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Broad neutralization of calcium-permeable amyloid pore channels with a chimeric Alzheimer/Parkinson peptide targeting brain gangliosides

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ABSTRACT

Growing evidence supports a role for brain gangliosides in the pathogenesis of neurodegenerative diseases including Alzheimer's and Parkinson's. Recently we deciphered the ganglioside-recognition code controlling specific ganglioside binding to Alzheimer's β-amyloid (Aβ1-42) peptide and Parkinson's disease-associated protein α-synuclein. Cracking this code allowed us to engineer a short chimeric Aβ/α-synuclein peptide that recognizes all brain gangliosides. Here we show that ganglioside-deprived neural cells do no longer sustain the formation of zinc-sensitive amyloid pore channels induced by either Aβ1-42 or α-synuclein, as assessed by single-cell Ca^{2+} fluorescence microscopy. Thus, amyloid channel formation, now considered a key step in neurodegeneration, is a ganglioside-dependent process. Nanomolar concentrations of chimeric peptide competitively inhibited amyloid pore formation induced by Aβ1-42 or α-synuclein in cultured neural cells. Moreover, this peptide abrogated the intracellular calcium increases induced by Parkinson's-associated mutant forms of α-synuclein (A30P, E46K and A53T). The chimeric peptide also prevented the deleterious effects of Aβ1-42 on synaptic vesicle trafficking and decreased the Aβ1-42-induced impairment of spontaneous activity in rat hippocampal slices. Taken together, these data show that the chimeric peptide has broad anti-amyloid pore activity, suggesting that a common therapeutic strategy based on the prevention of amyloid-ganglioside interactions is a reachable goal for both Alzheimer's and Parkinson's diseases.

1. Introduction

Alzheimer's and Parkinson's diseases are the most prevalent neurodegenerative diseases in humans, with millions of people affected worldwide [1,2]. Unfortunately, despite decades of research efforts we are still seeking a cure for these fatal diseases [3]. A category of proteins with exceptional conformational plasticity and aggregation properties have been identified as potential therapeutic targets. These proteins, collectively referred to as amyloid proteins [4], have been found in various deposits such as fibrils and plaques [5] that were initially suspected to be the causative agents of the diseases [6,7]. This concept has logically led to therapeutic strategies aimed at disrupting amyloid plaques, e.g. through immunization protocols [8,9]. Unfortunately, these attempts have not been successful and in some cases the trials had to be prematurely stopped due to severe toxic effects [10,11]. Moreover, it appeared that the presence of amyloid plaques in the brain of aged subjects was not always correlated with Alzheimer's disease or cognitive defect [12,13].

Overall, these data indicate that amyloid plaques can no longer be considered as responsible for neurodegenerative symptoms in Alzheimer's disease [12]. Instead, it is now considered that small oligomers are the main neurotoxic forms of Alzheimer's β-amyloid (Aβ1-42) peptide [14–16]. Several types of oligomers have been detected in the brain of patients with Alzheimer's disease [17,18]. In the plasma membrane of brain cells, these oligomers can form pore-like structures (the so-called 'amyloid pores') that behave as Ca^{2+}-selective ion channels [19–22]. The massive entry of Ca^{2+} through amyloid pores is believed to be one of the earliest steps in Alzheimer's, Parkinson's, and other neurodegenerative diseases [23,24]. For this reason, it is urgent to find compounds able to prevent amyloid pore formation in the plasma membrane of brain cells [3,25].

One possibility could be to inhibit the initial binding of amyloid proteins to the surface of brain cells. In this respect, one should exploit the common property of amyloid proteins to specifically interact with gangliosides in lipid raft domains [26,27]. However, this is not an easy task because Alzheimer's peptides Aβ1-42 and/or Aβ1-40 bind to several gangliosides including the main brain species GT1b, GD1a, GD1b, GM1

Abbreviations: ATCC, American Type Culture Collection; HPLC, high pressure liquid chromatography; GBD, ganglioside-binding domain; PPMP, 1R,2R-(+)-1-phenyl-2-palmitoylamino-3-N-morpholine-1-propanol.

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and GM3 [28–34]. Moreover, the Parkinson's associated protein α-synuclein has been shown to bind to GM1 [35–37] and to several other glycosphingolipids including ganglioside GM3 [36]. The measured avidity of amyloid proteins for specific gangliosides may vary according to various parameters, including protein concentration, protein–lipid ratio and the experimental approach used. Nevertheless, the data available in the literature indicate that both Aβ and α-synuclein are able to interact with several gangliosides (reviewed in [38]). Hence, it is widely admitted that gangliosides play a key role in the pathogenesis of neurodegenerative diseases [29,30,36,39]. Using the Langmuir film balance technology with reconstituted ganglioside monolayers probed by wild-type and mutant peptides derived from Aβ and α-synuclein, we have demonstrated that the binding of amyloid proteins to gangliosides obeys a biochemical code [40]. This code is based on a common structurally-conserved domain conferring ganglioside recognition, with minor sequence variations accounting for ganglioside specificity [40]. The deciphering of this code allowed us to create a universal peptide that is constructed as a chimer between the ganglioside-binding domains of α-synuclein and Aβ. This chimeric peptide, which combines the ganglioside binding properties of both proteins, behaves as a universal ganglioside-binding compound that interacts with all the gangliosides tested so far, including the main gangliosides expressed by neurons (GM1, GD1a, GD1b and GT1b) as well as the major glial ganglioside GM3 [40].

In the present study we have evaluated the activity of this universal anti-ganglioside peptide (referred to as 'chimeric peptide') on amyloid pore formation induced by Aβ1-42 and α-synuclein in brain cells. We show that nanomolar concentrations of both wild-type and disease-associated mutant forms of these proteins induce amyloid pore formation as assessed by Ca^{2+} flux studies. These channels were inhibited by Zn^{2+}, a specific inhibitor of amyloid pores [41,42]. Most importantly, ganglioside-depleted cells could no longer sustain the formation of these pores, which indicate that gangliosides are key actors of the process. Finally we studied the anti-pore activity of the chimeric peptide against various wild-type and mutant forms of Aβ1-42 and α-synuclein, as well as its ability to prevent Aβ-induced neurotoxicity in two functional assays, i.e. microscopy evaluation of synaptic vesicle trafficking and electrophysiological recordings of the spontaneous activity of rat hippocampal slices.

2. Materials and methods

2.1. Products

SH-SY5Y cells were purchased from ATCC. DMEM/F12, HBSS, glutamine and penicillin/streptomycin were furnished by Gibco. Fluo-4AM, FM1-43 and secondary antibody were purchased from Invitrogen. The anti-ganglioside GM1 antibody was purchased from Matreya. The full-length proteins α-synuclein1-140 (wild-type and mutants) and Aβ1-42 were from rPeptide. Synthetic peptides including α-synuclein 34–50, Aβ1-16 and the chimeric peptide were obtained from Schafer (Denmark). All peptides and proteins have a purity >95% as assessed by HPLC. The chimeric peptide used in this study has been patented under the number PCT/EP2015/054968: "A chimeric peptide that interacts with cell membrane gangliosides" (inventors: Nouara Yahi and Jacques Fantini, applicant: Aix-Marseille University). Three distinct batches of this peptide have been used over a 4-year period of time with fully reproducible data.

2.2. In silico studies

Molecular dynamics simulations of ganglioside–peptide interactions have been performed with the Hyperchem program as described [40].

2.3. Cell culture

Cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) supplemented with 10% fetal calf serum, glutamine (2 mM) and penicillin (50 U/mL)/streptomycin (50 μg/mL) and maintained at 37 °C with 5% CO_2. Cells were passaged twice a week and not used beyond passage 25.

2.4. Modulation of GM1 levels

Membrane GM1 levels were decreased by inhibiting cell glucosylceramide synthase with 10 μM 1R,2R-(+)-1-phenyl-2-palmitoylamino-3-N-morpholine-1-propanol (PPMP) for 48 h [43].

2.5. Ganglioside extraction and quantitation

Gangliosides were extracted and recovered from the upper phase of a Folch partition and analyzed by high performance thin layer chromatography (HPTLC) as previously described [44]. Neutral sphingolipids (ceramides, GalCer) and sphingomyelin were recovered from the lower phase of a Folch extract and analyzed by HPTLC as previously reported [44]. Glycosphingolipids were colored with orcinol and sphingomyelin and ceramide with Coomassie blue [45]. All sphingolipids were quantitated with a Gel Doc™ XR + Molecular Imager using the Image Lab™ software.

2.6. Immunocytochemistry

The cells were either submitted or not to PPMP treatment and then incubated with anti-ganglioside GM1 primary antibody (1:500) for 2 h. The cells were rinsed and subsequently treated with goat anti-rabbit Alexa Fluor 488 (1:400) for 1 h. Images were analyzed using Image J 1.45. No correction was applied to the images, and, for a better visualization, only contrast and brightness were adjusted in photography used for figures. The same adjustments were applied on all images to allow the comparison.

2.7. Lipid monolayer assay

Cell ganglioside–peptide interactions were studied with the Langmuir-film balance technique using a Kibron microtensiometer as previously described [36].

2.8. Calcium measurements

Cells were plated (45.000 cells/dish) in 35 mm culture dishes and grown during 72 h at 37 °C. They were loaded with 5 μM Fluo-4AM for 30 min in the dark, washed three times with HBSS and incubated 30 min at 37 °C [46]. The calcium fluxes were estimated by measuring the variation of cell fluorescence intensity after amyloid protein injection (220 nM) into the recording chamber directly above an upright microscope objective (BX51W Olympus) equipped with an illuminator system MT20 module. Fluorescence emission at 525 nm was imaged by a digital camera CDD (Hamanatsu ORCA-ER) after fluorescence excitation at 490 nm. Time-lapse images (1 frame/10 s) were collected using the CellR Software (Olympus). Fluorescence intensity was measured from region of interest (ROI) centered on individual cells. Signals were expressed as fluorescence after treatment (F_t) divided by the fluorescence before treatment (F_0) and multiplied by 100. The results were averaged and the fluorescence of control untreated cells is subtracted of each value. All experiments were performed at 30 °C during 1 h.

2.9. FM1-43 fluorescence measurements

For FM1-43 fluorescence measurements, we used the protocol developed by Shote and Seelig [47] with minor modifications. In brief, SH-SY5Y cells were washed with calcium imaging medium. Cells were then mounted above an upright microscope objective and incubated with FM1-43 probe (2 μM; Invitrogen) for 10 min. After amyloid protein injection directly into the recording chamber, the cells were illuminated with excitation light at 490 nm for 83 msec every 30 min. We did not observe any deleterious effect during the experiment. Images were analyzed using the Image J 1.45 software. For a better visualization, only contrast and brightness were adjusted in photography used for figures. The same adjustments were applied on all images to allow the comparison. Fluorescence intensity was measured from ROI centered on individual cells. For each cell, data were expressed as fluorescence after treatment (F_t) divided by the fluorescence value before treatment (F_0) and the results were averaged.

2.10. Acute hippocampal slices preparation

Six week old rats (Wistar rats, Charles River Laboratories) were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and decapitated. The brain was removed rapidly, the hippocampi were dissected, and transverse 400 μm slices were cut in oxygenated (95% O_2 and 5% CO_2) modified artificial cerebrospinal fluid (mACSF) containing the following (in mM): 132 choline, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgCl_2$, 0.5 $CaCl_2$, and 8 D-glucose; using a Leica VT1000S tissue slicer (Leica VTS1200S, Germany). Slices were then transferred for rest at room temperature (1-3 h) in oxygenated (95% O_2 and 5% CO_2) normal artificial CSF (ACSF) containing the following (in mM): 126 NaCl, 3.5 KCl, 1.2 NaH2PO4, 26 NaHCO3, 1.3 MgCl2, 2.0 CaCl2, and 10 D-glucose, pH 7.4.

2.11. Electrophysiological recordings

Hippocampal slices were incubated for 30–60 min with Aβ1-42 peptide (500 nM) with or without chimeric peptide (500 nM). Then, hippocampal slices were transferred to a recording chamber maintained at 30–32 °C and continuously perfused (2 ml/min) with oxygenated ACSF. Local field potentials were recorded in the CA1 region with glass electrodes (2–3 MΩ; filled with normal ACSF), using a DAM-80 amplifier (lowfilter, 0.1 Hz; highpass filter, 3 KHz; World Precision Instruments, Sarasota, FL). To ensure that the spontaneous events were post-synaptic events 50 μM CNQX and 5 μM D-APV was used to block recorded events. Signals were analyzed off-line using Clampfit 9.2 (PClamp) and MiniAnalysis 6.0.1 (Synaptosoft, Decatur, GA).

2.12. Statistical analysis

All data were expressed as mean ± S.E.M. and the statistical significance was tested using Student-test or Kruskal–Wallis test (non-parametric test).

3. Results

Cultures of human neuroblastoma SH-SY5Y showing numerous neuritic contacts were used to study the effect of Aβ1-42 and α-synuclein on calcium fluxes. To this end, the cells were loaded with the calcium-sensitive fluorescent probe Fluo-4 AM before the incubation with nanomolar concentrations of freshly prepared Aβ1-42 (Fig. 1A) or full-length α-synuclein (α-syn1-140) (Fig. 1B). In both cases, the amyloid protein induced a progressive increase of intracellular calcium concentration. This calcium rise could be detected in the minutes following protein addition and reached a plateau within 1 h of incubation. This effect was strongly inhibited (α-synuclein) or even abrogated (Aβ1-42) in the presence of Zn^{2+} (Fig. 1C), a classic blocker of amyloid pores [19,41,42,48]. These data strongly suggested that both Aβ1-42 and α-syn1-140 induced the formation of Ca^{2+}-permeable amyloid pores in these cells. Interestingly, rat Aβ1-42, which is not neurotoxic, did not affect intracellular Ca^{2+} concentrations (*Data in Brief*). Not only these data indicate that the effects of Aβ1-42 and α-syn1-140 of human origin are specific, but they also suggest a molecular mechanism for the formation of amyloid pores. Indeed, rat Aβ1-42 differs from its human counterpart at only three amino acid positions: Gly-5, Phe-10 and Arg-13 in rat Aβ1-42 vs. Arg-5, Tyr-10 and His-13 in the human sequence. Interestingly, all these amino acid residues have been involved in the binding of human Aβ1-42 to ganglioside GM1 [36,40]. In fact, these residues belong to the ganglioside binding domain allowing both Aβ1-42 and α-syn1-140 to interact with cell surface gangliosides [40]. To assess whether brain cell gangliosides are required for the formation of amyloid pores, we treated the cells with PPMP, a specific GlcCer synthase inhibitor that affects the metabolism of all GlcCer-derived glycosphingolipids including gangliosides [43,49]. After 48 h of treatment with a non-toxic concentration of PPMP (10 μM), which corresponds to mild treatment conditions [50], gangliosides and related sphingolipids were extracted from the cells and analyzed by high performance thin layer chromatography (HPTLC). These lipids were quantitated in both control and PPMP-treated cells (Fig. 1D). As expected for an inhibitor of GlcCer synthase, PPMP induced a marked increase of ceramides. Sphingolipids synthesized from ceramides independently of GlcCer synthase (GalCer, sphingomyelin) were also increased in PPMP-treated cells. In contrast, PPMP-treated cells showed a general decrease in ganglioside content (Fig. 1D). The main gangliosides detected in SH-SY5Y cells were GM1, GD1a, GM3 and GT1b, which is globally consistent with previous characterizations [50–52]. The expression of ganglioside GM1 was reduced by 87% and GD1a by 50% in PPMP-treated cells. Visual examination of the HPTLC plates suggested that GM3 and GT1b levels were also markedly decreased by PPMP but in these particular cases the quantitation was not possible with the software used (see Materials and methods), due to a poor contrast between the bands and the background. To further assess the effect of PPMP on GM1 expression we performed a surface immunofluorescence labeling with anti-GM1 antibodies (Fig. 1E). This study revealed that in control cells, GM1 is highly expressed in neuritic extensions, in agreement with both immunocytochemical studies and the well-known effect of GM1 on neurite outgrowth [53,54]. Most importantly, we observed that cell surface expression of GM1 was strongly reduced in PPMP-treated cells, which fully confirmed the data obtained by lipid quantitation. This depletion had a marked effect on the ability of neural cells to sustain amyloid pore formation. Indeed, the increase in intracellular calcium concentration induced by α-syn1-140 was significantly lower in PPMP-treated cells compared with control cells (Fig. 1F). A similar effect of ganglioside depletion was observed in PPMP-treated cells incubated with Aβ1-42 (Fig. 1F).

One could argue that PPMP, which interferes with sphingolipid metabolism (Fig. 1D), could globally affect the physicochemical properties of the plasma membrane, thereby rendering the cells resistant to amyloid pore formation. To rule out this possibility, we analyzed the pore-forming capacity of Aβ22-35, a synthetic Aβ peptide that lacks the ganglioside-binding domain and thus does not interact with GM1 [30]. Indeed, recent data from our group have demonstrated that Aβ22-35 interacts with cholesterol and forms Ca^{2+}-permeable pores through a ganglioside-independent mechanism [22,46]. AS shown in Fig. 1G, the synthetic Aβ22-35 peptide induced a progressive increase of intracellular Ca^{2+} in SH-SY5Y cells. When treated with PPMP (10 μM for 48 h) before Aβ22-35 incubation, these cells were still able to sustain amyloid pore formation as assessed by intracellular Ca^{2+} determinations (Fig. 1G). The micrographs taken under the fluorescence microscope in control and PPMP-treated cells showed that the percentage of cells responding to Aβ22-35 was even higher upon PPMP treatment (Fig. 1H), which is consistent with the kinetics of Fig. 1G (compare the initial phase of the Ca^{2+} response in control and PPMP-treated cells). Thus, these data showed that PPMP did not induce a global resistance

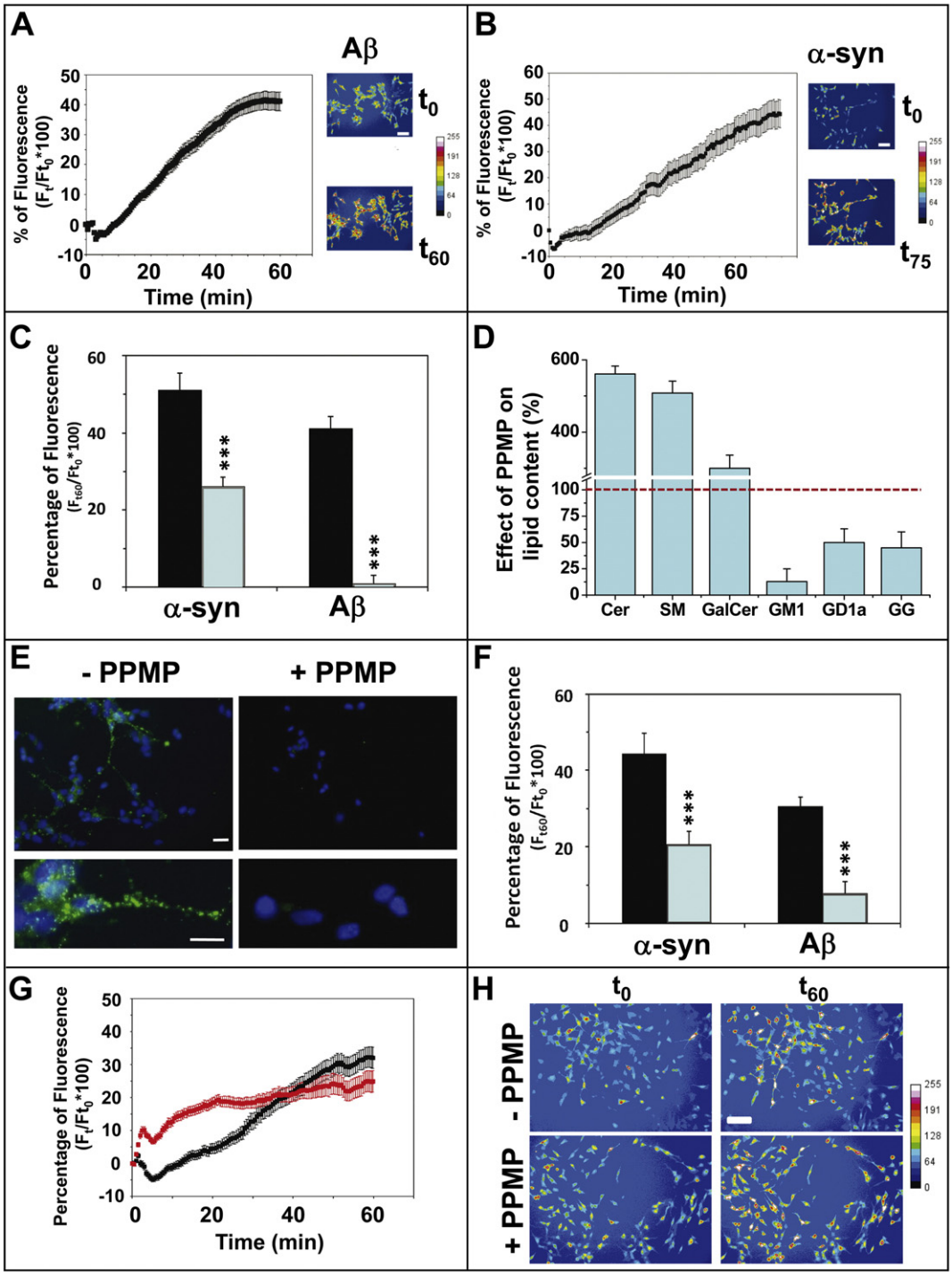

Fig. 1. Mechanistic studies of amyloid pore formation induced by Aβ1-42 and α-syn1-140. SH-SY5Y cells were loaded with the Ca^{2+} indicator Fluo-4 AM and then incubated with 220 nM of either Aβ1-42 (A) or α-syn1-140 (B). Basal Ca^{2+} fluxes, which did not exceed 5% of the whole response, were subtracted from the kinetics. The micrographs taken at the time of amyloid peptide injection (t_o) and at the end of the experiment after 60 or 75 min (t_{60} and t_{75}) showed that the increase in intracellular Ca^{2+} concerns most cells of the population. Data were expressed as fluorescence after treatment (F_t) divided by fluorescence before treatment (F_{t0}) and multiplied by 100. The images show pseudocolor representations of cells (scale bar: 100 μm), warmer colors corresponding to higher fluorescence. Data were expressed as mean ± SEM (n = 70 in A, and 57 in B). For both Aβ and α-synuclein (C), the Ca^{2+} response (black bars) was strongly decreased in the presence of 50 μM of the amyloid pore inhibitor Zn^{2+} (blue bars). Data were expressed as mean ± SEM. Student's t-test was used to compare the statistical significance on calcium dependent fluorescence after α-synuclein (***; $p = 1.6759 \times 10^{-6}$) or Aβ peptide (***; $p = 9.6898 \times 10^{-25}$) treatment between control and Zn^{2+} conditions. Biochemical quantitations showed that the metabolic inhibitor of glycosphingolipid biosynthesis PPMP (10 μM, 48 h) markedly decreased the cellular expression of total gangliosides (GG), especially GM1 and GD1a (D). In contrast, PPMP increased the expression of ceramides (Cer), GalCer and sphingomyelin (SM). Results are expressed as mean % ± S.E.M. of lipid content in PPMP-treated cells vs. control cells (the dotted red line indicates the control value for each lipid). Immunofluorescence studies with anti-GM1 antibody (E) showed that PPMP almost abrogated the cell surface expression of GM1 (green fluorescence), both in cell bodies and dendrites (bars: 10 μm). Cell nuclei were visualized by using Dapi (blue). Amyloid pore formation was decreased by PPMP treatment (F). Student's t-test was used to compare the statistical significance on calcium dependent fluorescence after α-synuclein (***; $p = 0.00035$) or Aβ peptide (***; $p = 4.0459 \times 10^{-8}$) treatment between control (black bars) and PPMP (blue bars) conditions. The kinetics of Ca^{2+} fluxes induced by Aβ22-35 (220 nM) in PPMP-treated cells (red curve, n = 118 cells) and in control untreated cells (black curve, n = 108 cells) are shown in G. The corresponding micrographs taken at time 0 (t_0) and 60 min (t_{60}) are shown in H (scale bar: 100 μm).

to amyloid pore formation but specifically affected ganglioside-dependent mechanisms of oligomerization.

Overall, these data strongly suggest that amyloid pore formation by both Aβ1-42 and α-syn1-140 is a ganglioside-dependent process. This is consistent with the known ability of these proteins to interact with various brain gangliosides [30,36]. However, although the interaction of both Aβ1-42 and α-syn1-140 with individual gangliosides has been extensively studied, it remained to establish whether these proteins could bind to natural gangliosides expressed by amyloid pore-forming neural cells such as SH-SY5Y cells. To assess this point, we studied the interaction of Aβ1-42 and α-syn1-140 with gangliosides extracted from these cells. In these experiments, the natural gangliosides were prepared as monolayers at the air-water interface and the interaction of the amyloid proteins with these gangliosides was studied by real-time surface pressure measurements [46,55]. Under these conditions, we could demonstrate that both Aβ1-42 and α-syn1-140 bind to natural brain cell gangliosides (Fig. 2A).

At this point, these data suggested that both Aβ1-42 and α-syn1-140 share a common pathway of amyloid pore formation controlled by an initial interaction of these proteins with cell surface gangliosides. This interaction is mediated by a common ganglioside-binding domain (GBD) [40]. This domain has sufficient structural homology to ensure ganglioside recognition, but also significant sequence variation to confer a high specificity in the repertory of gangliosides recognized by each protein [36]. In Aβ1-42, the pair of His residues is mandatory for GM1 binding. These His residues do not exist in the GBD of α-syn1-140 (they are replaced respectively by Ser and Lys). As a consequence, α-syn1-140 has a lower avidity for GM1, and interacts preferentially with GM3 through multiple contacts involving the terminal basic residues and the central Tyr of the motif. On the basis of an exhaustive analysis of the ganglioside-binding properties of Aβ1-42 and α-syn1-140 with the Langmuir technique, we created a chimeric peptide that combined the presence of the His pair derived from Aβ1-42 in the framework of the α-synuclein motif, i.e. KEGVLYVGHHTK [40]. This chimeric peptide displayed universal ganglioside-binding properties [40]. As expected, it readily interacted with gangliosides extracted from SH-SY5Y cells (Fig. 2A). Molecular dynamics simulations gave some light on this remarkable feature (Fig. 2B). In the case of gangliosides with only one sialic acid (e.g. GM1), each His of the pair interact with the sialic acid of one GM1, so that the peptide-GM1 complex involves two GM1 molecules in a typical chalice-like geometry [40]. When the ganglioside has more than one sialic acid (e.g. for the di-sialyl GD1a), the His pair forms a clamp around the sialic acid residues of the same ganglioside. Then, the remaining part of the chimeric peptide adapts its shape to the ganglioside dimer as shown in Fig. 2B. Besides these primary interactions with His residues, the terminal basic residues and, in some cases, the central aromatic residue of the chimeric peptide, further stabilize the complex.

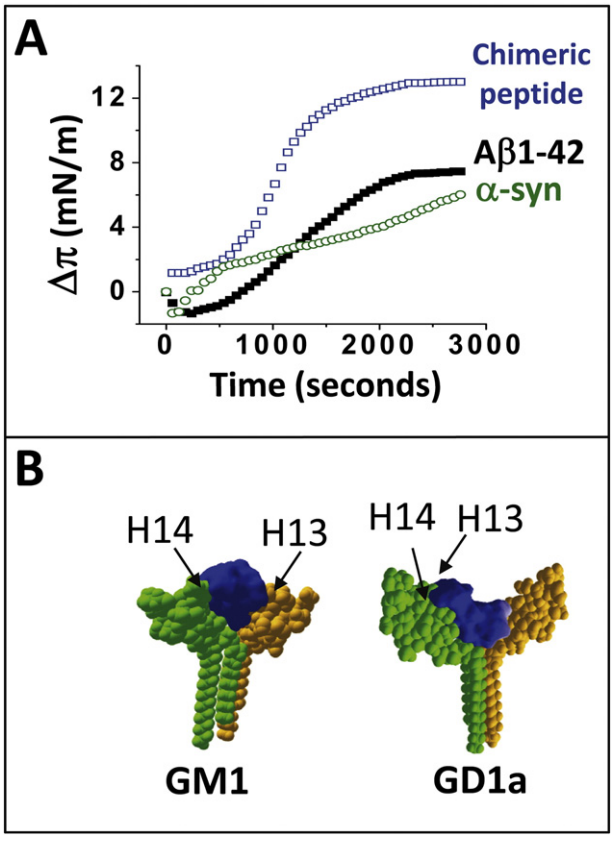

Fig. 2. Ganglioside binding to Aβ1-42 and to the chimeric peptide. A. Microtensiometry measurements indicated that both Aβ1-42 (black curve) and α-syncuclein (green curve) interacted with reconstituted monolayers of total gangliosides purified from SH-SY5Y cells. The chimeric peptide (blue curve) also interacted with these gangliosides, but at a higher rate and more quickly. B. Models of the chimeric peptide (in blue) docked onto chalice-shaped dimers of gangliosides GM1 and GD1a (in each ganglioside dimer, one ganglioside is in green and the other in orange). The ganglioside sites bound to His-13 and His-14 are indicated.

Next we investigated the effect of the chimeric peptide on amyloid pore formation induced by Aβ1-42 and α-syn1-140. For the sake of comparison, we also studied the effects of the respective ganglioside-binding motif of each protein, i.e. Aβ1-16 [55] and α-syn34-50 [56]. In these experiments, the cells were first loaded with Fluo-4AM and then incubated with 220 nM of either Aβ1-42 or α-syn1-140. The real ganglioside-binding domains or the chimeric peptide were tested at the same concentration (220 nM), in competition with these proteins.

In the case of Aβ1-42, amyloid pore formation was strongly inhibited by its homologous ganglioside-binding domain, i.e. Aβ1-16 (Fig. 3, upper panels). However, the ganglioside-binding domain of α-synuclein (α-syn34-50) had no effect on the intracellular calcium fluxes induced by Aβ1-42. This is expected because Aβ1-16 and α-syn34-50 do not have the same ganglioside-binding properties. Then we tested the activity of the chimeric peptide on this process. Remarkably, we observed that the chimeric peptide had a strong inhibitory effect on Aβ1-42-induced Ca^{2+} fluxes (Fig. 3, upper panels). Compared with the ganglioside-binding domain of wild-type α-synuclein, the chimeric peptide has a pair of His residues in place of Ser-42 and Lys-43. As a matter of fact, this pair of His residues, which confers to the chimeric an extended recognition of gangliosides, is a key feature of its biological activity.

In the case of α-syn1-140, the data are even more conclusive because although the real ganglioside-binding domain (α-syn34-50) inhibits amyloid pore formation, it is not able to totally prevent the intracellular calcium rise induced by the full-length protein (Fig. 3, lower panels). Most importantly, the chimeric peptide proved to be significantly more active than the original α-syn34-50 peptide. These data further emphasize the central role played by the pair of His residues in the chimeric peptide. The chimeric peptide has been rationally designed on the basis of thorough physicochemical studies of protein–ganglioside interactions [40]. Here we show that this peptide is also a universal inhibitor of amyloid pore formation that is able to block the intracellular Ca^{2+} fluxes induced by both Aβ1-42 and α-syn1-140.

Inherited forms of Parkinson disease have been associated to a set of mutations that arise in α-synuclein. In particular, three mutant proteins have been involved, including Ala-30-Pro (A30P), Glu-46-Lys (E46K) and Ala-53-Thr (A53T). Since none of these mutations affect the ganglioside binding domain of α-synuclein, it is surmised that all these mutant proteins can still interact with cell surface gangliosides, as demonstrated for the E46K mutant [56]. Moreover, atomic force microscopy studies showed that mutant forms of amyloid proteins could indeed form typical donut-like amyloid pores [20,57]. When incubated with SH-SY5Y cells preloaded with Fluo-4 AM, all three α-synuclein mutant proteins (A30P, E46K and A53T) readily induced an increase

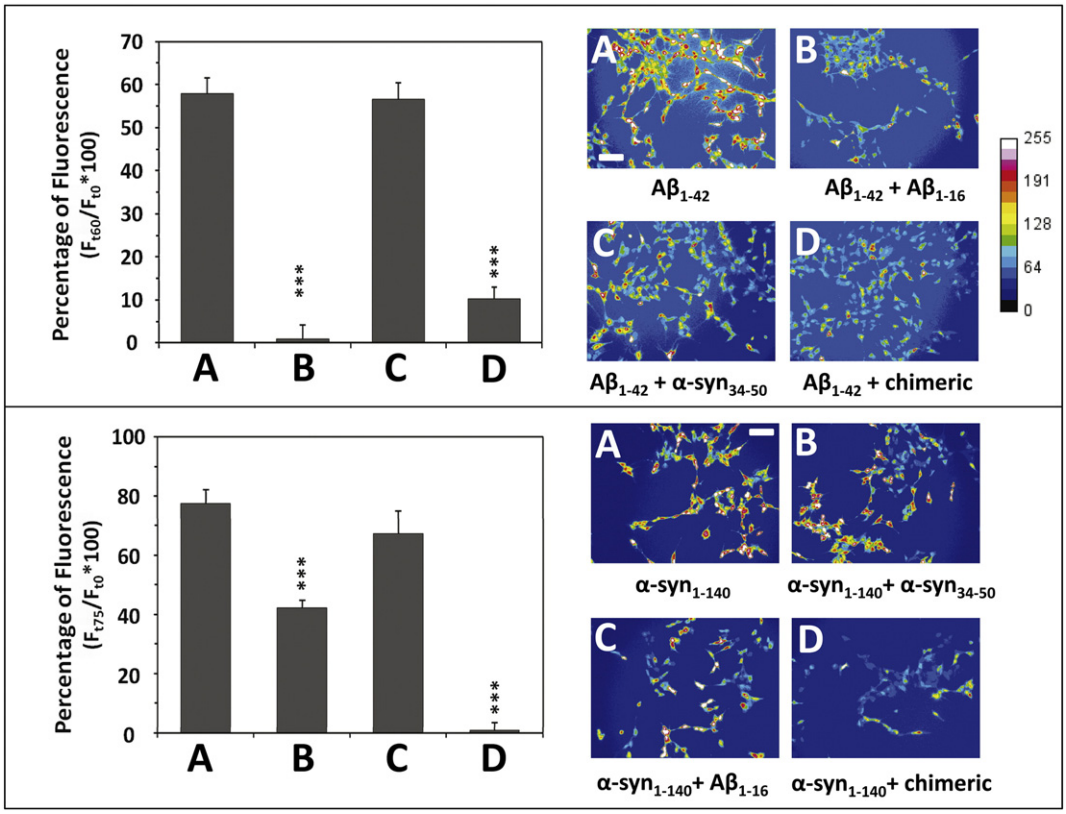

Fig. 3. The chimeric peptide has both anti-Alzheimer and anti-Parkinson properties. Upper panels — Cells were loaded with Fluo-4 AM and Ca^{2+}-dependent fluorescence was measured after injection of Aβ1-42 peptide alone (A, n = 80), or with Aβ1-16 peptide (B, n = 75), or with α-syn34-50 (C, n = 100), or with chimeric peptide (D, n = 85). Solutions of Aβ1-42 and Aβ1-16, Aβ1-42 and α- syn34-50, and Aβ1-42 peptide and chimeric peptide (both 220 nM) were mixed extemporaneously and directly injected onto the cells. Student's t-test was used to compare the statistical significance on fluorescence between Aβ1-42 with or without Aβ1-16 (***; $p = 6.4893 \times 10^{-18}$) or between Aβ1-42 with or without chimeric peptide (***; $p = 7.9218 \times 10^{-19}$). Lower panels — Cells were loaded with Fluo-4 AM and Ca^{2+}-dependent fluorescence was measured after injection of α-syn1-140 alone (A, n = 125), or with α-syn34-50 (B, n = 117), or with Aβ1-16 peptide (C, n = 116), or with chimeric peptide (D, n = 75). Solutions of α-synuclein1-140 and α-syn34-50, α-synuclein1-140 and Aβ1-16 peptide, and α-synuclein1-140 and chimeric peptide (both 220 nM) were mixed extemporaneously and directly injected onto the cells. Results were expressed as mean ± SEM. Student's t-test was used to compare the statistical significance on fluorescence between α-synuclein with or without α-syn34-50 (***; $p = 2.9853 \times 10^{-10}$) or between α-synuclein with or without chimeric peptide (***; $p = 2.5653 \times 10^{-46}$). The images show pseudocolor representations of cells (scale bar: 100 μm), warmer colors corresponding to higher fluorescence.

in intracellular Ca^{2+} concentration (Fig. 4). The chimeric peptide, used in competition at equimolar concentrations, abrogated the effect for all mutants. Thus, the chimeric peptide has broad anti-pore properties that affect amyloid pore formation induced by both wild-type and mutant amyloid proteins.

Since amyloid pores are now considered as the main neurotoxic species of amyloid in neurodegenerative diseases, these data suggested that the chimeric peptide could logically antagonize post-calcium deleterious effects induced by amyloid proteins in neural cells. To assess this point, we first analyzed the ability of the chimeric peptide to restore

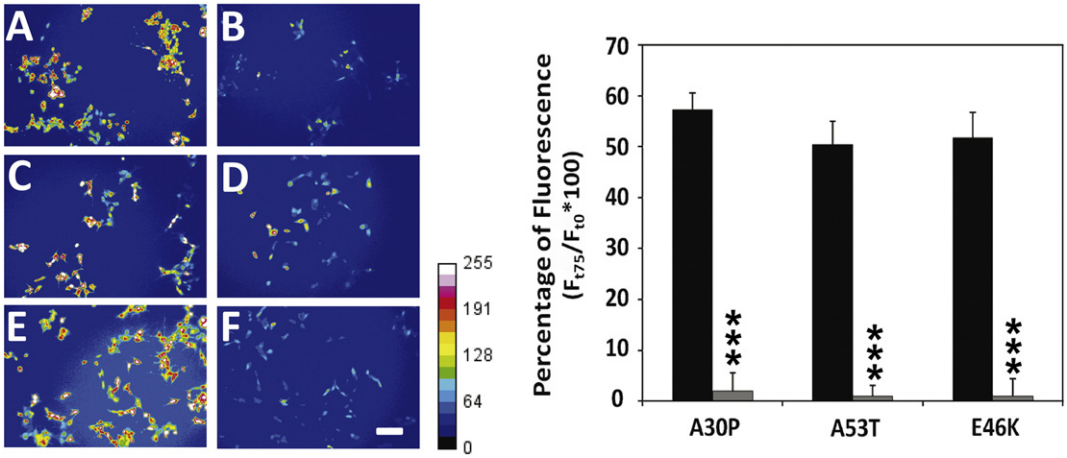

Fig. 4. The chimeric peptide is active against Parkinson's disease-associated mutant forms of α-synuclein. Cells loaded with Fluo-4 AM were treated with α-synuclein mutants A30P (n = 121 and 171), A53T (n = 121 and 174) or E46K (n = 103 and 150) in the presence or in the absence of chimeric peptide (A, C, E: cells incubated with mutant proteins alone, respectively A30P, A53T and E46K; B, D, F, cells incubated with both mutant proteins and chimeric peptide, respectively A30P + peptide, A53T + peptide and E46K + peptide). The images show pseudocolor representations of cells (scale bar: 100 μm), warmer colors corresponding to the higher fluorescence. Results in the histogram are expressed as mean ± SEM. Student's t-test was used to compare the statistical significance on fluorescence between: A30P and A30P + chimeric peptide (***; $p = 7.53 \times 10^{-20}$), A53T and A53T + chimeric peptide (***; $p = 1.42 \times 10^{-29}$) and E46K and E46K + chimeric peptide (***; $p = 5.91 \times 10^{-19}$).

synaptic functions that are notoriously perturbed by Aβ1-42 oligomers [58]. In these experiments, we used the uptake of the fluorescent dye FM1-43 into synaptic recycling vesicles as a measure of synaptic function [47,59]. Secretory neurotransmitter vesicles have been previously detected in the SH-SY5Y cell line [60,61] so that these cells, which sustain amyloid pore formation, can also be used to study synaptic vesicle recycling. We found that acute treatment of SH-SY5Y cells with Aβ1-42 increased the resting pool of vesicles, as assessed by a marked increase in the fluorescent signal, especially in submembrane areas of both cellular bodies and neuritic extensions (Fig. 5). Such an effect was not observed in control cells. In the presence of the chimeric peptide, Aβ1-42 did no longer induce this typical fluorescence increase and the cells recovered control levels. Therefore, the chimeric peptide is a potent antagonist of Aβ1-42-induced impairment of synaptic functions.

Finally, we investigated the effect of Aβ1-42 on the spontaneous network activity of the hippocampal CA1 region, a parameter that is considered to be related to the cognitive impairment observed during the early phases of Alzheimer's disease. Extracellular population recordings of rat hippocampal slices showed that Aβ1-42 markedly affected the frequency of hippocampal network activity. The chimeric peptide could efficiently antagonize these deleterious effects since the above-mentioned perturbations induced by Aβ1-42 were no longer observed in the presence of equimolar concentrations of chimeric peptide (Fig. 6).

4. Discussion

Until recently, amyloid fibers and plaques have been considered as the main culprits of several neurodegenerative diseases, including Alzheimer's and Parkinson's. Following the failure of tentative therapeutic strategies targeting amyloid plaques, an alternative paradigm is progressively emerging [3]. In this new model, amyloid proteins are still considered as responsible of the diseases, but now in the form of small oligomeric structures [12]. Among this neurotoxic oligomers, those forming Ca^{2+}-permeable annular channels, referred to as 'amyloid pores', have retained much attention [21,62]. Because these oligomers recall bacterial pore-forming toxins [62], several authors have underscored the fact that amyloid proteins could be considered as 'infectious proteins' [3] that perturb ion homeostasis [23] through their ability to perforate the plasma membrane of brain cells [63,64]. At this stage it is not clear whether monomeric forms of amyloid proteins penetrate the plasma membrane and then oligomerize into channels or whether preformed oligomers punch the membrane (or both). Nevertheless, in all cases it seems that the cell surface binding of amyloid monomers/oligomers involves a primary interaction with a ganglioside [29,65]. Among these potential ganglioside binding sites, GM1 has received special attention [28–37,55]. Most importantly, a recent study indicates that GM1 mediates the binding and neurotoxicity of soluble Aβ1-42 oligomers in mouse hippocampal slices [29]. We have recently deciphered the molecular mechanisms that control amyloid binding to gangliosides [40]. We showed that this binding obeys a biochemical code that determines the ganglioside-binding specificity of each amyloid protein [40]. This code is based on the presence of a common ganglioside-binding motif that consists of a common hairpin domain [66] exhibiting both common amino acid residues at critical locations and specific amino acid variations that control the repertory of gangliosides recognized by each protein [36]. Deciphering this code has allowed the creation of a universal ganglioside-binding peptide based on a chimer between the ganglioside-binding domains of Aβ1-42 and α-synuclein [40]. Specifically, the chimeric peptide combines the framework of the minimal ganglioside-binding domain of α-synuclein (fragments 34–45) with the presence of a pair of contiguous His residues derived from Aβ1-42 (His-13/His-14). It is important to note that the mechanistic interpretations that have led to the design of this peptide are based on data obtained with the Langmuir film balance technology, using reconstituted ganglioside monolayers and a series of wild-type and mutant peptides derived from both Aβ and α-synuclein [36,40]. This approach has proved to be useful for deciphering numerous lipid–protein interactions with low amounts of both lipids and proteins [22,67]. The involvement of His-13 and His-14 of Aβ in GM1 binding and amyloid pore formation has independently been established by other studies performed with bilayer membrane systems [68,69]. The rat Aβ protein, which differs from human Aβ at three positions (5, 10, and 13) has a lower avidity for GM1 and is far less toxic for neural cells than its human counterpart [34]. This is consistent with our finding that at nanomolar concentrations, rat Aβ does not induce Ca^{2+} fluxes in SH-SY5Y cells (Data in brief). It has also been shown that Aβ1-40 could form aggregates that remain located at the polar-apolar interface of GM1 clusters in a topology that seems inconsistent with the formation of a pore channel [31]. The apparent discrepancy between these data and our study might be due to the fact that these authors used Aβ1-40 and not Aβ1-42 (Aβ1-40 has a shorter apolar domain than Aβ1-42). The circular dichroism spectra recorded by Utsumi et al. also required high concentrations of Aβ (50 μM instead of 220 nM in our study) which may favor the amyloid aggregation at the surface of the GM1 cluster. Finally, the interaction of Aβ with bicelles, i.e. lipid structures with a high curvature [31] might as well induce specific modes of aggregation.

In any case, although both Aβ1-42 and α-synuclein have their own repertory of gangliosides, the chimeric peptide recognizes all gangliosides tested so far [40], including the four major gangliosides expressed in brain, i.e. GM1, GD1a, GD1b and GT1b [70,71]. The aim of the present study was to evaluate the ability of this chimeric peptide to prevent amyloid pore formation induced by both Aβ1-42 and α-synuclein.

In a first series of experiments we showed that both Aβ1-42 and α-synuclein could, at nanomolar concentrations, induce the formation of

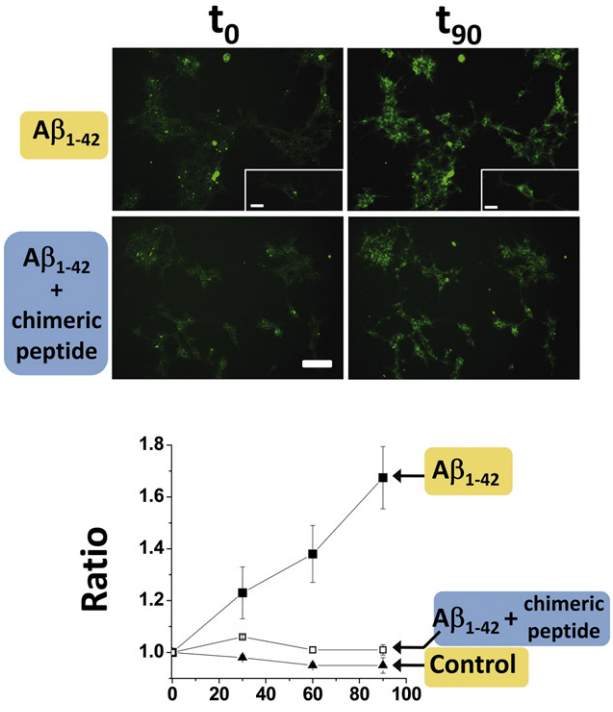

Fig. 5. The chimeric peptide prevents Aβ1-42-induced impairment of synaptic vesicles recycling. Cells were treated with FM1-43 dye, added directly under the lens of the microscope. Fluorescence was measured before and 30, 60 and 90 min on control cells (n = 59) or after injection of Aβ1-42 peptide (220 nM; n = 55), or Aβ1-42 peptide/chimeric peptide (both 220 nM; n = 60). Data were expressed as fluorescence after treatment divided by fluorescence before treatment. The images show representations of cells (scale bar: 10 μm) before injection and 90 min later, on cells after treatment with Aβ1-42 peptide (220 nM), or Aβ1-42 peptide/chimeric peptide (220 nM). Results were expressed as mean ± SEM. Student's t-test was used to compare the statistical significance on fluorescence between control and Aβ peptide conditions ($p = 1.1940 \times 10^{-8}$) or between Aβ peptide and Aβ peptide/chimeric peptide conditions ($p = 1.9577 \times 10^{-8}$).

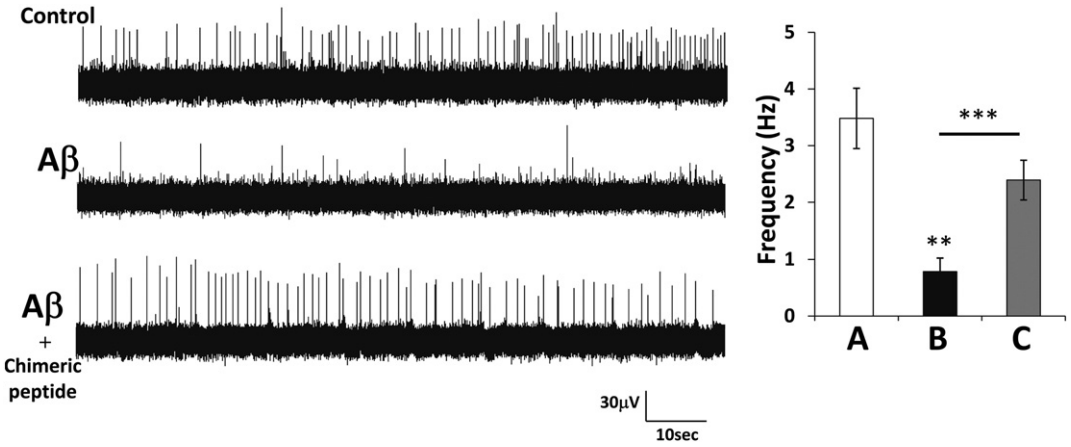

Fig. 6. The chimeric peptide prevents the deleterious effects of Aβ1-42 on the spontaneous activity of rat hippocampal slices. Hippocampal slices were incubated during 30–60 min with Aβ1-42 (n = 12; 500 nM) with or without chimeric peptide (n = 8; 500 nM) and then transferred to a recording chamber. In all cases, local field potentials were recorded and traces on the left are representative recordings of spontaneous activity of CA1. For all conditions, frequency was determined. Aβ1-42 produces a significant reduction of the spontaneous activity, which is counterbalanced in the presence of chimeric peptide. Legend of the histogram: A, control; B, Aβ1-42; C, Aβ1-42 + chimeric peptide. The values are indicated as the mean ± SEM.

amyloid pores in our cellular model, the human neuroblastoma cell line SH-SY5Y. These cells have been selected because we previously established that they represent a valuable model for studying lipid-dependent amyloid pores [46] and testing anti-pore compounds [25] at physiologically relevant (nanomolar) concentrations of both amyloid proteins and inhibitors. Moreover, these cells express several gangliosides including GM1, GM3, GD1a, and GT1b that are also expressed by the human brain [50–52]. The formation of amyloid pores was followed by intracellular Ca^{2+} measurements in cultured cells. The strong inhibitory effect of Zn^{2+}, a classic amyloid pore channel blocker [41,42], confirmed the formation of amyloid pores, in agreement with previous data [25,48].

The major outcomes of the present study can be summarized as follows: i) both wild-type Aβ1-42 and α-synuclein, as well as Parkinson's disease-associated mutant forms of α-synuclein readily induced amyloid pores in neural cells; ii) amyloid pore formation was specifically abrogated in ganglioside-deprived cells; iii) in the presence of equimolar concentrations of the chimeric peptide, the formation of amyloid pores induced by any of these proteins was no longer observed. In contrast, neither the ganglioside-binding domain of Aβ1-42 nor the one of α-synuclein could abrogate amyloid pore formation induced by heterologous proteins (i.e. the ganglioside-binding domain of Aβ had no effect on the pores formed by α-synuclein, and reciprocally). Altogether, these data indicate that cell surface gangliosides are required for the formation of amyloid pores and that the designed chimeric peptide, which has universal ganglioside-binding properties, also has a broad anti-pore activity. Moreover, we showed that by inhibiting amyloid pore formation, the chimeric peptide could also prevent more global deleterious effects induced by Aβ1-42 in neural cells. Specifically, the chimeric peptide blocked Aβ-associated impairments of synaptic functions by restoring normal vesicle recycling. Moreover, the chimeric peptide protected hippocampal slices from Aβ-induced disruption of spontaneous network activity, a parameter that is considered as a marker of the cognitive impairment observed during the course of Alzheimer's disease, especially in the early phases [72].

The originality of this approach is that the chimeric peptide that we developed can be used as a tentative therapeutic approach for *both* Alzheimers's and Parkinson's disease, including sporadic and inherited forms of these diseases. Indeed, our data show that this peptide is a universal blocker of amyloid pore formation induced by the wild-type and mutant forms of these proteins. The relationship between the broad activity of our peptide against various amyloid pores pertains to the involvement of gangliosides in the pathophysiology of most neurodegenerative diseases, including Alzheimer's [65,73], Parkinson's [56,74], but also Creutzfeldt-Jakob's [39,67]. Inasmuch as gangliosides mediate the interaction of any amyloid protein with the plasma membrane of brain cells, the chimeric peptide will bind to these gangliosides and competitively inhibit the attachment of the amyloid proteins. Since the adhesion of 'infectious' amyloid proteins precedes the formation of a functional amyloid pore within the membrane, the chimeric peptide is thought to act at the earliest steps of the disease. This is supported by a recent report describing the inhibitory effects of cholera toxin, a GM1-binding protein, on the deleterious effects of Aβ oligomers on long term potentiation (LTP) in mouse hippocampal slices [29]. These data are remarkably convergent with our demonstration that the chimeric peptide efficiently prevented Aβ-induced perturbations of hippocampal network activity. Nevertheless, it is important to emphasize that in addition to GM1, Aβ1-42 can interact with various gangliosides that can facilitate its binding on neuronal membranes (e.g. GM3 or GD1a) [29]. Because the chimeric peptide used in the present study has universal ganglioside-binding properties [40], it could theoretically prevent the binding of Aβ1-42 to any brain ganglioside.

We previously showed that the chimeric peptide is not toxic for neural cells, even at concentrations up to 200 μM (i.e. 1000-fold the active dose for blocking amyloid pores) [40]. Given the importance of gangliosides in brain development and functions [38], one could ask why a ganglioside-targeting peptide could be devoid of toxicity. In fact, there are two arguments that may explain the harmlessness of the chimeric peptide for brain cells. On one hand, structure–function relationship studies have demonstrated that the neurotoxicity of amyloid peptides is due to the presence of an apolar membrane-inserting domain which is distant from the ganglioside binding domain [75]. Indeed, the 25–35 fragment of Aβ1-42 and α-syn67-78 are neurotoxic, whereas Aβ1-16 and α-syn34-50 are not [40,75–77]. Thus, it is not surprising that the chimeric peptide, which is derived from the 34–45 sequence of α-synuclein, is devoid of neurotoxicity. On the other hand, it is likely that the chimeric peptide targets the same ganglioside population as the 'infectious' amyloid proteins, i.e. receptor-free gangliosides that are not associated with membrane receptors [38]. Since one of the main functions of brain gangliosides is to regulate neurotransmitter receptor functions through direct receptor–ganglioside interactions [54,78], the chimeric peptide is not expected to perturb synaptic functions [38].

Interestingly, one should note that the chimeric peptide (KEGVLYVGHHTK) contains both basic and aromatic residues that are a hallmark of a series of cell-penetrating peptides which are used as brain-delivery cargos. In particular, the SynB3 (RRLSYSRRRF) and the SynB5 (RGGRLSYSRRRFSTSTGR) vectors derived from the antimicrobial protein protegrin I are efficiently transported though the blood–brain barrier [79]. Moreover, it has recently been showed that small

peptides that recognize GM1 (e.g. the histidine-containing G23 peptide HLNILSTLWKYRC selected through a phage library strategy) are able to cross the blood–brain barrier into the brain parenchyma [80]. Taken together, these data suggest that the chimeric peptide described in the present study could probably cross the blood–brain barrier. This important issue is currently under investigation.

The possibility of a common therapy for neurodegenerative diseases involving amyloid proteins has been previously suggested by Glabe et al. who developed an anti-oligomer antibody able to recognize all amyloid oligomers through a common structural epitope [81]. The strategy that we propose here is a symmetric approach that targets, in a similar 'common structure-based' strategy, cell surface gangliosides. The analogy between both approaches may be even deeper if we consider that the common epitope identified by Glabe et al. could be related to the common ganglioside-binding domain expressed by all these proteins. This point warrants further investigation.

In conclusion we show here that a short chimeric peptide, designed after deciphering the biochemical code controlling ganglioside/amyloid interactions, is a potent blocker of amyloid pore formation induced by Aβ1-42 and α-synuclein. This peptide is a prototype whose in vitro properties suggest that a single 'silver bullet' drug curing several neurodegenerative diseases [82] can be seriously considered.

Transparency document

The Transparency document associated with this article can be found, in online version.

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