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Role of membrane GM1 on early neuronal membrane actions of Aβ during onset of Alzheimer's disease

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Abstract

The ability of beta-amyloid peptide (Aβ) to disrupt the plasma membrane through formation of pores and membrane breakage has been previously described. However, the molecular determinants for these effects are largely unknown. In this study, we examined if the association and subsequent membrane perforation induced by Aβ was dependent on GM1 levels. Pretreatment of hippocampal neurons with D-PDMP decreased GM1 and Aβ clustering at the membrane (Aβ fluorescent-punctas/20 μm, control = 16.2 ± 1.1 vs. D-PDMP = 6.4 ± 0.4, $p < 0.001$). Interestingly, membrane perforation

with Aβ occurred with a slower time course when the GM1 content was diminished (time to establish perforated configuration (TEPC) (min): control = 7.8 ± 2 vs. low GM1 = 12.1 ± 0.5, $p < 0.01$), suggesting that the presence of GM1 in the membrane can modulate the distribution and the membrane perforation by Aβ. On the other hand, increasing GM1 facilitated the membrane perforation (TEPC: control = 7.8 ± 2 vs. GM1 = 6.2 ± 1 min, $p < 0.05$). Additionally, using Cholera Toxin Subunit-B (CTB) to block the interaction of Aβ with GM1 attenuated membrane perforation significantly. Furthermore, pretreatment with CTB decreased the membrane association of Aβ (fluorescent-punctas/20 μm, Aβ: control = 14.8 ± 2.5 vs. CTB = 8 ± 1.4, $p < 0.05$), suggesting that GM1 also plays a role in both association of Aβ with the membrane and in perforation. In addition, blockade of the Aβ association with CTB inhibited synaptotoxicity. Taken together, our results strongly suggest that membrane lipid composition can affect the ability of Aβ to associate and subsequently perforate the plasma membrane thereby modulating its neurotoxicity in hippocampal neurons.

Keywords

Alzheimer's disease, Amyloid beta, Membrane perforation, Amyloid pore, Membrane lipids, Ganglioside GM1

1. Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disease that leads to debilitating cognitive deficits. AD is characterized histopathologically by the presence of senile plaques composed of amyloid-β peptide (Aβ), believed to be the major pathological agent of the disease.[1] Despite that molecular and cellular mechanisms are not completely understood, one of the key events leading to the synaptic failure seen in Alzheimer's disease (AD) is the disruption of the plasma membrane due to the formation of a pore-like structure permeable to cations, anions and other small molecules.[2] For example, it was described that the synaptic failure induced by Aβ, characterized by an acute and fast release of neurotransmitters and a delayed decrease in pre and postsynaptic proteins, is due to this membrane disruption.[3,4] The hypothesis of an amyloid pore is supported by a large amount of evidence.[3,5,6,7,8] However, it is still unclear what cellular components are involved in these synaptotoxic events and if membrane components can significantly affect the formation of this pore.

Presently, there is evidence suggesting that lipid rafts might be important in Aβ aggregation and toxicity.[9] For example, it is known that the production of Aβ can occur in these membrane structures[10] and a large level of peptide has been detected in areas of the membrane resistant to detergents (DRMs) and rich in proteins, cholesterol and sphingolipids.[11,12]

Gangliosides are a type of sphingolipid found in all vertebrate animals and have been widely described in the central nervous system where they are expressed in the external face of the cell membrane. They are predominantly located in lipid raft regions probably because their highly packed acyl chains are more stable in the liquid order phase of the membrane.[13] They appear to be important for regulating synaptic transmission,[14] especially in the hippocampus,[15,16] making them an important target in the study of AD. In 1995, Yanagisawa et al. showed that Aβ was strongly associated to ganglioside GM1 in AD patients. Also, Aβ can bind to ganglioside GM1 and this interaction acts like a seed accelerating the process of oligomerization.[9,17] Therefore, the interaction between GM1 and Aβ in the cell membrane could be important in the regulation of aggregation and binding of Aβ to the membrane, as well as in the neurotoxic consequences derived from membrane disruption. In this regard, there are some unresolved questions that need to be answered in order to better understand how Aβ causes its toxic effects: What is the role of GM1 in the membrane with regard to Aβ toxicity? Does GM1 play a role in the phenomenon of Aβ association and insertion into the membrane? Do augmented GM1 levels predispose the cell to membrane disruption and synaptotoxicity? In this regard, it is essential that the peptide be capable of interacting with the cell membrane and herein rests the importance of studying certain molecular elements present in lipid membranes, like GM1, that could

be modulating Aβ actions on plasma membranes and triggering the subsequent neurotoxic actions in hippocampal neurons.

On the basis of the available evidence, we hypothesize that the effects of Aβ, such as association and subsequent membrane disruption that are important for its synaptotoxicity, are somewhat dependent on the GM1 content in the cell membrane. Therefore, the aim of this study is to characterize the role of GM1 on the capacity of Aβ to bind and perforate the plasma membrane producing synaptotoxicity.

2. Material and methods

2.1. Primary cultures of rat hippocampal neurons

Hippocampal neurons were obtained from 18-day embryos from pregnant Sprague-Dawley rats and cultured for 10–14 days in vitro (DIV) as previously described.[18]

2.2. HEK-293 cells culture

HEK cells were cultivated in Dulbecco's modified Eagle's medium (D-MEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Life Technologies) and streptomycin/penicillin (200 units each, Life Technologies). Cells were maintained with 5% CO_2 at 37 °C.

2.3. Preparation of beta amyloid peptide

Human Aβ42 fluorescently labeled with FAM at the N-terminal or without fluorescence were bought from Biomatic (USA) and GenicBio (China), respectively. Aβ was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (10 mg/mL) (Merck Millipore, USA) and incubated in a parafilm sealed tube at 37 °C for 2 h. Then, the solution was incubated at 4 °C for 20 min and aliquots of 5 μL were placed in 1.5 mL Eppendorf tubes. The tubes were left with the lids open inside the chamber to evaporate the solvent (about 20 min) and until the appearance of a thin clear film in the bottom of the tube. After evaporation was complete, aliquots were stored at − 20 °C. In order to dissolve the films to form oligomer-rich solution to be used in the experiments, Nanopure water was added to obtain a final concentration of 80 μM and the tubes were incubated at room temperature for 20 min. Subsequently, a Teflon-coated magnetic stir bar was added to the solution (size: 2 × 5 mm), stirring at room temperature at 500 rpm for 24–48 h. This solution was used to perform the experiments.

2.4. Changes in GM1 levels in the cell membrane

To increase the levels of GM1 in the plasma membrane, cells were incubated with media containing liposomes composed of ganglioside GM1 and phosphatidylcholine. To form the liposomes, stock solutions of l-α-phosphatidylcholine (PC; 20 mg/mL) (Sigma, USA) and GM1 (1 mM) (Sigma, USA) (both in chloroform) at a 9:1 ratio were used. The chloroform was evaporated under a gentle flow of nitrogen in the hood, and then the film was resuspended in 500 μL of PBS and stirred vigorously using a vortex for 3–5 min. The cells were immediately incubated in this solution for 3–4 h at 37 °C in culture medium and washed with PBS before adding Aβ. To decrease the content of GM1 in the membrane, cells were incubated with 10 mU Vibrio cholera neuraminidase (Sigma, USA)[19] for 1 h at 37 °C in culture medium. Alternatively, we used d-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) (10 and 20 μM) for 72 h, which is a glucosyl ceramide synthase inhibitor effective at diminishing GM1 levels.[20,21] Cells were washed with PBS and then treatments were performed with Aβ.

2.5. Quantification and distribution of GM1

Cholera toxin subunit B (CTB) conjugated to Alexa Fluor 555 (Invitrogen, USA), which binds with high affinity to GM1, was used for fluorescent quantification.[22,23] 100 µg of CTB-Alexa Fluor 555 was dissolved in 100 µL PBS giving a stock solution of 1 mg/mL which was stored at − 20 °C. Cells were incubated for 15–30 min with Alexa Fluor 555-CTB (0.5 µg/mL) at 37 °C in culture medium and cells were washed with PBS. The fluorescence of each well, indicative of the level of GM1 on the cell surface, was read on a plate reader NovoStar (BMG Labtech, Germany) with a filter Ex = 544 nm/Em = 590 nm.

2.6. Electrophysiology

Electrophysiological recordings were carried out using the patch clamp technique as previously described.[2] Briefly, Aβ aggregates were used at 0.5–1 µM/L. Perforated recordings were obtained as follows: the perforating agent was added into the pipette solution and a 5 mV pulse was used to monitor the formation of the perforation at a holding potential of − 60 mV using an Axopatch 200B amplifier (Molecular Devices, USA). Data were displayed and stored using a 1322A Digidata acquisition board and analyzed with electrophysiological software pClamp 10.1 (Molecular Devices, USA). The external solution contained the following (in mmol/L): 150 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4, 330 mOsm). The standard internal solution in the patch pipette contained the following (in mmol/L): 120 KCl, 4.0 $MgCl_2$, 10 BAPTA, and 2.0 Na_2-ATP (pH 7.4, 310 mOsmol). Some experiments involved an internal solution containing NA7 peptide (20 µmol/L) (Peptide 2.0, USA) and CTB-Alexa Fluor 555 (0.5 µg/mL).

2.7. Immunocytochemistry

Hippocampal neurons were fixed for 15 min with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Monoclonal anti-SV2 (1:200; Developmental Studies Hybridoma Bank, Iowa City, IA) and rabbit anti-MAP2 (1:300; Santa Cruz Biotechnology, Dallas, TX CA) were incubated overnight, followed by incubation with a secondary anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated with Cy3 and anti-mouse IgG conjugated with FITC (1:500; Jackson ImmunoResearch Laboratories) for 2 h. All antibodies were diluted with horse serum (10%) in PBS. Samples were mounted in DAKO mounting medium (Dakocytomation, USA) and observed under a spectral confocal laser scanning microscope (LSM780, Zeiss, Germany).

2.8. Localization and quantification of Aβ fluorescent aggregates

Aβ42 labeled with FAM (Exmax/Emmax = 494/521 nm, Biomatic, USA) at the N-terminal was prepared as the unlabeled peptide. To determine the cellular localization of fluorescent Aβ, anti-MAP2 mouse monoclonal antibody (1:300, Santa Cruz Biotechnology, Dallas, TX CA) was used to identify the neurons. Cells were treated with different concentrations of fluorescent aggregates (0.5–1 µM) for up to 1 h at 37 °C in culture medium and then cells were washed with PBS. The fluorescence of each well, indicative of the Aβ-fluorescence level at the cell surface, was read on a NovoStar plate reader (BMG Labtech, Germany) with a filter = 485 nm Ex/Em = 520 nm for FAM.

2.9. Data analysis

All data obtained from the measurements of capacitive current, fluorescence and all other parameters were analyzed and plotted using OriginPro 8.0 (Microcal, Origin Lab, Northhampton, MA). Experiments with PDMP, GM1 and CTB showed that they did not have any effect on their own, so unless otherwise

indicated, the control condition reported in the study was considered in the presence of the modulator. Membrane charge was calculated by integrating the transient capacitive current after subtracting the pipette capacitance; under this condition, the area under the capacitive current represented the membrane charge transferred (fC). Moreover, we fitted the current with a standard exponential function using Chebyshev algorithm to calculate the tau for the decay of the current. Using this strategy, we calculated the total charge transferred to the membrane under the patch pipette. The curves of charge transferred were fitted using a sigmoidal function algorithm. Unless otherwise indicated, the results, including image analysis, are presented as mean ± standard error of the mean (SEM) from at least 5 to 8 neurons or cells. Statistical differences were determined using 1-way analysis of variance (ANOVA) or paired Student's *t*-tests, followed by the Bonferroni post hoc test in some cases. A probability level ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Increased association of Aβ correlated with increased GM1 membrane content

GM1 is a lipid that plays an important role in the interaction of Aβ with the cell membrane. Also, the interaction of Aβ with GM1 is a process that involves negatively charged sialic acid residues present in the lipid bilayer that interact electrostatically with N-terminal positively charged basic amino acids of Aβ.[24] Therefore, we examined if increasing GM1 also increased association of Aβ to the membrane. To achieve this, we supplemented the cell membrane with phosphatidylcholine liposomes containing GM1 and then incubated the cells with fluorescent Aβ and quantified the changes in the association pattern. Under this experimental condition, we observed more Aβ-FAM associated to the membrane at the soma and neurites (Fig. 1A,B). The latter result is better revealed in the ROIs that correspond to the first 20 µm of the primary process (Fig. 1C,D). The quantification of the Aβ aggregates in these ROIs showed a notable increase in the size of the amyloid clusters of > 100% compared to control aggregates (Fig. 1E). Regarding the number of Aβ clusters, the change was also significant, almost three times the number of clusters reflecting a marked increase in the association of Aβ to the neurons (Fig. 1F**)**. These results correlate well with data obtained from fluorescence measurements in culture plates in which the level of GM1 was quantified by the fluorescently labeled CTB.[25] The data show that the total level of fluorescence associated to Aβ augmented in parallel with the number of clusters in the membrane (Fig. 1G). This data strongly suggests that increasing membrane GM1 content favors Aβ

association and affects the way the clusters form in the membrane by augmenting their size and numbers.

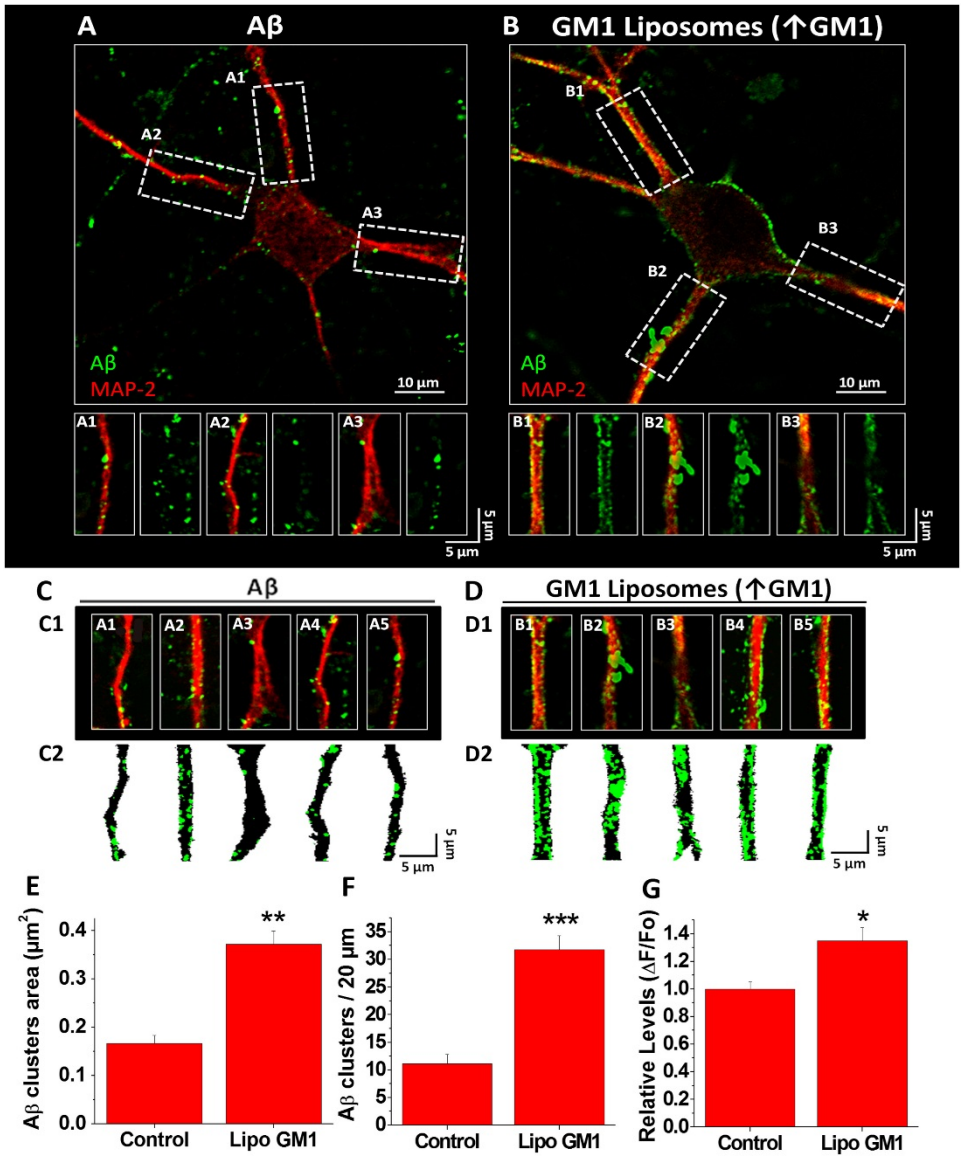

Fig. 1. Effects of increasing GM1 levels on Aβ association to neuronal membranes. **A** and **B**, Confocal microscopy images of hippocampal neurons exhibiting fluorescence for MAP-2 and the distribution of $A\beta_{42}$-FAM aggregates on the cell membrane after treatment with GM1 and phosphatidylcholine liposomes (1:9) for 3–4 h. **A1**–**A3** and **B1**–**B3,** Magnification of representative neurites showing the association of Aβ to neuronal processes in more detail. **C** and **D**, Five representative traces of the experiment: 3 primary processes shown in **A1**–**A3** and **B1**–**B3** plus 2 other primary processes of different micrographs (**C1** and **D1**) Underneath are the segmented fluorescent signal of Aβ for each of these processes (**C2** and **D2**). **E** and **F**, Quantification of Aβ clusters (area and number) in the first 20 µm of hippocampal neuron primary processes showing that both parameters were increased after treatment with GM1-containing liposomes. **G**, Quantification of the total fluorescence of **CTB-Alexa Fluor 555** showing the relative levels of GM1 for both conditions. The bars represent the mean ± SEM. * denotes $p < 0.05$, ** $p < 0.005$ and *** $p < 0.001$.

3.2. Decreasing GM1 reduced the association of Aβ in hippocampal neurons

After observing that increased GM1 levels in the membrane resulted in more Aβ association, we wanted to evaluate the association of Aβ to the neuronal membrane under conditions where the synthesis of GM1 was reduced using D-PDMP (Fig. 2). Under control conditions, Aβ associated to the soma and neuronal processes (Fig. 2A, green signal), while neurons pretreated with D-PDMP and subsequent incubation with Aβ exhibited a reduced association of the peptide (Fig. 2B). A more detailed representation for the association of Aβ to the neurites was evident in close-up images of hippocampal neuronal processes **(**Fig. 2C,D**)**. Quantification of the area and number of Aβ clusters in the first 20 µm of primary processes revealed a reduction in these two parameters when GM1 levels were decreased in the neuronal membrane. The number of Aβ clusters in control cells was reduced in treated cells, and their size was also decreased by approximately 50%, from 0.8 ± 0.05 μm^2 in control cells to 0.4 ± 0.03 μm^2 in treated cells **(**Fig. 2E,F**)** indicating a decline in clusters at the membrane. Quantification of the total fluorescence of Aβ in the culture wells containing the neurons correlated with the immunofluorescence data (Fig. 2G). This data showed that after D-PDMP treatment there was a significant decrease in the association of Aβ to the neuron in parallel to the reduction in the relative levels of GM1 in the membrane **(**Fig. 2**)**. These data suggest that the levels of GM1 in the membrane are important for the association and cluster formation of Aβ on the surface of the neuronal membrane.

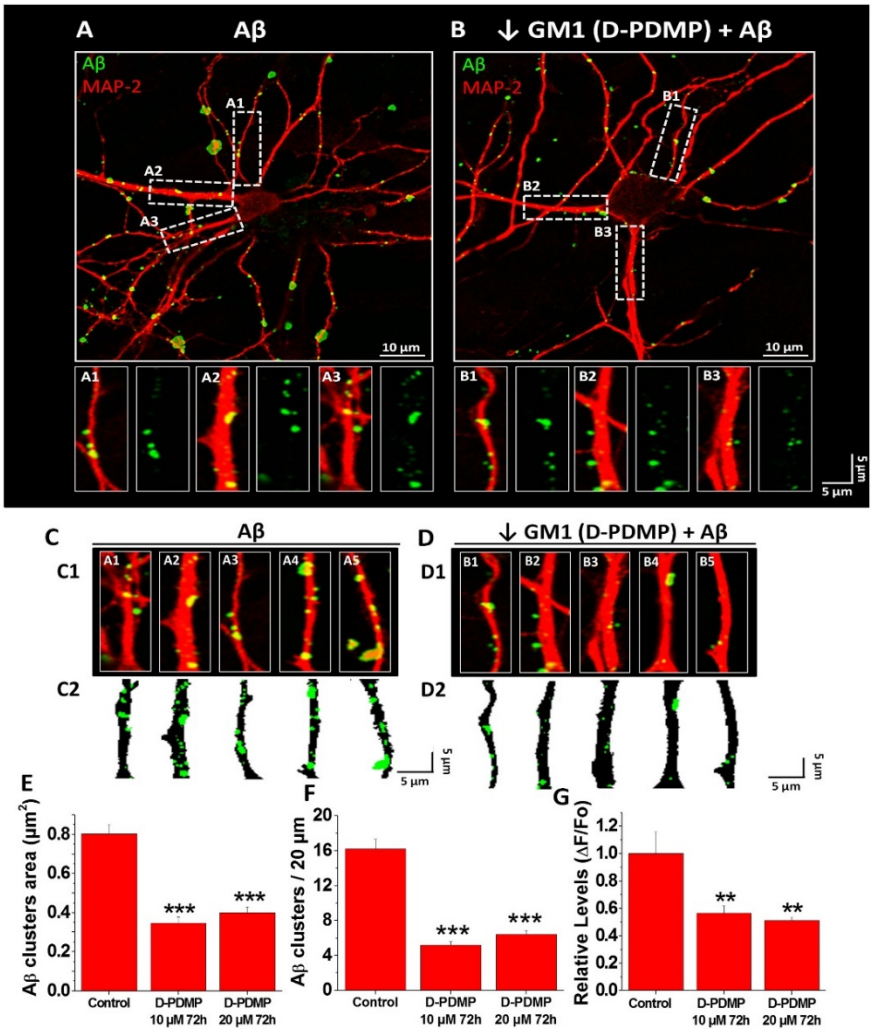

Fig. 2. Decrease in the association of $A\beta_{42}$ following the reduction of GM1. **A** and **B,** Confocal microscopy images of hippocampal neurons exhibiting fluorescence for MAP-2 and demonstrating the distribution of $A\beta_{42}$-FAM aggregates on the

cell membrane after treatment with D-PDMP (20 μM) for 72 h. **A1**–**A3** and **B1**–**B3**, Magnification of typical neurites showing the association of Aβ to neuronal processes in more detail. **C** and **D**, Five representative traces of the experiment: 3 primary processes shown in **A1**–**A3** and **B1**–**B3** plus 2 other primary processes of different micrographs (**C1** and **D1**) Underneath are the segmented fluorescent signal of Aβ for each of these processes (**C2** and **D2**). **E** and **F**, Quantification of Aβ clusters (area and number) in the first 20 μm of hippocampal neuron primary neuronal processes in control and after treatment with D-PDMP (10 and 20 μM) for 72 h. **G**, Quantification of the total fluorescence of CTB-Alexa Fluor 555 showing the relative levels of GM1 for each of the conditions tested. The bars represent the mean ± SEM. ** denotes $p < 0.005$, ***$p < 0.001$.

3.3. Altering levels of GM1 affects the membrane perforation induced by Aβ

Next, and under the same experimental conditions described before in Fig. 1, Fig. 2, we performed perforated patch clamp with Aβ in order to study the perforation of the neuronal membrane, as previously described by our laboratory.[2] Fig. 3 shows representative recordings of capacitive currents when the levels of GM1 were increased or decreased in the plasma membrane, and the time course for the perforation induced by Aβ (Fig. 3A,B**)**. The data demonstrate that increasing the levels of GM1 in the membrane produced a small acceleration in the perforation (Fig. 3B**,** white squares) compared to Aβ condition (Fig. 3B**,** light gray squares). For example, using sister cell cultures, 50% of the maximal effect (fC_{MAX}) of Aβ occurred at 7.8 min ($t_{A\beta}$), with 69 fC of charge transferred in this experimental condition. In the presence of GM1, the same amount of charge transferred was reached at 6.2 min ($t_{\uparrow GM1}$), indicating that the cells were undergoing a somewhat faster perforation. When cells were treated to diminish GM1 content (Fig. 3B, black squares) we found that this time was increased from 7.8 min ($t_{A\beta}$) to 12.1 min ($t_{\downarrow GM1}$), meaning that it took 1.55 times longer for Aβ to perforate the cell membrane when the cells had less GM1 in the membrane. The increase in GM1 produced the same effect on the charge transferred at the end of the experiment (14 min) as in control conditions, demonstrating that the Aβ effect could not be further potentiated by GM1 (Fig. 3C). On the other hand, the decrease in GM1 significantly reduced the effect of Aβ at the cell membrane, decreasing the extent of perforation by 40% compared to control conditions (fC_{MAX} (fC) = 141 ± 17.6 vs. ↓ GM1 + Aβ = 85.9 ± 13.5) (Fig. 3C). To demonstrate that the effect of Aβ was actually related to the formation of pores in the membrane, we performed experiments in the presence of NA7 a mini-peptide that has been widely studied by our and other laboratories and is capable of interfering with the formation of amyloid pores in cell membranes.[2,5,26] Co-applying Aβ with the mini-peptide NA7 in the same experimental condition when we decreased GM1 prevented membrane perforation as seen by the significant difference in the membrane charge transferred between the two conditions **(**Fig. 3C**)**, suggesting that the higher membrane charge seen in the presence of Aβ when GM1 membrane levels are low was produced by an increase in membrane permeability caused by Aβ channels.

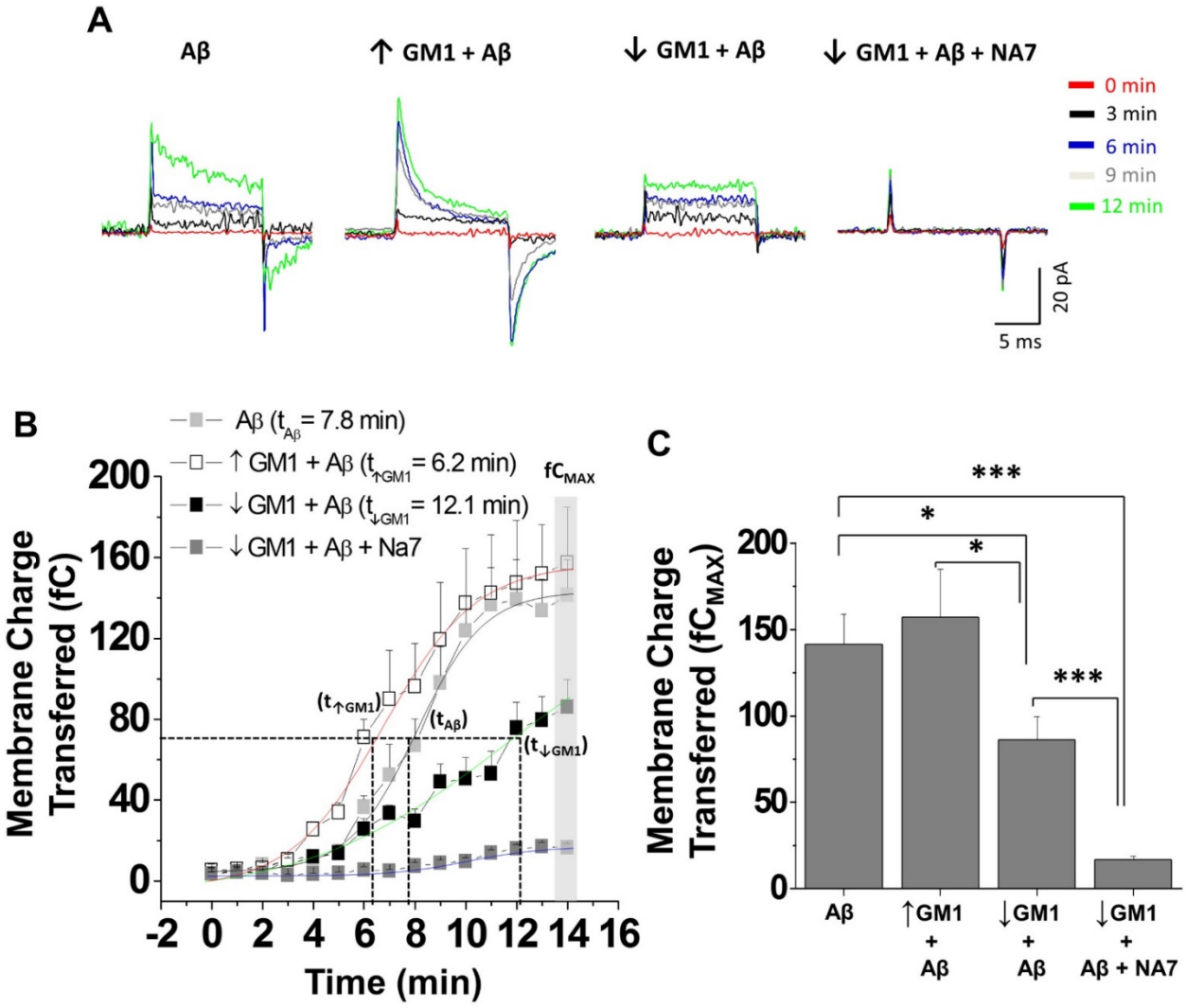

Fig. 3. Membrane levels of GM1 affect the membrane perforation induced by Aβ. **A,** Representative traces of capacitive currents after increasing or decreasing the levels of GM1 in HEK-293 cells. **B,** Time course of Aβ-induced perforation for each of the conditions described in **A** showing the time at which 50% of the maximal effect of Aβ (68.7 fC of charge transferred) was reached ($t_{A\beta}$), and the time at which decreasing or increasing GM1 levels reached the same amount of charge transferred ($t_{\uparrow GM1}$ and $t_{\downarrow GM1}$). **C,** Quantification of membrane charge transferred at the end of the experiment. The effects of Aβ were blocked by the NA7 peptide. Bars represent the mean ± SEM. * denotes $p < 0.05$, ***$p < 0.001$.

These data show that altering the levels of GM1 affects the rate of perforation, suggesting a key role for GM1 in this process. Therefore, less GM1 in the membrane correlates with less association and formation of amyloid pores in the cell membrane, while increased levels of GM1 favors the association of Aβ and facilitates cell membrane perforation.

3.4. Cholera Toxin (CTB) pre-treatment reduced the association of Aβ

Our previous data showed that treating the cells with D-PDMP (10 and 20 µM for 72 h) reduced the levels of GM1 and the association of Aβ to the neuronal membrane, suggesting that GM1 could be an important determinant to mediate the association of Aβ to the neuron. It is well recognized that CTB is a large protein that presents high affinity and selectivity for GM1 gangliosides in the cell surface.[22,27,28] In addition, its large molecular weight should provide a steric hindrance at GM1 sites. Thus, these characteristics should offer an advantage over other mechanisms to reduce the availability of GM1 in the membrane. Therefore, we examined the effect of CTB on Aβ association and perforation of the

cells. The data show that in control conditions there was a high amount of Aβ association to the neuron, especially to the dendrites, with a punctate distribution of the peptide and this was decreased when the cells were pre-incubated with CTB **(**Fig. 4A,B**)**. Higher resolution images show a marked reduction in Aβ association in the neuronal processes (Fig. 4C,D) and quantification of the data demonstrated an almost 40% decrease in the size and 45% decrease in the number of Aβ clusters with respect to control neurons (Fig. 4E,F). This reduction was also reflected in the quantification of the Aβ-FAM fluorescence in the wells containing control and pre-treated cells which decreased ~ 30% **(**Fig. 4G**)**. In conclusion, pre-treatment with CTB affected the association of Aβ indicating that GM1 plays a role in Aβ binding.

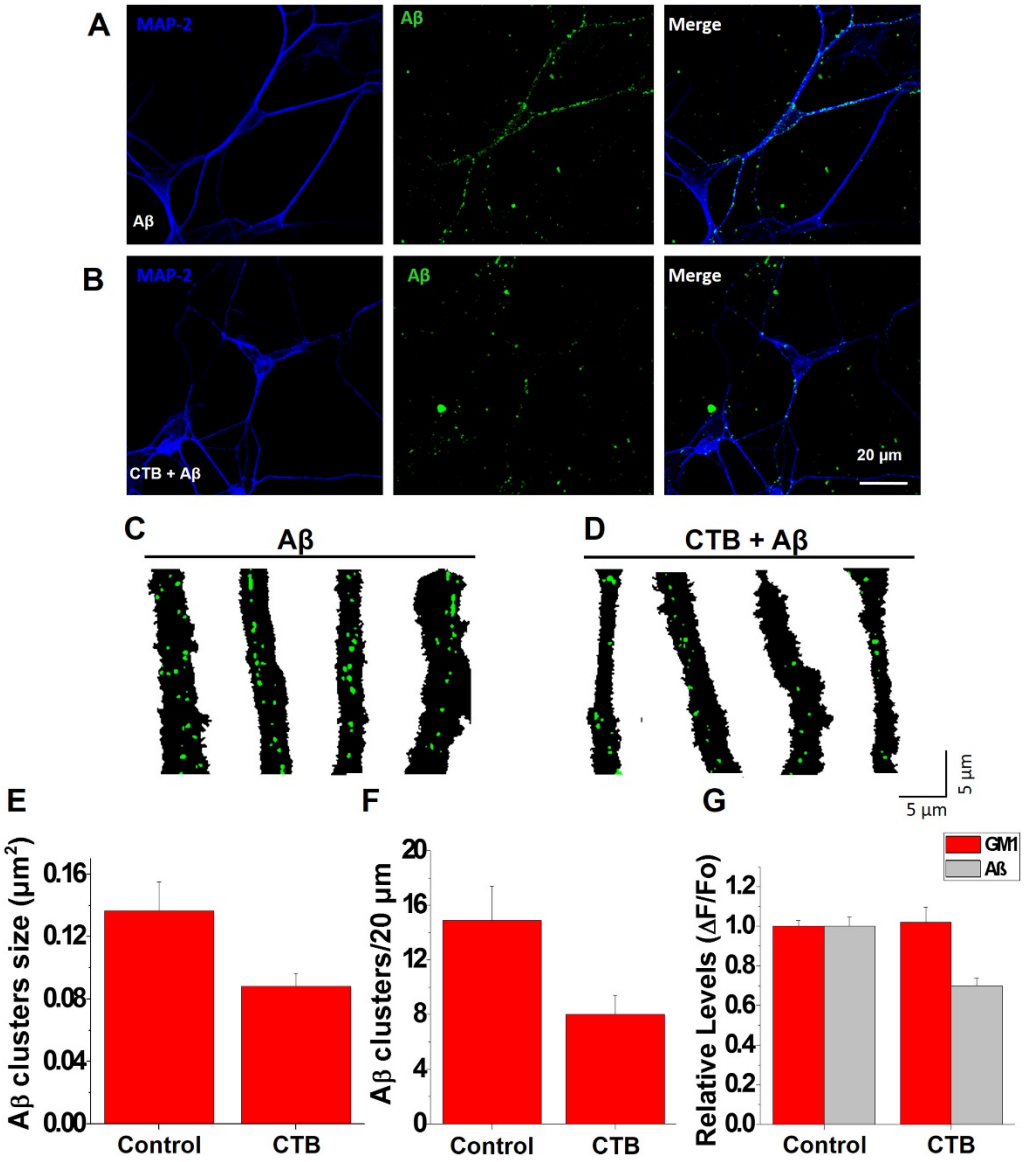

Fig. 4. Reduction in $A\beta_{42}$ association after blocking GM1 with CTB. **A** and **B,** Confocal micrographs of hippocampal neurons showing the distribution of Aβ42-FAM aggregates on the cell surface and MAP-2 immunoreactivity after pretreatment with CTB (0.5 µg/ml). **C** and **D**, Segmented fluorescent signal of Aβ showing a more detailed representation for the association and distribution of Aβ clusters to primary processes of different micrographs. **E** and **F**, Quantification of the size and number of Aβ-FAM clusters on the neuronal membrane in the primary process. **G**, Quantification of Aβ-FAM fluorescence and CTB-Alexa Fluor 555 in the wells containing the culture of hippocampal cells showing that after pre-incubation with CTB the relative levels of Aβ are decreased, while the relative levels of GM1 remains unchanged. Bars represent the mean ± SEM. * denotes $p < 0.05$, ***$p < 0.001$.

3.5. CTB inhibited the membrane perforation induced by Aβ in hippocampal neurons

The previous results showed that decreasing GM1 in the cell membrane reduced the capacity of Aβ to interact with the cell and generate membrane pores. Therefore, blockade of the GM1 site with the beta subunit of Cholera toxin (CTB) should attenuate the Aβ effect. Indeed, the data in Fig. 5 show that Aβ was able to perforate the neurons in control condition as seen in the representative traces (Fig. 5A, center) showing an increase in capacitive currents and charge transferred at 30 min. However, pre-incubation with CTB (0.5 μg/ml) for 15–30 min fully prevented the formation of pores as demonstrated by the much reduced effect of Aβ on membrane resistance and charge transferred **(**Fig. 5B, C**)**, thus confirming that the presence of GM1 in the membrane is fundamental to induce membrane perforation.

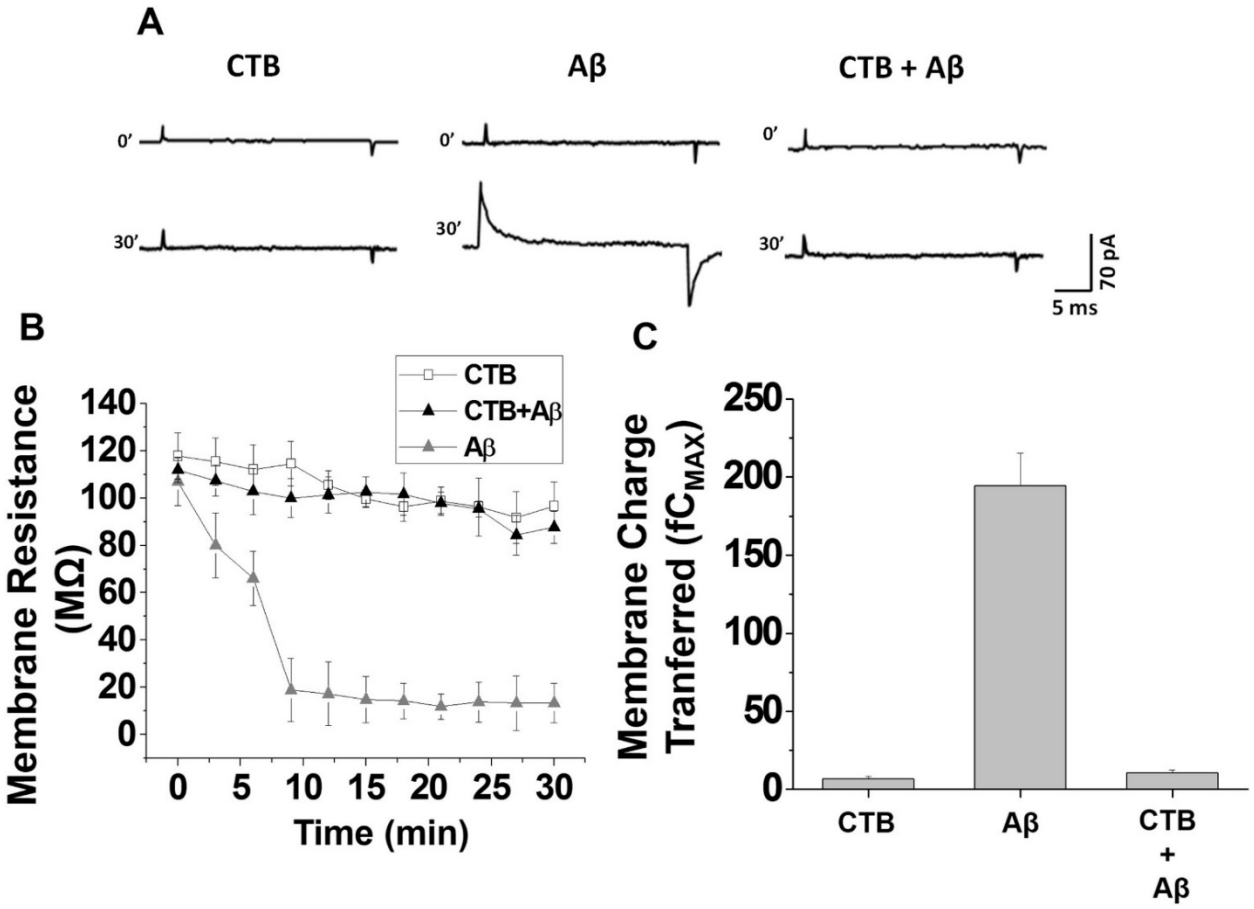

Fig. 5. CTB treatment inhibits membrane perforation induced by Aβ. **A**, Representative traces of capacitive currents in hippocampal neurons at the beginning and end of the experiment showing that Aβ perforated the cell membrane at 30 min, whereas pre-incubation of hippocampal neurons with CTB prevented it. **B**, Time course quantification of membrane resistance during the experiment showing the Aβ-induced perforation for each of the conditions described in **A**. **C**, Quantification of the charge transferred at the end of experiment (30 min) demonstrating a significant difference between pretreatment with CTB and no treatment with CTB. Bars represent the mean ± SEM. *** Denotes $p < 0.001$.

Synaptic deficit is a good indicator of cognitive dysfunction in AD and Aβ-induced neurotoxicity.[2,29,30,31,32] We previously reported that Aβ applied for 24–48 h caused a parallel decrease in synaptic transmission and levels of SV2, a synaptic vesicle protein.[4] Therefore, we quantified SV2 protein clusters to determine if a change in GM1 level in the cellular membrane is important for the synaptotoxic actions of Aβ. In order to do this, we first proceeded to increase or decrease the levels of

GM1 and then treated the cells with fluorescent Aβ for 48 h. Also, to interfere with GM1 in the membrane, we used a co-incubation of CTB with Aβ for 48 h. Lastly, we quantified the number and size of SV2 clusters in the first 20 µm of primary processes along with the Aβ fluorescence in these three experimental conditions.

3.6. Increase in GM1 enhanced clustering and long-term synaptotoxicity induced by Aβ

Culturing the neurons with longer exposure of GM1 containing liposomes increased the number of Aβ clusters from 7.3 ± 1.5 to 15.0 ± 2.4 ($p < 0.005$), without changes in their cluster size (Fig. 6A–C). The increase in Aβ clusters to the neurons found after increasing GM1 was correlated to a reduction in the number of SV2 (Fig. 6D), but not in their size (data not shown). For example, the number of SV2 clusters/20 µm was 31.8 ± 3.9 in control and 15.5 ± 1.6 in presence of Aβ ($p < 0.001$). The number of SV2 clusters was even smaller in presence of Aβ and GM1, compared to Aβ alone (8.1 ± 1.1) ($p < 0.05$) (Fig. 6D). Thus, increased GM1 levels in the cell membrane potentiated the synaptotoxic effect of the Aβ peptide.

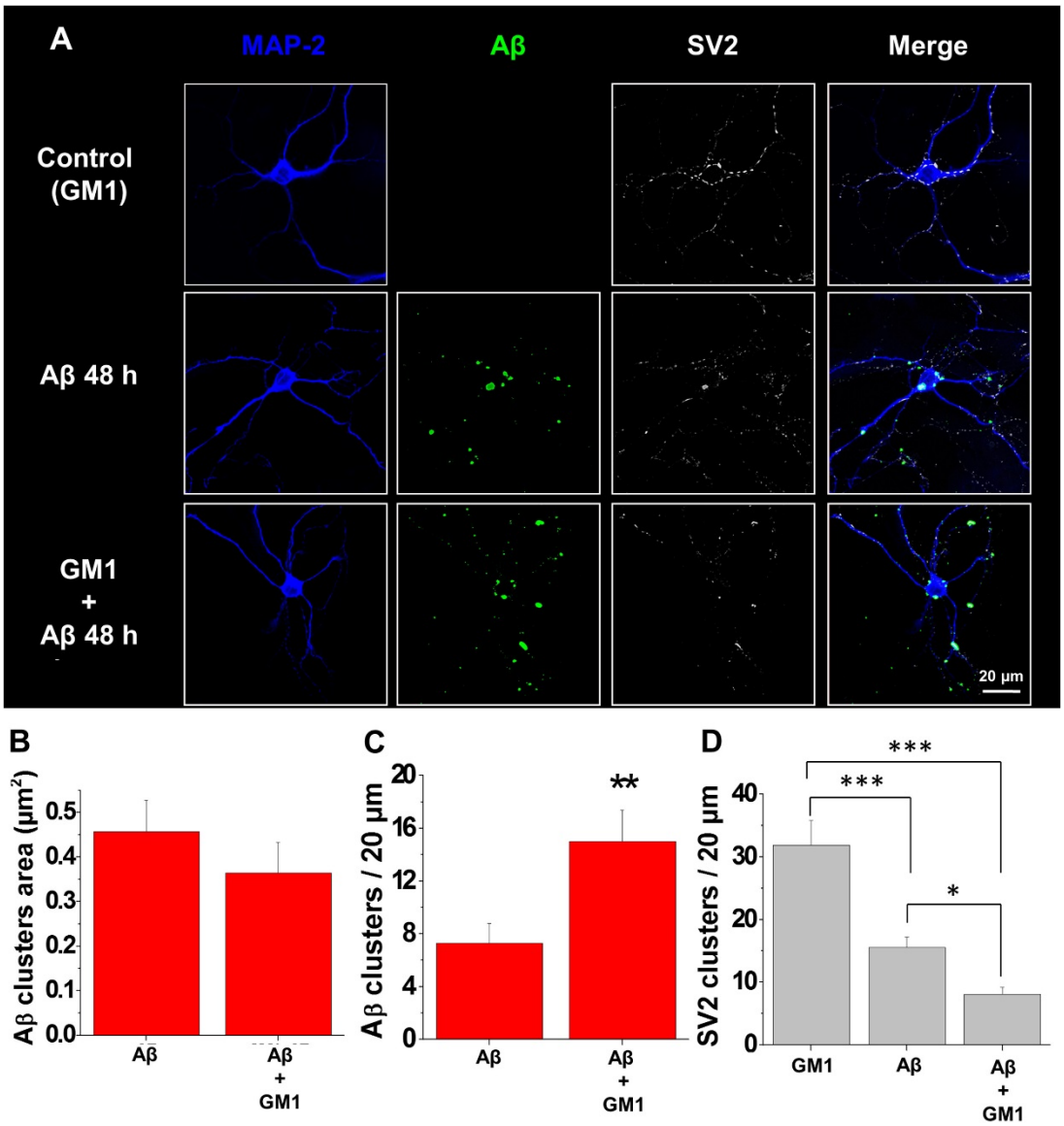

Fig. 6. Long term increase in GM1 enhances clustering of Aβ and synaptotoxicity. **A**, Confocal micrographs showing the distribution of Aβ clusters on the cell membrane surface along with the SV2 immunoreactivity in hippocampal neurons treated with Aβ-FAM in three different experimental conditions: GM1:PC liposomes (1:9) for 3–4 h (no Aβ incubation), Aβ-FAM 0.5 µM for 48 h and GM1:PC liposomes (1:9) for 3–4 h + Aβ-FAM 0.5 µM for 48 h. **B** and **C**, Quantification of fluorescent Aβ clusters (number) in the first 20 µm of hippocampal primary neuronal processes showing that they increased in number but not in size after treatment with GM1 liposomes. **D**, Graph summarizing the number of SV2 clusters in the same ROI (region of interest) used in **B** and **C**, under the three different conditions used for this experiment. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

3.7. Decreased levels of GM1 did not affect Aβ-induced synaptotoxicity

Confocal microscopy data showed that decreasing the levels of GM1 with PDMP during a longer time of exposure resulted in a reduction in the association of the neurotoxic Aβ peptide to the neuronal membrane (Fig. 7A). The quantification for Aβ clusters showed that the area was reduced from 0.55 ± 0.08 in µm^2 to 0.16 ± 0.04 in µm^2 (Fig. 7B). The number of Aβ clusters/20 µm was also reduced from 7.3 ± 1.5 in control to 1.76 ± 0.50 after treatment with PDMP for 72 h (Fig. 7C). Despite reducing the association of Aβ to the membrane, reducing GM1 did not affect the synaptotoxicity of the peptide on the reduction of SV2 cluster size (data not shown), but partially prevented the reduction of cluster number. For example, application of Aβ for 48 h caused a diminution in the number of SV2 clusters by

about 50% with respect to control (Fig. 7A, middle micrographs and Fig. 7D). Nevertheless, when the cells were in presence of Aβ plus PDMP, SV2 cluster was ~ 30% (Fig. 7D), highlighting the importance of the presence of GM1 for the synaptotoxicity of Aβ.

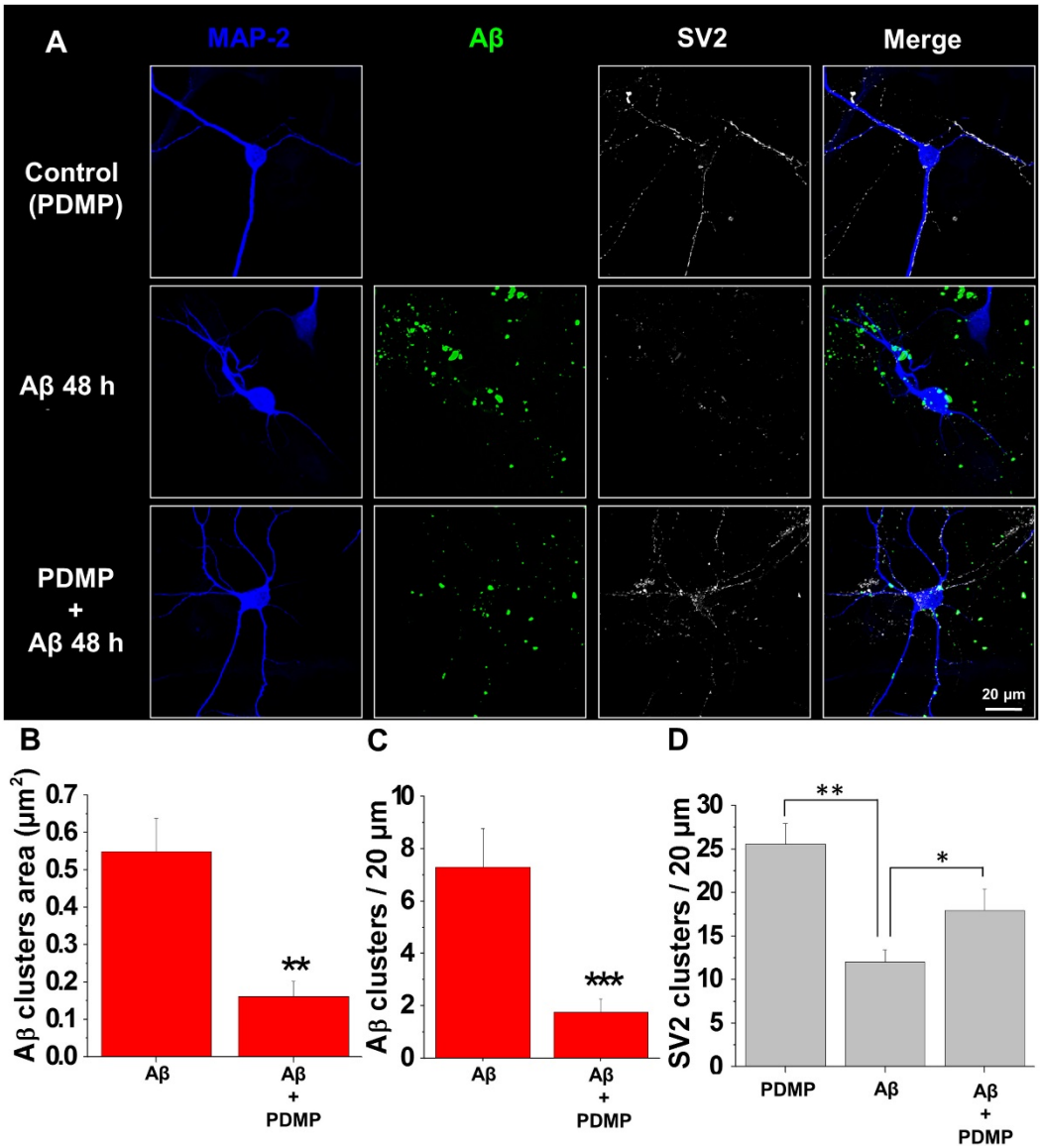

Fig. 7. Decreased levels of GM1 reduced Aβ aggregation and partially abates synaptotoxicity. **A**, Confocal micrographs showing the distribution of Aβ clusters on the cell membrane surface along with the SV2 immunoreactivity in hippocampal neurons treated with Aβ-FAM in three different experimental conditions: PDMP 10 µM for 72 h (no Aβ incubation), Aβ-FAM 0.5 µM for 48 h and PDMP 10 µM for 72 h + Aβ-FAM 0.5 µM for 48 h. **B** and **C**, Quantification of fluorescent Aβ clusters (area and number) in the first 20 µm of hippocampal primary neuronal processes showing that both parameters were reduced after treatment with D-PDMP. **D**, Graph summarizing the number of SV2 clusters in the same ROI (region of interest) used in **B** and **C**, under the three different conditions used for this experiment. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

3.8. CTB reduced Aβ aggregation and blocked synaptotoxicity

Treatment of the cells with CTB was very effective diminishing the size and number of Aβ aggregates on the neuronal processes (Fig. 8A–C). For example, the Aβ clusters area in μm^2 was reduced from 0.25 ± 0.02 to 0.13 ± 0.02 in presence of CTB. In addition, the number of Aβ clusters/20 μm was reduced from 16.1 ± 2.5 to 3.6 ± 0.5 in presence of Aβ plus CTB (Fig. 8B,C). In addition, in the CTB + Aβ condition, the synaptotoxic effect of Aβ on SV2 cluster size was similar to control (CTB: 0.15 ± 0.01; Aβ: 0.09 ± 0.01; Aβ + CTB: 0.12 ± 0.01; Fig. 8D). A more pronounced neuroprotective effect was observed on the number of SV2 clusters when Aβ was incubated with CTB showing an increase from 5.5 ± 0.7 in the Aβ condition to 34 ± 4.0 in the presence of CTB, a value corresponding to approximately 70% of control (Fig. 8E).

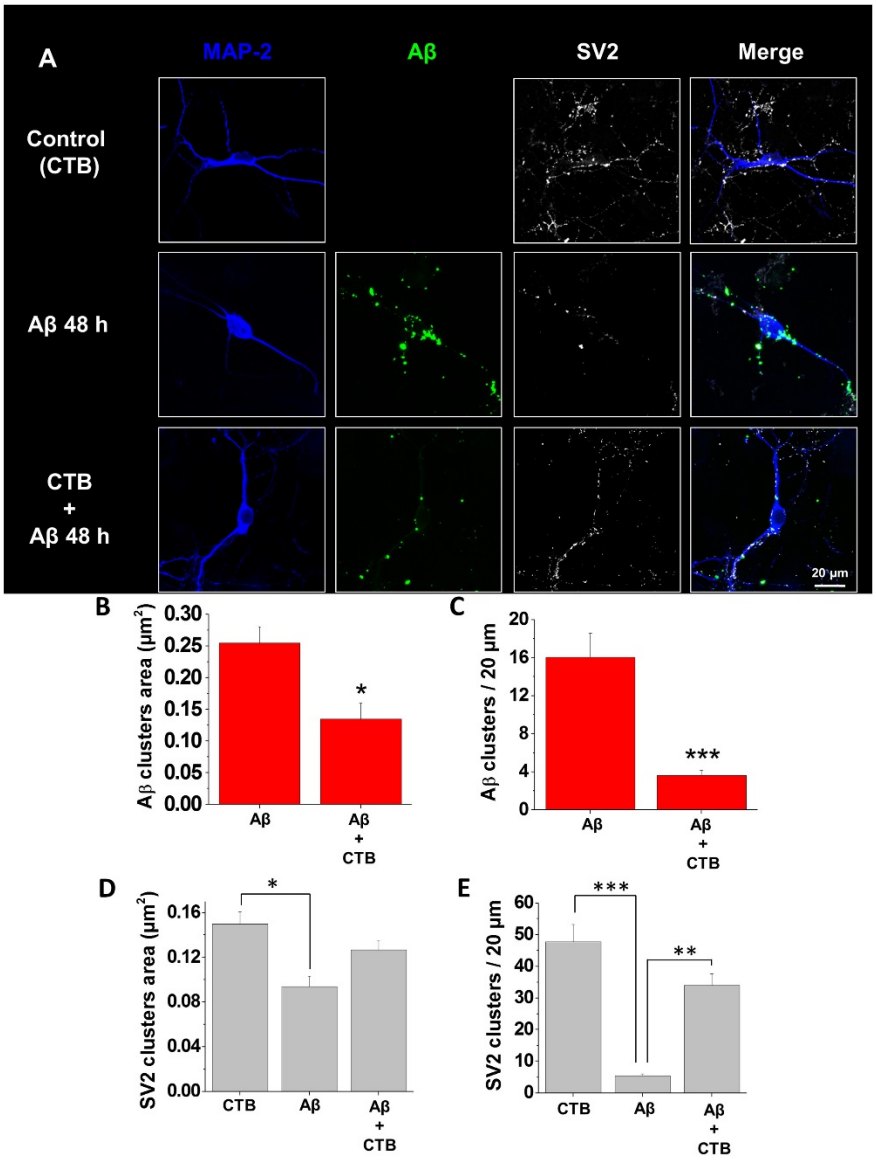

Fig. 8. CTB reduces Aβ aggregation and completely blocks synaptotoxicity. **A**, Confocal micrographs showing the distribution of Aβ clusters on the cell membrane surface along with the SV2 immunoreactivity in hippocampal neurons treated with Aβ-FAM in three different experimental conditions: CTB 0.5 μg/mL for 48 h (no Aβ incubation), Aβ-FAM 0.5 μM for 48 h and co-incubation of CTB 0.5 μg/mL + Aβ-FAM 0.5 μM for 48 h. **B** and **C**, Quantification of fluorescent Aβ clusters (area and number) in the first 20 μm of hippocampal primary neuronal processes showing that both parameters were decreased after

CTB treatment. **D** and **E**, Graphs summarizing the size and number of SV2 clusters in the same ROI (region of interest) used for the quantifications done in **B** and **C**, under the three different conditions used for this experiment. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$.

4. Discussion

The results showed that GM1 plays a major role in early effects of Aβ (association and membrane perforation), as well as synaptotoxicity. A more definite role of Aβ in Alzheimer's disease initiation and progression is still unresolved. Presently, it is not well understood how this neurotoxic peptide can assemble in vivo, since even in patients with AD the concentration of soluble Aβ in the cerebral spinal fluid seems to be several orders of magnitude below the micromolar concentration required for in vitro oligomerization.[33] From this, one can suggest the existence of cellular determinants able to facilitate this process, and part of the evidence points to components on the membrane surface. For example, one important characteristic of Aβ is its capacity to bind to cell membranes and initiate synaptic failure, and it is currently accepted that this process is strongly affected by the membrane lipid composition (negatively charged lipids, clusters of gangliosides, cholesterol, lipid rafts, and lipid:protein ratio) (Fig. 9).

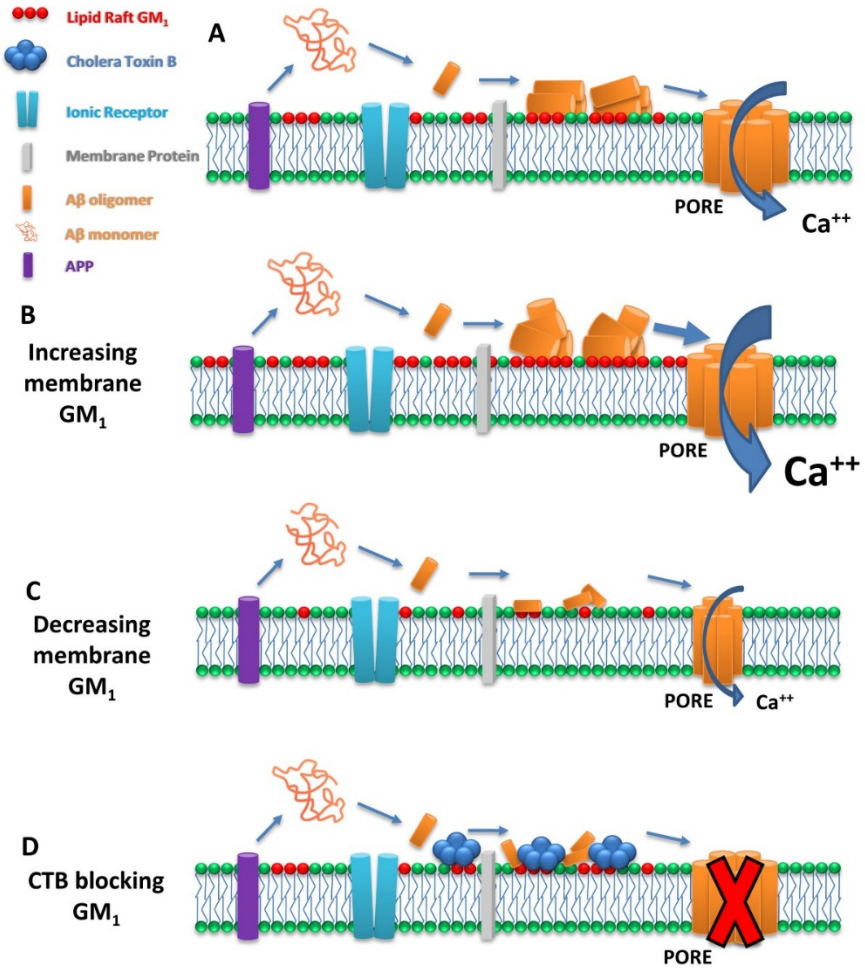

Fig. 9. Proposed GM1-dependent early neurotoxic membrane actions of amyloid-β. **A**, In the amyloid cascade, the Amyloid Precursor Protein (APP) is cleaved and Aβ is released to the extracellular space as a monomer, which, under conditions not well understood, goes through a process of aggregation resulting in the formation of oligomers. The association of oligomers to the membrane triggers the insertion of the peptide, two processes in which GM1 is relevant. The consequence of this is the formation of a pore in the neuronal membrane that disrupts cell homeostasis (intracellular Ca^{++} imbalance) and leads to synaptotoxicity. **B,** When the membrane levels of GM1 are increased, the process of association and clustering of

Aβ are enhanced, speeding the process of perforation. Thus, more charge is transferred in less time meaning that more ions, such as Ca^{++}, enter the cytoplasm and produce ionic dyshomestasis and synaptotoxicity. **C**, On the contrary, decreasing GM1 reduces Aβ clustering and association, slowing down perforation and decreasing the neurotoxic effect of Aβ on synapses. **D**, Blocking GM1 sites in the membrane by CTB also reduces association and clustering of Aβ and almost completely reduces the perforation of the cell membrane and the synaptotoxic effect of Aβ. This highlights the importance of GM1 on the neurotoxic effects of Aβ in the cell membrane and how these early actions can be modulated to enhance or decrease the subsequent synaptotoxic effects caused by Aβ.

Accumulating evidence suggests that membrane charge can alter the binding of Aβ and influence aggregation processes and amyloid pore formation.[34] For example, various amino acid residues in Aβ such as arginine 5, lysine 16 and lysine 28 display a positive charge at neutral pH and therefore possess affinity for negatively charged lipids like phosphatidylglycerol, phosphatidylserine or ganglioside GM1, but not for neutral lipids like phoshphatidylcholine (Seelig et al., 1995).[35] Moreover, examination of the ability of GM1 to associate with Aβ revealed that Aβ exhibits a significantly higher binding affinity for sialic acid residues (hydrophylic) than for ceramide residues, suggesting that its association to GM1 is principally due to electrostatic interactions.[36] Additionally, interaction between sialic acid residues and Aβ induces a reordering in the conformation of the Aβ peptidic chain[37] which could potentiate the oligomerization process. Also, it was proposed that gangliosides mediate the formation of toxic amyloid fibrils of Aβ with an antiparallel β-sheet structure by providing less polar environments.[38] Additionally, in physiological in vitro conditions, another study confirmed that Aβ does not bind specifically to other lipids as it does to GM1,[39] and amyloid fiber formation was accelerated in the presence of liposomes with a composition very similar to sphingomyelin-associated lipid rafts. Thus, facilitation of the interaction between Aβ and the membrane occurs allowing the C-terminus end of the peptide to deform and interact with the C-terminus of other Aβ peptides favoring the oligomerization process and leading to the formation of toxic species on the cell surface.[8] These toxic species are important in the disruption of the neuronal membrane through the formation of a pore permeable to cations, anions and small molecules.[2,3] This is the same mechanism that has been proposed for pore forming toxins, which behave very similarly to Aβ,[3,32,40] in which membrane binding occurs following a catalyzing conformational change mediated by the membrane and finally oligomerization leading to the formation of a pore.[41] This pore will lead to ionic dyshomeostasis affecting intracellular signaling, action potential generation and synaptic transmission, among others.[2,4]

In the present study, we show that increasing the size and number of Aβ clusters by increasing the level of GM1 correlates negatively with the onset in perforation time, e.g. the bigger the cluster size and number, the faster the membrane perforation. On the other hand, by selectively blocking the access of Aβ to this lipid with CTB resulted in blockade of plasma membrane disruption. We suggest that actually the increase in membrane GM1 favors Aβ association and the size of the cluster (which we interpret as increased oligomerization on the surface of the membrane). From a functional point of view, increasing GM1 levels in native membranes caused a slight acceleration of Aβ pore formation indicating that although there is more Aβ clustering (Fig. 1), the effects of GM1 on membrane perforation leveled off (Fig. 3).

The present results indicate that GM1 is not only important for the association and clusterization of Aβ on the membrane, but also in the perforation, a well-known mechanism involved in the subsequent synaptotoxic events.[2,29,30,31,32] Yamamoto et al. suggested that Aβ assembly initiates at synaptic sites,[42] therefore the perforation of the plasma membrane is also likely to start at these sites. Thus, one of the main consequences of the membrane perforation is the reduction in various synaptic (pre- and post) proteins, such as the SV2 protein.[4,43] In this study, we demonstrate that the increase in association and perforation due to augmented levels of GM1 in the membrane was associated with a marked decrease

in SV2 cluster levels, indicating that under these conditions Aβ becomes more neurotoxic. On the contrary, decreasing the levels of GM1 with PDMP, or blocking the GM1 sites with CTB reduced this effect (in the case of CTB the values reached levels close to control). This suggests that Aβ actions at the cellular membrane might be in part responsible for this decrease in SV2, which initiated with a change in lipid composition.

Additionally, we can also hypothesize that membrane components could be regulating the Aβ pore, thus altering the neurotoxic properties of Aβ. Our results show that indeed the membrane composition is somehow controlling the perforation process.

The present results suggest that the association and subsequent perforation of the plasma membrane by Aβ occurs in GM1-rich microdomains. Thus, the lipid composition, the surface charge and fluidity of the membrane, as well as the binding, conformation and oligomeric state of Aβ are all variables that influence the perforation mediated by the peptide and the subsequent synaptotoxic effects. Therefore, it can be postulated that the composition of the membrane appears to be crucial in the development and progression of AD. These results have led to the hypothesis that depletion of lipid raft components could have an effect on Aβ production. Thus, the importance of GM1 sites on the cell surface for the association and clustering of Aβ on the neuronal membrane and CTB as a neuroprotective molecule against the synaptotoxicity of Aβ could provide insight for possible therapy to treat AD.

The following are the supplementary data related to this article.

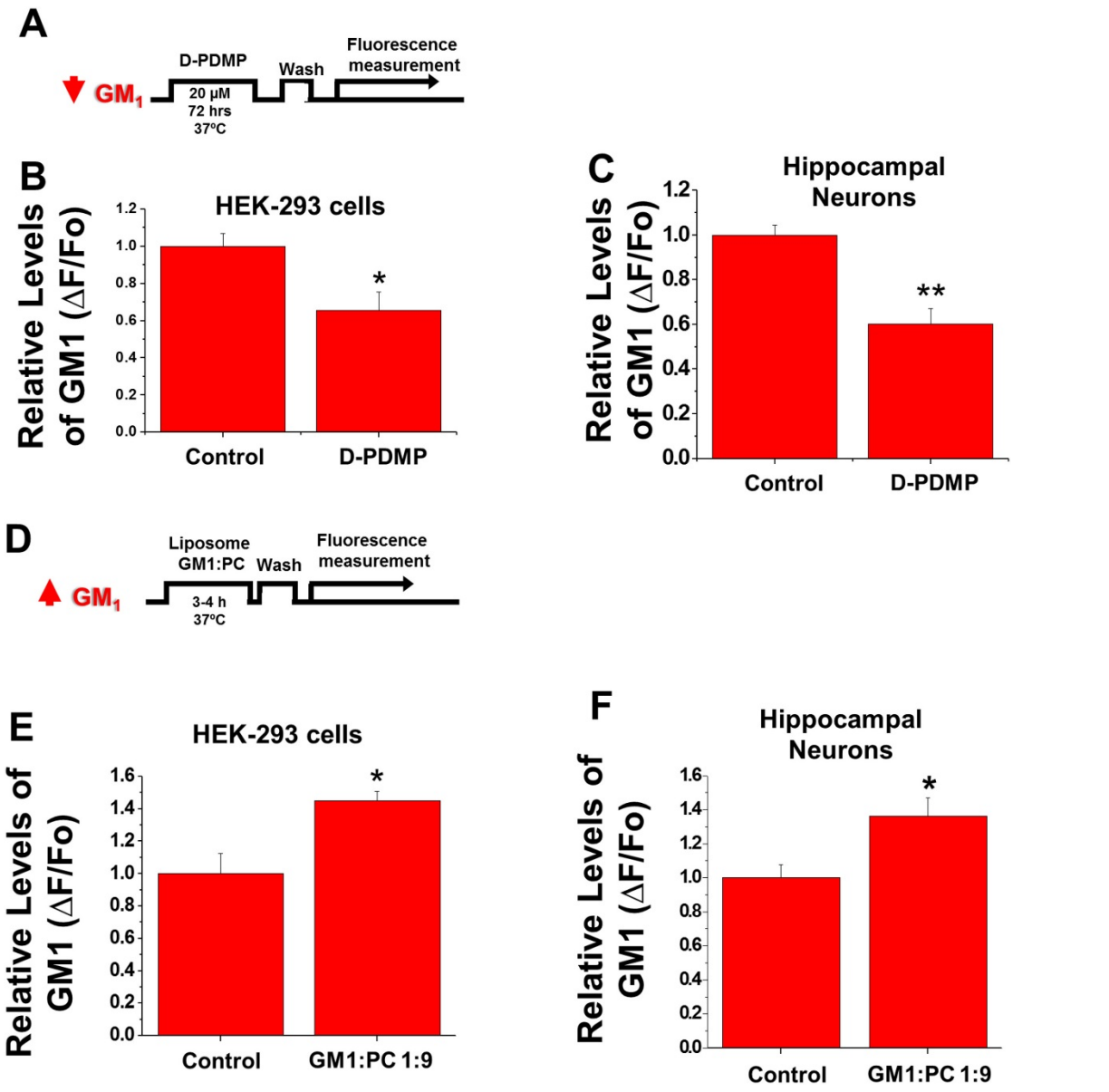

Fig. S1. Increase and decrease in relative levels of GM1 in HEK-293 cells and hippocampal neurons. **A**, Schematic protocol used to decrease the content of GM1 in the membrane with D-PDMP. **B**, **C**, Quantification of CTBAlexa Fluor 555 relative fluorescence, indicating the relative levels of GM1 in control and treated cells with D-PDMP (20 µM for 72 h) in HEK-293 (**B**) and hippocampal neurons (**C**). **D**, Schematic protocol used to increase the membrane levels of GM1 using GM1:PC liposomes (1:9). **E**, **F**, Quantification of 30 CTB-Alexa Fluor 555 relative fluorescence in control and treated cells with GM1/phosphatidylcholine liposomes showing the enrichment of GM1 content in the membrane of HEK-293 cells (**E**) and hippocampal neurons (**F**). Bars represent the mean ± SEM. * denotes $p < 0.05$, ** $p < 0.005$.

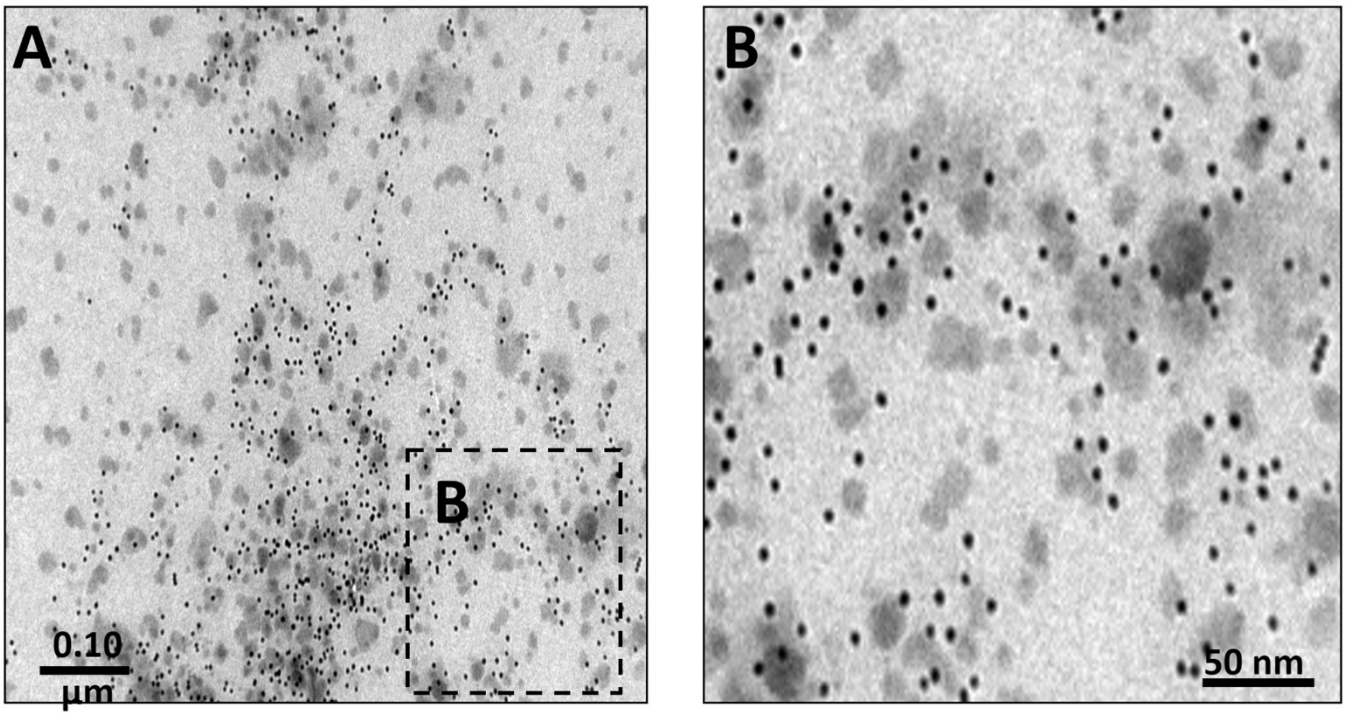

Fig. S2. Electron micrographs that demonstrate the presence of amyloid aggregates in the preparations used with immunogold (5 nm particle). A, Micrographs of Aβ oligomer preparations showing the distribution of different size oligomers. B, Zoom of the section showed in A, demonstrating the presence of spherical or disc shaped oligomeric species.

Modification of relative GM1 levels in hippocampal neurons and HEK-293 cells. The objective of our study was to evaluate the association and perforation of Aβ under conditions in which the relative levels of GM1 were increased or decreased. Our first approach was to assess the effectiveness of the treatments used. Modification of the relative levels of this lipid was done in both HEK-293 cells and hippocampal neurons. For this we used PDMP 20 µM for 72 h to decrease GM1 levels (Fig. S1 A) and a media enriched with phosphatidylcholine/GM1 liposomes (9:1) for 3–4 h in order to increase the amount of GM1 in the membranes (Fig. S1 D). Fluorescent quantification, using CTB, indicated that PDMP treatments were able to decrease the levels of GM1 by about 40% in both types of cells (Fig. S1 B,C). On the other hand, GM1 liposomes increased the levels in HEK cells and hippocampal neurons by approximately 40% (Fig. S1 E, F). These data demonstrate that our treatments were effective at decreasing or increasing the relative levels of GM1 in cell membranes. Supplementary data associated with this article can be found in the online version, at doi.

Transparency document

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Contributions

L.G.A., R.P., E.J.F.P and F.S. contributed to experimental design. E.J.F.P. and F.S. carryout the study and analyzed data. L.G.A. and E.J.F.P. wrote the manuscript.

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