may result from combined effects of perlecan deficiency on distinct signaling pathways, which renders their identification difficult. A cumulative effect of perlecan deficiency on distinct signaling pathways may also account for the increased amount of fast VGKCs at the juxtaparanodes. Juxtaparanodal clustering of Kv1 channels depends on their association with the Caspr2/TAG-1 adhesion complex.^{47,48} Expression of TAG-1 was unaltered in PLN mice, suggesting a normal organization of juxtaparanodes. Cellular pathways involving endoplasmic reticulum chaperones, G-protein-coupled receptors, and phosphorylation also play a role in fast VGKCs clustering and could be indirectly modified when perlecan is lacking through a mechanism that is yet to be determined.⁴⁹

Whatever the origin of increased fast VGKC expression when perlecan is lacking, it is functionally silent because peripheral nerve excitability parameters, depending on fast VGKCs, are normal in PLN mice. The more probable explanation is the poor involvement of the fast VGKCs, located under the myelin sheath, in the action potential repolarization.² Another possibility is a functional compensation by the increased number of SLIs. SLIs provide a direct radial route for the transport of ions and metabolites between the cytoplasmic compartments on the abaxonal and adaxonal sides of Schwann cells. The increased number of SLIs could partially compensate for the increased physiologic demands that result from the increased density of fast VGKCs so that nerve functions are preserved when perlecan is lacking. The factors involved and the mechanism for the interrelated formation of SLIs and juxtaneurites are poorly understood. P0 is one critical molecular element for SLI formation.⁵⁰ An increased number of SLI has also been observed in mice deficient for proteins with unrelated functions. These proteins include MBP, desert hedgehog, Schwannomin/merlin, cerebroside sulfotransferase, and Caspr.^{44,51–53} If these mutant mice share defective axoglial interactions, they develop increased numbers of SLIs through different mechanisms that remain to be identified and that may also occur when perlecan is lacking.

Despite the structural changes that result from perlecan deficiency, the excitability properties (ie, ion conductance and axonal membrane properties) of motor axons assessed *in vivo* along the nerve trunk did not differ from normal. Threshold tracking techniques provide an assessment of nerve excitability at the point of stimulation.³⁶ Unless the assessment can be performed at the lesion site, they may not be so useful for detecting focal changes. In addition, they only test axons with thresholds close to the level chosen for tracking. Our data thus confirm that the changes in hypomorphic PLN mutant mice are more focal in nature. Hence, we detected a greater undershoot that developed in parallel with PNH. Our analyses suggested a greater activity of slow VGKCs under conditions when the slow K⁺ current is more prone to be activated due to the slow time course of its activation and deactivation. However, the normality of the other threshold electrotonus parameters that depend on slow VGKCs rules out general changes in their activity.³⁸ Nerve conduction requires fine tuning of ionic currents

through delicate interactions between axons and Schwann cells. A local increase in extracellular K⁺ concentration shifts the voltage dependence of activation of slow VGKCs to more negative membrane potentials. Consequently, the greater activity of slow VGKCs detected *in vitro* at high stimulation rates could reflect changes in K⁺ homeostasis of nerve fibers when perlecan is lacking. The exaggerated effect of axonal depolarization induced by high extracellular K⁺ concentration on CNAP attenuation occurring in PLN mice versus WT mice supports this assumption. Schwann cells via their paranodal loops and their SLI play a major role in the uptake and storage of K⁺ ions accumulated in the periaxonal space during trains of AP.⁵⁴ An appealing hypothesis is a reduced ability of Schwann cells to assume this function when perlecan is reduced. Although the increased paranodal length and higher number of SLIs correlate with the development of PNH, the molecular organization of these structures was unchanged. This finding suggests that more subtle physiologic changes are taking place. Schwann cells express a variety of K⁺ channel subtypes and different co-transporters involved in the maintenance of cellular osmotic homeostasis, including the K⁺-Cl⁻ and Na⁺-K⁺-2Cl⁻ cotransporters.^{54–56} Therefore, we could assume that the function of one or several of these ion channels and/or co-transporters is altered when perlecan is lacking. Another exciting hypothesis is a role of perlecan in the local accumulation of ions by the diffusion barrier function of the extracellular matrix thanks to its high net negative charge due to its glycosaminoglycan chains and its long (up to 200 nm) length, as recently reported for Bral1 in the central nervous system.⁵⁷

Our data do not argue for an exclusive role of these physiologic changes in the genesis of PNH in SJS because the nerve trunk does not display hyperexcitability. Combined with our previous works showing that spontaneous activity in SJS is sensitive to NMJ blockade by curare but not to proximal axotomy, this result strongly argues for a preterminal generator site of PNH.⁸ The transition zone between myelinated and nonmyelinated preterminal segments is a particularly vulnerable site for spontaneous impulse generation.⁵⁸ Accordingly, PNH observed in Kv1.1-deficient and KCNQ2-deficient mice originates from the distal part of the motor nerve.^{30,31} Perlecan-deficient mice develop preterminal dysmyelination with reduced MBP thickness of the last internode and increased length of the nonmyelinated preterminal segment. These changes occur with age and are associated with denervation-reinnervation processes. Remodeling of the preterminal segments may result from developmental defects leading to demyelination and axonal retraction or be induced by defects in the maintenance of the NMJ presynaptic component, leading to terminal nerve sprouting. We favor the second hypothesis because no signs of demyelination were seen along the nerve trunk when perlecan is lacking. Patterning of motor nerve terminal is sequentially organized by three independent sets of molecules among which are synaptic BM components: FGF 7, 10, and 22 subfamilies (formation), laminin β 2 (maturation), and α 3–6 collagen IV chains (maintenance).⁵⁹ Interestingly, loss of collagens α 3–6(IV) predominantly affects the diaphragm, which is nearly the sole striated

muscle to be healthy in SJS.⁸ Perlecan could be the counterpart of collagens $\alpha 3$ –6(IV) for the NMJ maintenance in the skeletal muscles, resulting in NMJ denervation-reinnervation processes and dysmyelination of the preterminal segment when perlecan is lacking.

More severe remodeling of preterminal and terminal segments are seen in congenital peripheral neuropathies, with distinct changes depending on the mutation: preterminal axonal swelling, demyelination, and nodal sprouting leading to failure of action potential conduction are observed in periaxin-null mice, whereas P0-overexpressing mice displayed retraction of motor neurons leading to numerous nodal and terminal sprouting with defective neuromuscular transmission with age.^{60,61} If we cannot exclude conduction blocks along the long non-myelinated preterminal segments in perlecan-deficient mice, we did not find physiologic evidence for action potential transmission failure *in vivo* (ENMG) or *in situ* (muscle force recording).^{8,17} This finding suggests that the severity of preterminal modifications that result from perlecan deficiency is not sufficient to induce conduction blocks recordable when studying whole motor units or muscles. In the adult, the major determinants rendering the preterminal myelinated segment prone to reexcitation are the gradual internodal shortening that precedes the nonmyelinated nerve terminal and slow VGKCs.⁶² Therefore, the reduced ILs and the potential changes in K^+ homeostasis, which can induce a focal increase in extracellular K^+ concentration and axonal membrane depolarization, that result from perlecan deficiency in the Schwann cell BM could favor preterminal nerve hyperexcitability. The hyperexcitable properties of the preterminal nerve segment probably benefit from the synaptic AChE deficiency, which may generate presynaptic antidromic activity, to generate spontaneous activity.⁶³ In summary, our data suggest that PNH in SJS results from a synergistic interaction between changes that affect several determinants of distal nerve excitability, due to critical neuromuscular functions of perlecan in longitudinal organization of myelinating Schwann cells (ILs and K^+ homeostasis), NMJ formation (synaptic AChE anchoring), and NMJ maintenance (stability of the presynaptic component) (see Supplemental Figure S5 at <http://ajp.amjpathol.org>).

Acknowledgments

We thank the Plateforme d'Imagerie Cellulaire Pitié Salpêtrière, especially Drs. Dominique Langui and Aurélien Dauphin for use of the electron and confocal microscopes and Drs. Markus Rüegg (University of Basel, Basel, Switzerland), Elior Peles (The Weizmann Institute of Science, Rehovot, Israel), Laurence Goutebroze (Institut du Fer à Moulin, Paris, France), and Domna Karagogeos (University of Crete Medical School and Institute of Molecular Biology and Biotechnology, Heraklion, Greece) for providing antibodies. The monoclonal Caspr antibody was obtained from the UC Davis/National Institute of Neurological Disorders and Stroke/National Institute of Mental Health NeuroMab Facility.

References

- Hart I, Newsom-Davis J: Generalized peripheral nerve hyperexcitability (neuromyotonia). Edited by A Engel, C Franzini-Armstrong. New York, McGraw-Hill Medical Publishing Division, 2004, pp 1301–1309
- Waxman SG, Ritchie JM: Molecular dissection of the myelinated axon. *Ann Neurol* 1993, 33:121–136
- Browne DL, Gancher ST, Nutt JG, Brunt ER, Smith EA, Kramer P, Litt M: Episodic ataxia/myokymia syndrome is associated with point mutations in the human potassium channel gene, KCNA1. *Nat Genet* 1994, 8:136–140
- Jen JC, Graves TD, Hess EJ, Hanna MG, Griggs RC, Baloh RW: Primary episodic ataxias: diagnosis, pathogenesis and treatment. *Brain* 2007, 130:2484–2493
- Dedek K, Kunath B, Kananura C, Reuner U, Jentsch TJ, Steinlein OK: Myokymia and neonatal epilepsy caused by a mutation in the voltage sensor of the KCNQ2 K^+ channel. *Proc Natl Acad Sci U S A* 2001, 98:12272–12277
- Wuttke TV, Jurkat-Rott K, Paulus W, Garncarek M, Lehmann-Horn F, Lerche H: Peripheral nerve hyperexcitability due to dominant-negative KCNQ2 mutations. *Neurology* 2007, 69:2045–2053
- Nicole S, Stum M, Fontaine B: Perlecan: Schwartz-Jampel syndrome (SJS, MIM 255800) and dyssegmental dysplasia, Silverman-Handmaker type (DDSH, MIM 224410). Edited by Epstein C, Erickson R, Wynshaw-Boris A. Oxford, Oxford University Press, 2008, pp 1435–1442
- Echaniz-Laguna A, Rene F, Marcel C, Bangratz M, Fontaine B, Loeffler JP, Nicole S: Electrophysiological studies in a mouse model of Schwartz-Jampel syndrome demonstrate muscle fiber hyperactivity of peripheral nerve origin. *Muscle Nerve* 2009, 40:55–61
- Taylor RG, Layzer RB, Davis HS, Fowler WM, Jr.: Continuous muscle fiber activity in the Schwartz-Jampel syndrome. *Electroencephalogr Clin Neurophysiol* 1972, 33:497–509
- Aberfeld DC, Namba T, Vye MV, Grob D: Chondrodystrophic myotonia: report of two cases. Myotonia, dwarfism, diffuse bone disease, and unusual ocular and facial abnormalities. *Arch Neurol* 1970, 22:455–462
- Nicole S, Davoine CS, Topaloglu H, Cattolico L, Barral D, Beighton P, Ben Hamida C, Hammouda H, Cruaud C, White P, Samson D, Urtizbereja JA, Lehmann-Horn F, Weissenbach J, Fontaine B: Perlecan, the major proteoglycan of basement membranes, is altered in patients with Schwartz-Jampel syndrome (chondrodystrophic myotonia). *Nat Genet* 2000, 26:480–483
- Arikawa-Hirasawa E, Le AH, Nishino I, Nonaka I, Ho NC, Francomano CA, Govindraj P, Hassell JR, Devaney JM, Spranger J, Stevenson RE, Iannaccone S, Dalakas MC, Yamada Y: Structural and functional mutations of the perlecan gene cause Schwartz-Jampel syndrome, with myotonic myopathy and chondrodysplasia. *Am J Hum Genet* 2002, 70:1368–1375
- Stum M, Davoine CS, Vicart S, Guillot-Noel L, Topaloglu H, Carod-Artal FJ, Kayserili H, Hentati F, Merlini L, Urtizbereja JA, el Hammouda H, Quan PC, Fontaine B, Nicole S: Spectrum of *HSPG2* (Perlecan) mutations in patients with Schwartz-Jampel syndrome. *Hum Mutat* 2006, 27:1082–1091
- Arikawa-Hirasawa E, Watanabe H, Takami H, Hassell JR, Yamada Y: Perlecan is essential for cartilage and cephalic development. *Nat Genet* 1999, 23:354–358
- Costell M, Gustafsson E, Aszodi A, Morgelin M, Bloch W, Hunziker E, Addicks K, Timpl R, Fassler R: Perlecan maintains the integrity of cartilage and some basement membranes. *J Cell Biol* 1999, 147:1109–1122
- Whitelock JM, Melrose J, Iozzo RV: Diverse cell signaling events modulated by perlecan. *Biochemistry* 2008, 47:11174–11183
- Stum M, Girard E, Bangratz M, Bernard V, Herbin M, Vignaud A, Ferry A, Davoine CS, Echaniz-Laguna A, Rene F, Marcel C, Molgo J, Fontaine B, Krejci E, Nicole S: Evidence of a dosage effect and a physiological endplate acetylcholinesterase deficiency in the first mouse models mimicking Schwartz-Jampel syndrome neuromyotonia. *Hum Mol Genet* 2008, 17:3166–3179
- Arikawa-Hirasawa E, Rossi SG, Rotundo RL, Yamada Y: Absence of acetylcholinesterase at the neuromuscular junctions of perlecan-null mice. *Nat Neurosci* 2002, 5:119–123
- Rasminsky M: ectopic impulse generation in pathological nerve fibres. *Trends Neurosci* 1983, 6:388–390

20. Zielasek J, Toyka KV: Nerve conduction abnormalities and neuromyotonia in genetically engineered mouse models of human hereditary neuropathies. *Ann N Y Acad Sci* 1999, 883:310–320
21. Salzer JL, Brophy PJ, Peles E: Molecular domains of myelinated axons in the peripheral nervous system. *Glia* 2008, 56:1532–1540
22. Chiu SY, Ritchie JM: Potassium channels in nodal and internodal axonal membrane of mammalian myelinated fibres. *Nature* 1980, 284:170–171
23. Devaux JJ, Kleopa KA, Cooper EC, Scherer SS: KCNQ2 is a nodal K⁺ channel. *J Neurosci* 2004, 24:1236–1244
24. Yurchenko PD, Patton BL: Developmental and pathogenic mechanisms of basement membrane assembly. *Curr Pharm Des* 2009, 15:1277–1294
25. Saito F, Moore SA, Barresi R, Henry MD, Messing A, Ross-Barta SE, Cohn RD, Williamson RA, Sluka KA, Sherman DL, Brophy PJ, Schmelzer JD, Low PA, Wrabetz L, Feltri ML, Campbell KP: Unique role of dystroglycan in peripheral nerve myelination, nodal structure, and sodium channel stabilization. *Neuron* 2003, 38:747–758
26. Occhi S, Zambroni D, Del Carro U, Amadio S, Sirkowski EE, Scherer SS, Campbell KP, Moore SA, Chen ZL, Strickland S, Di Muzio A, Uncini A, Wrabetz L, Feltri ML: Both laminin and Schwann cell dystroglycan are necessary for proper clustering of sodium channels at nodes of Ranvier. *J Neurosci* 2005, 25:9418–9427
27. Arnaud E, Zenker J, de Preux Charles AS, Stendel C, Roos A, Medard JJ, Tricaud N, Kleine H, Luscher B, Weis J, Suter U, Senderek J, Chrast R: SH3TC2/KIAA1985 protein is required for proper myelination and the integrity of the node of Ranvier in the peripheral nervous system. *Proc Natl Acad Sci U S A* 2009, 106:17528–17533
28. Court FA, Hewitt JE, Davies K, Patton BL, Uncini A, Wrabetz L, Feltri ML: A laminin-2, dystroglycan, utrophin axis is required for compartmentalization and elongation of myelin segments. *J Neurosci* 2009, 29:3908–3919
29. Boerio D, Greensmith L, Bostock H: Excitability properties of motor axons in the maturing mouse. *J Peripher Nerv Syst* 2009, 14:45–53
30. Devaux JJ: The C-terminal domain of α IV-spectrin is crucial for KCNQ2 aggregation and excitability at nodes of Ranvier. *J Physiol* 2011, 588:4719–4730
31. Zhou L, Zhang CL, Messing A, Chiu SY: Temperature-sensitive neuromuscular transmission in Kv1.1 null mice: role of potassium channels under the myelin sheath in young nerves. *J Neurosci* 1998, 18:7200–7215
32. Hall SM, Williams PL: Studies on the “incisures” of Schmidt and Lanterman. *J Cell Sci* 1970, 6:767–791
33. Poliak S, Peles E: The local differentiation of myelinated axons at nodes of Ranvier. *Nat Rev Neurosci* 2003, 4:968–980
34. Court FA, Sherman DL, Pratt T, Garry EM, Ribchester RR, Cottrell DF, Fleetwood-Walker SM, Brophy PJ: Restricted growth of Schwann cells lacking Cajal bands slows conduction in myelinated nerves. *Nature* 2004, 431:191–195
35. Jessen KR, Mirsky R: Negative regulation of myelination: relevance for development, injury, and demyelinating disease. *Glia* 2008, 56:1552–1565
36. Bostock H, Cikurel K, Burke D: Threshold tracking techniques in the study of human peripheral nerve. *Muscle Nerve* 1998, 21:137–158
37. Bostock H, Rothwell JC: Latent addition in motor and sensory fibres of human peripheral nerve. *J Physiol* 1997, 498:277–294
38. Schwarz JR, Glassmeier G, Cooper EC, Kao TC, Nodera H, Tabuena D, Kaji R, Bostock H: KCNQ channels mediate IKs, a slow K⁺ current regulating excitability in the rat node of Ranvier. *J Physiol* 2006, 573:17–34
39. Gorassini M, Eken T, Bennett DJ, Kiehn O, Hultborn H: Activity of hindlimb motor units during locomotion in the conscious rat. *J Neurophysiol* 2000, 83:2002–2011
40. Eshed Y, Feinberg K, Poliak S, Sabanay H, Sarig-Nadir O, Spiegel I, Bermingham JR Jr, Peles E: Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. *Neuron* 2005, 47:215–229
41. Lonigro A, Devaux JJ: Disruption of neurofascin and gliomedin at nodes of Ranvier precedes demyelination in experimental allergic neuritis. *Brain* 2009, 132:260–273
42. Waxman SG: Determinants of conduction velocity in myelinated nerve fibers. *Muscle Nerve* 1980, 3:141–150
43. Sherman DL, Brophy PJ: Mechanisms of axon ensheathment and myelin growth. *Nat Rev Neurosci* 2005, 6:683–690
44. Denisenko N, Cifuentes-Diaz C, Irinopoulou T, Carnaud M, Benoit E, Niwa-Kawakita M, Chareyre F, Giovannini M, Girault JA, Goutebroze L: Tumor suppressor schwannomin/merlin is critical for the organization of Schwann cell contacts in peripheral nerves. *J Neurosci* 2008, 28:10472–10481
45. Grossman Y, Parnas I, Spira ME: Differential conduction block in branches of a bifurcating axon. *J Physiol* 1979, 295:283–305
46. Meier T, Masciulli F, Moore C, Schoumacher F, Eppenberger U, Denzer AJ, Jones G, Brenner HR: Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. *J Cell Biol* 1998, 141:715–726
47. Poliak S, Salomon D, Elhanany H, Sabanay H, Kiernan B, Pevny L, Stewart CL, Xu X, Chiu SY, Shrager P, Furley AJ, Peles E: Juxtaparanodal clustering of Shaker-like K⁺ channels in myelinated axons depends on Caspr2 and TAG-1. *J Cell Biol* 2003, 162:1149–1160
48. Traka M, Goutebroze L, Denisenko N, Bessa M, Nifli A, Havaki S, Iwakura Y, Fukamauchi F, Watanabe K, Soliven B, Girault JA, Karageorgos D: Association of TAG-1 with Caspr2 is essential for the molecular organization of juxtaparanodal regions of myelinated fibers. *J Cell Biol* 2003, 162:1161–1172
49. Gu C, Barry J: Function and mechanism of axonal targeting of voltage-sensitive potassium channels. *Prog Neurobiol* 2011, 94:115–132
50. Yin X, Kidd GJ, Nave KA, Trapp BD: P0 protein is required for and can induce formation of Schmidt-Lanterman incisures in myelin internodes. *J Neurosci* 2008, 28:7068–7073
51. Gould RM, Byrd AL, Barbarese E: The number of Schmidt-Lanterman incisures is more than doubled in shiverer PNS myelin sheaths. *J Neurocytol* 1995, 24:85–98
52. Sharghi-Namini S, Turmaine M, Meier C, Sahni V, Umehara F, Jessen KR, Mirsky R: The structural and functional integrity of peripheral nerves depends on the glial-derived signal desert hedgehog. *J Neurosci* 2006, 26:6364–6376
53. Hoshi T, Suzuki A, Hayashi S, Tohyama K, Hayashi A, Yamaguchi Y, Takeuchi K, Baba H: Nodal protrusions, increased Schmidt-Lanterman incisures, and paranodal disorganization are characteristic features of sulfatide-deficient peripheral nerves. *Glia* 2007, 55:584–594
54. Baker MD: Electrophysiology of mammalian Schwann cells. *Prog Biophys Mol Biol* 2002, 78:83–103
55. Sun YT, Lin TS, Tzeng SF, Delpire E, Shen MR: Deficiency of electroneutral K⁺-Cl⁻ cotransporter 3 causes a disruption in impulse propagation along peripheral nerves. *Glia* 2010, 58:1544–1552
56. Alvarez-Leefmans FJ, Leon-Olea M, Mendoza-Sotelo J, Alvarez FJ, Anton B, Garduno R: Immunolocalization of the Na⁺(⁺)-K⁺(⁺)-2Cl⁻ cotransporter in peripheral nervous tissue of vertebrates. *Neuroscience* 2001, 104:569–582
57. Bekku Y, Vargova L, Goto Y, Vorisek I, Dmytrenko L, Narasaki M, Ohtsuka A, Fassler R, Ninomiya Y, Sykova E, Ohashi T: Bral1: its role in diffusion barrier formation and conduction velocity in the CNS. *J Neurosci* 2010, 30:3113–3123
58. Sivak M, Ochoa J, Fernandez J: Positive manifestations of nerve fiber dysfunction: clinical, electrophysiologic, and pathologic correlates. Edited by WF Brown, CF Bolton. Boston, Butterworth-Heinemann, 1993, pp 117–147
59. Fox MA, Sanes JR, Borza DB, Eswarakumar VP, Fassler R, Hudson BG, John SW, Ninomiya Y, Pedchenko V, Pfaff SL, Rheault MN, Sado Y, Segal Y, Werle MJ, Umemori H: Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. *Cell* 2007, 129:179–193
60. Yin X, Kidd GJ, Pioro EP, McDonough J, Dutta R, Feltri ML, Wrabetz L, Messing A, Wyatt RM, Balice-Gordon RJ, Trapp BD: Dysmyelinated lower motor neurons retract and regenerate dysfunctional synaptic terminals. *J Neurosci* 2004, 24:3890–3898
61. Court FA, Brophy PJ, Ribchester RR: Remodeling of motor nerve terminals in demyelinating axons of periaxin-null mice. *Glia* 2008, 56:471–479
62. Zhou L, Messing A, Chiu SY: Determinants of excitability at transition zones in Kv1.1-deficient myelinated nerves. *J Neurosci* 1999, 19:5768–5781
63. Aizenman E, Bierkamper GG, Stanley EF: Botulinum toxin prevents stimulus-induced backfiring produced by neostigmine in the mouse phrenic nerve-diaphragm. *J Physiol* 1986, 372:395–404