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Partitioning and confinement of GM1 ganglioside induced by amyloid aggregates

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ABSTRACT

Growing evidence shows that GM1 ganglioside is involved in amyloid deposition and toxicity. By means of real-time single particle tracking, we show that amyloid oligomers and aggregates formed by Aβ1–42 and amylin, two peptides associated, respectively, with the development of Alzheimer's disease and type II diabetes, interact with GM1 and decrease dramatically its lateral diffusion on the plasma membrane of living neuroblastoma cells. The confinement of GM1, a constituent of membrane rafts involved in neuroprotection, at the level of both types of amyloid aggregates can interfere with cell signaling pathways and contribute to the loss of neuroprotection.
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1. Introduction

Neuronal impairment in Alzheimer's disease (AD) is currently attributed to a complex cascade of events triggered by the interaction of amyloid oligomers, constituted primarily by Aβ1–42 peptide, with the plasma membrane [1]. Amongst the variety of toxic mechanisms proposed, one involves the binding of amyloid species to GM1 gangliosides [2–6]. GM1 takes part into the formation of membrane rafts, dynamic and specialized membrane microdomains responsible for the compartmentalization of cellular processes such as signalling and protein trafficking [7,8]. Altered distribution of GM1 and GM2 gangliosides has been recently found in AD brains [9]. The interaction with GM1 has been demonstrated to be a crucial factor also in mediating the aggregation and toxicity of other amyloidogenic proteins and peptides [10–12], such as amylin (also known as human islet polypeptide, hIAPP), whose aggregation and binding to the plasma membrane is thought to be the main factor determining the death of pancreatic β-cells in type II diabetes [13]. In particular, in vitro experiments show that the aggregation and seeding of Aβ and amylin peptides on synthetic membranes are enhanced in the presence of GM1 [10,14,15], as well as in vivo studies demonstrate increased assembly at the level of membrane rafts [12,16,17]. Conversely, GM1 is fundamental in mediating the binding of preformed Aβ oligomers and amylin aggregates to synthetic lipid vesicles and causing their subsequent permeabilitzation [12,18]. Exogenously applied oligomeric Aβ1–42 has been shown to accumulate on the membrane of cultured neurons at the level of rafts enriched in GM1 [17]. Nevertheless, experiments providing compelling evidence of interaction between Aβ oligomers and GM1 in living cells are still missing. The consistent, although sometimes conflicting, body of literature on the interaction of GM1 with amyloid species relies on averaged results obtained using bulk methods. In this case, many important details can be missed and only the most prominent features are eventually taken into account.

Here we take advantage of single particle tracking (SPT) techniques to monitor in real-time in living cells the dynamics of GM1 following the binding of amyloid aggregates of Aβ1–42 and amylin to the plasma membrane. We demonstrate that a direct interaction takes place in vivo, heavily affecting the diffusion properties of a subpopulation of GM1 molecules. Our results might imply an additional mechanism of toxicity, where amyloid aggregates alter cellular processes dependent on membrane raft mobility and clustering.

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2. Materials and methods

2.1. Cell culture

Human SH-SY5Y neuroblastoma cells were obtained from A.T.C.C. (Manassas, VA) and cultured in 1:1 DMEM/F-12 supplemented with 10% FBS and 1.0% antibiotics. Cell cultures were maintained in a 5.0% CO_2 humidified atmosphere at 37 °C and grown until 80% confluence. Cells were used for a maximum of 20 passages.

2.2. Oligomers preparation and cells treatment

Aβ1–42 (A9810) and amylin (D2162) were purchased from Sigma. Amylin or Aβ1–42 peptides were initially incubated in hexafluoroisopropanol (HFIP) at room temperature for 1 h. The HFIP was evaporated under a gentle stream of N_2. Dried peptide aliquots were dissolved in anhydrous DMSO to a final concentration of 5.0 mM, diluted into ice-cold phenol red-free F12 medium to 100 μM and incubated at 4 °C for 24 h. Aβ1–42 and amylin samples were transferred directly to the cell medium to a final concentration of 10 μM monomer equivalent for 1 h at 37 °C.

2.3. Immunolabeling

Following standard protocols, cells previously exposed to amyloid aggregates were fixed in 4% paraformaldehyde for 10 min at room temperature, and then blocked with bovine serum albumin (BSA, 1 mg/ml, Sigma–Aldrich). Mouse monoclonal anti-Aβ (β-Amyloid DE2B4, Santa Cruz Biotechnology Inc., 1:100 dilution) and anti-amylin (Amylin R10/99, Santa Cruz Biotechnology Inc., 1:50 dilution) antibodies were used as primary antibodies (50 min). Alexa 568 anti-mouse antibodies (Invitrogen) were used as secondary antibodies at 1:500 dilution (30 min). No labeling was observed when cells were incubated in the absence of aggregates. GM1 was labeled by incubating cells for 20 min with 10 μg/ml biotinylated ctxb (C9972, Sigma) and subsequentely with streptavidin-Atto 488 (Atto Tec) at 1:500 dilution (20 min). Finally, we incubated cover slips for 1 min with 1 μg/ml Hoechst 33342 (Invitrogen). Washing was performed between each step in PBS. The cover slips were mounted on glass microscope slides with a Mowiol 4-88 solution (9% Mowiol w/w, 22.7% glycerol w/w, 0.2 M Tris–HCl, pH 8.5, 3 mM NaN_3.

Cells were monitored with a Nikon Eclipse TE300 inverted microscope equipped with a Nikon Plan-Apo 60XA/1.40 n.a. DICH WD 0.21 oil immersion objective. Excitation light was provided by a mercury lamp (Hg 100 W). Fluorescence signal was detected by a Hamamatsu Photonics K.K. Japan high sensitivity silicon-intensified target (SIT) camera, coupled to a Hamamatsu Argus Image Processor and a Hamamatsu C2400 camera controller. Digital images were captured using a custom-made Virtual Instrument on Labview 7.1.

2.4. PLA

In situ Proximity Ligation Assay (PLA) was performed with Duolink kit (Olink, Bioscience) with minor modifications at the level of primary antibody labeling. Basically, two primary antibodies raised in different species recognize the target antigen or antigens of interest. Species-specific secondary antibodies, called PLA probes, each with a unique short DNA strand attached to it, bind to the primary antibodies. When the PLA probes are in close proximity (<40 nm), the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides. After joining of the two added oligonucleotides by enzymatic ligation, they are amplified via rolling circle amplification using a polymerase. After the amplification reaction, several-hundredfold replication of the DNA circle has occurred, and labeled complementary oligonucleotide probes highlight the product. The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a distinct bright dot. Here, cells previously exposed to amyloid aggregates were fixed following the protocol above and incubated with anti-Aβ1–42 or anti-amylin (1:100 or 1:50, respectively) for 20 min, and anti-mouse PLA probe. Regarding the anti-rabbit PLA probe the labeling procedure was more elaborated but still feasible. Cells were incubated for 10 min with 10 μg/ml biotinylated ctxb, subsequentely with 10 μg/ml streptavidin (Invitrogen) for 1 min, with biotinylated anti-rabbit Fab antibodies (1:400, Abcam) for 15 min, then with a randomly chosen rabbit antibody (in this case we used anti-hypf-N, 1:100), and finally with the anti-rabbit PLA probe.

2.5. Single particle imaging and tracking

Quantum dots (QDs) labeling and live imaging has been extensively described in [19]. Briefly, living cells previously exposed to amyloid aggregates were incubated in phenol red-free Leibovitz's L-15 medium 10% FBS at 37 °C first with anti-Aβ1–42 or anti-amylin (1:100 or 1:50, respectively) for 20 min, then for 5 min with anti-mouse Alexa 488 (1:500) and 10 μg/ml biotinylated ctxb, and finally with streptavidin QDs (Invitrogen) in QD binding buffer for 1 min. QDs emitting at 655 nm were used at a 1:10 000 dilution. Cells were monitored with a custom-made wide-field epifluorescence microscope equipped with an oil-immersion objective (Nikon Plan Apo TIRF 60×/1.45), a Reliant 150 Select argon ion laser (excitation line 488 nm) and a heating chamber. A FF499-Di01-25 dichroic, and FF01-655/15-25 (for QDs) and FF01-530/43-25 (for Alexa 488) emission filters (Semrock) were used. 250 or 100 consecutive frames were acquired with an integration time of 330 ms, respectively, with an Electron Multiplying Charge-Coupled device camera PI-Max (Roper Scientific) using WinView (PI Acton, Roper Scientific). Recording sessions did not last more than 30 min.

Tracking of single QDs, which were identified by their fluorescence intermittence, was performed with MATLAB (MathWorks, Natick, MA) using a homemade macro that accounts for blinking in the fluorescence signal [19–21]. In brief, the method consisted of two main steps, applied successively to each frame of the sequence. First, fluorescent spots were detected by cross-correlating the image with a Gaussian model of the Point Spread Function. A least-squares Gaussian fit was applied (around the local maximum above a threshold) to determine the center of each spot with a spatial accuracy of 10–20 nm (depending on the signal-to-noise ratio). Second, QD trajectories were assembled automatically by linking, from frame to frame, the centres of the fluorescent spots likely coming from the same QD. The association criterion was based on the assumption of free Brownian diffusion and took into account short blinking events. After completion of the process, a manual association step was performed, in which QD trajectories of maximal length were assembled from smaller fragments separated by longer blinking events that were not taken into account by the automatic linking procedure. A high concentration of pentavalent B subunit of cholera toxin can in principle induce crosslinking of GM1 [22]. For SPT experiments, however, we incubated the cells with ctxb for times shorter than for standard immunolabeling experiments, thus obtaining a lower level of labeling. The concentration of strep-QDs was largely in excess with respect of biotin-ctxb. Most if not all the ctxb molecules bound to the plasma membrane are therefore expected to be labeled. In our analysis we only monitored the dynamics of all the blinking QDs, indicative of single, not crosslinked, ctxb molecules, and avoided permanently

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