Anomalous Surface Distribution of Glycosyl Phosphatidyl Inositol-anchored Proteins in Neurons Lacking Acid Sphingomyelinase

Cristian Galvan,^{*†} Paola G. Camoletto,^{*†§||} Flavio Cristofani,^{*}
Paul P. Van Veldhoven,^{||} and Maria Dolores Ledesma^{*†§}

^{*}Cavalieri Ottolenghi Scientific Institute, Università degli Studi di Torino, A.O. San Luigi Gonzaga, Regione Gonzole 10, 10043 Orbassano (Torino), Italy; [†]Department of Molecular and Developmental Genetics, Flanders Institute for Biotechnology, 3000 Leuven, Belgium; [§]Center for Human Genetics, Katholieke Universiteit Leuven, 3000 Leuven, Belgium; and ^{||}Department of Molecular Cell Biology, Laboratorium for Lipid Biochemistry and Protein Interactions, Katholieke Universiteit Leuven, 3000 Leuven, Belgium

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Acid sphingomyelinase (ASM) converts sphingomyelin (SM) into ceramide. Mutations in the ASM gene cause the mental retardation syndrome Niemann Pick type A (NPA), characterized as a lysosomal disorder because of the SM accumulation in these organelles. We here report that neurons from mice lacking ASM (ASMKO) present increased plasma membrane SM levels evident in detergent-resistant membranes. Paralleling this lipidic alteration, GPI-anchored proteins show an aberrant distribution in both axons and dendrites instead of the axonal enrichment observed in neurons from wild-type mice. Trafficking analysis suggests that this is due to defective internalization from dendrites. Increasing the SM content in wild-type neurons mimics these defects, whereas SM reduction in ASMKO neurons prevents their occurrence. Moreover, expression of active RhoA, which membrane attachment is affected by SM accumulation, rescues internalization rates in ASMKO neurons. These data unveil an unexpected role for ASM in neuronal plasma membrane organization and trafficking providing insight on the molecular mechanisms involved. They also suggest that deficiencies in such processes could be key pathological events in NPA disease.

INTRODUCTION

Loss-of-function mutations in the gene encoding for the sphingomyelin (SM)-converting enzyme acid sphingomyelinase (ASM) cause the Niemann Pick type A (NPA) disease, a severe neurological disorder characterized by mental retardation and early death (Brady *et al.*, 1966). In ASM knock out (ASMKO) mice, which develop a phenotype essentially identical to the human NPA disease (Horinouchi *et al.*, 1995; Otterbach and Stoffel, 1995), the presence of axonal dystrophy (Kuemmel *et al.*, 1997) and neurodegeneration with a differential susceptibility depending on the molecular phenotypes of the neurons (Sarna *et al.*, 2001) have been described. How the lack of ASM activity leads to these alterations, which can certainly define the severe neurological course of the disease, remains however poorly understood.

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[†] These authors contributed equally to this work.

Address correspondence to: Maria Dolores Ledesma (lola.ledesma@med.kuleuven.be).

Abbreviations used: Pr^{PC}, cellular prion protein; DRMs, detergent-resistant membranes; ASM, acid sphingomyelinase; NPA, Niemann Pick type A disease; SM, sphingomyelin; TLC, thin-layer chromatography; GPI, glycosyl phosphatidyl inositol; MBP, maltose binding protein.

The major site of ASM activity is in lysosomes (Stoffel, 1999) and in fact, the cells of NPA-affected individuals and ASMKO mice present abnormally large lysosomes filled with SM. This particular feature has been utilized to classify NPA as a lysosomal storage disease (Futerman and van Meer, 2004). Still, it is not directly proven that abnormal SM accumulation in lysosomes leads to brain dysfunction and neurodegeneration. Although the neutral sphingomyelinase (Smase) has been considered the main responsible of SM turnover at the cell surface, the remarkable enrichment of SM at the plasma membrane of neurons and the presence of a pool of the ASM at this cellular site, particularly in detergent-resistant membranes (DRMs; Liu and Anderson, 1995; Grassme *et al.*, 2001), suggest that at least some of the alterations in NPA disease might be the consequence of plasma membrane defects.

Although to certain extent their biological significance remains conflictive (Munro, 2003; Douglass and Vale, 2005), the existence of plasma membrane-derived DRMs has been described in most cell types (Parton and Richards, 2003). DRMs are biochemically defined by their resistance to extraction with nonionic detergents at low temperature (Simons and Toomre, 2000). This is due to their particular lipid composition enriched in SM and cholesterol, which cluster together with the ganglioside GM1 and certain proteins, most notoriously, glycosyl phosphatidyl inositol (GPI)-anchored, dually acylated and cholesterol-associated, and palmitoylated proteins (Simons and Toomre, 2000). Numerous experimental evidences support that clustering within DRMs is important for a number of functions, including

Figure 1. Increased SM levels in the plasma membrane- but not Golgi-derived DRMs from ASMKO neurons. (Aa) Preparation of total and Golgi lysosomal-free membranes from wt and ASMKO brains. Three steps of low-speed centrifugations (see *Materials and Methods*) were used to eliminate lysosomes from brain homogenates. The supernatant of the pellet P3 was considered the total extract. Further centrifugations in

certain types of endocytosis (Parton and Richards, 2003), modulation of intracellular signaling (Simons and Toomre, 2000), and as a way to keep segregated plasma membrane territories with different protein composition, such as the apical and basolateral surface in epithelial cells or the axonal and dendritic surface in neurons (Simons and Ikonen, 1997; Ledesma *et al.*, 1998). Given the potential biological relevance of DRMs and that ASM has been found in these kind of membranes and controls the turnover of a major DRM lipid, SM, we have in this work investigated DRM composition, trafficking and function in ASMKO mice-derived neurons in vitro and in situ.

MATERIALS AND METHODS

Antibodies

Antibodies against the following molecules were used: γ -adaptin (BD Transduction Laboratories, Lexington, KY); Bip (BD Transduction Laboratories); GM-130 (BD Transduction Laboratories); Lamp-2 (BD Transduction Laboratories); synaptophysin (Clone Sy-38, Boehringer Mannheim, Indianapolis, IN); Transferrin Receptor (Clone CD-71, Santa Cruz Biotechnology, Santa Cruz, CA); amyloid precursor protein (APP; Clone 22C11, Chemicon, Temecula, CA); Flotillin-1 (BD Transduction Laboratories); PrP^c (Pom-1, clone Ag1193, kindly provided by Dr. A. Aguzzi, Institute of Neuropathology, Zurich, Switzerland); MAP2 (Peninsula Laboratories, Belmont, CA, polyclonal); RhoA (clone 26C4, Santa Cruz Biotechnology); Cdc-42 P1 (Santa Cruz Biotechnology); α -tubulin (Calbiochem, La Jolla, CA).

Mice

A breeding colony of ASM heterozygous C57BL/6 mice (Horinouchi *et al.*, 1995), kindly donated by Dr. E. Schuchman (Mount Sinai School of Medicine, New York), was established. The experiments were performed by comparing littermates of wild-type (wt) or ASMKO mice (7 mo of age), which genotype

was determined from genomic DNA in a PCR reaction as described in Horinouchi *et al.* (1995). All procedures involving the use of animals were performed under the supervision of a licensed veterinarian (Dr. F. Cristofani) according to guidelines specified for the animal protection and welfare by the Italian Ministry of Health (DDL 116/92).

Cell Culture

Cultures of hippocampal neurons were prepared from brains of 16-d-old mouse embryos as described in Goslin and Banker (1991). These neurons survive for several weeks and undergo full polarization when cultured in serum-free medium in the presence of a supporting layer of astrocytes. For our experiments hippocampal neurons were kept in culture for more than 8 d when they reach full maturation (Dotti *et al.*, 1988).

Isolation of Lysosomal Free Membrane Fractions and Golgi-enriched Membranes

Total mice brains were homogenized in ice-cold 0.5 M sucrose-PKM buffer (100 mM potassium phosphate, pH 6.5, 5 mM MgCl₂, and 3 mM KCl). Samples were centrifuged for 10 min at 2500 rpm. The postnuclear supernatant was centrifuged two more times for 10 min at 8000 rpm to get a lysosomal-free fraction. The supernatant after these series of low-speed centrifugations was considered total membrane extract as it contained markers for all membranes tested (i.e., endoplasmic reticulum, Golgi apparatus, plasma membrane, and endosomes; Figure 1A and data not shown). To obtain Golgi-enriched membranes, this supernatant was treated as indicated by Fath and Burgess (1993). Thus, it was layered onto a step gradient containing 1.3 M sucrose-PKM and 0.7 M sucrose-PKM and then centrifuged at 17500 rpm in a SW40 rotor for 60 min. Membranes that concentrated at the 0.7/1.3 sucrose interface were collected and brought to 1.25 M sucrose-PKM overlaid with 1.1 M sucrose-PKM, 0.5 M sucrose-PKM, and centrifuged at 15000 rpm in a SW40 rotor for 90 min. Golgi membranes were collected at the 0.5/1.1 M interface, adjusted to 0.7 M sucrose-PKM, and finally centrifuged at 10400 rpm for 15 min to pellet the Golgi stacks.

Gel Electrophoresis and Immunoblotting

Proteins were resolved on 12% SDS-PAGE gels and electroblotted to nitrocellulose membranes. Immunoblots were incubated with appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using enhanced chemiluminescence reagents. For quantitation scanned autoradiographies were analyzed with the NIH Image J program (<http://rsb.info.nih.gov/ij/>) under conditions of nonsaturated signal.

DRM Isolation

Either total or Golgi-enriched membranes without lysosomes were incubated for 1 h at 4°C in 1% Triton X-100, 25 mM MES, pH 7.00, 5 mM DTT, 2 mM EDTA, and protease inhibitors (CLAP: chymostatin, leupeptin, antipain, pepstatin, each at a final concentration of 25 μ g/ml). The extracts were mixed with 90% sucrose prepared in MBS buffer (25 mM MES, pH 7.00, 150 mM NaCl, and CLAP) to reach a final concentration of 60% and overlaid in an SW40 centrifugation tube with a step gradient of 35 and 5% sucrose in MBS. After overnight centrifugation at 35,000 rpm and 4°C, DRMs were obtained in fractions 4 and 5.

Lipid Extraction and Analysis by Thin Layer Chromatography

Membrane pellets were obtained after centrifugation at 100000 g at 4°C for 1 h and lipids were extracted according to Bligh and Dyer (1959). Extracted lipids were analyzed by thin-layer chromatography (TLC) on silica gel 60 HPTLC plates using two running solvents subsequently (hydrophilic: chloroform/acetone/acetic acid/methanol/water [50:20:10:10:5] and hydrophobic: hexane/ethyl acetate [5:2]). Standards of cholesterol, ceramide, and SM (Sigma, St. Louis, MO) were run in each TLC to identify the different lipid species. GM1 was analyzed by slot-blot using cholera toxin subunit B linked to peroxidase (Sigma). Scanned TLCs or slot-blots were quantified using the ImageJ under conditions of nonsaturated signal.

Mass Analysis of Lipids

The amount of major lipids were measured in lipid extracts prepared from one brain hemisphere in chloroform/methanol/water (1/2/0.8, vol/vol) and phase-separated in the presence of salt or prepared from monolayers scraped in methanol (Van Veldhoven and Bell, 1988), followed by extraction with chloroform/methanol and phase separation. Aliquots of the extracts were analyzed for phospholipids (organic phosphate; Van Veldhoven and Bell, 1988), ceramide by means of [γ -³²P]ATP and recombinant ceramide kinase (Van Overloop *et al.*, 2006) or subjected to TLC (0.25 mm Silica gel 60, Merck, Rahway, NJ; solvent hexane/diethylether/acetic acid 70/30/1, vol/vol) followed by elution and enzymatic quantification of triglycerides, cholesteryl esters, and cholesterol as described in Van Veldhoven *et al.*, (1997, 1998) except that cholesteryl esters and triglycerides were hydrolyzed chemically (5% 5 M

Figure 1 (cont). sucrose gradients were performed to isolate Golgi membranes. The figure shows Western blots of the different fractions of the experimental protocol using antibodies against markers of several membrane compartments: γ adaptin and GM130 (Golgi), Bip (endoplasmic reticulum), Lamp-2 (lysosomes), and synaptophysin (synaptic vesicles/plasma membrane), which confirm the initial elimination of lysosomes and the enrichment of Golgi membranes in a specific fraction. (Ab) Comparison of the SM content in lysosomal enriched and lysosomal free fractions. The amount of SM in fractions P1–P3 and in the supernatant of pellet 3 that gathers nonlysosomal fractions (nonlys) was analyzed by TLC. The bottom panel shows a representative example of the SM staining in the TLC. The graph shows mean values and SD of data corresponding to three ASMKO and three wt brains expressed as percentage over the wt values that were considered 100% (**p < 0.001). (B) Lipid analysis of total membrane DRMs from wt and ASMKO mice brains (a) and 10⁶ cultured hippocampal neurons (b) or Golgi membrane DRMs from wt and ASMKO mice brains (c). Bottom panels show representative images of the TLC for cholesterol and SM or slot-blot for GM1. Graphs in the top panels show mean values and SD of five ASMKO and five wt brains (a and c) or three independent neuronal cultures (b) expressed as percentage over the wt values that were considered 100% (*p < 0.05; **p < 0.001). (C) Levels of DRM-enriched lipids at the neuronal surface. DIC and fluorescence images of nonpermeabilized wt and ASMKO neurons labeled with Lysenin-MBP, filipin, and cholera-toxin subunit B to stain cell surface SM, cholesterol, and GM1, respectively. Graphs show mean values and SD of the fluorescence intensity/area unit expressed as percentage over wt values that were considered 100%. Twenty cells in each of three independent cultures were analyzed per condition (**p < 0.005). (D) Levels of SM in cell surface DRMs. Fluorescence images of representative wt and ASMKO neurons extracted with cold Triton X-100 after incubation with Lysenin-MBP. Graph shows mean values and SD of the fluorescence intensity/area unit expressed as percentage over wt values that were considered 100%. Twenty cells in each of three independent cultures were analyzed per condition (**p < 0.001). Bars, 10 μ m in all panels.

KOH in ethanol, 75°C, 90 min). Main phospholipids, also separated by TLC (solvent chloroform/methanol/formic acid 65/25/10, vol/vol), were visualized by iodine staining followed by ashing and phosphate analysis (Van Veldhoven and Bell, 1988). Recovery of standards after chromatography is estimated at 80–85%. Analysis of minor lysosphingolipids, including sphingosine, will be described in detail elsewhere. Briefly, acidic methanolic tissue or cell extracts were fortified with a suitable internal standard (C_{17} -sphinganine, Toronto Research Chemicals, Toronto, ON, Canada), diluted with water and applied to a hydrophobic SPE cartridge (60 mg HLB-Oasis, Waters Associates, Millipore, Milford, MA). Compounds, eluted with methanol, were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Lester and Dickson, 2001) with some modifications and subjected to normal phase SPE (100 mg NH₂-BondElut, Varian, Sunnyvale, CA) to separate the derivatized sphingoid bases/lysosphingolipids. After two selective hydrolysis steps, samples were separated by reversed phase HPLC (Symmetry C18-column 4.6×150 ; $5 \mu\text{m}$; $100 \text{ \AA}$; Waters) with an increasing gradient of buffered methanol/acetonitrile coupled to fluorometric analysis (Van Veldhoven, unpublished data).

Treatments with SM or Smase

A stock of SM was prepared in 2:1 ethanol/Me₂SO as described in Puri *et al.* (2003). The stock was added to living 8-d neurons reaching a final concentration of $40 \mu\text{g}/\mu\text{l}$. Thirty minutes before the addition of SM the cells were incubated with 50 mM Na₂HPO₄ that inhibits ASM (Testai *et al.*, 2004). The bacterial SMA from *bacillus aureus* Smase (Sigma) was directly added to living 8-d neurons at 0.1 unit/100 μl medium. After 48 h of SM or Smase treatments cells were collected for biochemical analysis or fixed with 4% paraformaldehyde (PFA) for immunofluorescence. Cell viability was tested by measuring the levels of apoptosis in a total of hundred cells for every treatment in each

independent culture. Apoptosis was scored by TUNEL (terminal deoxynucleotidyl transferase [Tdt]-mediated nick end labeling) assay as described in Estus *et al.* (1997).

Immunofluorescence

For surface lipid staining neurons were incubated with either the worm toxin lysenin linked to MBP protein (kind gift from Dr. T. Kobayashi, RIKEN Institute, Saitama, Japan), which binds specifically to SM (Kiyokawa *et al.*, 2004); with filipin (Sigma), which binds to cholesterol; or with cholera toxin subunit B linked to fluorescein (Sigma), which binds with high affinity to GM1 (Orlandi *et al.*, 1993). The same protocol of fixation without permeabilization using 4% PFA for 15 min was used in all cases. To visualize the amount of SM specifically on plasma membrane DRMs, living cells were extracted in 1% Triton X-100 on ice for 5 min (Ledesma *et al.*, 1998) after treatment with lysenin-MBP. Then cells were fixed and incubated with anti MBP polyclonal antibody and anti-rabbit rhodamine.

For the analysis of the polarized distribution of molecules double-labeling immunofluorescences were performed using cholera toxin subunit B fluorescein-linked or monoclonal antibodies against PrP^C, APP, or transferrin receptor (TfRc) in combination with the polyclonal antibody against MAP2. Cells were fixed as indicated above in 4% PFA and then permeabilized with 0.1% Triton X-100 for 3 min in order to detect MAP2. Fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibodies (Alexa, Molecular Probes, Eugene, OR) were used as secondary antibodies. For quantitation, areas were chosen randomly in each neurite. The phase-contrast images and the MAP2 staining were analyzed to unequivocally identify the areas as dendritic or axonal. Pixel intensity of the GM1, PrP^C, APP, or TfRc labeling in these areas was measured with the ImageJ. The mean intensity was calculated per area unit. Data are given as the average percentage obtained for the axons

Figure 2. Aberrant distribution of GM1 and PrP^C in ASMKO neurons *in vitro* and *in vivo*. (A) Phase-contrast/DIC images and double fluorescence images (merge) of representative examples of mature wt and ASMKO neurons incubated with anti PrP^C, cholera toxin subunit B to label GM1, anti APP, or anti TfRc (green labeling). In all cases neurons were also incubated with anti MAP2 (red) to label the dendrites. The axons (MAP2 negative) are indicated by arrows. (B) Immunohistochemical analysis using antibodies against PrP^C (red) and MAP2 (green) of hippocampal slices from wt and ASMKO mice brains. The dendrites (MAP2 positive) are indicated by arrowheads.

Figure 3. SM accumulation promotes the unpolarized distribution of GM1 and PrP^C. (Aa) TLC analysis of SM, cholesterol, and ceramide levels in wt neurons treated or not with exogenous SM. Graphs show quantitative data from three independent cultures as percentage over wt values considered as 100% (*** $p < 0.001$). (Ab) Fluorescence image of nonpermeabilized wt neurons treated or not with exogenous SM and incubated with Lysenin-MBP to visualize cell surface SM. (B) Representative images of double immunofluorescence analysis for GM1 (a) or PrP^C (b) and MAP2 in wt neurons treated or not with exogenous SM. Numbered squares are magnified on the right to better show the axonal (MAP2 negative) distribution of the molecules in the nontreated neurons in contrast to their appearance in dendrites (MAP2 positive) in the treated neurons.

(MAP2 negative) versus dendrites (MAP2 positive). Seventy to 80 dendrites and 50–60 axons were analyzed for each experimental condition. All samples were analyzed in a Leica fluorescence microscope (Deerfield, IL).

In Situ Immunostaining

Brains from wt and ASMKO mice were fixed by transcardiac perfusion with 4% PFA followed by cryoprotection in 30% sucrose. Fifteen-micrometer cryostat sections were obtained, washed in phosphate-buffered saline (PBS) for 10 min, and then incubated in blocking solution (2% fetal calf serum, 2% bovine serum albumin, 0.2% fish skin gelatin solution in PBS) and 0.5% Triton X-100 for 30 min. Incubation with primary antibodies at 4°C overnight was followed by washings in PBS and incubation with fluorescein- and rhodamine-conjugated secondary antibodies for 1 h at RT. Samples were analyzed in a Leica fluorescence microscope.

GPI-GFP Expression and Trafficking

Cultured mature hippocampal neurons (more than 8 d in vitro) from wt and ASMKO mice were transfected with the GFP-GPI plasmid DNA using calcium phosphate, as described in Köhrmann *et al.* (1999). The cells were fixed in 4% PFA at different time points after transfection and were incubated with the antibody against MAP2 to determine the polarized distribution of the GFP-GPI. Samples were examined in a Leica fluorescence microscope. The phase-contrast images and the MAP2 staining were analyzed to unequivocally identify the areas as dendritic or axonal. For quantitation the mean pixel intensity of the GFP-GPI labeling in the proximal segment of dendrites (maximum 20 μ m away from the cell body) and in the axons was measured per area unit with ImageJ. Thirty different neurons from two independent experiments were analyzed in each condition.

Internalization Assays

Living neurons were incubated for 10 min with cholera toxin subunit B fluorescein-linked, styryl dye FM 4-64 (Molecular Probes, T3166) or the mAb against PrP^C. Cells were then extensively washed, fixed with 4% PFA, and permeabilized with 0.1% Triton X-100. After blocking anti-mouse secondary antibodies rhodamine- or fluorescein-linked (Alexa) were used to label the anti PrP^C antibody. Samples were analyzed in a confocal scanning microscope (LSM 510 on an Axiovert 100M platform). Fifteen different neurons from three independent experiments were analyzed in each condition. To evaluate the internalization the following parameters were quantified for FM 4-64, PrP^C and GM1: number of clusters, total fluorescence associated to all clusters, fluorescence, and size of each cluster. The measurements were performed using the ImageJ software and are given per area unit in the cell body (excluding the clusters at the periphery, as determined in the phase-contrast image, which were considered cell surface staining). Data represent the average numbers from all the intracellular stacks (at least 4) analyzed in each cell.

RhoA and Cdc42 Activity Assays

The EZ-Detect Rho Activation Kit (Pierce, Rockford, IL) was used to determine the affinity of RhoA to its downstream effector Rhotekin and thus its activity. The EZ-Detect Cdc42 Activation Kit (Pierce) was used to determine the binding of cdc42 to the p21-binding domain (PBD) of p21-activated kinase 1 (Pak1), which only occurs when Cdc42 is active. Fresh brain homogenates from age-matched wt and ASMKO mice containing 500 μ g of protein were processed, in parallel, following the manufacturer's instructions.

Figure 4. Normal exocytosis but delayed endocytosis of the GPI-moiety occur in ASMKO neurons. (A) Phase-contrast/DIC and merged fluorescence (red, MAP2; green, GFP-GPI) images of representative examples of the cell body and/or initial segment of dendrites from wt and ASMKO neurons at 8, 12, 18, 24, and 36 h after transfection with the GPI-GFP construct. Arrows indicate axons that run in parallel to dendrites. Bars, 10 μ m. (B) Graph shows the mean values and SD of the GPI-GFP associated fluorescence/area unit in the initial segment of dendrites from wt and ASMKO transfected neurons. Data correspond to the analysis of 20 cells from each of two independent cultures (* $p < 0.05$).

RhoA Overexpression

ASMKO neurons were transfected at day 8 in vitro with the active form of RhoA (L63) cloned into the BamHI-EcoRI site of the pmRFPC1 vector using the Effectene kit (Qiagen, Santa Clarita, CA). At 15 h after transfection living neurons were incubated for 10 min with the mAb against PrP^C. Transfected neurons were identified by the red fluorescence signal from the red fluorescence protein (RFP) encoded by the vector. Levels of PrP^C internalization were compared in transfected and nontransfected neurons by confocal scanning microscope as described above.

Statistical Analyses

The Student's *t* test was used to analyze the data. *p* < 0.05 was considered statistically significant.

RESULTS

The Plasma Membrane of ASMKO Neurons Presents High Levels of SM in DRMs

To determine to which extent ASM deficiency affects the lipidic composition of neuronal DRMs we quantified the levels of SM, cholesterol, and GM1 in DRMs from ASMKO and wt mice brains. To discriminate between the contribution of lysosomal membranes, which are expected to have increased SM in ASMKO conditions, and the rest of cellular membranes, we set a protocol in which these organelles remain in the initial fractions while they were absent from the others (see *Materials and Methods* and Figure 1Aa). This experimental approach revealed that although the lysosomal-enriched ASMKO membranes indeed contained 6.1-fold more SM than the wt nonlysosomal membranes also presented a significant, 4.8-fold, SM increase in ASMKO conditions (Figure 1Ab). It is important to note that the amount of other lipids (i.e., triglycerides, phospholipids, gangliosides, cholesterol, cholesteryl esters, and ceramide) analyzed by TLC and a series of analytical/biochemical assays (see *Materials and Methods*) was similar in lysosomal and nonlysosomal membranes and in total brain extracts from wt and ASMKO mice, except for a slight decrease and increase of phosphatidylserine and ceramide, respectively, in ASMKO conditions (Supplementary Figure A). On the other hand, analysis of less abundant lipids (see *Materials and Methods*) revealed that not only the levels of SM were drastically increased in ASMKO brains but also those of its N-deacylated derivative (sphingosylphosphorylcholine) and of sphingosine (Supplementary Figure A). To analyze whether the rise in SM found in ASMKO intact membranes affected DRMs, the nonlysosomal membranes were extracted in Triton X-100 at 4°C followed by sucrose gradient centrifugation. DRMs were obtained in fractions 4 and 5 of the gradients according to the accumulation of the DRM-enriched proteins flotillin1 and PrP^C (see Supplementary Figure B). TLC and slot-blot analyses of these fractions revealed similar levels of cholesterol and slightly higher amounts of GM1 (116 ± 20 and $127 \pm 3.1\%$, respectively) in ASMKO membranes with respect to wt membrane values (considered as 100%; Figure 1Ba). In contrast, a strong, 4.1-fold, increase in SM was found in DRMs from ASMKO mice brains (Figure 1Ba).

To assess to which extent the SM increase in whole brain membrane reflects that of neuronal membranes, these were obtained from mature hippocampal neurons grown in primary culture from wt and ASMKO mice. These cultures contain <5% of glial contamination (Goslin and Banker, 1991). Lipidic measurement performed as above revealed that the DRM fractions of ASMKO-derived hippocampal neurons present similar levels of cholesterol and GM1 (95.5 ± 12 and $90 \pm 3.3\%$, respectively, with wt values

considered as 100%) yet significantly high SM levels ($460 \pm 50\%$; Figure 1Bb).

DRMs are formed in the Golgi apparatus, where SM is obtained from both de novo synthesis and from ceramide recycled from lysosomes due to the action of ASM on SM. To test whether ASM deficiency affects DRM formation in the Golgi apparatus, we measured the levels of cholesterol, SM, and GM1 in DRMs derived from Golgi-enriched membrane fractions of wt and ASMKO mice brains (obtained as described in Figure 1Aa). TLC/slot-blot analysis revealed that cholesterol, GM1, and SM are at similar levels in both conditions (87 ± 11 , 101 ± 4.2 , and $108 \pm 7.2\%$, respectively, in ASMKO with respect to wt brains; Figure 1Bc). DRM alteration in nonlysosomal total membranes but not in Golgi-derived membranes was also supported by the DRM protein profile obtained in sucrose gradients after cold detergent extraction. Thus, although flotillin1 and PrP^C are predominantly found in fraction 5 in wt mice total membranes (6.6- and 4.8-fold more than in fraction 4, respectively), they are enriched in the lighter fraction 4 in ASMKO membranes (1.7- and 1.9-fold more than in fraction 5, respectively; Supplementary Figure B). In contrast, no density shift was evident for these proteins when DRMs from Golgi-derived membranes were analyzed, appearing similarly enriched in fraction 5 in both wt and ASMKO conditions (Supplementary Figure B).

Given that a small pool of ASM had been described at the plasma membrane (Grassme *et al.*, 2001), we hypothesized that this cellular site could contribute to the SM increase observed in nonlysosomal/non-Golgi membranes. To test this directly, we visualized cell surface lipid levels by adding to nonpermeabilized neurons, specific fluorochrome-conjugated, lipid-binding probes: lysenin-MBP for SM, filipin for cholesterol, and cholera toxin subunit B for GM1. In agreement with SM accumulation in the plasma membrane, the staining for SM was 3.8-fold higher in ASMKO-derived hippocampal neurons compared with wt neurons, whereas cholesterol and GM1 staining appeared at similar levels (106 ± 24 and $105 \pm 37\%$, respectively; Figure 1C). Furthermore, addition of cold Triton X-100 to ASMKO neurons (Ledesma *et al.*, 1998) labeled with lysenin-MBP resulted in the retention of a much stronger SM labeling than in wt, confirming the elevated content of this lipid in cell surface DRMs of ASMKO neurons ($398 \pm 22\%$ with respect to wt; Figure 1D). Altogether, the data obtained by microscopy and biochemical means confirm that the lack of ASM activity produces a dramatic increase in SM on plasma membrane-derived DRMs of neurons.

Aberrant Distribution of DRM-enriched Molecules in ASMKO Neurons

Accepting the notion that DRMs are required for the proper sorting of certain types of proteins of the neuronal surface such as those that are GPI-anchored (Ledesma *et al.*, 1998; Galvan *et al.*, 2005), the above results would be consistent with alterations in their spatial distribution. To test this, we determined the localization of DRM (GPI-anchored PrP^C and GM1) and non-DRM (TfRc and APP) constituents. In wt mice-derived, fully mature, hippocampal neurons in culture $90 \pm 5.5\%$ of PrP^C was found on the axonal domain as revealed by surface immunofluorescence microscopy (Figure 2A). Similar axonal enrichment ($95 \pm 6\%$) was observed for GM1 (Figure 2A). In contrast, in ASMKO neurons PrP^C and GM1 were also found in the dendritic surface (45 ± 18 and $51 \pm 4\%$, respectively; Figure 2A). To rule out that morphological alterations could account for the changes in molecular distribution, the overall morphology, number,

thickness, and length of neurites were compared in ASMKO and wt neurons (see Supplementary Figure C for quantitative data), and no major differences were found. Importantly, the perturbation in the distribution of PrP^C observed in cultured neurons was also evident in situ in sections of ASMKO mice brains (Figure 2B). On the other hand, the non-DRM proteins APP and TfRc, which in cultured hippocampal neurons are markers for axons (Yamazaki *et al.*, 1995) and dendrites (West *et al.*, 1997), respectively, presented a similar axonal or dendritic enrichment, respectively, in wt and ASMKO neurons (Figure 2A). This last result further supports that DRMs are more affected by the lack of ASM activity than more fluid membranes occupied by APP or TfRc. This is consistent with the fact that the vast majority of SM is present in DRMs in mature neurons (Galvan *et al.*, 2005) and that the pool of ASM at the plasma membrane has been described in these kind of membranes (Grassme *et al.*, 2001).

To test if the abnormal distribution of PrP^C and GM1 in the ASMKO neurons is the direct consequence of the excess of SM, this lipid was experimentally augmented in hippocampal neurons from wt mice through the addition of purified SM (see *Materials and Methods*). Incubation with 40 $\mu\text{g}/\mu\text{l}$ exogenous SM in the presence of phosphate ion, an inhibitor of ASM (Testai *et al.*, 2004), for 48 h resulted in 5.9-fold increase in plasma membrane SM without a noticeable effect in cholesterol and a slight increase (1.2-fold) in ceramide content (Figure 3Ab). In our experimental conditions a slight increase in the SM-derivative sphingosine (0.22 and 0.32 pmol/nmol PC in nontreated and treated wt neurons, respectively) but not in sphingophosphorylcholine (0.071 and 0.073 pmol/nmol PC in nontreated and treated neurons, respectively) were also detected (see *Materials and Methods*). Noteworthy, the increase produced in SM is similar to that observed in ASMKO-derived neurons (see Figure 1 and Supplementary Figure A). Immunofluorescence microscopy evidenced that the treatment resulted in abnormally high PrP^C and GM1 amounts on the dendritic surface ($46.5 \pm 2.7\%$ of PrP^C and $51 \pm 5\%$ of GM1 appear in dendrites; Figure 3B, a and b). Because the exogenous SM did not perturb neither cell viability (2.9 ± 1.1 and $3.1 \pm 0.9\%$ of apoptotic cells in treated and nontreated cultures, respectively) nor the normal morphology of axons and dendrites (see Figure 3 and Supplementary Figure C) this last series of results suggest that altered spatial distribution of DRM-enriched molecules in ASMKO neurons is a direct consequence of high SM levels.

Exocytic Trafficking of the GPI Moiety Is Not Affected in ASMKO Neurons

Two types of mechanisms have been described for the polarized distribution of axonal membrane proteins in neurons: selective delivery and selective retention (Sampo *et al.*, 2003). In the former the molecules are transported exclusively to the axonal domain, whereas in the latter the proteins are delivered to both axons and dendrites but appear only in the axonal membrane because of rapid endocytosis from the dendritic surface. To determine which of these are defective in the neurons lacking ASM, the distribution of newly synthesized GPI moieties was compared in wt and ASMKO hippocampal neurons by expressing a GFP-tagged GPI cDNA (Figure 4). This construct was chosen because it emulates the intracellular trafficking of endogenous GPI-anchored proteins (Keller *et al.*, 2001). At a short posttransfection time, 8 h, a similar level of GFP signal was detected in tubular structures restricted to the cell body in wt and ASMKO neurons. By 12 h after transfection, both wt and

Figure 5. Endocytosis of GM1 and PrP^C is reduced upon SM accumulation. (A and B) Representative confocal images of the cell body of wt neurons treated (B) or not (A) with SM and nontreated ASMKO neurons (A) after 10-min internalization of the cholera toxin subunit B that labels GM1 (green in merge image) and the PrP^C antibody (red in merge image). Z values indicate in μm the depth of the stack in each image. Arrowheads designate the surface of the cell body. (C) Graphs show quantitative analysis of 15 neurons from each of three independent cultures per condition. Data are expressed as mean value and SD of the number of intracellular structures positive for GM1 and PrP^C per cell body area or as total fluorescence in arbitrary units (** $p < 0.005$).

Figure 6. Enhancement of SMA activity re-establishes the normal polarized distribution and internalization of GM1 and PrP^C. (Aa) TLC analysis of SM levels in nontreated wt neurons and ASMKO neurons treated with Smase. Graphs show quantitative data from three independent cultures as percentage over wt values considered as 100%. (Ab) Fluorescence image of nonpermeabilized ASMKO neurons

ASMKO neurons presented intense GFP labeling in the axon, with some GFP signal also evident in dendrites, especially in the initial segment, at a similar extent (1.1 ± 0.3 -fold higher in ASMKO with respect to wt; Figure 4). Because endogenous GPI-anchored proteins such as PrP^C are largely confined to the axonal domain (Galvan *et al.*, 2005), the above results suggest that the unpolarized distribution observed in ASMKO neurons may be due to defective endocytosis from dendrites. In support of this we found higher GFP-GPI labeling intensity in the dendrites of transfected ASMKO neurons than in wt at longer times after transfection: 4.7-fold at 18 h and 23-fold at 24 h (Figure 4).

ASMKO Neurons Present Reduced PrP^C and GM1 Internalization

To directly test if ASM deficiency leads to impaired endocytosis of GPI-anchored proteins, wt and ASMKO hippocampal neurons were incubated with the anti PrP^C mAb followed by chase periods of various lengths of time, to monitor the internalization rate of this protein. Neurons were simultaneously incubated with fluorescein-linked cholera toxin subunit B to follow GM1 internalization as well (Orlandi and Fishman, 1998; Puri *et al.*, 2001). Confocal microscope analysis of wt neurons revealed efficient internalization of both types of probes and their high degree of colocalization within intracellular structures in the cell body (Figure 5, A and C). When the number of intracellular structures positive for PrP^C and GM1 was analyzed in ASMKO neurons incubated and chased for the same lengths of time as wt neurons, a strong reduction was observed (17 ± 2.9 and 2 ± 1.2 intracellular structures in wt and ASMKO neurons, respectively). The endocytosed material was further analyzed by monitoring the size, the fluorescence associated with each structure, and the fluorescence associated with all intracellular structures per cell as an indication of the total endocytosed material. Although the individual intensity of the structures was similar (1.5 ± 0.7 and 1.7 ± 0.2 a.u. in wt and ASMKO neurons, respectively), a tendency to have bigger size was detected in ASMKO neurons compared with wt (22 ± 10 vs. 13 ± 4 a.u.). Still, and in agreement with the lower number of structures, the total fluorescence associated was clearly reduced in ASMKO conditions (41.1 ± 3.7 and 18.5 ± 3.4 a.u. in wt and ASMKO neurons, respectively; Figure 5C). This inefficient internalization rate was however not detected for non-DRM constituents. In fact, the endocytic rate of the fluid-phase lipid probe FM4-64 was not different in wt and ASMKO neurons (25 ± 2.6 and 23.7 ± 5.7 intracellular structures and 118.8 ± 13 and 121.1 ± 7.7 a.u. total fluorescent intensity in wt and ASMKO neurons, respectively; Supplementary Figure D).

To assess if defective endocytosis of DRM components is the consequence of SM excess the internalization of PrP^C

and GM1 was measured in wt neurons with experimentally increased SM (see above and *Materials and Methods*). In these conditions the efficiency of internalization was drastically reduced (3.6 ± 1.3 positive intracellular structures compared with 17 ± 2.9 in nontreated cells; Figure 5, B and C). Similar to the situation in the ASMKO neurons, the fluorescence intensity of each structure was not significantly altered (1.5 ± 0.5 in wt; 1.7 ± 0.2 in ASMKO), but the average size was slightly bigger in SM-treated neurons (21 ± 5 and 13 ± 4 a.u. in treated and nontreated neurons, respectively). The total fluorescence in such conditions was clearly reduced (42.1 ± 2.3 and 19.6 ± 5.7 in nontreated and treated neurons, respectively).

To further prove the involvement of high SM levels in defective endocytosis, ASMKO-derived neurons were treated with Smase. This treatment resulted in the reduction of plasma membrane SM in the ASMKO neurons to levels similar to that of wt neurons (Figure 6A, a and b). Although the levels of ceramide were increased (70 and 20 pmol/nmol PC in treated and nontreated neurons, respectively), this did not result in diminished cell viability (2.7 ± 0.6 and $2.3 \pm 1.5\%$ of apoptotic cells in treated and nontreated neurons, respectively). The morphology of the treated neurons was not significantly affected either (Supplementary Figure C). However, Smase addition led to an improvement of PrP^C and GM1 internalization in the ASMKO neurons that showed rates similar to those of wt neurons (15.6 ± 3.2 , 17 ± 2.9 , and 2 ± 1.2 intracellular structures and 45.5 ± 11 , 42.8 ± 5 , and 16 ± 2.1 total fluorescence intensity in ASMKO neurons treated with Smase, wt neurons, and nontreated ASMKO neurons, respectively; Figure 6B). Notably, Smase addition also had an effect on the distribution of PrP^C and GM1. Thus, Smase-treated ASMKO neurons presented a striking axonal enrichment of PrP^C and GM1, comparable to that of untreated wt neurons ($87 \pm 4.7\%$ of PrP^C and $91.8 \pm 7\%$ of GM1 is in axons of ASMKO neurons treated with Smase; Figure 6C, a and b; compare with Figure 2).

High SM Impairs PrP^C Internalization via RhoA Inactivation

Very recent evidence has shown the requirement of SM for the targeting to the plasma membrane and thus activation of the small GTPases RhoA and cdc42 in fibroblasts (Cheng *et al.*, 2006). In turn, these GTPases have been reported to drive clathrin-independent mechanisms for the internalization of GPI-anchored proteins in nonneuronal cells (Sabharanjak *et al.*, 2002). To gain insight into the molecular alterations that impair endocytosis in ASMKO neurons, we analyzed the levels of membrane attachment of these GTPases in wt and ASMKO conditions. A moderate, still significant, reduction ($20 \pm 8\%$) of membrane-bound RhoA was found in ASMKO-derived membranes, whereas the amount of bound cdc42 was not different from wt membranes (Figure 7A). Total levels of these two proteins were not altered. To confirm that diminished RhoA membrane attachment reflected less activity of the protein a Rhotekin binding assay, which detects specifically the active GTP-bound state of RhoA, was performed (see *Materials and Methods*). In agreement with an impaired activation the amount of RhoA with affinity for its downstream effector, Rhotekin was $39.8 \pm 6\%$ less in ASMKO brains than in wt (Figure 7B). In contrast and consistent with the normal membrane attachment observed for cdc42, the activity of this GTPase was similar in wt and ASMKO brains (Figure 7B). To determine if SM accumulation is responsible for the reduction of RhoA membrane attachment, exogenous SM was added to wt neurons. In such conditions $36 \pm 11\%$ less RhoA was membrane bound

Figure 6 (cont). treated or not with Smase and incubated with Lysenin-MBP to visualize cell surface SM. (B) Representative confocal images of the cell body of ASMKO neurons treated or not with Smase after 10-min internalization of PrP^C antibody (red in merge image) or cholera toxin subunit B that labels GM1 (green in merge image). Z values indicate in μm the depth of the stack in each image. Arrowheads designate the surface of the cell body. (C) Representative images of the double-immunofluorescence analysis for PrP^C (a) or GM1 (b) and anti-MAP2 of ASMKO neurons treated or not with Smase. Numbered squares are magnified to better show the re-establishment of PrP^C and GM1 axonal distribution (MAP2 negative processes) in the treated neurons, in contrast to the ASMKO nontreated neurons where these molecules colocalize in dendrites with MAP2. Bars, 10 μm in all panels.

compared with nontreated neurons (Figure 7C). Finally, to test whether membrane-bound RhoA reduction and thus

deficient RhoA activation leads to the impaired endocytosis observed in ASMKO neurons, these were transfected with a

constitutively active form of RhoA. In such conditions the levels of PrP^C internalization was clearly enhanced (10.6 ± 1.8 and 4.5 ± 0.6 intracellular structures/ 60 ± 7 and 10 ± 3.5 a.u. total fluorescence intensity, in transfected ASMKO neurons and in nontransfected ASMKO neurons, respectively; Figure 7D).

DISCUSSION

A main discovery of this work, based on three different experimental approaches: biochemical lipid quantification, fluorescence microscopy, and protein flotation profile, is that the hippocampal neurons from ASMKO mice have an excess of SM in plasma membrane-derived DRMs. Although ASM is mainly a lysosomal enzyme this novel finding is in agreement with the reported presence of a small pool of ASM in DRMs at the cell surface (Liu *et al.*, 1995; Grassme *et al.*, 2001). The observation that the SM increase in lysosomal and nonlysosomal membranes is in a similar range suggests that nonlysosomal alterations could be of relevance in NPA pathology. A second important finding is that in ASMKO neurons the PrP^C protein and the glycolipid GM1, which are highly enriched in DRMs, are not restricted to the axonal surface but abnormally enriched in the dendrites, most likely because of their deficient endocytosis from this domain after initial delivery from the Golgi apparatus. In support of this conclusion we observed that 1) ASMKO neurons present defective spatial distribution and internalization of PrP^C and GM1; 2) reduction of the excess SM in the ASMKO neurons, through the addition of SMase, restored the internalization rate and the axonal polarization of these molecules; and 3) SM increase in wt neurons produced their missorting and deficient internalization. The fact that aberrant distribution and high SM levels were also evident in ASMKO neurons *in situ*, strongly suggest that similar alterations might also exist in the neurons of NPA patients. The observation that the lack of ASM activity leads to impaired endocytosis and misdistribution of GPI-anchored proteins such as PrP^C, raises the question of which other neuronal functions are affected. We envision that these cells could have survival and differentiation defects. This appears a logical assumption considering that several neurotrophic factor receptors are GPI-anchored proteins (Saarma, 2000). The results here presented pave the way to analyze this possibility.

Our work provides with novel information on how GPI-anchored proteins acquire polarization in neurons. This is a controversial issue in epithelial cells where two opposite views have been raised to explain the apical distribution of GPI-anchored proteins. Either transcytosis from the basolateral to the apical domain (Polishchuk *et al.*, 2004) or direct delivery to the apical surface (Paladino *et al.*, 2006) have been proposed. Our results, showing GPI-GFP in axons and dendrites of fully polarized primary hippocampal neurons at short times after transfection but its exclusive presence in the axons at longer times, support an indirect mechanism based in the selective axonal retention rather than in selective delivery. In such mechanism rapid endocytosis of GPI-anchored proteins from dendrites becomes an essential step to achieve axonal polarization, as has been shown for proteins such as VAMP2 (Sampo and Banker, 2003). In agreement, we show that reestablishment of normal internalization rates in ASMKO neurons restores the axonal distribution of the GPI-anchored protein PrP^C.

The relevance of lipids in the regulation of endocytic trafficking has been only recently discovered in a series of studies performed in fibroblasts. Pioneer work demon-

strated that cholesterol levels regulate the endocytosis of sphingolipids (Puri *et al.*, 1999) and late endosome motility (Lebrand *et al.*, 2002). Moreover, elevated endosomal cholesterol levels in NPA fibroblasts inhibit Rab4 function, leading to altered LacCer and transferrin recycling (Choudhury *et al.*, 2004). On the other hand, addition of cholesterol and glycosphingolipids, but not that of ceramide or phosphatidylcholine, specifically stimulates caveolar endocytosis (Sharma *et al.*, 2004). By contrast, endosome motility is not affected in Tay Sachs disease (Lebrand *et al.*, 2002), a storage disorder where ganglioside turnover is altered (Futerman and van Meer, 2004). Our data highlight the importance of SM in the regulation of endocytosis in neurons. Interestingly, accumulation of SM at the plasma membrane has an effect opposite of that described for cholesterol and glycosphingolipids (Sharma *et al.*, 2004), leading to the impaired endocytosis of certain molecules instead of having a stimulatory effect. Taken together all the above argue in favor of different roles for specific lipids in the various steps of the internalization process that could also depend on the cell type. How different lipids affect the protein machinery involved in endocytosis is a relevant issue to address. Our work provides insights into the previously unexplored mechanism of endocytosis of GPI-anchored proteins in neuronal cells. In contrast to what happens in other cell types where GPI-APs are internalized in a cdc42-dependent but RhoA-independent pathway (Sabharanjak *et al.*, 2002), the latter seems to be crucial for PrP^C endocytosis in primary neurons. We show that SM accumulation impairs the membrane targeting and activation of RhoA and that overexpression of a RhoA active form restores PrP^C internalization rate in ASMKO neurons. Interestingly, impairment of RhoA membrane targeting is also induced by SM depletion in nonneuronal cells (Cheng *et al.*, 2006), suggesting that adequate levels of SM are crucial for the membrane targeting of RhoA and that both an excess or a deficiency of this lipid leads to RhoA inactivation and defects on endocytosis. Different from RhoA, we detect no alterations in the membrane attachment and activity of cdc42 upon SM accumulation. Nevertheless, further work is needed to determine the precise role of this GTPase in neuronal endocytosis.

It has been reported that changes in the levels and/or distribution of one lipid might affect such parameters in other lipids (Marks and Pagano, 2002). Thus, the accumulation of sphingolipids leads to alterations in the intracellular distribution of cholesterol in fibroblasts and macrophages of several storage diseases including NPA (Puri *et al.*, 1999; Leventhal *et al.*, 2001). It was speculated that cholesterol might be trapped within sphingolipid-laden compartments as a result of the affinity of the association between these lipids (Pagano *et al.*, 2000). However, our results show that the levels of cholesterol in the plasma membrane of NPA neurons from ASMKO mice are not higher despite the clear increase in SM. This was also the case when the levels of plasma membrane SM were elevated by exogenous addition of the lipid, suggesting that neurons react in a cell-specific manner to the accumulation of membrane SM. Alternatively, it is possible that neurons without ASM respond with a defective efflux of cholesterol from intracellular stores (i.e., lysosomes) to the plasma membrane, as has been shown in macrophages from patients with Niemann Pick type C disease (Leventhal *et al.*, 2001). This would compensate for the reduced endocytosis, resulting in normal steady-state levels of cholesterol at the plasma membrane of NPA neurons. On the other hand, the data revealing that in hippocampal neurons most of SM accumulates in membranes with the biochemical characteristics assigned to DRMs (Galvan *et al.*,

2005) and that the deleterious consequences observed in this work upon ASM deficiency (i.e., aberrant distribution and reduced internalization) seem to affect DRM-enriched molecules specifically suggest the involvement of a particular membrane microenvironment in the pathology of NPA. However, it is possible that the effects of SM accumulation are independent of its clustering in DRMs. We envision that changes in the thickness and fluidity of the plasma membrane due to SM increase could alter, per se, not only the retrieval of membrane but also the reception and transmission of signals across it. In this regard, it is important to highlight that ASM deficiency not only increases brain SM levels but also those of its metabolites like sphingosylphosphorylcholine, which have been shown to participate in signaling events regulating cellular responses such as differentiation, survival, or cytoskeletal rearrangements (Kostenis, 2004). Taken together all the above, further research appears essential to address how SM metabolism defects influence the trafficking and distribution of other lipids and the functional properties of membranes in neuronal cells.

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