Sialic Acid on the Glycosylphosphatidylinositol Anchor Regulates PrP-mediated Cell Signalling and Prion Formation

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Running Title: Composition of GPI affects prion formation

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SUMMARY

The prion diseases occur following the conversion of the cellular prion protein (PrP^C) into disease-related isoforms (PrP^{Sc}). In this study the role of the glycosylphosphatidylinositol (GPI) anchor attached to PrP^C in prion formation was examined using a cell painting technique. PrP^{Sc} formation in two prion-infected neuronal cell lines (ScGT1 and ScN2a cells), and in scrapie-infected primary cortical neurons, was increased following the introduction of PrP^C. In contrast, PrP^C containing a GPI anchor from which the sialic acid had been removed (desialylated PrP^C) was not converted to PrP^{Sc}. Furthermore, the presence of desialylated PrP^C inhibited the production of PrP^{Sc} within prion-infected cortical neurons, ScGT1 and ScN2a cells. The membrane rafts surrounding desialylated PrP^C contained greater amounts of sialylated gangliosides and cholesterol than membrane rafts surrounding PrP^C. Desialylated PrP^C was less sensitive to cholesterol depletion than PrP^C and was not released from cells by treatment with glimepiride. The presence of desialylated PrP^C in neurons caused the dissociation of cytoplasmic phospholipase A_2 ($cPLA_2$) from PrP-containing membrane rafts and reduced the activation of $cPLA_2$. These findings show that the sialic acid moiety of the GPI attached to PrP^C modifies local membrane microenvironments that are important in PrP-mediated cell signalling and PrP^{Sc} formation. These results suggest that pharmacological modification of GPI glycosylation might constitute a novel therapeutic approach to prion diseases.

Introduction

The prion diseases are fatal neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy in cattle and Creutzfeldt-Jakob disease in man. A key event in these diseases is the conversion of a normal host protein designated PrP^C into disease-associated isoforms (PrP^{Sc}). In this conversion process a portion of PrP^C that is mostly α-helix and random coil becomes refolded into a β-pleated sheet in the PrP^{Sc} molecule (1). Although the presence of PrP^C is essential for prion formation (2), not all cells that express PrP^C are permissive for PrP^{Sc} replication. The reasons why some cells that express PrP^C do not replicate PrP^{Sc} are not fully understood. Reports that the targeting of PrP^C to specific membranes is required for efficient PrP^{Sc} formation (3,4) indicate that factors that affect the cellular targeting and intracellular trafficking of PrP^C are critical in determining PrP^{Sc} replication.

The majority of PrP^C molecules are linked to membranes via a glycosylphosphatidylinositol (GPI) anchor (5). GPI anchors play a complex role in the regulation of cell membrane composition, cell signalling and protein trafficking (6). For example, the presence of a GPI anchor targets PrP^C to specific membrane micro-domains called rafts that are required for efficient PrP^{Sc} formation (3,4). The role of the GPI anchor in prion diseases is

controversial as transgenic mice that produce anchorless PrP^{C} produce large amounts of soluble PrP^{Sc} (7), suggesting that the GPI anchor was not essential for PrP^{Sc} formation. In contrast, observations that neuronal cells producing anchorless PrP^{C} were resistant to scrapie infection (8), and that monoacylated PrP^{C} was not converted to PrP^{Sc} within neuronal cells (9), indicate that the GPI is a critical factor in facilitating membrane-associated PrP^{Sc} formation.

GPI-anchored molecules, including PrP^{C}, are rapidly incorporated into living cells (10,11) and introducing PrP^{C} to prion infected cells by cell painting increased PrP^{Sc} formation (9). This technique was used to examine the role of specific GPI structures on PrP^{C} and PrP^{Sc} formation. The GPI anchor attached to PrP^{C} is unusual amongst mammalian GPIs in that it contains sialic acid (12). Neuraminidase digestion was used to produce a PrP^{C} with a GPI anchor lacking sialic acid (desialylated PrP^{C}), a modification that could not be achieved by genetic manipulation methods (13). We report 3 major observations; firstly that desialylated PrP^{C} behaves differently from PrP^{C} with regards to its effects on membrane composition and cell signalling, secondly that desialylated PrP^{C} is not converted to PrP^{Sc} and thirdly that desialylated PrP^{C} inhibits the conversion of endogenous PrP^{C} to PrP^{Sc}.

Experimental procedures

Cell lines – Prion-infected ScN2a and ScGT1 cells were grown in Ham's F12 medium containing 2 mM glutamine and 2% foetal calf serum. To determine the effect of test preparations on PrP^{Sc} formation, ScN2a or ScGT1 cells were added to 6 well plates, allowed to adhere overnight and then cultured in the presence or absence of test PrP^{C} preparations. Cells were grown with daily changes of media (± PrP^{C} preparations) and the amounts of PrP^{Sc} were evaluated after 7 days. In other experiments ScGT1 and ScN2a cells were pre-treated for 1 hour with control medium or desialylated PrP^{C} and then incubated with phospholipase A_2-activating peptide (PLAP) (Bachem) for a further 1 hour.

Primary neurons - Cortical neurons were prepared from the brains of mouse embryos (day 15.5) derived from Prnp wild type$^{(+/+)}$ and Prnp knockout$^{(0/0)}$ mice after mechanical dissociation. Neurons (10^6) were added to 6 well plates that had been coated with poly-L-lysine and incubated in Ham's F12 medium containing 5% foetal calf serum for 2 hours. Cultures were shaken (600 rpm for 5 minutes) and non-adherent cells removed by 2 washes in PBS. Neurons were grown in neurobasal medium (NBM) containing B27 components and 5 ng/ml nerve growth factor (Sigma) for 10 days. Immunostaining showed that the cells were greater than 95% neurofilament positive. In some experiments neurons were pre-treated for 24 hours with squalestatin (GlaxoSmithKline). The fate of PrP^{C} preparations was determined by incubating neurons with 10 ng PrP^{C} or desialylated PrP^{C}. Cells were washed to remove unbound PrP^{C} and incubated in fresh culture medium for different time periods. For infection studies neurons from either Prnp$^{(0/0)}$ or Prnp$^{(+/+)}$ mice were pulsed with 1 ng PrP^{Sc} (derived from ScGT1 cells (14)) and then incubated with 10 ng PrP^{C} or 10 ng desialylated PrP^{C}. Media containing PrP^{C} preparations were replaced daily for 10 days and the amount of PrP^{Sc} in neurons was measured by enzyme-linked immunosorbent assay (ELISA). All experiments were performed in accordance with European regulations (European Community Council Directive, 1986, 56/609/EEC) and approved by the local authority veterinary service/ethical committee.

Cell extracts - Treated cells were washed 3 times with ice cold PBS and homogenised in an extraction buffer containing 10 mM Triz-HCl pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS at 10^6 cells/ml and nuclei and large fragments were removed by centrifugation (1000 x *g* for 5 minutes). Mixed protease inhibitors (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, Aprotinin, Leupeptin, Bestatin, Pepstatin A and E-46) (Sigma) and a phosphatase inhibitor cocktail including PP1, PP2A, microcystin LR, cantharidin and p-bromotetramisole (Sigma) were added to some membrane extracts. To determine the amount of PrP^{Sc} in cells extracts were digested with 1 µg/ml proteinase K for 1 hour at 37°C, heated to 95°C for 5 minutes and tested in a PrP ELISA as described (9).

Isolation of detergent-resistant membranes (DRMs)/rafts - Cells were homogenised in an ice-cold buffer containing 1% Triton X-100, 10 mM Triz-HCl pH 7.2, 100 mM NaCl, 10 mM EDTA and mixed protease inhibitors (10^6 cells/ml) and nuclei and large fragments were removed by centrifugation (1000 x *g* for 5 minutes). The supernatant was incubated on ice for 60 minutes prior to further centrifugation (16,000 x *g* for 30 minutes at 4°C). The detergent-soluble material (DSM) was reserved as the normal cell membrane. The insoluble DRMs were homogenised in extraction buffer (10 mM Triz-HCL pH 7.4, 10 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS and mixed protease inhibitors. After further centrifugation (10 minutes at 16,000 x *g*) the supernatant was collected as the raft fraction.

Sucrose density gradients - Cells were harvested with a Teflon scraper and homogenised in a buffer containing 250 mM sucrose, 10 mM Triz-HCl, pH 7.2 and 1 mM EDTA, mixed protease inhibitors, 1 mM dithiothreitol. Nuclei and cell fragments were removed by centrifugation (1000 x *g* for 5 minutes). Membranes were washed by centrifugation at 16,000 x g for 20 minutes at 4°C and solubilised in an ice-cold buffer containing 1% Triton X-100, 10 mM Triz-HCl pH 7.2, 150 mM NaCl and 10 mM EDTA. 5, 10, 15, 20, 30 and 40% sucrose solutions were prepared and layered to produce a gradient at 4°C. Solubilised membranes were layered on top and centrifuged at 50,000 x *g* for 18 hours at 4°C. Serial 0.8 or 0.25 ml volumes were collected from the bottom of gradients.

Isolation of PrP^C - PrP^C molecules were isolated from murine GT1 neuronal cell membranes using a combination of immunoaffinity columns, size exclusion chromatography (Superdex) and reverse-phase chromatography on C18 columns (Waters) as described (9). PrP^C was digested with 2 units/ml endoglycosidase F (PNGase) (Sigma) and/or 0.2 units/ml neuraminidase (*Clostridium perfringens* - Sigma) for 2 hours at 37°C. Trifluoroacetic acid (TFA) was added (final concentration - 0.1%) and digested PrP^C was loaded onto C18 columns. Samples were eluted under a gradient of acetonitrile and water containing 0.1% TFA. 1 ml samples were collected, split into two and lyophilized. One sample was solubilised in an extraction buffer (as above) and tested in a PrP ELISA. PrP-containing fractions were solubilised in culture medium by sonication prior to use.

Test PrP^{Sc} preparations. PrP^{Sc} preparations consisted of supernatants collected from ScGT1 cells which were centrifuged (500 x *g* for 5 minutes) to remove cell debris and concentrated using a 10 kDa filter (Sartorius Vivaspin). Preparations were digested with 1 µg/ml proteinase K for 1 hour at 37°C to remove PrP^C and the remaining protease-resistant PrP was measured by ELISA, see below. Samples were frozen at -20°C for storage. On the day of use, PrP^{Sc} preparations were diluted to 1 ng/ml in culture medium and sonicated before addition to cells.

PrP ELISA - Maxisorb immunoplates (Nunc) were coated with mAb ICSM18 and blocked with 5% milk powder. Samples were applied and detected with biotinylated mAb ICSM35, followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate (Sigma). Absorbance was measured on a microplate reader at 405 nm and the amount of PrP in samples was calculated by reference to serial dilutions of recombinant murine PrP (Prionics).

$cPLA_2$ ELISA - Maxisorb immunoplates were coated with 0.5 µg/ml of mouse mAb anti-$cPLA_2$, clone CH-7 (Upstate) and blocked. Samples were incubated for 1 hour and the amount of $cPLA_2$ was detected using a goat polyclonal anti-$cPLA_2$ (Santa-Cruz Biotech) followed by biotinylated anti-goat IgG, extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate. Absorbance was measured at 405 nm and the amount of $cPLA_2$ protein expressed in units, 100 units = amount of $cPLA_2$ in extracts or immunoprecipitates from 10^6 untreated cells.

Activated $cPLA_2$ ELISA - The activation of $cPLA_2$ is accompanied by phosphorylation of the 505 serine residue which creates a unique epitope that was measured in an ELISA using an anti-$cPLA_2$ mAb (clone CH-7) combined with rabbit polyclonal anti-phospho-$cPLA_2$ (Cell Signalling Technology) followed by biotinylated anti-rabbit IgG (Dako), extravidin-alkaline phosphatase and

1mg/ml 4-nitrophenyl phosphate). Absorbance was measured on a microplate reader at 405 nm. Results were expressed as "units activated $cPLA_2$" with 100 units defined as the amount of activated $cPLA_2$ in extracts derived from 10^6 untreated cells.

Isolation and analysis of GPIs – GPIs were isolated and analysed as described (13). Briefly, PrP^C preparations were digested with proteinase K (100 µg/ml) overnight at 37°C. Digested products were mixed with water-saturated butanol. The butanol phase was collected and washed with water a further 3 times before being loaded onto C18 columns. GPIs were eluted from C18 columns under a gradient of propanol and water. The presence of phosphatidylinositol in GPIs was determined using mAb (5AB3-11) and specific glycans were detected with biotinylated lectins. Isolated GPIs bound to nitrocellulose membranes by dot blot and were blocked with 5% milk powder. Samples were probed with mAb 5AB3-11, biotinylated *Sambucus nigra (S. nigra)* lectin (detects terminal sialic acid residues bound α-2,6 or α-2,3 to galactose), biotinylated concanavalin A (detects mannose) or biotinylated *Ricinus Communis* Agglutinin I (RCA I) (detects terminal galactose) (Vector Labs). Bound lectins were visualised using extravidin peroxidise and enhanced chemiluminescence. The mAb was visualised by incubation with a horseradish peroxidise conjugated anti-murine-IgM and chemiluminescence. In other studies GPIs were separated on silica gel 60 high performance thin layer chromatography (HPTLC) plates in a mixture of chloroform/methanol/water (10/10/3 v/v/v) and detected by mAb 5AB3-11. The concentrations of GPIs were measured by ELISA. Maxisorb immunoplates were coated with 0.5 µg/ml concanavalin A (binds mannose) and blocked with 5% milk powder in PBS-tween. Samples were added and any bound GPI was detected by the addition of mAb 5AB3-11 (15), followed by a biotinylated anti-mouse IgM (Sigma), extravidin-alkaline phosphatase and 1mg/ml 4-nitrophenyl phosphate. Absorbance was measured on a microplate reader at 405 nm.

Immunoprecipitations of PrP-specific rafts - Treated cells were homogenised in ice cold 1% Triton X-100, 10 mM Triz-HCl, pH 7.2, 100 mM NaCl, 10 mM EDTA at 10^6 cells/ml. Cell debris was removed from by centrifugation (1000 x *g* for 5 mins) and the supernatant incubated with a mAb to PrP (4F2) for 30 minutes at 4°C on rollers. Magnetic microbeads containing protein G (Miltenyi Biotech) were added (10 µl/ml) for 30 minutes and protein G bound antibody complexes isolated using a µMACS magnetic system (Miltenyi Biotech) at 4°C. The amounts of sialylated gangliosides in precipitates were determined by diluting precipitates from 10^6 cells in 1 ml carbonate buffer and plating in Maxisorb immunoplates overnight. Plates were blocked with 5% milk powder in PBS-tween and sialylated gangliosides were detected by the addition of biotinylated *S. nigra* lectin followed by extravidin-alkaline phosphatase and 1mg/ml 4-nitrophenyl phosphate. Absorbance was measured on a microplate reader at 405 nm. Gangliosides within immunoprecipitates were separated by HPTLC in a solvent containing chloroform/methanol/water (6/4/1). Plates were blocked and sialic acid containing gangliosides were detected by incubation with biotinylated *S. nigra* lectin, extravidin-peroxidase and chemiluminesence.

Western Blot - Samples were dissolved in 50 µl of Laemmli buffer, boiled, and subjected to electrophoresis on a 15 % polyacrylamide gel. Proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biotech) by semi-dry blotting. Membranes were blocked using 10% milk powder in PBS containing 0.2 % Tween 20. PrP was detected by incubation with mAb ICSM18, β-actin by mAb AC-74 (Sigma) and $cPLA_2$ with mAb CH-7, followed by a secondary anti-mouse IgG conjugated to peroxidase. Bound antibody was visualised by chemiluminescence.

Cholesterol and protein content - Cholesterol and protein content were determined in cell membrane extracts. Protein concentrations were measured using a micro-BCA protein assay kit (Pierce). The concentrations of cholesterol were measured using the Amplex Red cholesterol assay kit (Invitrogen). Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide which reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin. Fluorescence was measured by excitation at 550 nm and emission detection at 590 nm. The concentration of

cholesterol was calculated by reference to cholesterol standards.

Statistical Analysis - Comparison of treatment effects was carried out using Student's paired t-tests, one-way and two-way ANOVA with Bonferroni's post-hoc tests (IBM SPSS statistics 20). Error values are standard deviation (SD) and significance was determined where $P<0.01$. Correlations between data sets were analysed using Pearson's bivariate coefficient (IBM SPSS statistics 20).

Results

Deglycosylated PrP^C is converted to PrP^{Sc} – Initial studies were performed to determine whether the N-linked glycans that are attached to PrP^C affect PrP^{Sc} formation in ScGT1 cells. N-linked glycans were removed from PrP^C by digestion with PNGase (16) (Figure 1A) and deglycosylated PrP^C was isolated on C18 columns (Figure 1B). The addition of PrP^C or PNGase-digested PrP^C (deglycosylated PrP^C) increased the PrP^{Sc} content of ScGT1 cells in a dose-dependent manner (Figure 1C); there were no significant differences in the amounts of PrP^{Sc} in cells incubated with PrP^C or with deglycosylated PrP^C. Similarly, the addition of 10 ng deglycosylated PrP^C increased the amount of PrP^{Sc} in ScN2a cells (4.7 ng $PrP^{Sc}/10^6$ cells ± 1.1 compared with 1.5 ng PrP^{Sc} ± 0.7, n=15, $P<0.05$). As the absence of N-linked glycans did not significantly affect PrP^{Sc} formation in these cells all the following experiments were performed with deglycosylated PrP^C preparations.

Sialic acid on the GPI anchor of PrP^C is necessary for PrP^{Sc} formation - The GPI anchor attached to PrP^C is unusual for mammalian GPIs in that it contains sialic acid as first described by Stahl and colleagues (12) and illustrated in Figure 2A. To confirm the presence of sialic acid in our PrP^C preparations, GPIs were isolated from PrP^C and desialylated PrP^C (neuraminidase-digested) by reverse phase chromatography (Figure 2B). GPIs derived from PrP^C and desialylated PrP^C eluted at different concentrations of propanol. HPTLC analysis confirmed that neuraminidase digestion altered the migration of the GPI isolated from PrP^C consistent with the loss of sialic acid (Figure 2C). Isolated GPIs were blotted onto nitrocellulose membranes and probed with mAb 5AB3-11 and biotinylated lectins. The digestion of PrP^C with neuraminidase did not affect the binding of mAb 5AB3-11 (binds to phosphatidylinositol) or concanavalin A (binds to mannose) to the GPIs showing that similar amounts of GPIs were added to blots. *S. nigra* lectin (which reacts with terminal sialic acid bound either α-2,6 or α-2,3 to galactose) bound to GPIs isolated from PrP^C, but not to GPIs derived from neuraminidase-digested PrP^C indicating that sialic acid had been removed (Figure 2D). The lectin RCA I bound to neuraminidase-digested GPIs indicating that terminal galactose had been exposed.

The observation that PrP^{Sc} does not replicate in $Prnp^{(0/0)}$ neurons (14) allowed us to examine whether desialylated PrP^C was converted to PrP^{Sc}. Both PrP^C and desialylated PrP^C bound readily to recipient $Prnp^{(0/0)}$ neurons; following the addition of 10 ng PrP^C preparations for 2 hours there was no significant difference in the amounts of PrP^C and desialylated PrP^C bound (9.4 ng PrP^C ± 0.9 compared with 9.7 ng ± 0.9, n=9, P=0.7). Neurons from $Prnp^{(0/0)}$ mice were pulsed with 1 ng PrP^{Sc} for 2 hours and incubated with culture media containing control medium, 10 ng PrP^C or 10 ng desialylated PrP^C. Media containing the PrP^C/desialylated PrP^C was replaced daily for 10 days when the amounts of PrP^{Sc} in neurons were measured. We did not detect any PrP^{Sc} in neurons that had been pulsed with 1 ng PrP^{Sc} and incubated with control medium, suggesting that original inoculum was degraded by these neurons. However, PrP^{Sc} pulsed neurons incubated daily with 10 ng PrP^C contained 25.7 ng PrP^{Sc} ± 4.2, n=12 indicating that some of the added PrP^C was converted to PrP^{Sc}. In contrast, no PrP^{Sc} was detected in neurons pulsed with 1 ng PrP^{Sc} and incubated daily with 10 ng desialylated PrP^C indicating that it was not converted to PrP^{Sc} (Figure 3A). Immunoblots confirmed the ELISA data and showed that PrP^{Sc} was formed in $Prnp^{(0/0)}$ neurons pulsed with PrP^{Sc} and incubated daily with PrP^C but not in neurons pulsed with PrP^{Sc} and incubated daily with control medium or with desialylated PrP^C (Figure 3B).

Desialylated PrP^C reduced PrP^{Sc} formation – Next, the effects of desialylated PrP^C on the

conversion of endogenous PrP^{C} to PrP^{Sc} were determined For this study neurons derived from $Prnp^{(+/+)}$ mice were pulsed with 1 ng PrP^{Sc} for 2 hours and incubated for a further 10 days with 10 ng PrP^{C} or 10 ng desialylated PrP^{C}. Neurons pulsed with PrP^{Sc} and incubated in control medium contained 40.2 ng PrP^{Sc} ± 5.2, n=12. The addition of PrP^{C} increased the amount of PrP^{Sc} in neurons to 68.5 ng PrP^{Sc} ± 7.1, n=12, whereas the addition of desialylated PrP^{C} reduced the amount of PrP^{Sc} to 5.2 ng/ml (Figure 4A&B) indicating that desialylated PrP^{C} suppressed the conversion of PrP^{C} to PrP^{Sc}. Furthermore, when neurons from $Prnp^{(0/0)}$ mice were pulsed with 1 ng PrP^{Sc} and incubated with 10 ng PrP^{C} or a mixture of 10 ng PrP^{C} and 10 ng desialylated PrP^{C} for 10 days the presence of desialylated PrP^{C} reduced PrP^{Sc} production (Figure 4C). Similarly, the daily addition of desialylated PrP^{C} reduced the PrP^{Sc} content of recipient ScN2a cells (Figure 4D) and ScGT1 cells (Figure 4E).

Sialic acid on the GPI anchor affects the fate of PrP^{C} – To determine the mechanism(s) involved in desialylated PrP^{C}-induced suppression of PrP^{Sc} formation its properties in non-infected cells were examined. Both PrP^{C} and desialylated PrP^{C} bound readily to recipient $Prnp^{(0/0)}$ neurons. The targeting of PrP^{C} to DRMs is necessary for efficient prion formation (4) and following the addition of 10 ng PrP^{C} preparations similar amounts of PrP^{C} and desialylated PrP^{C} were found within DRMs (8.2 ng PrP^{C} ± 0.9 compared with 8.1 ng desialylated PrP^{C} ± 1.2, n=9, P=0.7). PrP^{C} and desialylated PrP^{C} were found within different membrane rafts; after $Prnp^{(0/0)}$ neurons pulsed with 10 ng of PrP^{C} or 10 ng desialylated PrP^{C} were homogenised in ice cold triton X-100 (which maintains the integrity of membrane rafts) and subjected to sucrose density gradient analysis, the desialylated PrP^{C} was found in lower density rafts than those containing PrP^{C} (Figure 5A). These rafts were examined in detail after immunoprecipitation. Similar amounts of PrP^{C} and desialylated PrP^{C} were obtained in each precipitate (Figure 5B). However, the rafts isolated with PrP^{C} contained significantly less cholesterol than rafts surrounding desialylated PrP^{C} (Figure 5C). HPTLC analysis of immunoprecipitates demonstrated that rafts containing desialylated PrP^{C} contained more sialylated gangliosides than did membranes surrounding PrP^{C} (Figure 5D). ELISA studies confirmed that rafts co-precipitated with desialylated PrP^{C} contained significantly more sialylated gangliosides than rafts co-precipitated with PrP^{C} (Figure 5E).

Reducing cellular cholesterol concentrations destabilised some rafts resulting in the relocation of PrP^{C} from rafts to DSMs (4). Here we examined whether the targeting of desialylated PrP^{C} to DRMs was also sensitive to cholesterol depletion. $Prnp^{(0/0)}$ neurons pre-treated with the cholesterol synthesis inhibitor squalestatin for 24 hours were pulsed with 10 ng PrP^{C} or 10 ng desialylated PrP^{C} and DRMs (rafts) isolated. Whereas squalestatin reduced the amounts of PrP^{C} in rafts, desialylated PrP^{C} remained within rafts (Figure 6A). The concentrations of cholesterol within cell membranes were reduced by squalestatin in a dose-dependent manner. There was a significant correlation between amounts of raft PrP^{C}, but not desialylated PrP^{C}, and concentrations of cholesterol in squalestatin-treated cells (Figure 6B). Neurons incubated with PrP^{C}/desialylated PrP^{C} were also treated with PI-PLC for 1 hour. While digestion with PI-PLC reduced the amounts of cell-associated desialylated PrP^{C} to less than 0.5 ng (indicating that all desialylated PrP^{C} was expressed at the cell surface), 3.6 ng PrP^{C} ± 0.4 remained cell-associated after PI-PLC digestion (Figure 6C). Increasing the concentration of PI-PLC, or incubation period, did not affect the amounts of cell-associated PrP^{C} suggesting that there is a pool of PrP^{C} that is not expressed at the surface of neurons. As proteins with different GPI anchors traffic via different pathways (17,18), the effect of the GPI anchor on the fate of PrP^{C} within cells was investigated. $Prnp^{(0/0)}$ neurons were pulsed with 10 ng of PrP^{C} preparations and the amounts of PrP^{C} measured at time points thereafter. Whereas PrP^{C} was rapidly cleared from neurons, and was absent after 48 hours, desialylated PrP^{C} remained in neurons for up to 96 hours (Figure 6D). Some GPI-anchored proteins are released from cells following the glimepiride-induced activation of an endogenous GPI-PLC (19,20). Treatment with 5 µM glimepiride caused the release of PrP^{C} but not desialylated PrP^{C} (Figure 6E).

Desialylated PrP^{C} reduced the activation of $cPLA_2$ in prion-infected cells – Since the concentration of cholesterol within cell membranes

affects PrP^{Sc} formation (4) the effects of PrP^{C} and desialylated PrP^{C} on cholesterol in prion-infected cells were also examined. The amount of cholesterol in ScGT1 cell membranes was significantly increased after incubation with 10 ng PrP^{C} (744 ng cholesterol/10^6 cells ± 84 compared with 534 ± 48, n=9, P<0.05) or with 10 ng desialylated PrP^{C} (771 ng cholesterol/10^6 cells ± 76 compared with 534 ± 48, n=9, P<0.05). Critically there was no significant difference in the amounts of cholesterol between ScGT1 cells treated with PrP^{C} or with desialylated PrP^{C} (744 ng cholesterol/10^6 cells ± 84 compared with 771 ± 76, n=9, P<0.36).

As PrP^{Sc} formation is dependent upon the activation of $cPLA_2$ (21) the effects of desialylated GPI on this enzyme were studied. The addition of 25 ng desialylated PrP^{C} significantly reduced the amounts of activated $cPLA_2$ in both ScN2a and ScGT1 cells (Table 1). However, desialylated PrP^{C} did not affect PLAP-induced activation of $cPLA_2$ showing that it did not have a direct effect upon $cPLA_2$. The possibility that the addition of PLAP could restore PrP^{Sc} formation in cells treated with desialylated PrP^{C} was investigated PrP^{Sc} was not detected in ScGT-1 cells treated with a combination of 25 ng desialylated PrP^{C} and 1 μM PLAP (PrP^{Sc}<0.05 ng/10^6 cells) showing that non-selective activation of $cPLA_2$ was not sufficient to reverse desialylated PrP^{C}-induced suppression of PrP^{Sc} formation.

The clustering of GPIs in the membrane that arises as a consequence of the self-aggregation of PrP^{Sc} molecules can be mimicked by mAb-mediated cross-linkage of PrP^{C} which also activates $cPLA_2$ (22). When $Prnp^{(0/0)}$ neurons were pulsed with PrP^{C} or desialylated PrP^{C} and incubated with mAb 4F2 cross-linkage of PrP^{C}, but not desialylated PrP^{C}, caused a dose-dependent increase in activated $cPLA_2$ (Figure 7A). Furthermore, when $Prnp^{(+/+)}$ neurons were pulsed with desialylated PrP^{C} and incubated with mAb 4F2 to cross-link PrP^{C}, the presence of desialylated PrP^{C} reduced activated $cPLA_2$ in a dose-dependent manner (Figure 7B).

Sialic acid on the GPI anchor stabilised $cPLA_2$ within PrP-containing rafts - Upon activation $cPLA_2$ migrates from the cytoplasm to rafts (9,23). Sucrose density gradients showed that in untreated ScGT1 cells most of the $cPLA_2$ was in low density membranes (fractions 9 to 12) while in cells that had been incubated with 25 ng of desialylated PrP^{C} a proportion of $cPLA_2$ had relocated to fractions 4 to 7 (Figure 8A). Immunoprecipitation studies showed that $cPLA_2$ was found within PrP^{Sc}-containing rafts (9) raising the possibility that the sialic acid moiety on the GPI attached to PrP^{C} or PrP^{Sc} is required to capture $cPLA_2$ within lipid rafts. This hypothesis was tested by treating $Prnp^{(+/+)}$ neurons with 10 ng of PrP^{C} or 10 ng desialylated PrP^{C} followed by the cross-linkage of PrP^{C} by mAb 4F2. Although precipitates contained similar amounts of PrP^{C}, the precipitates from neurons incubated with PrP^{C} contained more $cPLA_2$ than precipitates from neurons containing desialylated PrP^{C} (Figure 8B).

Discussion

Recent studies suggest that differential glycosylation of the GPI anchor can affect the properties of some proteins, including protein structure (24), membrane localisation and intracellular trafficking (25). Here we show that sialic acid in the GPI affected the composition of the membranes surrounding PrP^{C}, cell signalling and the conversion of PrP^{C} to PrP^{Sc}.

Desialylated PrP^{C} was created by the sequential digestion of PrP^{C} with a PNGase (to remove N-linked glycans) followed by neuraminidase to remove sialic acid. The removal of N-linked glycans was necessary to show that the effects of the subsequent neuraminidase digestion was upon sialic acid contained within the GPI anchor, and not due to the removal of sialic acid contained within the N-linked glycans. $Prnp^{(0/0)}$ neurons exposed to PrP^{Sc} demonstrated that while PrP^{C} was converted to PrP^{Sc}, desialylated PrP^{C} was not. Consequently, the factors that prevented the conversion of desialylated PrP^{C} to PrP^{Sc} were examined. Both PrP^{C} and desialylated PrP^{C} bound to recipient cells, increased the concentrations of cholesterol in cell membranes and were targeted to rafts. However, membrane rafts are heterogeneous (26) and the composition of the GPI anchor is thought to target proteins to specific rafts. For example, PrP^{C} and Thy-1 have different GPI anchors and are targeted to different rafts (27) and replacing the GPI of PrP^{C} with that of Thy-1

altered the trafficking of PrP^C to apical membranes (28). Therefore it is possible that the differences in their GPIs direct PrP^C and desialylated PrP^C to different rafts and consequently their trafficking within cells.

GPI-anchored proteins are surrounded by specific phospholipids, glycolipids and cholesterol that constitute a raft, the composition of which is dependent upon multiple interactions between the protein, glycans and membrane lipids (29). Thus, a change in the composition of the GPI anchor attached to a protein could affect the composition of the surrounding raft. This hypothesis is supported by observations that the composition of GPIs attached to Thy-1 differs from those attached to PrP^C (12,30) and the membranes surrounding these molecules have different lipid compositions (31). We hypothesise that the presence of sialic acid in the GPI has a direct effect upon the composition of the surrounding membrane rafts as illustrated in Figures 9A & B. Our observation that the rafts surrounding desialylated PrP^C contained more cholesterol and sialylated gangliosides than those surrounding PrP^C supported this hypothesis. In addition; treatment with glimepiride caused the release of PrP^C, but not desialylated PrP^C, suggesting that only PrP^C is associated with a raft containing endogenous GPI-PLC. How sialic acid affects the composition of rafts is unknown. One possibility is that the sialic acid contained within the GPI competes with gangliosides for sialic acid-binding proteins within rafts. Thus the removal of sialic acid from the GPI would allow the incorporation of gangliosides, which are associated with the suppression of PrP^{Sc} formation (32), into rafts. Gangliosides help sequester cholesterol and stabilise rafts (33,34) especially in brain tissue (35). The increased amounts of gangliosides that surround desialylated PrP^C would be expected to form a raft containing more cholesterol, as was demonstrated in these experiments. The concentrations of cholesterol in cell membranes affects membrane rigidity and helps stabilise membrane rafts (36). We noted that desialylated PrP^C remained within rafts in cholesterol-depleted cells whereas PrP^C was relocated to DSMs, a finding indicating that the rafts surrounding desialylated PrP^C are more cholesterol dense and hence more resistant to cholesterol depletion than those surrounding PrP^C. Such observations are compatible with reports the amount of gangliosides in rafts affects the expression and function of some GPI-anchored proteins (37,38) including PrP^C (39).

Our observation that desialylated PrP^C remained in cells longer than PrP^C suggests that sialic acid in the GPI anchor affects the trafficking of PrP^C. The endocytosis of PrP^C involves a step in which it moves out of rafts and enters clathrin-coated pits (17,40). We speculate that desialylated PrP^C stabilised within cholesterol-dense rafts does not to move out of rafts nor undergo the same endocytic process as PrP^C. It is possible that the cellular location of desialylated PrP^C results in reduced interactions with PrP^{Sc}, which would explain why desialylated PrP^C was not converted to PrP^{Sc}.

Desialylated PrP^C also inhibited the conversion of endogenous PrP^C to PrP^{Sc} in primary cortical neurons, ScN2a and ScGT1 cells. Previously we had shown that monoacylated PrP^C reduced prion formation (9) and others have demonstrated that co-expression of mutant prion proteins alters the cellular localisation of wild type PrP^C and portioning into DRMs/rafts (41). One possibility is that desialylated PrP^C competes with endogenous PrP^C for specific partner proteins involved in endocytosis and that the depletion of these partner proteins alters the trafficking of endogenous PrP^C and hence PrP^{Sc} formation. Here we explored the concept that the binding of desialylated PrP^C to PrP^{Sc} modifies the rafts that are involved in PrP^{Sc} formation (3). The composition and hence the function of rafts is dynamic and controlled by an "induced fit" model (26). Since the composition of membranes is affected by the glycan structure of GPIs (29,31) then the membrane surrounding a complex between PrP^{Sc} and PrP^C (Figure 9C) would be expected to differ from membranes surrounding PrP^{Sc} and desialylated PrP^C (Figure 9D). Studies in T and B cell receptor signalling show that the coalescence of raft outer membrane proteins affects the composition of the cytoplasmic leaflet and its association with signalling molecules (42-45). We propose that the binding of desialylated PrP^C to PrP^{Sc} changes the composition of the local membrane so that it is unfavourable for the conversion of PrP^C to PrP^{Sc}.

The hypothesis that the clustering of sialic acid-containing GPIs attached to PrP leads to the

activation of $cPLA_2$, a factor that promotes PrP^{Sc} formation (21) was explored. This occurs naturally as a consequence of PrP^{Sc} self-aggregation and experimentally following cross-linkage of PrP^{C} by mAbs. Whereas cross-linkage of PrP^{C} activated $cPLA_2$, cross-linkage of desialylated PrP^{C} had no affect demonstrating that sialic acid was required (13). Cross-linkage of a mixture of PrP^{C} and desialylated PrP^{C} reduced activation of $cPLA_2$ when compared with cross-linkage of PrP^{C} alone (13) indicating that homogeneity of the GPI anchors was critical. Notably the presence of desialylated PrP^{C} did not affect the activation of $cPLA_2$ by PLAP indicating that it did not have a direct effect upon this enzyme.

To understand how desialylated PrP^{C} inhibited activation of $cPLA_2$ the cellular location of $cPLA_2$ was examined. The activation of $cPLA_2$ causes it to migrate from the cytoplasm to membrane rafts (23). In control ScGT1 cells activated $cPLA_2$ was found predominantly in rafts, and more specifically $cPLA_2$ co-localised to PrP^{Sc}-containing rafts (9). The addition of desialylated PrP^{C} caused the dissociation of $cPLA_2$ from rafts. The targeting of $cPLA_2$ to membranes containing their substrates can regulate the formation of second messengers such as platelet-activating factor that facilitate PrP^{Sc} formation (21). We propose that the density of sialic acid attached to GPIs is critical to the stabilisation and activation of $cPLA_2$ in membrane rafts and that the binding of desialylated PrP^{C} to PrP^{Sc} changed the composition of the underlying membrane so that it no longer captured and activated $cPLA_2$. This reduced the activation of $cPLA_2$ by existing PrP^{Sc} and hindered the conversion of PrP^{C} to PrP^{Sc}. It is noteworthy that desialylated PrP^{C} is surrounded by gangliosides than PrP^{C} which is consistent with reports that gangliosides inhibit the activation of $cPLA_2$ (46-48).

Some of the PrP^{C} extracted from hamster brains have GPIs which did not contain sialic acid (12). The chemical composition of the GPI anchor is cell type specific (49) and the GPI anchor attached to PrP^{C} derived from a glial cell line (N9 cells) does not contain sialic acid (13). More recently we found that some neuronal cell lines produce predominantly desialylated PrP^{C} suggesting that these cells may be resistant to prion infection; an observation that may explain the different sensitivities of neurons to prion infection.

In conclusion we show that sialic acid attached to the GPI affects the properties of PrP^{C}, altering the surrounding cell membrane, trafficking of PrP^{C} and PrP^{C}-induced cell signalling. Critically the presence of desialylated PrP^{C} reduced the activation of $cPLA_2$ and PrP^{Sc} formation in prion-infected neuronal cells. We propose that sialic acid on the GPI anchor attached to PrP^{C} affects the membrane targeting and cell signalling that is conducive to its conversion to PrP^{Sc}. Consequently, therapeutics that block the incorporation of sialic acid into GPI anchors could prove useful in the treatment of prion diseases.

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Conflict of interest - The authors declare that there are no conflicts of interests.

Authors' contributions – CB: conception and design, data collection and analysis, manuscript writing and revision. WN: experimental design, data collection and analysis AW: conception and design and manuscript writing. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. N-linked glycans did not affect the conversion of PrP^C to PrP^{Sc} – (A) Immunoblot showing PrP^C (1) and deglycosylated PrP^C (2) separated by PAGE. (B) The amounts of deglycosylated PrP^C (●) in fractions from C18 columns eluted under a gradient of propanol and water. (C) The amounts of PrP^{Sc} in ScGT1 cells treated daily with concentrations of PrP^C (□) or deglycosylated PrP^C (■) as shown for 7 days. Values are means ± standard deviation (SD) from triplicate experiments performed 4 times, n=12.

Figure 2. Neuraminidase removed the sialic acid from the GPI anchor attached to PrP^C - (A) Cartoon showing the putative structures of the GPI anchor attached to PrP^C and the desialylated PrP^C. Glycan residues shown include mannose (Man), sialic acid (SA), galactose (Gal), inositol (Inos), N-acetyl galactosamine (GalNAc) and glucosamine (GlcN). (B) GPIs isolated from PrP^C (○) and desialylated PrP^C (●) were separated by reverse phase chromatography using a C18 column. GPIs were detected with mAb 5AB3-11 reactive with phosphatidylinositol. (C) HPTLC showing the migration of GPIs derived from PrP^C (1) or desialylated PrP^C (2) separated on silica 60 plates. (D) Dot blots showing the binding of mAb 5AB3-11 (phosphatidylinositol), biotinylated concanavalin A (mannose), biotinylated *S. nigra* lectin (sialic acid) and biotinylated RCA1 (galactose) to GPI anchors isolated from PrP^C or desialylated PrP^C.

Figure 3. Desialylated PrP^C is not converted to PrP^{Sc} – (A) The amounts of PrP^{Sc} in $Prnp^{(0/0)}$ neurons pulsed with 1 ng PrP^{Sc} and incubated daily with control medium, 10 ng of PrP^C or 10 ng desialylated PrP^C for 10 days. Values are means ± SD from triplicate experiments performed 4 times, n=12. (B) Immunoblots showing the amounts of β-actin and PrP^{Sc} in extracts of $Prnp^{(0/0)}$ neurons pulsed with 1 ng PrP^{Sc} and incubated daily with control medium (i), 10 ng of PrP^C (ii) or 10 ng desialylated PrP^C (iii) for 10 days.

Figure 4. Desialylated PrP^C inhibited PrP^{Sc} formation - (A) The amounts of PrP^{Sc} in $Prnp^{(+/+)}$ neurons pulsed with 1 ng PrP^{Sc} and incubated daily with control medium (□), 10 ng of PrP^C or 10 ng desialylated PrP^C (■) for 10 days. Values means ± SD from triplicate experiments performed 4 times, n=12. (B) Immunoblots showing amounts of β-actin and protease-resistant PrP in neurons pulsed with 1 ng PrP^{Sc} and incubated daily with control medium (1), 10 ng of PrP^C (2) or 10 ng desialylated PrP^C (3) for 10 days. (C) The amounts of PrP^{Sc} in $Prnp^{(0/0)}$ neurons pulsed with 1 ng PrP^{Sc} and incubated daily with control medium, 10 ng PrP^C or a combination of 10 ng PrP^C and 10 ng desialylated PrP^C for 10 days. Values are means ± SD from triplicate experiments performed 4 times, n=12. (D) The amounts of PrP^{Sc} in ScN2a cells treated daily with control medium (□) or desialylated PrP^C (■) as shown for 7 days. Values are means ± SD from triplicate experiments performed 4 times, n=12. (E) The amounts of PrP^{Sc} in ScGT1 cells treated daily with control medium (□) or desialylated PrP^C (■) as shown for 7 days. Values are means ± SD from triplicate experiments performed 4 times, n=12.

Figure 5. Sialic acid on the GPI affects the composition of membrane rafts – $Prnp^{(0/0)}$ neurons were pulsed with 25 ng PrP^C or 25 ng desialylated PrP^C and homogenised in ice cold triton X-100. (A) The amounts of PrP^C (□) and desialylated PrP^C (■) in membranes separated on a sucrose density gradient. Values are means ± SD from an experiment run in triplicate. Membrane rafts containing PrP^C were isolated by precipitation with the PrP^C-reactive mAb 4F2. (B) Blots showing the amounts of PrP^C and desialylated PrP^C in immunoprecipitates. (C) The concentrations of cholesterol in precipitates from PrP^C (□) or desialylated PrP^C (■). Values are means ± SD from 4 precipitates, each measured in triplicate, n=12. *=cholesterol significantly higher than in PrP^C precipitates ($P<0.05$). (D) Gangliosides in immunoprecipitates from cells incubated with PrP^C (1) or desialylated PrP^C (2) separated by HPTLC on silica 60 plates. (E) The concentrations of gangliosides in precipitates of PrP^C (□) or desialylated PrP^C (■). Values are means ±SD from 4 precipitates, each measured in triplicate, n=12. *=gangliosides significantly higher than in PrP^C precipitates ($P<0.05$).

Figure 6. Sialic acid on the GPI anchor affects the stability of PrP^C – (A) The amounts of PrP^C in rafts (DRMs) from $Prnp^{(0/0)}$ neurons pre-treated with squalestatin as shown and pulsed with 10 ng PrP^C (□) or desialylated PrP^C (■) for 2 hours. Values are means ± SD from triplicate experiments performed 4 times, n=12. *=significantly less PrP^C in rafts. (B) There was a significant correlation between concentrations of cholesterol and concentrations of PrP^C (○), but not desialylated PrP^C (●), in rafts derived from $Prnp^{(0/0)}$ neurons pre-treated with squalestatin and pulsed with 10 ng PrP^C/desialylated PrP^C for 2 hours, Pearson's coefficient=0.41, P<0.01. (C) The amounts of PrP^C in $Prnp^{(0/0)}$ neurons pulsed with 10 ng of PrP^C (□) or desialylated PrP^C (■) for 2 hours and incubated with control medium or PI-PLC for 1 hour. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=significantly less desialylated PrP^C after digestion with PI-PLC. (D) The amounts of PrP^C remaining $Prnp^{(0/0)}$ neurons pulsed with 10 ng PrP^C (□) or desialylated PrP^C (■) measured at time points thereafter. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=significantly more desialylated PrP^C remaining in neurons. (E) The amounts of PrP^C in $Prnp^{(0/0)}$ neurons incubated for 2 hours with 10 ng PrP^C (□) or 10 ng desialylated PrP^C (■) and treated with control medium or 5 μM glimepiride for 1 hour. Values are means ± SD from triplicate experiments performed 3 times, n=9. *=significantly less PrP^C in neurons after treatment with glimepiride.

Figure 7. Desialylated PrP^C reduces activation of $cPLA_2$ by cross-linkage of PrP^C- (A) The amounts of activated $cPLA_2$ in $Prnp^{(0/0)}$ neurons pre-treated with PrP^C (□) or desialylated PrP^C (■) as shown and incubated with mAb 4F2. Values are means ± SD from triplicate experiments performed 3 times, n=9. (B) The amounts of activated $cPLA_2$ in $Prnp^{(+/+)}$ neurons pre-treated with desialylated PrP^C as shown and incubated with mAb 4F2. Values are means ± SD from triplicate experiments performed 3 times, n=9.

Figure 8. Desialylated PrP^C reduced the targeting of $cPLA_2$ to rafts - (A) The amounts of $cPLA_2$ in membrane extracts separated on a sucrose density gradient from ScGT1 cells treated with control medium (□) or 25 ng desialylated PrP^C (■). Values are means ± SD from an experiment run in triplicate. (B) Immunoblots showing amounts of PrP^C and $cPLA_2$ precipitated by mAb 4F2 (reactive with PrP^C) from wildtype $Prnp^{(+/+)}$ neurons treated for 3 hours with control medium or 25 ng desialylated PrP^C.

Figure 9. PrP^C with sialic acid may alter the underlying cell membrane – A schematic showing the proposed membranes surrounding PrP^C (A) and desialylated PrP^C (B) that include cholesterol, saturated phospholipids, unsaturated phospholipids and gangliosides. (C) Proposed interactions between PrP^C and PrP^{Sc} and its effects on the surrounding membrane including the incorporation of $cPLA_2$ into membranes. (D) Proposed interactions between desialylated PrP^C and PrP^{Sc} and its effects on the surrounding membrane including gangliosides.

Tables

Treatment	Activated $cPLA_2$ (units/10^6 cells)	
	ScN2a cells	**ScGT1 cells**
None	1.0 ± 0.14	1.0 ± 0.11
desialylated PrP^C	0.48 ± 0.12	0.6 ± 0.18
PLAP	3.87 ± 0.58	2.42 ± 0.28
desialylated PrP^C + PLAP	3.52 ± 0.49	2.22 ± 0.29

Table 1. Sialic acid in the GPI anchor affected prion-mediated activation of $cPLA_2$ - The amounts of activated $cPLA_2$ in cell extracts from ScN2a or ScGT1 cells pre-treated with control medium or with 25 ng desialylated PrP^C and then incubated with 1 μM PLAP for 1 hour. Values are means ± SD from triplicate experiments performed 5 times, n=15. The amount of activated $cPLA_2$ in untreated cells was standardised as 1 for each cell line.

Figure 1.

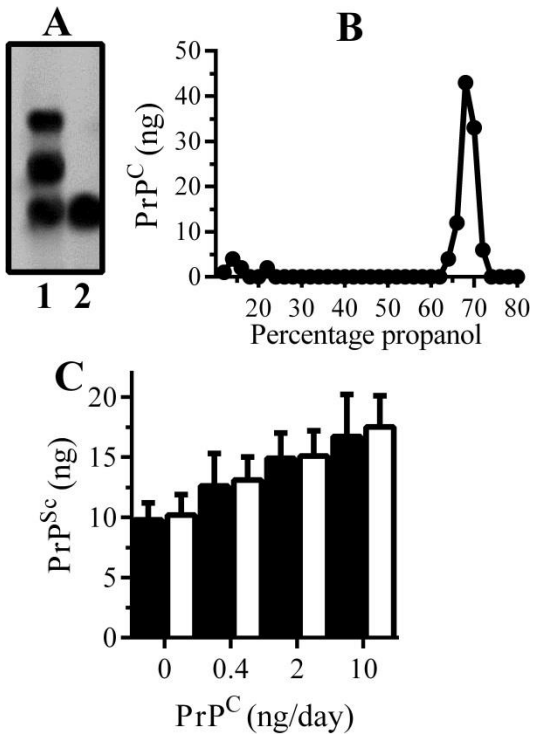

Figure 2.

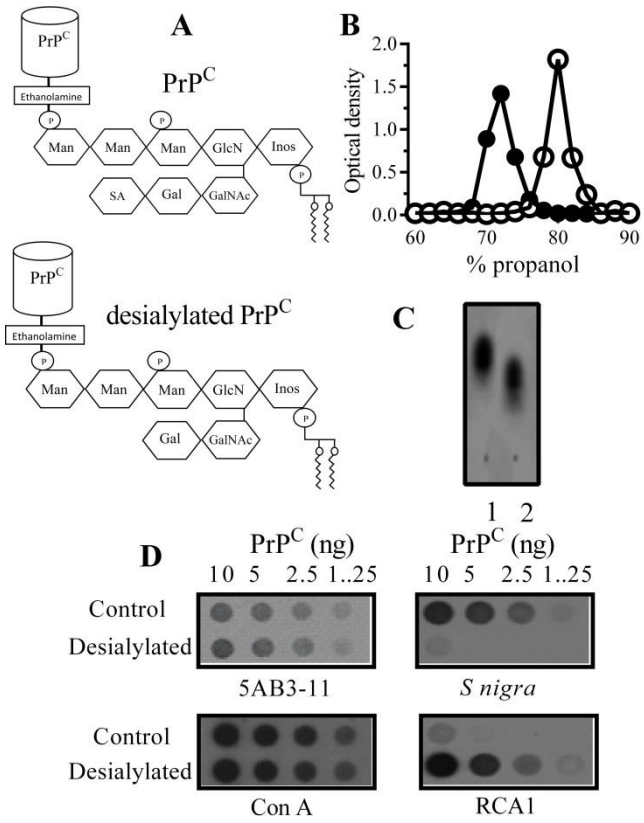

Figure 3.

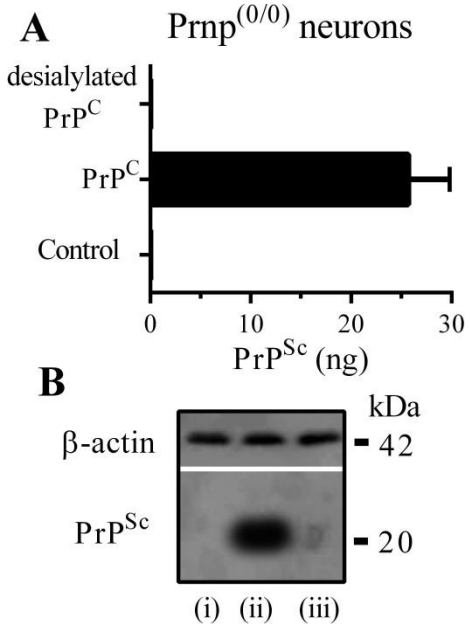

Figure 4.

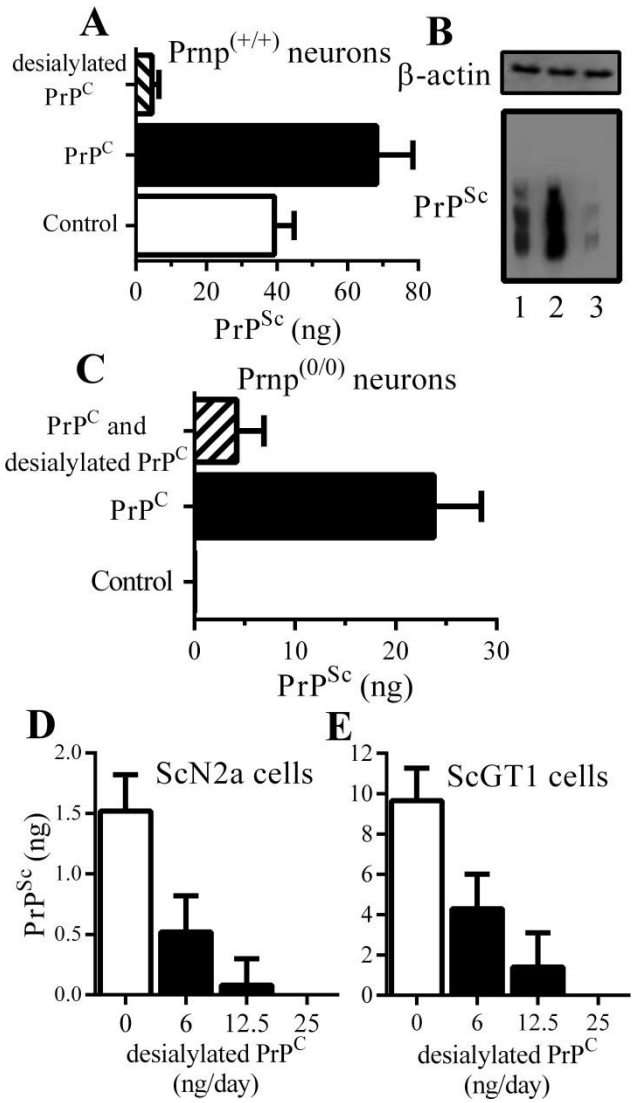

Figure 5.

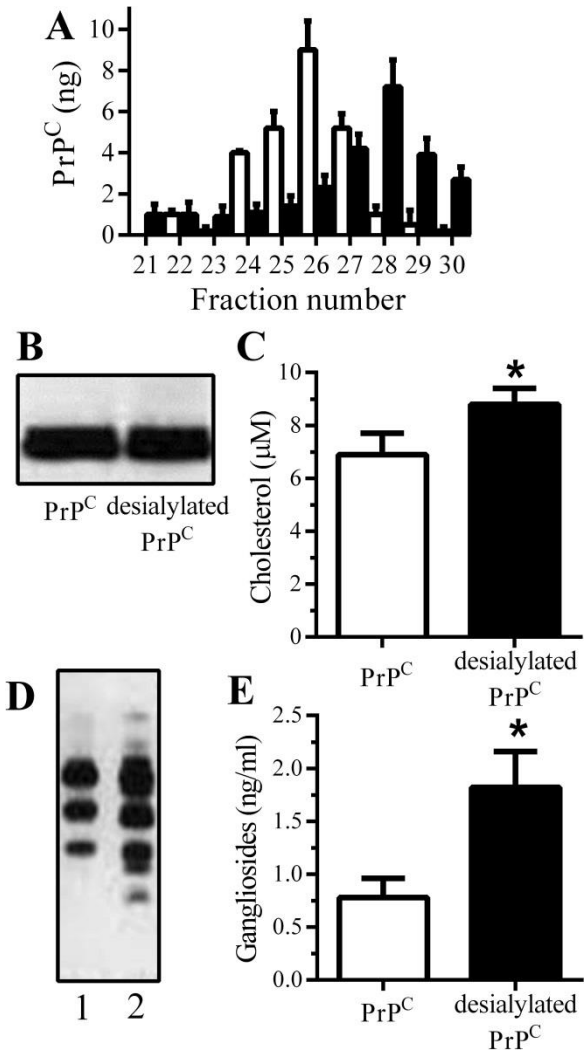
A
PrP^C (ng)
0
2
4
6
8
10
21 22 23 24 25 26 27 28 29 30
Fraction number
B
PrP^C
desialylated PrP^C
C
Cholesterol (μM)
0
2
4
6
8
10
*
PrP^C
desialylated PrP^C
D
1 2
E
Gangliosides (ng/ml)
0.0
0.5
1.0
1.5
2.0
2.5
*
PrP^C
desialylated PrP^C

Figure 6.

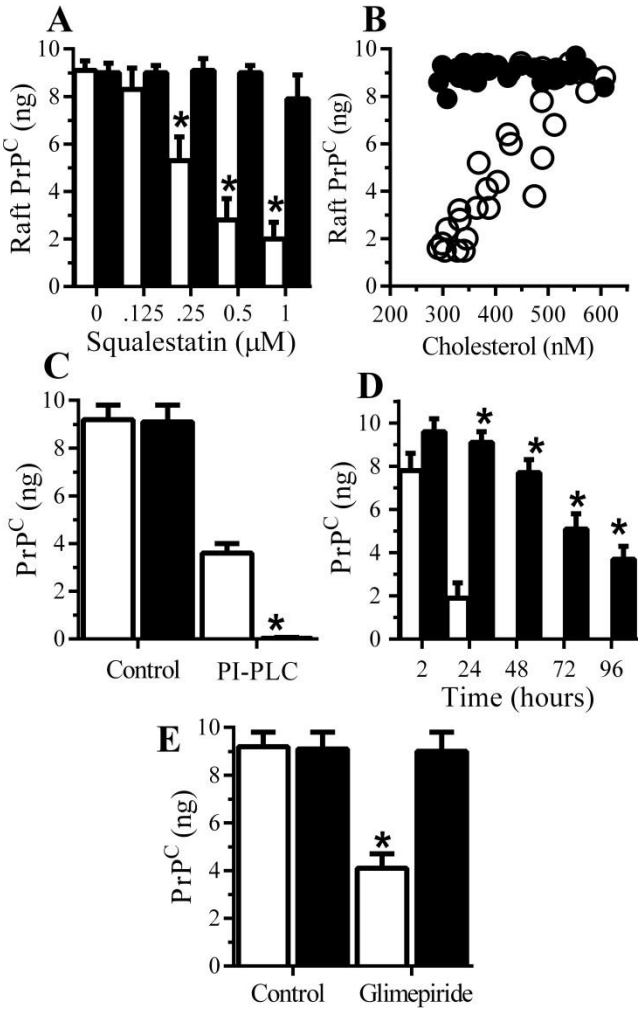

Figure 7.

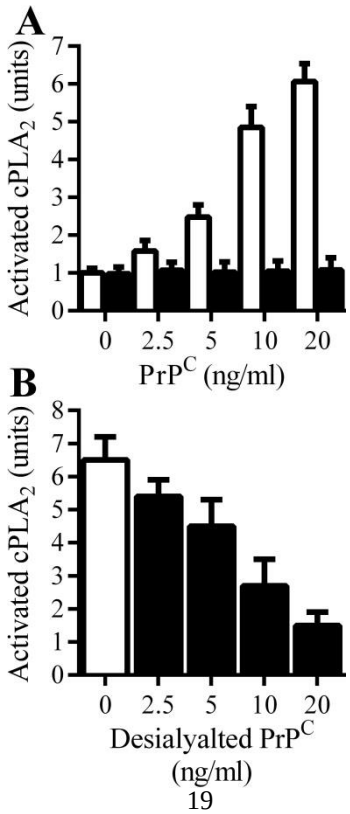

Figure 8.

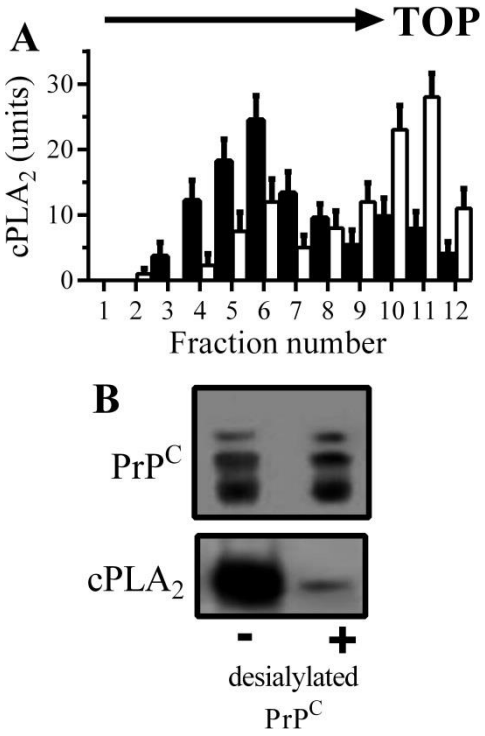

Figure 9.