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Effects of gangliosides on the activity of the plasma membrane Ca^{2+}-ATPase

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ABSTRACT

Control of intracellular calcium concentrations ($[Ca^{2+}]_i$) is essential for neuronal function, and the plasma membrane Ca^{2+}-ATPase (PMCA) is crucial for the maintenance of low $[Ca^{2+}]_i$. We previously reported on loss of PMCA activity in brain synaptic membranes during aging. Gangliosides are known to modulate Ca^{2+} homeostasis and signal transduction in neurons. In the present study, we observed age-related changes in the ganglioside composition of synaptic plasma membranes. This led us to hypothesize that alterations in ganglioside species might contribute to the age-associated loss of PMCA activity. To probe the relationship between changes in endogenous ganglioside content or composition and PMCA activity in membranes of cortical neurons, we induced depletion of gangliosides by treating neurons with D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP). This caused a marked decrease in the activity of PMCA, which suggested a direct correlation between ganglioside content and PMCA activity. Neurons treated with neuraminidase exhibited an increase in GM1 content, a loss in poly-sialoganglioside content, and a decrease in PMCA activity that was greater than that produced by D-PDMP treatment. Thus, it appeared that poly-sialogangliosides had a stimulatory effect whereas mono-sialogangliosides had the opposite effect. Our observations add support to previous reports of PMCA regulation by gangliosides by demonstrating that manipulations of endogenous ganglioside content and species affect the activity of PMCA in neuronal membranes. Furthermore, our studies suggest that age-associated loss in PMCA activity may result in part from changes in the lipid environment of this Ca^{2+} transporter.

1. Introduction

Precise regulation of free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in neurons is highly dynamic and serves as the basis for a vast array of signaling activities that are essential for neuronal viability and the integrity of the entire nervous system. Neurons express multiple systems that allow for rapid, transient Ca^{2+} pulses to initiate either short or long-term signaling events and to terminate equally rapidly the Ca^{2+}-mediated signals by returning $[Ca^{2+}]_i$ to normal resting levels. The extrusion of intracellular Ca^{2+} across the plasma membrane takes place against a 10,000-fold gradient of this ion [1,2]. Two integral membrane proteins mediate the extrusion of Ca^{2+}, the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX). The PMCA is a high affinity but low capacity system for pumping Ca^{2+} to the extracellular medium [3–5], while the NCX is a low affinity, high capacity antiporter. The PMCA is responsible for the fine-tuning of $[Ca^{2+}]_i$ and the maintenance of low resting $[Ca^{2+}]_i$ [3–5].

The aging process in the brain is accompanied by increases in overall Ca^{2+} content in brain neurons and synaptic terminals and a slower clearance of intracellular Ca^{2+} from nerve endings following a depolarizing stimulus [6,7]. Previously, we reported that the activity of PMCA in synaptic plasma membranes (SPMs) progressively decreases with age [8,9] and suggested that this loss in activity may contribute to the aberrant cellular Ca^{2+} overload observed in aging neurons.

The PMCA is an integral membrane protein with ten transmembrane domains [10]. The activity of this enzyme is regulated by multiple factors, including calmodulin (CaM) binding, proteolysis, protein kinases, and acidic phospholipids in the membrane environment of the protein [10,11]. In addition to the modulation of the enzyme by acidic phospholipids residing in the inner leaflet of the plasma membrane, the activity

Abbreviations: CaM, calmodulin; CTXB, cholera toxin subunit B; D/L-PDMP, D/L-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; LC–MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+}-ATPase; $[Ca^{2+}]_i$, intracellular calcium concentration

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of purified and reconstituted PMCA is also sensitive to exogenously introduced gangliosides, a class of glycosphingolipids that reside in the outer leaflet of the plasma membrane [12,13]. However, the influence of endogenous gangliosides on PMCA activity has not been explored.

Gangliosides are highly enriched in the central nervous system of vertebrates. This is particularly true in the brain where they constitute 20–25% of the outer leaflet of neuronal membranes or 10–15% of the total lipids in nerve ending (synaptosomal) membrane fractions [14]. Gangliosides participate in a variety of vertebrate cellular processes such as toxin uptake and cell adhesion, growth, mobility, and differentiation [15]. In the nervous system, gangliosides play critical roles in neuritogenesis [16,17], synaptic transmission [18,19], axonal regeneration, myelin stability [20], and signal transduction [21–24].

Due to their amphiphilic properties and their tendency to complex with Ca^{2+} ions, gangliosides are implicated in the modulation of Ca^{2+} signaling and the maintenance of cellular Ca^{2+} homeostasis during normal neuronal functioning [25]. Null mutant mice for GM2/GD2 synthase are deficient in GM2, GD2, and all gangliotetraose gangliosides and exhibit an impaired capacity for Ca^{2+} regulation, which supports the hypothesis that the maintenance of Ca^{2+} homeostasis is a key function of complex gangliosides [26]. In rabbit skeletal muscles, both GM3 and GM1 can induce a conformational change in the sarco/endoplasmic reticulum Ca^{2+}-ATPase and alter the activity of this Ca^{2+}-sequestering enzyme [27]. At the plasma membrane, GM1 activates indirectly a non-voltage-gated channel, the transient receptor potential C5 channel, by cross-linking with $\alpha5\beta1$ integrin, thereby activating the integrin signaling cascade and promoting Ca^{2+} influx and the initiation of neurite outgrowth [28,29]. However, unlike the information that exists with regard to regulation of Ca^{2+} influx into neurons by endogenous gangliosides, there is only limited information on the effects of endogenous gangliosides on Ca^{2+} efflux across the plasma membrane [25]. This is particularly true with regard to the regulation of PMCA by endogenous gangliosides.

In our studies of the PMCA activity in synaptic membranes, we found that the highest activity was present in the raft domains, i.e., membrane microdomains that are highly enriched in cholesterol and gangliosides [30]. This observation led us to hypothesize that the content or composition of gangliosides in brain synaptic plasma membranes (SPMs) may affect the activity of PMCA and that age-dependent alterations in endogenous ganglioside content or composition may contribute to the age-associated loss of PMCA activity. Therefore, our goals in these studies were: (1) to determine if endogenous SPM gangliosides change with age; (2) to assess whether decreases in neuronal membrane content of endogenous gangliosides lead to changes in PMCA activity; and, (3) whether alterations in certain ganglioside species can selectively increase or decrease the PMCA activity associated with neuronal membranes. Our results indicated that: (1) the composition of gangliosides in rat brain SPMs was altered during aging, and (2) the activity of PMCA was highly sensitive to alterations in both content and composition of endogenous gangliosides in membranes of primary cortical neurons. The findings from this study indicate a novel, CaM-independent mechanism for the regulation of PMCA activity and the modulation of Ca^{2+} signaling in the CNS.

2. Materials and methods

2.1. Reagents, antibodies and cell culture media

The following reagents were purchased from Matreya LLC (Pleasant Gap, PA): D- and L-threo-1-Phenyl-2-Decanoylamino-3-Morpholino-1-Propanol·HCl (D-PDMP and L-PDMP, respectively), purified bovine ganglioside mixture, and purified preparations of the ammonium salts of GD1a, GD1b, and GT1b. Purified preparations of the ammonium salt of GM1, all solvents (HPLC-grade only) used in lipid extraction or HPLC, neuraminidase (*Clostridium perfringens*, type V), ouabain, thapsigargin, oligomycin, ATP, horseradish peroxidase-coupled cholera toxin subunit B (CTXB), secondary anti-mouse and anti-rabbit antibodies, and Phosphatase Inhibitor Cocktail 3, were obtained from Sigma-Aldrich (St. Louis, MO). The bicinchoninic acid protein assay kit was from Pierce-Thermo Scientific (Rockford, IL), the Protease Inhibitor Cocktail 3 and the high performance thin layer chromatography (HPTLC) plates (Silica gel 60, 10 × 20 cm, Merck) were from Calbiochem-EMD (San Diego, CA), and the purified sodium salt of GQ1b and anti-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Tris-Glycine gradient gels (8–16%) and the serum-free Neurobasal™ cell media were purchased from Invitrogen (Carlsbad, CA), pan-PMCA antibodies (monoclonal) and antibodies to isoform-specific PMCA1–4 were obtained from Affinity Bioreagents (Golden, CO). The polyclonal rabbit pan-PMCA antibody, raised against a 21 amino acid sequence in the middle of the PMCA, was generated by our laboratory. All aqueous HPLC solvents were prepared in 18 MΩ resistivity water.

2.2. Animals and isolation of brain SPMs

Fisher 344/Brown Norway hybrid (F344/BNF1) male rats at 6, 23, and 34 months of age were obtained from the National Institute on Aging colony maintained by Harlan Industries. All protocols were implemented in accordance with NIH guidelines and approved by the University of Kansas Institutional Animal Care and Use Committee. The rats were quarantined for 2 weeks prior to their use in the studies. The rats were anesthetized by CO_2 inhalation, decapitated using a guillotine, and the brains recovered quickly. The brains from individual rats at each of the 3 ages were processed in parallel. Argon was bubbled through all solutions immediately preceding their use. Each whole brain was homogenized and processed for the isolation of synaptosomes by discontinuous ficoll density gradient centrifugation [30,31]. The synaptosomes were lysed in 3 mM Tris–HCl, at pH 8.5, buffer that contained a cocktail of protease inhibitors, were centrifuged at 64,200 ×*g* for 15 min, and the SPM pellet resuspended in 10 mM Tris–HCl, 50 μM $MgCl_2$, and 0.32 M sucrose, at pH 7.4.

2.3. Capillary HPLC mass spectrometric analysis (LC–MS) of gangliosides

Gangliosides were extracted from SPM samples using the Folch method [32]. Briefly, SPM samples (50 μl of each with an average protein concentration of 14.8 mg/ml) were made up to a volume of 250 μl in methanol:water (1:1, v/v) and mixed with 4 ml of chloroform:methanol (2:1, v/v). After the 25 min incubation, the solutions were centrifuged (1000 ×*g*, 5 min). The aqueous (upper) phase was collected while the chloroform layer was re-extracted three more times with 800 μl methanol:water (1:1, v/v) each time. The aqueous extracts were combined, dried under vacuum, and stored at −80 °C until use. Prior to LC–MS analysis, samples were resuspended in 500 μl methanol:water (1:1, v/v). HPLC analysis was performed on a nanoAcquity HPLC system (Waters, Milford, MA). SPM samples and the mixture of bovine brain gangliosides used as an external standard (5 μl of each) were initially loaded (45 μl/min, 99% acetonitrile, 1% of 50 mM ammonium acetate buffer, pH 5.6) onto a self-packed reversed phase C8 trap column (Zorbax RX-SB/C8, 5 μm, Agilent Technologies, Santa Clara, CA) for pre-concentration/desalting. After 3 min, the trap was switched in-line with an Acquity UPLC HILIC column (BEH-Amide column, 1.7 μm particle size, 1.0 × 50 mm, Waters). The gangliosides were resolved with an acetonitrile/ammonium acetate buffer gradient. The total column effluent was introduced into a SYNAPT G2 hybrid quadrupole time of flight mass spectrometer (Waters). The instrument was operated in sensitivity mode with a cone voltage of 45 eV and helium was admitted to the collision (trap) cell. Spectra were acquired at a mass range of 250–3000 m/z. Time to mass calibration was made with NaI cluster ions acquired under the same conditions, and lock mass corrected with an auxiliary sprayer presenting the doubly charged ion (m/z = 554.2614) from leucine enkephalin (YGGFL) peptide. Lock mass spectra were acquired every 30 s. Quantification of each class of

gangliosides was performed using Waters QuanLynx (ver. 4.1) to obtain peak-area data from extracted-ion-plots, targeting either the singly or doubly charged ions, or both, for each ganglioside species. Mean limits of detection and quantification for each class of ganglioside were calculated using RMS signal to noise data (generated by QuanLynx) for each injection.

Linear standard curves for each ganglioside were prepared as follows. Individual gangliosides from commercial sources were dissolved in DMSO at concentrations ranging between 1 and 4 mg/ml. The molar concentration of these solutions was determined by the University of Kansas Nuclear Magnetic Resonance Core laboratory using quantitative NMR techniques [33]. Nine-point linear standard curves for the gangliosides were generated by collecting data from triplicate injections of the appropriate serial dilutions (in 1:1 methanol:water) of the primary stock solutions. These standard curves were then used to calibrate the individual ganglioside abundance in a solution (40 μg/ml solution in 1:1 v/v methanol:water) made from the dried natural extract of gangliosides GM1, GD1a, GD1b and GT1b obtained from bovine brains.

Instrument sensitivity was monitored by regularly injecting 5 μl of the bovine–brain ganglioside mixture and using the calilbration data to attenuate the sample for the effects of instrument drift in a ganglioside specific manner. To correct for the effect of signal attenuation by the sample matrix, known amounts of bovine brain ganglioside mixture were spiked into three randomly selected SPM extracts, and these extracts were analyzed by LC–MS. The deficit between the theoretical and observed concentration served as an estimate of the matrix-dependent signal attenuation for each class of ganglioside.

2.4. Neuronal cell cultures and manipulation of ganglioside levels

Dissociated cortical neuron cultures were established from 18-day old Sprague Dawley fetuses as described previously [34,35]. Dissociated neurons were plated at a density of 1.5–1.75 × 10^6 cells/dish on 35 mm tissue culture dishes. After 24 h, the medium was replaced by serum-free Neurobasal™ medium [36]. Greater than 90% of the cells in the primary cultures were neurons, based on morphological characteristics and confirmation with immunofluorescent staining for glial fibrillary acidic protein and neuron specific enolase. Chronic depletion of cellular gangliosides was achieved by treating neurons with D-PDMP, an inhibitor of ganglioside synthesis [37]. Neurons were treated for 3 days with 10 μM D-PDMP added to the culture medium on day in vitro (DIV) 6. L-PDMP, the enantiomeric form of D-PDMP, was used as a negative control for the ganglioside depletion and was added to the neuronal cultures in the same way as D-PDMP. Changes in the sialic acid levels of the cell surface gangliosides were achieved through treatment with neuraminidase [25]. The neuraminidase was prepared in Neurobasal™ medium, filtered, and applied to each dish at 1 unit/ml on DIV 6. The neuraminidase was present in the cultures for 3 days before the cell collection.

2.5. HPTLC of gangliosides in neuronal membranes

Following exposure of neurons in culture to the agents noted above, the cell culture medium was removed, the cells were washed twice with 200 mM Tris–HCl, at pH 7.4, and then lysed in 3 mM Tris–HCl buffer, at pH 8.0, containing a cocktail of protease inhibitors. The plasma membrane-enriched fraction was obtained following centrifugation of the lysate at 10,000 ×*g* at 4 °C for 20 min, and the resuspension of the pellet in 3 mM Tris–HCl, at pH 8.0. For the ganglioside preparation, cell particulate fractions (1 mg protein) were treated the same as for the ganglioside extraction from SPM samples. All of the upper phases were combined and KCl was added (final concentration of 0.1 M). The ganglioside-containing extracts were applied to Sep-Pak C18 cartridges (Sep-Pak 1 cm^3, 100 mg, Waters) that were pre-washed three times alternately with 10 volumes of methanol and chloroform/methanol/0.1 M KCl in water (3:48:47) [38]. Salts and other contaminants were washed from the cartridge matrix with 10 volumes of water, the gangliosides were eluted with 8 volumes of methanol, dried in a SpeedVac, and re-dissolved in chloroform/methanol/water (2:1:0.1). HPTLC was performed as described previously [39]. Briefly, the tank was saturated overnight with the developing solvent chloroform/methanol/water (55:45:10) containing 0.02% $CaCl_2$. Pre-coated HPTLC plates were first activated by heating at 100 °C for 15 min and then cooled down to room temperature under desiccation. Ganglioside standards containing a total of 5 μg of 4 major brain gangliosides and the ganglioside samples from the cells were spotted as 7 mm streaks, 3 cm from the edge of the plate. The plate was developed for ~50 min until the solvent ascended to within 1 cm of the top of the plate. After being completely dried, the plate was sprayed with a fine mist of resorcinol/HCl/Cu^{2+} reagent [40] and heated in the oven at 110 °C for 1 h to allow visualization of the gangliosides. Plates were scanned in a Kodak Image Station 4000MM PRO (Carestream Health Inc., Rochester NY) using a colorimetric method at multi-wavelengths between 555 and 700 nm, and the density of each spot quantified using Carestream Molecular Imaging Software 5.x (Carestream Health Inc.). Finally, the level of ganglioside GM1 in 20 μg of each particulate sample protein was used as a general index of membrane-associated gangliosides and was determined by immunoblotting using CTXB (300 μg/ml) and procedures identical to those previously published [30].

2.6. PMCA activity and protein immunoblotting

The activity of PMCA in the particulate fraction was determined by monitoring the Ca^{2+}-activated generation of P_i from ATP as described previously [30,31,34]. For the immunoblot analyses of proteins, the sample proteins were separated by SDS-PAGE, transferred to methanol-activated PVDF membranes, and immunoreactivity measured as described previously [34,41].

2.7. Data analysis

Statistical significance of differences was assessed using Student's *t* test for unpaired samples, except for the LC/MS estimates of SPM-associated gangliosides in which one-way ANOVA with post-hoc Tukey–Kramer analysis was applied.

2.8. Co-immunoprecipitation

SPMs containing 1.2 mg protein in 0.32 M sucrose were diluted 1:10 in 50 mM Tris and 50 mM NaCl, at pH 7.4, containing protease inhibitors and phosphatase inhibitors. After centrifugation at 100,000 ×*g* for 1 h to remove the sucrose, the membrane proteins were extracted by resuspending the pellet in detergent-containing buffer (50 mM Tris, 50 mM NaCl, 0.1% NP-40, pH 7.4, protease inhibitors and phosphatase inhibitors). The membranes were frozen in liquid nitrogen and thawed at 37 °C 3–6 times and were spun at 12,000 ×*g* for 20 min. The supernatant was collected as the detergent soluble fraction and the starting material of the co-immunoprecipitation. A preclearing step was performed with 7–10 μg of irrelevant antibody (mouse IgG, Sigma I8765) for 2 h at 4 °C and 40 μl Protein A UltraLink™ resin (Thermo Scientific, 50% slurry). The remaining protein lysate was incubated with 2 μl (2–10 μg) of pan-PMCA monoclonal antibody overnight at 4 °C. The same sample co-incubated with mouse IgG served as a negative control. The antigen–antibody complexes were passed through the Protein A UltraLink™ resin and flow-through was collected. After three washes with the detergent-containing buffer (see above) and one with saline solution (150 mM NaCl), the resin was resuspended in 100 μl sample buffer (42 mM Tris, 6.7% Glycerol, 2.7% SDS, 0.05% Bromophenol blue) and boiled. The resuspended eluate was examined for the yield of PMCA and the presence of gangliosides by immunoblotting using the custom polyclonal pan-PMCA antibody to detect PMCA, and cholera toxin to detect GM1.

3. Results

3.1. Age-dependent changes in ganglioside content and species in rat brain SPMs

Gangliosides were extracted from SPMs prepared from the individual brains of 6-, 23-,and 34 month old F344/BNF1 rats and were quantified by LC–MS as described under Materials and methods. Five brain gangliosides: GM1, GD1a, GD1b, GT1b, and GQ1b, all containing the C18-sphingosine moiety, were detected. Quantification of the first four gangliosides was possible because their abundance in all samples exceeded their respective limits of quantitation (ranging between 0.5 and 20 pmol/injection, depending on the species). We were unable to accurately measure GQ1b because its concentration in all samples, regardless of age, barely exceeded the limit of detection for that species (7 pmol/injection). The two most abundant species in terms of the length of the fatty acid chain, i.e., 18:0 (Fig. 1A, n = 4) and 20:0 (Fig. 1B, n = 4), were detected for GM1, GD1a, GD1b, and GT1b. Two patterns of age-related change in SPM gangliosides were observed (Fig. 1). The abundance of the mono-sialoganglioside GM1, both C18 and C20 fatty acyl chain lengths, markedly increased with age (~1.5 fold at 34 months for). In contrast, the levels of the poly-sialogangliosides GD1a, GD1b, and GT1b, of both the species with C18 and the C20 fatty acyl chain length, decreased between 6 and 23 months and were restored to the 6-month level at 34 months.

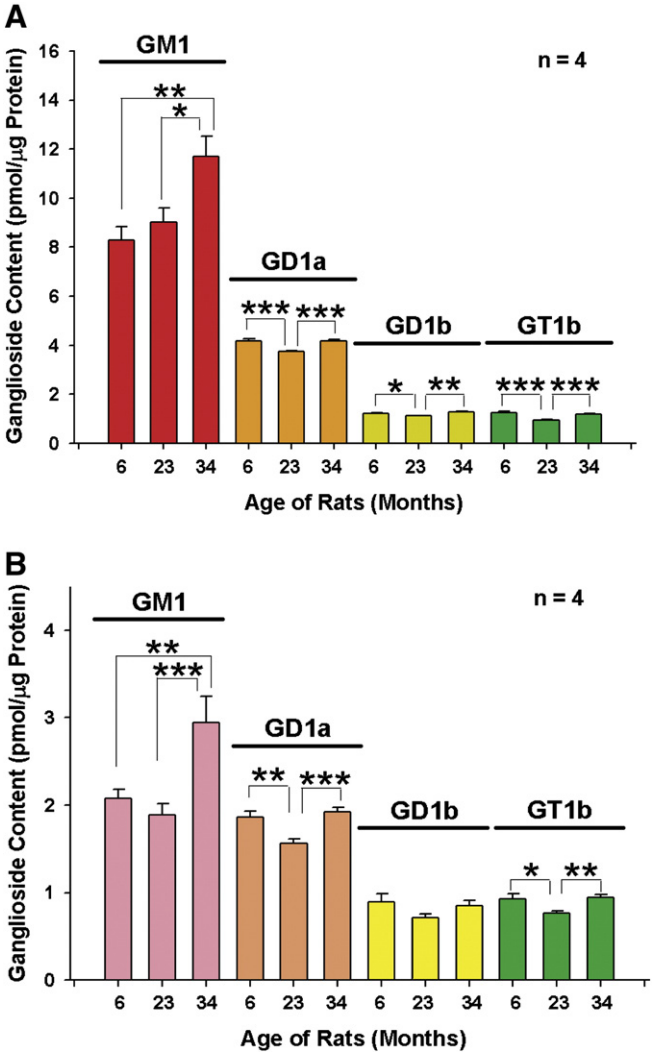

Fig. 1. Alterations in SPM gangliosides with aging. Gangliosides were extracted from SPMs isolated from the brains of F344/BNF1 rats at the ages of 6, 23, and 34 months and were quantified by HPLC–MS. The 2 most abundant constituents for each ganglioside species containing C18-spingosine but varying in the fatty acyl chain are presented. (A) Ganglioside species with C18-sphingosine and C18 fatty acyl chains; (B) Ganglioside species with C18-sphingosine and C20 fatty acyl chains. Data are means ± SEM from 4 preparations of SPMs per age. Statistical analysis was performed by one-way ANOVA with post-hoc Tukey–Kramer. *indicates $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.2. Effects of D- and L-PDMP treatment of primary neuronal cultures on neuronal structure, ganglioside and protein contents

To determine whether changes in the levels of endogenous gangliosides in the SPMs might affect the PMCA activity, we designed experiments in which we manipulated the endogenous levels of gangliosides in primary neurons. PDMP is a synthetic analog of the ceramide backbone of gangliosides. The D-threo enantiomer of PDMP is an inhibitor of UDP-glucose:ceramide glucosyltransferase, the enzyme that is required for the first step in glycosphingolipid synthesis. The L-threo-PDMP lacks such activity and was used as a control for the effects of treatment with D-PDMP. To assess whether PDMP caused toxicity in primary cortical neurons, we treated the cells with either 10 μM D-PDMP or 10 μM L-PDMP for 3 days as described under Materials and methods. We observed that the untreated cells had a normal neuronal structure with an intact neurite network, whereas the cells treated with either enantiomer of PDMP tended to have fewer, shorter, and beaded neurites (Fig. 2A). We have counted all recognizable neurites in 4 areas of equal size for each of the images we obtained. The neurites were counted in 4 images of cells per treatment condition. The results indicated that control cultures had an average of 27.0 ± 1.3 neuritic processes per 5.7 cm^2 area, D-PDMP-treated neuronal cultures had 17.7 ± 0.6 processes, and L-PDMP-treated neurons had 17.4 ± 1.0 processes. Both PDMP-treated neurons had significantly fewer neuritic processes than the control ($p < 0.01$ for both PDMP treatments) whereas there is no significant difference between D- and L-PDMP-treated neurons ($p = 0.82$). The effects of PDMP enantiomers on the morphology of primary neurons observed in our studies were consistent with the findings reported by others [42,43]. In addition to the changes in the morphology of neurons produced by D- or L-PDMP treatment, the total protein content of neurons treated for 3 days was significantly decreased compared with neurons that were grown in the absence of exposure to either PDMP enantiomer (n = 10, Fig. 2B). Both PDMP treatments led to a ~21% protein loss, indicating a modest and equivalent neurotoxicity of the two PDMP enantiomers.

We next examined the effect of D-PDMP on the ganglioside content of the membrane-enriched particulate fraction from primary neuronal cultures. In the initial studies, we used the binding of CTXB to GM1 as an index of ganglioside content in neuronal samples. Actin immunoreactivity was used as the loading control and the ratio of GM1 to actin was used to quantify the relative levels of gangliosides in the samples. D-PDMP reduced significantly the ganglioside content (~31%) compared with the untreated control samples (n = 4) (Fig. 3A and B). On the other hand, L-PDMP treatment did not alter the GM1 levels significantly ($p = 0.46$), compared with the untreated neuronal samples. The difference between D- and L-PDMP in terms of their effects on GM1 levels was also significant (n = 4). Furthermore, when we examined the effects of D-PDMP and L-PDMP treatments on the most abundant species of gangliosides present in neuronal membranes as revealed by HPTLC analyses, we determined that D-PDMP caused a greater reduction in the levels of most ganglioside species when compared with the effect of either L-PDMP or the no-treatment condition (Fig. 3C). The effect of D-PDMP on the combined abundance of the major poly-sialogangliosides (GD1a, GD1b and GT1b) was approximately equal to that on the mono-sialoganglioside GM1, and represented an ~38% decrease in the levels of these membrane gangliosides as compared with neuronal membranes from untreated cells (Fig. 3D). L-PDMP caused no significant decreases in either GM1 or the combined poly-sialoganglioside content ($p = 0.75$ for GM1; $p = 0.11$ for poly-sialogangliosides) when compared with the no-treatment condition. And, the differential effect of

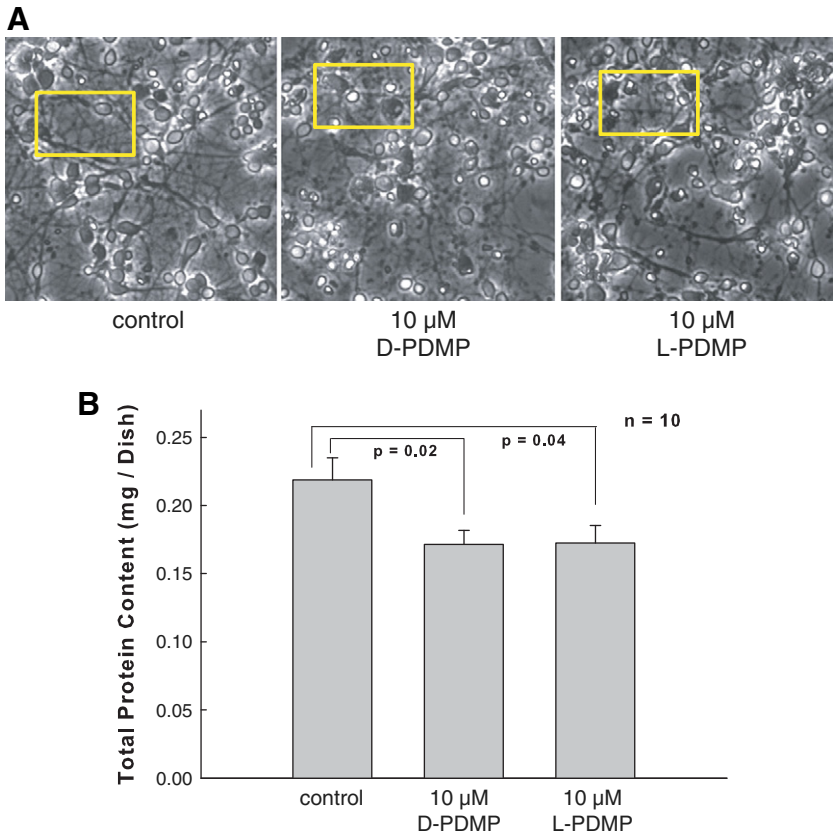

Fig. 2. Effects of D- and L-PDMP on neuronal cultures. (A) Representative phase contrast images of primary cortical neurons with no treatment (control) or treated with either 10 μM D-PDMP or L-PDMP for 3 days. Images were taken at a 20× magnification. A box used to count the neuritic processes is shown as an example on each image. The intact neurites in 4 boxes from each image obtained from 4 cultures per treatment condition were counted and the average number is calculated and reported in the text. (B) Effects of D- and L-PDMP on protein levels in neuronal cultures. After a 3-day treatment with PDMP, cells were collected and lysed, and the total protein/dish was determined by the bicinchoninic acid assay. Data are means ± SEM obtained from 10 experiments using different culture preparations. Statistical evaluation of the differences between control and treated cultures was determined using Student's *t*-test for unpaired samples.

D-PDMP vs. L-PDMP on ganglioside levels was also significant, thus the L-PDMP treatment condition was a good control for the effects of treatment with the enantiomer D-PDMP.

3.3. Effects of ganglioside depletion on PMCA content and activity of neuronal membranes

Having characterized the effects of the PDMP enantiomers on neuronal membrane gangliosides, we sought to determine whether decreases in ganglioside content affected either the levels of PMCA protein associated with neuronal membranes or the activity of this enzyme. An equivalent amount of protein from each particulate fraction was resolved by SDS-PAGE and probed with the pan-PMCA antibodies (Fig. 4A). The PMCA to actin ratio was not statistically different between the membranes obtained from the D-PDMP and those from neurons treated with L-PDMP. The same was also true for the levels of PMCA in D- or L-PDMP-treated neurons as compared with those of untreated cells.

We next determined the activity of PMCA in the membrane fractions of neurons treated with either the D- or L-enantiomers of PDMP. Because cells have several ATPase systems, we first validated that the majority of the activity measured using our method was from PMCA by applying the PMCA inhibitor to the assay. Carboxyeosin and lanthanum are operating as PMCA inhibitors through different mechanisms with carboxyeosin competing for the ATP binding site and lanthanum for Ca^{2+}. We tested the effect of the combination of the two inhibitors and determined that at 200 μM carboxyeosin plus 500 μM lanthanum, the maximal inhibition was 91.3 ± 1.6%, indicating that the enzyme activity we measured was primarily due to PMCA. The specific activity of PMCA in neuronal membranes from untreated cells was 13.13 ± 0.31 nmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$, whereas that in membranes from cells treated with D-PDMP was 26% lower than in the controls (9.70 ± 0.38 nmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$) (Fig. 4B). In contrast to the effects of D-PDMP, L-PDMP had no statistically significant effect on the PMCA activity when compared with untreated controls (12.24 ± 0.25 nmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$, $p = 0.06$). The differential effect of D-PDMP vs. L-PDMP on PMCA activity was significant and indicated that gangliosides either promoted or sustained the activation of PMCA.

We also examined whether the PMCA activator CaM was a major contributor to the sensitivity of PMCA to ganglioside depletion. The PMCA activity was only marginally stimulated by the addition of CaM to untreated primary neurons [34] and the D-PDMP-induced decrease in the activity of PMCA could not be reversed by the addition of 300 nM exogenous CaM to the assay (data not shown). Finally, to rule out the possibility that D-PDMP had inactivated PMCA by binding directly to it, we added 10 μM (final concentration) D-PDMP directly into an assay system setup with cells not previously treated with D-PDMP. The addition of D-PDMP into the PMCA assay medium had no effect on the activity of the enzyme, indicating that D-PDMP does not exert a direct effect on the enzyme (data not shown).

3.4. Neuraminidase-induced conversion of endogenous polysialyl ganlgiosides to GM1 and effects on PMCA

The treatment of neuronal cultures with D-PDMP decreased the levels of all species of membrane-associated gangliosides; thus no conclusions could be drawn with respect to any selective effects on

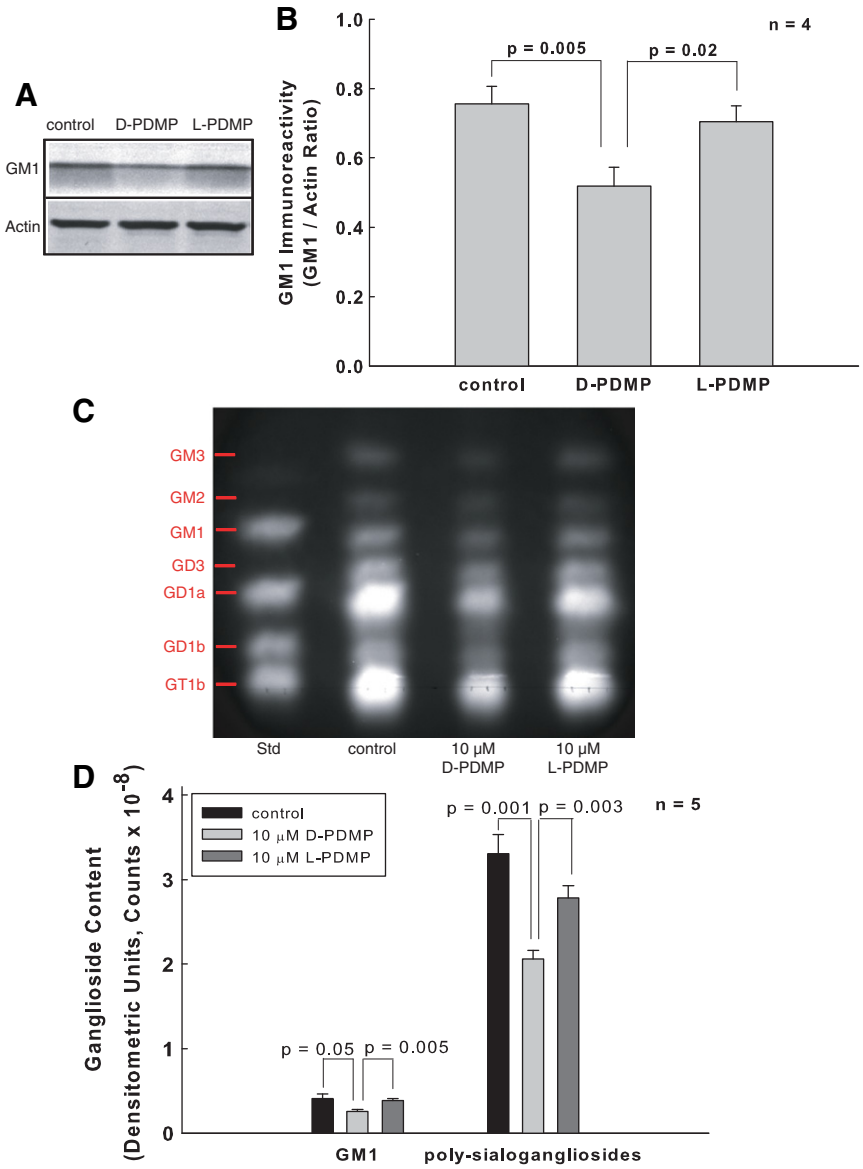

Fig. 3. Effects of PDMP treatments on GM1 levels. (A) Immunoblot analysis of GM1 following exposure to 10 μM D- or L-PDMP. Twenty micrograms of protein from cell particulate fractions were loaded to each gel lane, and GM1 was detected via CTXB. Anti-actin antibody was used at 1:1000 dilution. A representative immunoblot from 4 independent experiments with similar results is shown. (B) Densitometric analyses of the levels of GM1 in particulate fractions normalized to actin as a loading control. Data represent means ± SEM from 4 experiments using different cultures. (C) Ganglioside profiling by HPTLC. Gangliosides extracted from particulate fractions containing 1 mg protein were separated on an HPTLC plate. (D) Quantification of HPTLC images. Data are presented as means ± SEM from 5 experiments using different cultures. The total levels of GM1 vs. the combined content of polysialogangliosides (GD1a, GD1b, GT1b) expressed in the control, D-PDMP- and L-PDMP-treated neurons are shown.

PMCA associated with either mono- or poly-sialogangliosides. Because of the differential patterns of change in poly- vs. mono-sialogangliosides in SPMs from older animals, we explored the possible differential sensitivity of the enzyme to mono- vs. poly-sialogangliosides which might be important in the regulation of the PMCA during the aging process. To control the level of sialylation of gangliosides in the plasma membrane of neurons we treated the cells with neuraminidase. This enzyme hydrolyzes the α-glycosidic bonds between sialic acid and cell surface glycoconjugates [44], thus converting polysialyl gangliosides to GM1, a species resistant to neuraminidase-induced cleavage [25,45]. Neurons in culture were exposed to *C. perfringens* neuraminidase at a higher concentration than that employed in previous studies (1 unit/ml) [45] and for a prolonged period (3 days) in order to compensate for the fact that the incubations were conducted under non-optimal pH conditions for enzyme activity [46].

Examination of the neuronal cultures by phase contrast microscopy showed that the neuraminidase-treated primary neurons had darker, thickened neurites when compared with the untreated cells (Fig. 5). These morphological changes would fit the known induction of neuritogenesis following neuraminidase treatment [45]. Yet, despite the thickening of neuritic processes following neuraminidase treatment, the total protein content of neuraminidase-treated neurons was ~15% less than that of untreated controls ($p < 0.001$, $n = 8$). With respect to the ganglioside content of neuronal membrane preparations from neuraminidase-treated neurons, as expected, immunoblot labeling of GM1 with CTXB revealed that the GM1 to actin ratio in particulates of neuraminidase-treated neurons was ~2.5-fold higher than that in particulates from untreated neurons (Fig. 6A). Quantification of the ganglioside bands from HPTLC images (Fig. 6B) demonstrated that the major di- (GD1a, GD1b) and tri-sialogangliosides (GT1b) were reduced by a factor of 2–3 following exposure of neurons to neuraminidase, while GM1 increased by approximately the same factor of 2–3 (Fig. 6C, $n = 3$). Commercial preparations of neuraminidase are reported to catalyze the hydrolysis of the *N*-acetylneuraminic acid group from

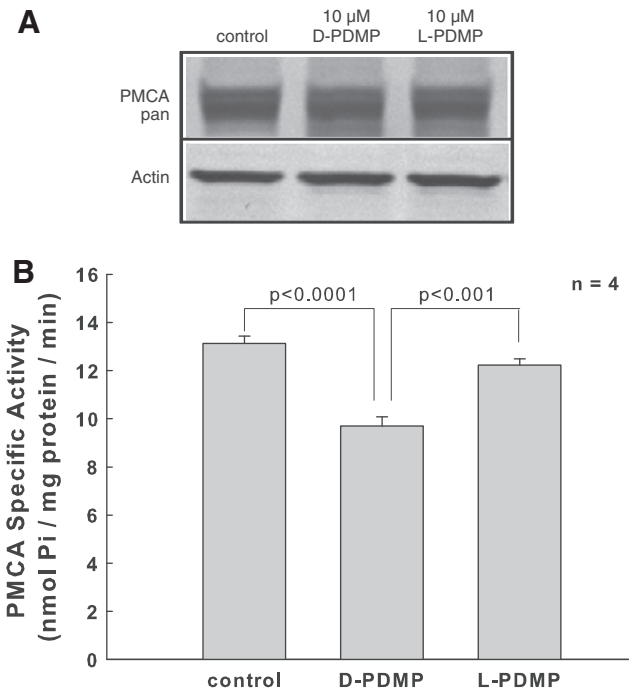

Fig. 4. Effects of ganglioside depletion on PMCA content and activity. (A) Immunoblot analysis of PMCA following exposure to 10 μM D- or L-PDMP. Twenty micrograms of protein from cell particulate fractions were loaded to each gel lane. Anti-pan-PMCA and anti-actin antibodies were used at 1:1000 dilution. A representative immunoblot from 4 independent experiments with similar results is shown. (B) The specific activity of PMCA in neuronal particulate fractions (9 μg protein) from control cultures and D- or L-PDMP treated cultures was assayed. The data represent means ± SEM from 4 different cell preparations.

GM2 gangliosides [47]. However, we observed only a marginal change in the GM2 level following exposure of primary cortical neurons to neuraminidase. This might have been due to the generally low level of expression of GM2 in neurons.

Immunoblot analyses using pan-PMCA antibodies indicated no significant changes in PMCA levels (PMCA to actin ratios, data not shown) in primary neurons treated with neuraminidase compared with controls. This suggests, once more, that alterations in endogenous ganglioside composition of neurons did not affect the PMCA levels in membranes. On the other hand, neuraminidase treatment did affect the PMCA activity in neuronal membranes (Fig. 7). Membranes from neurons treated with neuraminidase exhibited a ~39% decrease in the specific activity of PMCA (8.57 ± 0.64 nmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$) as compared with membranes from cultures not exposed to neuraminidase (13.95 ± 0.65 nmol $P_i \cdot mg^{-1}$ protein $\cdot min^{-1}$). The decrease in PMCA activity following neuraminidase treatment was coincident with an increase in GM1 content and the decreases in poly-sialogangliosides. The greater reduction in PMCA activity induced by neuraminidase (39%) as compared with that induced by D-PDMP (26%) might indicate that PMCA activity in neuronal membranes is up-regulated by poly-sialogangliosides and down-regulated by GM1.

3.5. The expression of PMCA isoforms in brain SPMs compared with neuronal particulates

The age-related changes in ganglioside content in neurons were observed using SPM preparations from rat brain, whereas the effects of manipulating ganglioside levels were determined in particulate fractions from primary neuronal cultures. Since all measurements of the effects of mono- and poly-sialogangliosides on PMCA activity were obtained using particulate preparations from primary neuronal cultures, it was important to assess the similarities in the expression of the four isoforms of PMCA in SPMs from the brain and the particulate fraction from primary neurons. To this end, we assessed the relative levels of each of the four PMCA isoforms in SPMs vs. neuronal particulates by immunoblot analysis (Fig. 8). We found that all isoforms were present in both sample types although they were relatively less abundant in the crude neuronal membrane fraction. This difference in PMCA abundance was likely related to the higher level of plasma membrane purification in the SPM fraction vs. the whole cell particulates. Despite the lower enrichment in PMCA in the cell particulate fraction, all 4 isoforms were present at similar levels proportionately represented in the two preparations.

3.6. Association of gangliosides with PMCA

To determine if there is any direct interaction between gangliosides and PMCA, we performed co-immunoprecipitation experiments with a solubilized protein fraction of the brain SPMs. The SPM protein complexes were extracted and solubilized by using the nonionic detergent, NP-40, at a low concentration of 0.1% in order to extract the complexes out of the membranes while maintaining the structures and their possible interacting partners intact. The majority of PMCA appeared in the detergent soluble fraction whereas GM1 showed an opposite trend (Fig. 9 lanes 1 and 2). Co-immunoprecipitation of PMCA was carried out using the monoclonal pan-PMCA antibody. Mouse IgG was used as a negative control. The flow-through and eluate were collected and examined by immunoblotting using the polyclonal pan-PMCA antibody to determine the PMCA yield and the specificity of the immunoprecipitation (lanes 3–6). In the eluate, in addition to the PMCA band at ~140 kDa, we observed two broad bands with heavy staining at higher molecular weights (lane 5). Protein identification via gel electrophoresis followed by Coomassie staining and MS analysis confirmed that PMCA was also present in those two bands,

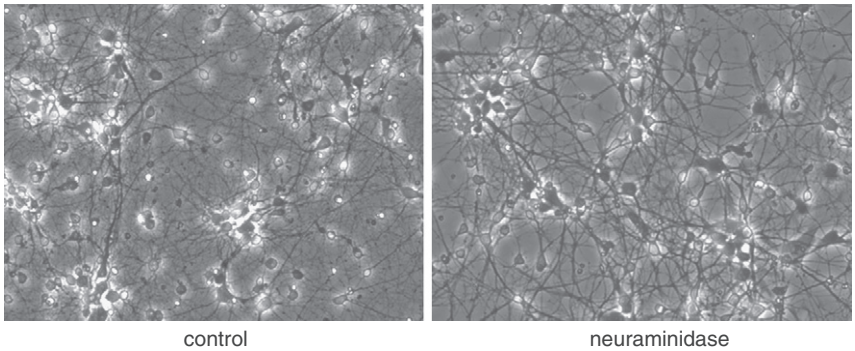

Fig. 5. Effects of neuraminidase on cultured neurons. Phase contrast images of primary cortical neurons with no treatment (control) or treated with neuraminidase at 1 unit/ml for 3 days were taken at a 20× magnification.

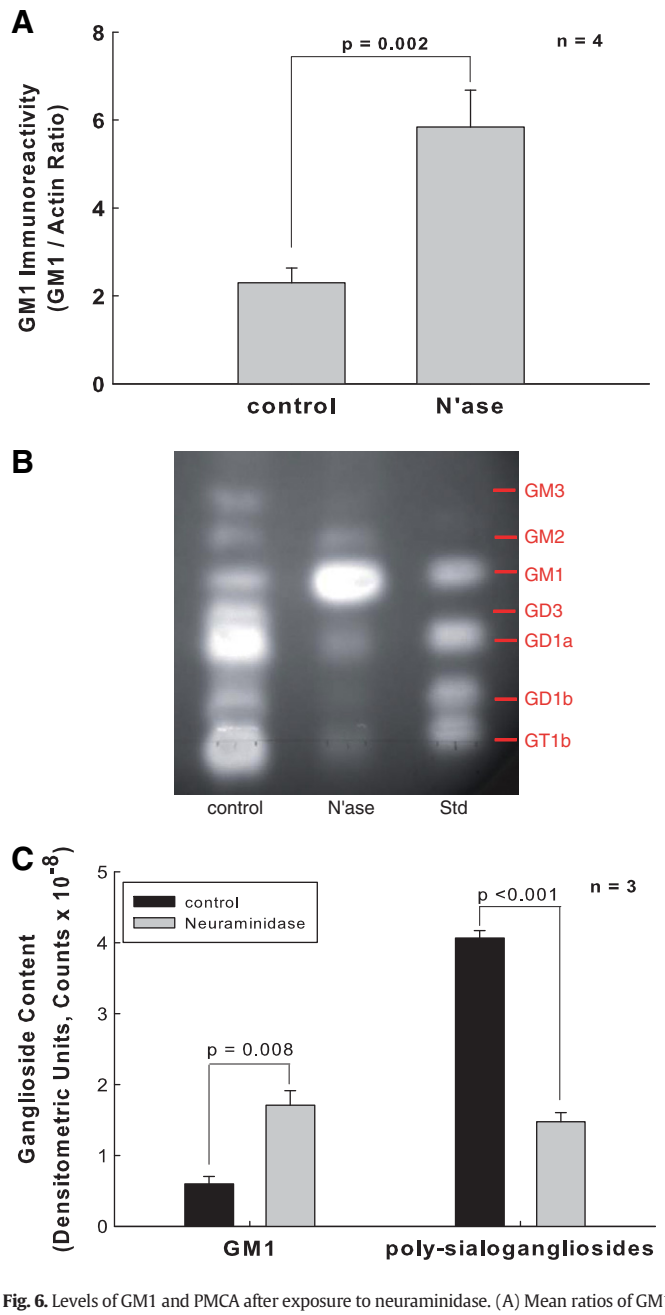

Fig. 6. Levels of GM1 and PMCA after exposure to neuraminidase. (A) Mean ratios of GM1 to actin after neuraminidase treatment. The results are from 4 experiments using different cultures. (B) Representative HPTLC image showing the effect of neuraminidase exposure on poly-sialoganglioside levels (n = 3 cultures). (C) Densitometric analysis of HPTLC images from 3 different cell preparations. Data are presented as means ± SEM of GM1 vs. the combined content of poly-sialogangliosides.

possibly representing PMCA aggregates and/or PMCA-interacting complexes. Densitometric measurement of PMCA bands in the starting material (lane 1) vs. eluate (lane 5), factored by the total volume of the sample, revealed that 52% of the total PMCA had been immunoprecipitated. There was virtually no immunoreactivity in the negative control (lane 6). GM1 did not seem to bind to the PMCA-linked resin and thus appeared in the flow-through (lane 3). No detectable amount of GM1 was observed in the eluate despite the loading of a larger volume of the eluate (lane 5). This indicated that GM1 was not interacting with the PMCA proteins directly. Immunoblots of flow-through and eluate were also run to probe for GD1a by using an anti-GD1a antibody, and results similar to those with GM1 were

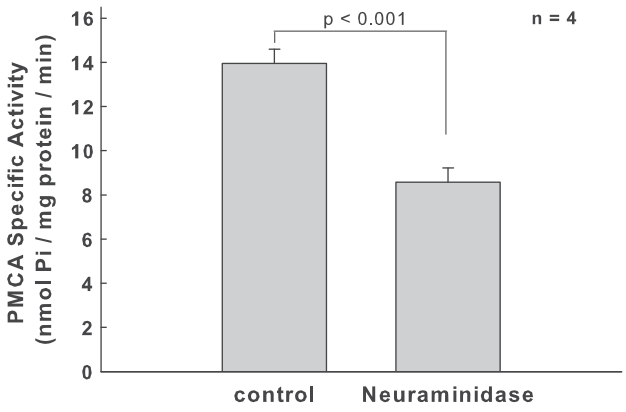

Fig. 7. Effects of neuraminidase treatment on PMCA activity. The specific activity of PMCA was measured in particulate fractions from control or neuraminidase-treated neurons. The data shown are the means ± SEM from 4 different cell preparations.

obtained (data not shown), further confirming that gangliosides did not directly associate with the PMCA in neuronal membranes through tight binding interactions.

4. Discussion

Using a high-resolution and high-sensitivity LC–MS method to assess age-related changes in the relative abundance of specific gangliosides in rat brain SPMs, we found that the levels of the major brain gangliosides did change with age and that such changes were different for the poly-sialogangliosides when compared with those of the mono-sialogangliosides. The results of our studies pointed to significant decreases in GD1a and GT1b at 23 months of age, and a

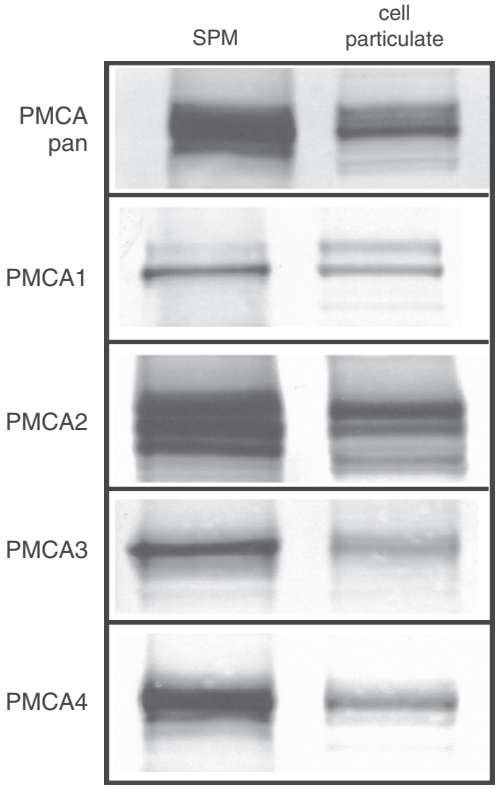

Fig. 8. PMCA isoforms in SPMs and neuronal particulates. Fifteen micrograms of protein from either SPMs or cell particulates were loaded to each gel lane for probing the levels of pan PMCA and the 4 isoforms. The dilution of each primary antibody is: 1:1000 for anti-pan-PMCA, 1:400 for anti-PMCA1, PMCA3, and PMCA4, and 1:2000 for anti-PMCA2.

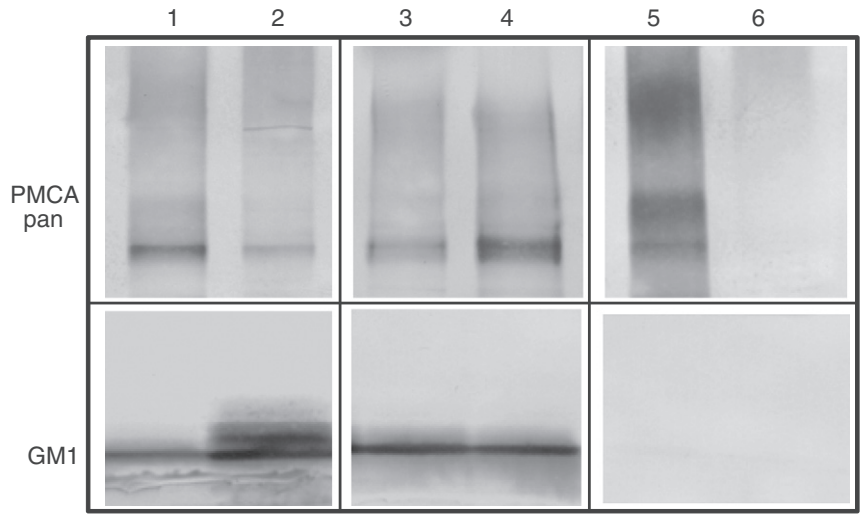

Fig. 9. Test for direct interaction between PMCA and gangliosides. The membrane proteins were extracted from SPMs and solubilized by 0.1% NP-40. Co-immunoprecipitation was performed by co-incubation of the detergent soluble fraction with monoclonal pan-PMCA antibody and Protein A UltraLink™ resin (see Materials and methods). Flow-through and eluate were both collected and analyzed via immunoblotting for the yield of PMCA using polyclonal pan-PMCA antibody (1:400 dilution) and the detection of GM1 using cholera toxin. Lane 1: detergent soluble fraction, 5 μg protein loading (3.32 μl); Lane 2: detergent insoluble fraction, 5 μg (3.10 μl); Lane 3: flow through, 12 μg (7.64 μl); Lane 4: flow through of the negative control, 12 μg (8.69 μl); Lane 5: eluate, 8 μl for PMCA and 40 μl for GM1 probing; Lane 6: eluate of the negative control, 8 μl for PMCA and 40 μl for GM1.

significant increase in GM1 content in synaptic membranes at 34 months of age. All values reported for changes in the content of various ganglioside species in older rats were compared with those in SPMs from 6 month old rats.

The LC–MS method employed in our studies utilized a trapping strategy to simultaneously desalt and focus the gangliosides prior to resolving them by HILIC. This approach enabled each of five major classes of gangliosides (GM1, GD1a, GD1b, GT1b, GQ1b) to be fully resolved, despite the fact that they are chemically similar amphipathic molecules and that GD1a and GD1b are isobaric. The high performance mass spectrometry (i.e., mass-resolution, accuracy, and fragmentation by MS^e) afforded substantive molecular speciation enabling us to differentiate between co-eluting gangliosides and the C18:1 sphingosine family, as well as between those that differed only in the length of their fatty acyl chains (18:0 vs. 20:0). We also noted that the use of the limit of quantitation (LoQ) as a measure of sensitivity was more appropriate than the standard ganglioside quantitative techniques. The measurement of the worst LoQ (20 pmol/injection for the GM1 with the 18:0 fatty acyl chain) was achieved in spite of the fact that this ganglioside co-eluted with the residual sucrose added during extraction of the SPMs (see Supplemental Table 1).

Changes in ganglioside levels during development and aging have been reported previously, but most of these measures were obtained from homogenates of rat [14], mouse [48], and human brains [16,49]. Therefore, it is not clear whether the changes in ganglioside content reported in previous studies reflect age-related neuronal or glial alterations. In the present study, we employed a fraction of neuronal SPMs and observed significant increases in the abundance of GM1 in SPMs from the oldest rats. These findings are consistent with the age-associated, high density clustering of GM1 at presynaptic terminals reported previously [50]. Our observations showing no age-associated changes in the fatty acid composition of either the C18:1 or C20:1 sphingosine component of all major brain gangliosides present in rat synaptosomal fractions are also consistent with those reported previously [51]. The reason why GD1a content and GT1b content were first decreased at 23 months and restored at 34 months is unclear and could be a compensatory mechanism for the neurons at 34 months to overcome the effects of lower levels of the poly-sialogangliosides at 23 months.

Different types of gangliosides have been reported to produce divergent effects on the activity of PMCA purified from either brain synaptosomes or erythrocyte membranes and reconstituted into liposomes [12,13]. In these studies, it was shown that the effects of gangliosides on the activity of PMCA are related to the number of sialic acid residues present in the gangliosides, with GD1b stimulating, GM1 reducing, and asialo-GM1 markedly inhibiting the PMCA activity. If changes in the content of endogenous GM1 and GD1b in SPMs have effects on the activity of membrane-attached PMCA that are similar to those of exogenously added gangliosides, then we would predict that the age-associated increases in GM1 and transient decreases in GD1b in rat brain SPMs would lead to an overall age related reduction in neuronal PMCA activity. To assess the validity of this idea, we chose to manipulate the total content of endogenous gangliosides as well as the levels of the various ganglioside species.

We performed two manipulations to alter endogenous ganglioside levels. One was aimed at suppressing the synthesis of both mono- and poly-sialogangliosides by treating neurons with D-PDMP. The other was designed to reduce the content of poly-sialogangliosides while increasing the level of GM1 through treatment of primary neurons with neuraminidase. Both manipulations decreased the activity of PMCA without having a significant effect on PMCA protein levels. Both D-PDMP and L-PDMP, as well as the neuraminidase treatments, induced slight morphological changes in the neuronal cultures and caused similar but small losses of total protein when treated cultures were compared with untreated controls. But, whereas D-PDMP inhibited PMCA activity significantly, L-PDMP had no significant effect on PMCA activity. This selectivity suggests that it is the reduction in overall ganglioside content in neurons treated with D-PDMP that caused decreases in enzyme activity. Neuraminidase treatment of primary neurons led to an even greater loss of PMCA activity than that seen with D-PDMP (39% vs. 26%). As neuraminidase markedly increased GM1 while causing significant decreases in poly-sialogangliosides, it appears that poly-sialogangliosides facilitate and GM1 reduces PMCA activity. Based on these observations we suggest that the naturally occurring decreases in the disialo- and trisialo-gangliosides at 23 months of age combined with the increases in GM1 at 34 months of age might both contribute to the loss of activity of neuronal PMCA during the aging process.

Conclusions about the role of gangliosides in PMCA regulation based on the results of treatment of neurons with neuraminidase should be tempered by the fact that such enzymatic treatment would cleave not only sialic acid residues in gangliosides but also sialic acid residues in glycoproteins. Thus, unlike the D-PDMP treatments that affect only the

biosynthesis of gangliosides, neuraminidase treatments may potentially alter the environment of PMCA by changing the structure of both gangliosides and glycoproteins. Moreover, with both D-PDMP and neuraminidase treatments, gangliosides other than the ganglio series, such as GD3 and GM3, were also decreased, which could contribute to the PMCA activity loss. Finally, since all of the studies of neuraminidase and D-PDMP treatments on PMCA activity were focused on the Ca^{2+}-activated ATP hydrolysis, additional studies are planned to also measure the effects of such manipulations on ATP-dependent Ca^{2+} transport in neuronal preparations.

The mechanisms underlying the modulation of PMCA by gangliosides remain undetermined. Gangliosides may modulate PMCA function either through direct interaction with the enzyme or through a broader effect on the biophysical properties of the membrane lipid bilayer. Previous studies of the effects of adding exogenous gangliosides to liposome-reconstituted preparations of PMCA indicated that direct interactions between gangliosides and the protein do occur [12]. However, our results from co-immunoprecipitation studies revealed that endogenous gangliosides in SPMs may not interact directly with PMCA or that the affinity of such binding interactions is low and the binding is readily reversible. This finding is consistent with our assumption that gangliosides, residing in the outer leaflet of the membrane with their head-groups extending toward the extracellular medium, may have transient, possibly low affinity interactions with the PMCA extracellular domain or they may have an indirect effect on lipid bilayer organization. It should be noted that 70% of the mass of the PMCA protein and nearly all of the functional domains are on the cytoplasmic side of the plasma membrane [52].

With regard to the effects of gangliosides on PMCA that may be produced through changes in the overall membrane structure, it is important to point out that an ordered lipid environment, such as that in raft domains, increases the PMCA activity [30,34,53]. In the present study, neither D-PDMP nor neuraminidase treatments altered the membrane cholesterol levels (data not shown). Thus the effects of the treatments could not be ascribed to cholesterol depletion or enrichment of the membranes. It has been suggested that the large hydrophilic head-groups of gangliosides may help to form and stabilize membrane lipid domains that exhibit a positive curvature [54]. Therefore, changes in the carbohydrate units could affect the membrane curvature and intermolecular packing, thereby altering the three dimensional structure of membrane proteins and leading to either their activation or inhibition [54,55]. Also, the geometry of the gangliosides, which is determined by the size of the head-groups, can modulate the fluidity of the membrane lipid core [54] and thus affect the activity of membrane-associated enzymes. A mechanistic resolution of the various alternatives must await more definitive biophysical measurements of ganglioside effects on membrane structure. The results of such studies might shed light on the mechanisms underlying the effects of compositional changes in gangliosides and their relationship to the loss of PMCA activity in aging brain membranes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2014.01.003.

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