Canine myocilin is associated with lipid modified by palmitic acid

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Purpose: Myocilin, the product of the GLC1A locus in humans, has been associated with primary open angle glaucoma (POAG). Mutations in myocilin exon 3 correlate with age of onset and severity of POAG. Myocilin has been under investigation in experimentally induced glaucoma in rats, mice, and monkeys, however no animal model of inherited POAG exists except in beagles. Our aim was to determine whether canine myocilin more closely resembles human myocilin than does myocilin from other species.

Methods: Canine myocilin was cloned and sequenced from beagle ocular tissues and Madin-Darby Canine Kidney (MDCK) cells, a well characterized canine cell line. Immunohistochemistry was performed on beagle tissues and cultured canine ocular primary cells. RT-PCR was employed to analyze genes expressed by tissues and cells. Myocilin protein synthesized by MDCK and canine optic nerve head cells (ONH) was analyzed by metabolic radioisotope labeling. Immunoprecipitation, enzymatic digestion, and Triton X-114 phase separation were performed to determine the carbohydrate content and the hydrophobicity of canine myocilin, respectively.

Results: Canine ONH astrocytes cultured from a beagle eye expressed myocilin and markers of type 1B astrocytes observed also in human ONH cells in culture. The sequence of canine myocilin showed close similarity with the human sequence but lacked the additional upstream coding region found only in human and rat. Myocilin was labeled with 3H palmitic acid. This label co-eluted with 35S label in hydroxylapatite chromatography despite purification by phase separation in Triton X-114, immunoprecipitation with myocilin-specific sera, and denaturing conditions of heat, SDS, and reducing agent.

Conclusions: Canine myocilin revealed no striking sequence differences from myocilin of other species. Mutations in myocilin of glaucomatous beagles may suggest a similar pattern of involvement in inherited POAG. This represents the first report of lipid associated with the myocilin protein. Many lipid-modified proteins reside in lipid rafts. This modification of myocilin with lipids supports the unusual secretion properties and may suggest a function of myocilin in sorting, cell signaling and communication between the extracellular matrix and the cell membranes.

In the 1970s, primary open angle glaucoma (POAG) as well as secondary glaucoma was found to exist in certain dogs [1-5]. Kirk Gelatt and colleagues identified a colony of beagles with inherited primary open angle glaucoma [6-9]. Since its identification in the human [10], myocilin has been cloned and sequenced in Macaque [11], rabbit [12], cow [13], pig [14], rat [15], mouse [16], and cat [17], but not dogs. Myocilin has been under investigation in experimentally induced glaucoma in rats [18], mice [19], and monkeys [20]. Because no animal model of inherited POAG exists except in beagles, determining the biochemical properties of myocilin in canine DNA would provide a further basis for developing this potential model for the study of the mechanisms and pathology of POAG in humans.

The function of myocilin is unclear, however the influence of extracellular myocilin on cell adhesion [21], effect on neurite outgrowth [22], and interaction with fibronectin [23,24] suggest that extracellular myocilin shares characteristics of an extracellular matrix protein. Myocilin upregulation by glucocorticoids [25], mechanical stress in organ and cell culture [26], heat shock in the worm homolog [27], and myocilin involvement in the unfolded protein response suggest that intracellular myocilin is stress responsive [28-30]. The amino terminal portion of human myocilin does not appear to function as a typical signal peptide, suggesting that it uses a different secretion pathway [31]. It has been reported that the leucine zipper region is necessary and may [32] facilitate secretion. This study reports the sequence of canine myocilin. This represents the first report of covalent lipid modifications to myocilin protein, suggesting a mechanism that promotes unconventional secretion and retention at membrane surfaces.

METHODS

Dissection, cell culture: A pet beagle dog without eye disease was euthanized for medical (not research) reasons by Philip L. Wagenknecht, DVM, and with consent from the pet owner. The tissues were harvested on a one-time basis approved by the Saint Louis University Animal Care Committee. Beagle tissues were dissected, fixed in 4% freshly made paraformaldehyde in PBS, embedded in paraffin, and sectioned by the Saint Louis University pathology department (Figure 1A).
Beagle optic nerve head (ONH) cell cultures (Figure 1B,C) were established using the method of Yang and coworkers [33] except that the tissue pieces were treated five minutes with dispase at room temperature before plating. Cells were maintained until passage 5 in Dulbecco’s Modified Essential Medium (DMEM; Mediatech, Herndon VA) containing 10% fetal bovine serum (FBS; MP Biomed Irvine CA). MDCK (NBL-2) CCL-34 cells were purchased from American Type Culture Collections (ATCC, Manassas VA) at passage 55.

**Chemicals, antibodies, and western blotting:** Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal rabbit antibodies to myocilin were purchased from Santa Cruz Biotech. Inc. (Santa Cruz, CA). Antimyocilin peptide was custom made by Sigma-Genosys (Woodlands, TX) and affinity purified with the peptide that was used to generate the antibodies. Sheep polyclonal antibody pAb3.1 was raised to human recombinant myocilin olfactomedin domain (generous gift of Dr. Tom Steely, Alcon Labs, Ft Worth, TX).

**Cloning and sequencing:** Genomic DNA from a normal beagle was the generous gift of Drs. Susan Pierce-Keller and Gustavo Aquirre (Cornell University, Ithaca NY). A genomic DNA library was generated using a Genome-Walker universal kit according to manufacturer’s directions (Clontech Laboratories, Inc. Mountain View, CA). Tissues dissected from the beagle were stored in RNAse-away until use. Total RNA was purified from tissues and also from MDCK cells using Trizol according to the manufacturer’s directions (Invitrogen Corp., Carlsbad, CA) and DNase I treated to remove genomic DNA. First strand cDNA was transcribed using both oligo dT and random hexamers and superscript II for 3 h at 45 °C, heat-killed at 70 °C for 15 min, and chilled. Reactions were incubated with 2 units RNAse H at 37 °C for 20 min, then heated at 70 °C for 15 min. PCR was performed with primers (Table 1), and products were ligated into pCR2.1 TopoTA vector, transformed into chemically competent TOP 10 cells according to the manufacturer’s directions (all from Invitrogen, Carlsbad, CA), and candidate colonies were screened. Posi-

![Image 1](http://www.molvis.org/molvis/v12/a161/)

**Figure 1.** Canine optic nerve head and cells expressed myocilin protein. A: Sagittal section of H&E stained beagle optic nerve showing normal morphology. Dark pink shows central vessels, light blue indicates nuclei of glial columns, light pink stains nerve bundles, dark blue stains pigmented layers. Original magnification 10X. B: Canine optic nerve head glia migrating from explanted optic nerve head (lower right). Original magnification 20X. C: Canine optic nerve head cells after passage. Original magnification 40X.
tive candidates were grown and plasmid DNA gas purified for sequencing. Sequencing was performed by Retrogen, Inc. (San Diego, CA). Multiple overlapping clones were sequenced on both strands and the consensus of at least three independent clones was submitted to GenBank (DQ149972). This sequence was independently confirmed a few months later (GenBank DQ303878).

Metabolic labeling: steady state: Either MDCK cells or canine ONH cells, passage 2, were seeded in T-75 culture flasks, and were used 3 days later when subconfluent. Cells were washed with Dulbecco’s PBS, then incubated in 2 ml methionine and cysteine-free DMEM (MP Biomedicals, Irvine, CA). After 30 min, the cysteine and methionine-free medium was removed and replaced with 5 ml cysteine and methionine-free DMEM (MP Biomedicals, Irvine, CA). After 30 min, the cysteine and methionine-free medium was removed and replaced with 5 ml cysteine and methionine-free medium containing 100 µCi/ml TranS-label (MP Biomedicals, Irvine, CA). Following a 30 min pulse, cells were washed then placed into 1.5 ml DMEM containing 10X methionine and cysteine and 10% FBS for 0, 0.5, 1, 2, 6, 9, 15, 18, 24, and 48 h. Medium was harvested, cells were washed with cold PBS and scraped into 25 mM Tris HCl, pH 7.0, 150 µM NaCl (TBS) containing 5 mM MgCl2, 1% Triton X-100, 1 mM DTT, and Complete® protease inhibitors (Roche Diagnostic, Mannheim Germany). Cell nuclei and debris were removed by centrifugation at 20,000x g at 4°C.

Immunoprecipitation: Samples were immunoprecipitated by pAb3.1 at 4°C in the presence of protein-G agarose beads. Beads were centrifuged and washed with TBS containing 5 mM MgCl2, 0.1% Triton X-100, 0.1% SDS, 1 mM DTT, and Complete® protease inhibitors. Beads were resuspended in Laemml SDS sample buffer containing 100 mM DTT and heated at 95°C for 10 min.

Triton X-114 phase separation: Triton X-114 (Sigma-Aldrich, St. Louis, MO or Fisher Scientific, Pittsburgh, PA) was precondensed and equilibrated in TBS according to the method of Bordier [34]. Washed cell monolayers were scraped into ice-cold TBS containing 1/5 volume condensed Triton X-114, incubated 15 min, and centrifuged at 10,000x g for 10 min at 4°C. Supernatant was transferred to a fresh tube and warmed to 37°C for 15 min and centrifuged at 10,000x g for 5 min at room temperature. The upper phase was transferred to a fresh tube. Immunoprecipitations were performed in TBS without detergents.

Hydroxylapatite chromatography: Hydroxylapatite (HAP; BioRad Laboratories, Richmond, CA) chromatography columns were prepared using a protocol described in references [35,36]. Spin columns were prepared with 400 µl HAP and equilibrated in 0.01 M sodium phosphate buffer pH 6.8, containing 0.1% SDS and 1 mM DTT (PB). Samples were diluted with equilibration buffer until refractive index was that of 0.01 M PB, 1.3340 nD (VeeGee digital refractometer) Samples were applied to columns, washed with 4 column volumes of 0.01 M PB and eluted with gradient 0.10 to 0.5 M PB: 1.3350-1.3430 nD. Fractions were collected, refractive indices measured, and radioactivity measured by liquid scintillation counting (Packard Tri-Carb 2900TR). Graphs were generated by Kaleidagraph 3.6 (Synergy Software, Reading PA), and exported to Adobe Photoshop (Adobe Systems, Inc., San Jose CA) for figure preparation with a Macintosh G4 computer (Apple Computer, Inc.).

Electrophoresis: Proteins were analyzed on 4-20% mini gradient gels (Invitrogen) or large format 15% SDS polyacrylamide gels that were 20 cm in length [37] and electrophoresed at 175 V. Gels were soaked in water, stained in GelCode (Pierce, Rockford IL), destained in water, and soaked in 1 M sodium salicylate to enhance for fluorography [38], then dried and exposed to x-ray film (Kodak, Rochester NY) at -80°C.

## RESULTS

Canine optic nerve head cells expressed myocilin protein: Figure 1A shows paraffin-embedded, sectioned, and stained beagle ONH. Hematoxylin&eosin stained nerve bundles light...
pink, central vessels dark pink, nuclei of glial columns light blue, and pigmented cells dark blue. The contralateral ONH was dissected and cultured until cells emerged from the explanted tissue, lower right (Figure 1B; lower right). Cultured cells are shown in Figure 1C.

Gene expression in canine ocular cells was similar to that observed in human cells: We wanted to determine whether gene expression of canine ocular tissues and cells was comparable to that published for human tissues and cell cultures. Human ocular tissues and ONH cells in culture express the 140 kDa form of neural cell adhesion molecule (NCAM) as well as glial fibrillary acidic protein (GFAP) and were thus classified type 1B astrocytes [39]. Because gene sequences were more readily available than antibodies to dog proteins, RT-PCR was employed to further characterize canine ocular tissues and cells (Table 2; Figure 2). RNA extracted from the ocular tissues, ciliary body and retina (Figure 2A), and ONH (Figure 2B), confirmed that these tissues expressed canine myocilin. In addition, NCAM and GFAP were expressed. Whereas the ONH expressed both NCAM-140 and 180 isoforms (Figure 2B, lane 3) cultured beagle ONH cells expressed only the 140 kDa form of NCAM (Figure 2C, lane 3). The retina and ONH tissue expressed several GFAP species, whereas cultured ONH cells expressed only the spliced form, and MDCK did not express GFAP (Figure 2D, lane 4). Rab3 guanine nucleotide dissociation inhibitor (GDI), involved in vesicle trafficking, was expressed in all except retina. As a control, beta-actin expression was confirmed.

Canine myocilin was cloned and sequenced: The mRNA for myocilin was cloned and sequenced using several primers spanning exon boundaries (Table 2). Primers were based on intron-exon junction sequences conserved in human, porcine, and bovine myocilin sequences. For sequencing, PCR products were ligated into a TA vector (Invitrogen) and sequenced using vector-specific primers flanking the inserts. When the canine genome was published, the sequence of predicted myocilin was used to design additional primers. At least three independent clones were sequenced in both directions for complete coverage of the coding region (Figure 3; GenBank DQ149972). The nucleotide sequence was 82.3% identical to human. The canine myocilin amino acid sequence was 82.7% identical to that of human. Canine myocilin encodes 483 amino acids, predicting a protein backbone of 54,228 Dalton with isoelectric point 5.42. The sequence closely resembles that of other species; however, it lacks the 14 residues found at the NH2-terminus of human and rat myocilin. The N-glycosylation sequon was present, but was followed by a seven-residue deletion in exon 1. Residues of consensus represent identity across all species sequenced to date, although canine and human sequences are identical at many additional positions. Our sequence was independently confirmed six months later by K. Kato, N. Sasaka, S. Matsunaga, R. Nishimura, and H. Ogawa, and appeared under accession number DQ303878. The

| Dog tig dn | 5'gcac gta cag tct cgg ccc caa atc ac3' |
| Dog tig up | 5'cag cct cac tgg cgc ccc ttc tc3' |
| Clon1UP | 5'gc cgc tgc agg ccc aag atg3' |
| Clon1DN | 5'c ccc ctc tgc att ctt acc3' |
| Clon2UP | 5'ttg tgt ctc tct ctt tta at3' |
| Clon2DN | 5'gaa acc tta act ctc taa3' |
| Clon3UP | 5'ttc cgg aat tta cca gg3' |
| ClonTigaDN | 5'a cag ctt gga ggc ttc tca3' |
| ClonTigtDN | 5'a gcg ctt ggt ggc ttc tca3' |
| Beag-dn | 5'ct gac atg ggg ggc tct tca3' |
| Beag-1A | 5'cga tgc cag tatt ttc ag3' |
| Beag-1B | 5'tgg gcc aga tcc tca ttt tc3' |
| Beag-2A | 5'agc agc cag gga gta gca ag3' |
| Beag-2B | 5'gca tgc gga gaa ctt gat gtc tc3' |
| Beag-3A | 5'gca gga acc tca gtt aac tc3' |
| Beag-3B | 5'gga gga acc cag cat gat gtc13' |
| Beag-3C | 5'cga cag cag cag cat gat gtc3' |

Cloning and sequencing primer design. Primers were based on intron-exon junction sequences conserved in human, porcine, and bovine myocilin sequences. When the canine genome was published, the sequence of the predicted myocilin was used to design additional primers.
nucleotide sequence of MDCK (cocker spaniel) myocilin was identical to that of the beagle.

Myocilin protein was expressed by transformed canine cells: Because primary beagle ONH was nonrenewable, MDCK were utilized as an alternate source of canine myocilin. MDCK were established from a normal cocker spaniel in 1958. Although the myocilin sequence was identical to the beagle sequence, at some point during this long history the cells may have become cross-contaminated with another cell line, [40,41]. To rule that out, MDCK cells were sent for independent authentication by multiplex PCR using 16 markers for both human and canine alleles at specific loci (Veterinary Diagnostics Center, Fairfield, OH). The cells possessed only the canine markers, confirming canine origin and the absence of contamination with human cell lines. As shown in Figure 2, MDCK expressed myocilin mRNA, providing a convenient source for canine myocilin.

Myocilin was N-glycosylated in MDCK: Myocilin immunoprecipitated from MDCK medium showed a mobility shift to about 54 kDa after treatment with peptide N-glycanase F, confirming that the glycosylation sequon was functional under culture conditions (Figure 4C). The band after enzyme treatment was less intense, probably due to a loading artifact. In contrast, SDS-PAGE failed to resolve a mobility shift of

![Figure 4. Myocilin was N-glycosylated in MDCK. ^3S labeled extracellular myocilin protein was immunoprecipitated from the medium of canine cells, and a portion was treated with peptide endoglycanase F (PNGase F). A: Fluorography of untreated and treated passage 2 beagle ONH cell myocilin. B: Western blot of beagle ONH cell myocilin. C: Fluorography of MDCK myocilin. Untreated (upper arrow) and an approximately 54 kDa protein backbone remained after treatment (lower arrow). Immunoprecipitates were separated by electrophoresis on 4-20% gradient SDS gels, and visualized by fluorography on x-ray film. The western blot was visualized with chemiluminescence.](image-url)
Myocilin was secreted continuously in pulse chase experiments: The amino terminus of canine myocilin was presumed to serve as a functional leader sequence, because labeled myocilin protein appeared in the culture medium in less than one hour in pulse chase experiments (Figure 5A). Nevertheless the labeled protein continued to be detectable intracellularly and was not completely chased out to the medium even after a 48-hour experimental period (Figure 5). Treatment with peptide endoglycosidase F produced a mobility shift in both cell-associated and extracellular MDCK myocilin, suggesting rapid glycosylation and exit from cells (not shown).

Myocilin protein was modified by palmitic acid: Hardy and coworkers [31] suggested that myocilin may utilize an unconventional secretion pathway and our pulse-chase experiments resulted in continuous secretion as well as continuous cell retention, myocilin appeared to be more hydrophilic than its amino acid sequence would predict. We asked the question whether myocilin was modified by lipid. MDCK cells were labeled with 35S cysteine and methionine for 24, 48, and 72 h. Sister cultures were labeled with 3H myristic acid. Because palmitoylation is reversible by cellular enzymes and because palmitic acid can be metabolized, 3H palmitic acid labeling period was only 5 h. Myocilin was not detectably labeled with myristic acid, but palmitic acid labeled myocilin.

Myocilin from passage 2 beagle ONH cells. A detectable mobility shift was not observed by fluorography (Figure 4A) or by western blot (Figure 4B).

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DISCUSSION
Canine ocular tissues were cultured using methods developed for human cells. Canine gene expression was similar to that of human ocular tissues, and gene expression of the canine ONH cell cultures resembles that of type 1B astrocytes [39,42]. The expression of NCAM 140 was shown in normal human cultured ONH astrocytes that have not been subjected to biomechanical stress [42]. NCAM 140 is the polysialated embryonic form and is re-expressed in glaucomatous tissues [43]. Like myocilin, Rabs and vesicular trafficking cofactors are widely expressed in many tissues, but are associated with disease only in those tissues that contain limited types of binding partners. Rab3-GDI was not detected in the beagle retina. It is premature to say whether all dog retinas lack this type of GDI. There are many overlapping sets of Rabs, GDIs, and other vesicle escort proteins. Rab3-GDI [44] expression was of interest because it is a homolog of the choroideremia-associated gene, Rab escort protein-1 (REP1). Mutated REP-1 is unable to bind to and prenylate the Rab27b protein found predominantly in the retina [45] therefore causing choroideremia.

The canine myocilin gene was cloned and sequenced from both primary beagle and the MDCK cell line. The sequence resembles the human gene with the exception of the 3' start site and a seven residue deletion in the first exon. The canine translational start site could provide a reasonable signal peptide. The presence of a signal peptide generally encodes a secreted protein that is not glycosylated. Mammalian myocilin sequences possess at least one potential N-glycosylation site, except for pig, which possesses two, and rabbit, rat, and cow, which possess none. One N-linked glycosylation motif is present in canine myocilin cDNA. Its occupancy was confirmed by enzyme treatment of protein secreted by MDCK cells, which are a transformed canine cell line. In contrast, myocilin from passage 2 canine cells did not undergo a mobility shift. The MDCK pulse chase results for secreted myocilin also showed PNGase F sensitivity. The pulse chase results suggest that myocilin processing in the transformed cell line, MDCK, may be subject to many steric and interfering factors [46]. Although PNGase F treatment was performed in the presence of protease inhibitor cocktail, an approximately
35 kDa species also appeared after enzyme treatment of chase medium. This may correspond to the 35 kDa band observed by others and that cosecreted with myocilin [13]. It is possible that proteolytic digestion occurred during treatment, or that a myocilin fragment was coimmunoprecipitated with the secreted full length protein, and was released by the enzyme treatment.

Although human myocilin also contains a glycosylation sequon, neither ocular tissue in vivo [47] nor primary cultured cells secreted detectably glycosylated myocilin, whereas myocilin from brain astrocytes and transformed astrocytes was heavily glycosylated (C.S. Ricard et al. ARVO abstracts, and unpublished). Occupancy of glycosylation sequons is a function of protein conformation, the available pool of charged dolichol oligosaccharide carriers, and the metabolic state of the cells [48-52]. Transformed cells are metabolically active, whereas resident cells of differentiated tissue, such as ONH or trabecular meshwork, are less active until stressed. Myocilin in trabecular meshwork cells induced by dexamethasone results in several spots in two-dimensional gels and many bands in one-dimensional gels [25]. Myocilin protein is both secreted and retained intracellularly as many experiments have shown (unpublished and [14,15]). The hydrophobic behavior of myocilin, such as aggregate formation, cosecretion, and coimmunoprecipitation with other species, is similar to observations of lipid-modified viral proteins [36].

This represents the first report of lipid associated with myocilin of any animal species. Protein palmitoylation is a

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**Figure 6.** Myocilin protein was modified by palmitic acid. Hydroxylapatite chromatograms of phase-separated myocilin. Protein labeled with $^{35}$S (dashed blue squares) and $^3$H palmitic acid (solid red crosses) was immunoprecipitated, treated with SDS, reduced and heated, then adsorbed to HAP columns and eluted with 0.1 to 0.5 M phosphate gradient. Y-axis=dpm; X-axis=fraction. A: Myocilin from culture medium resolved in two peaks, each containing palmitate and protein label. B: The upper phase from Triton X-114 resolved as three or more peaks, each containing palmitate and protein label. C: The lower phase from Triton X-114 resolved as one lipid and one protein-lipid peak. D: Y-axis=refractive indices (nD) measured for fractions eluted from HAP chromatography and showing identical elution gradients for samples in A (blue squares), B (black crosses), and C (green triangles).
posttranslational and reversible modification. This modification is regulated and, in turn, may regulate conformation, membrane association, protein-protein interactions, and intracellular localization of the target protein. The hydrophobic behavior of myocilin protein has been observed by many workers and the leucine heptad repeat was regarded as the source for this characteristic [53]. In our study, several peaks eluted from HAP, each containing different proportions of label. Predominantly palmitate-labeled peaks may be composed of membrane lipid and Triton X-114 micelles or detergent-resistant rafts. Myocilin may be lipid modified at more than one site. Canine myocilin possesses six cysteine residues that may be candidates for thioester linkages. Protein palmitoylation and depalmitoylation reactions are considered to be primarily enzymatic and catalyzed by membrane-bound palmitoyl acyltransferases [54-56], and cytoplasmic and lysosomal forms of palmitoyl thioesterases [57].

Regulation of the protein palmitoylation and depalmitoylation cycle is thought to be important in regulating protein localization, conformation, protein-protein interaction, and activity [58,59]. Palmitoylation is a determinant for sorting to myelin membranes [60], and myocilin has been observed in myelin of both optic [20] and sciatic nerves and nodes of Ranvier [61]. Palmitoylation is a determinant for sorting to either apical or basal membranes in polarized epithelial cells [62]. MDCK are polarized cells that have been well characterized and used in the study of protein trafficking, lipid rafts, and cell-cell junctions. That they also express myocilin adds even more to their value as a cell model. MDCK were derived from canine kidney, and myocilin was indeed detected and characterized in cells of the kidney [61-63].

Experiments are currently underway to analyze the lipid that is attached to myocilin protein to determine whether it is the original 16-carbon fatty acid, or whether it has been metabolized by the cell. Neutral hydroxylamine, treatment with acylprotein thioesterase or phosphatidylinositol-specific phospholipase C will distinguish between amide and ester linkages. Limited proteolysis experiments will localize which cysteine residue contains the lipid modification, and then mutagenesis experiments will investigate the processing of myocilin transfected into cells. Of interest will be the possible function of myocilin as a lipid receptor, binding oxidized low density lipoprotein or free fatty acids. Of particular interest will be the role of lipids in targeting myocilin to specialized membranes such as myelin, nodes of Ranvier, and lipid rafts.

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