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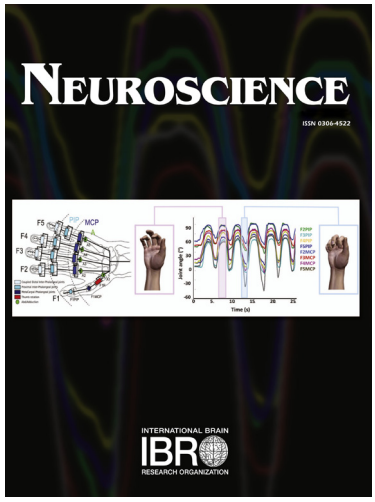

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Ana C. Pascual, Virginia L. Gaveglio, Norma M. Giusto, Susana J. Pasquaré

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2-arachidonoylglycerol metabolism is differently modulated by oligomeric and fibrillar conformations of amyloid beta in synaptic terminals

Ana C. Pascual, Virginia L. Gaveglio, Norma M. Giusto and Susana J. Pasquaré*

Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB, UNS-CONICET), Edificio E1, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina

TEL: ++54 291 4861201. FAX: ++54 291 4861200

e-mail: pasquare@criba.edu.ar

***Correspondence to:** S.J. Pasquaré, Instituto de Investigaciones Bioquímicas de Bahía Blanca (INIBIBB), CONICET-Bahía Blanca and Universidad Nacional del Sur (UNS), Edificio E1, Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina. TEL: ++54 291 4861201. FAX: ++54 291 4861200. e-mail: pasquare@criba.edu.ar

Abstract

Alzheimer´s disease (AD) is the most prevalent disorder of senile dementia mainly characterized by amyloid-beta peptide (Aβ) deposits in the brain. Cannabinoids are relevant to AD as they exert several beneficial effects in many models of this disease. Still, whether the endocannabinoid system is either up- or down-regulated in AD has not yet been fully elucidated. Thus, the aim of the present paper was to analyze endocannabinoid 2-arachidonoylglycerol (2-AG) metabolism in cerebral cortex synaptosomes incubated with Aβ oligomers or fibrils. These Aβ conformations were obtained by "aging" the 1-40 fragment of the peptide under different agitation and time conditions. A diminished availability of 2-AG resulting from a significant decrease in diacylglycerol lipase (DAGL) activity was observed in the presence of large $A\beta_{1-40}$ oligomers along with synaptosomal membrane damage, as judged by transmission electron microscopy and LDH release. Conversely, a high availability of 2-AG resulting from an increase in DAGL and lysophosphatidic acid phosphohydrolase activities occurred in the presence of $A\beta_{1-40}$ fibrils although synaptosomal membrane disruption was also observed. Interestingly, neither synaptosomal mitochondrial viability assayed by MTT reduction nor membrane lipid peroxidation assayed by TBARS formation measurements were altered by $A\beta_{1-40}$ oligomers or fibrils. These results show a differential effect of $A\beta_{1-40}$ peptide on 2-AG metabolism depending on its conformation.

Keywords: Alzheimer´s disease, amyloid-beta peptide, 2-arachidonoylglycerol, synaptosomes

Introduction

Alzheimer´s disease (AD) is a neurodegenerative process that depends on aging but differs from physiological aging. The first event in the pathogenesis of AD is the deposition of amyloid-beta peptide (Aβ) which precedes the aggregation of hyperphosphorylated tau protein generating neurofibrillary tangles (LaFerla, 2010). AD progression leads to synaptic loss, reduced dendritic arbors and neuronal loss in several brain regions, thus affecting multiple neurotransmitter systems (Duyckaerts and Dickson, 2011). The progressive accumulation of Aβ is due to an imbalance between its production and clearance (Crews et al., 2010; Querfurth et al., 2010). Aβ derives from the amyloid precursor protein (APP) by a proteolytic process (Haass et al., 1999; Walter et al., 2001) that generates peptides of 40 and 42 amino acid residues. The assembly of monomeric Aβ into multimeric structures leads first to the formation of oligomers which finally, by an elongation phase, form larger polymers called fibrils (Kumar et al., 2011). Although deposits of Aβ fibrils into plaques have been proposed as the neurotoxic agents of AD pathology, current evidence relates oligomers to the initial state of this disease. In this context, it has been suggested that synaptic plasticity is inhibited by oligomers (Cleary et al., 2005). This leads to a different hypothesis on the neuropathology of AD whose first event could be a consequence of oligomer-induced synaptic dysfunction which causes memory loss (Hardy et al., 2002; Lacor et al., 2004). On the other hand, oligomer aggregations into fibrils and their deposition into plaques could represent an inactive reservoir of neurotoxic oligomers (Lacor et al., 2004).

Endocannabinoid-triggered signaling may modulate several processes that occur prior to the onset of dementia in neurodegenerative pathologies including AD (Stella et al., 1997; Marsicano et al., 2003; Aso et al., 2014). The endocannabinoid system (ECS) is a cell communication mechanism which comprises endogenous ligands, mainly anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) (Devane et al., 1992; Stella et al., 1997), cannabinoid receptors (CBR) (Matsuda et al., 1990; Munro et al., 1993), and enzymes which are involved in the biosynthesis and inactivation of endocannabinoids (Jonsson et al., 2006; Piscitelli et al., 2012)

Endocannabinoids have been shown to be involved in a large number of important pathophysiological processes (Cravatt et al., 2004; Di Marzo et al., 2005; Viveros et al., 2005). In line with this, it has been observed that the ECS plays an important role in AD (Ramirez et al., 2005). It has been reported that two key brain regions implicated in learning and memory, such as cortex and hippocampus, which are targets of this pathology, express high levels of some ECS components (Hopper et al., 1976; Mackie, 2005). Interestingly, many components of the ECS undergo different changes depending on the stage of AD (Basavarajappa et al., 2017), which are detailed in the Discussion section. It has also been suggested that the activation of CBR by synthetic and plant cannabinoids has beneficial effects on AD by reducing the detrimental Aβ action and tau phosphorylation as well as by activating repair mechanisms in the brain (Aso et al., 2014). As stated above, AD is an age-dependent neurodegenerative process. In this respect, we have previously demonstrated that aging modifies 2-AG metabolism decreasing its availability (Pascual et al., 2013) and also decreases CB1 and CB2 protein expression (Pascual et al., 2014a), in rat cerebral cortex (CC) synaptosomes. Furthermore, we have shown that 2-AG metabolism is modulated by CB1 and/or CB2 receptor antagonists in adult and aged rat CC synaptosomes (Pascual et al., 2014a). We have also observed that fatty acid amidohydrolase (FAAH) activity, an enzyme involved not only in AEA but also in 2-AG hydrolysis, decreases in the frontal cortex from AD patients and that this effect is mimicked by $A\beta_{1-40}$ (Pascual et al., 2014b). Taking into account that certain cannabinoid compounds exert neuroprotection against Aβ, (Ruiz-Valdepenas et al., 2010) and that 2-AG metabolism in neurodegenerative processes has been only partially explored, the main purpose of the present study was to analyze if the activities of the enzymes involved in 2-AG synthesis (lysophosphatidic acid phosphohydrolase-LPAase- and diacylglycerol lipase -DAGL- activities) and hydrolysis (mainly monoacylglycerol lipase -MAGL- activity) are modified by the presence of $A\beta_{1-40}$ peptide in an oligomeric or fibrillar conformation. To this end, we analyzed 2-AG metabolism in CC synaptic terminals, which are highly vulnerable neuronal structures in AD pathology.

Experimental procedure

Materials

[2-^{3}H]Glycerol (2 Ci/mmol) was obtained from PerkinElmer (Boston, MA, USA). Preblended Dry Fluor (98 % PPO and 2 % bis-MSD) was obtained from Research Products International Corp. (Mt. Prospect, IL, USA). Lysophosphatidic acid, 1-oleoyl [oleoyl-9,10-^{3}H(N)]-(54 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). Oleoyl-L-α lysophosphatidic acid, N-ethylmaleimide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton X-100, thiobarbituric acid (TBA) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). $A\beta_{1-40}$ was obtained from PolyPeptide (Strasbourg, France) and $A\beta_{1-42}$ from was obtained from Sigma-Aldrich (St. Louis, MO, USA). The kit (LDH-P UV AA) for measuring lactate dehydrogenase (LDH) activity was generously supplied by Wiener Laboratory (Rosario, Santa Fe, Argentina). Monoclonal antibody against Aβ peptide (sc-28365) and the horseradish peroxidase (HRP)-conjugated mouse IgGκ light chain binding protein (m-IgGκ BP-HRP, sc-516102) were generously supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of the highest purity available.

Preparation of synaptosomes

Wistar-strain adult rats (4 months old) were kept and killed, and CC was dissected, as previously described (Pascual et al., 2013). All procedures were carried out following the guidelines issued by the Animal Research Committee of the *Universidad Nacional del Sur* (Argentina) in accordance with the Guide of the Care and Use of Laboratory Animals of the Institute for Laboratory Animal Research (ILAR) of the National Academy of Science (Bethesda, MD). Synaptosomal isolation protocol was followed as described elsewhere (Pascual et al., 2013).

Preparation of human amyloid β_{1-40} and amyloid β_{1-42}

Oligomers and fibrils were obtained from monomers, based on the protocols described by Uranga and co-workers (Uranga et al., 2010) and by Martin-Moreno and co-workers (Martin-Moreno et al., 2011), respectively, introducing minor modifications. Aβ was resuspended in DMSO at a concentration of 10 mg/ml. From this solution, dilutions in PBS were prepared at a concentration of 80 µM and were "aged" at 37 °C for 2 hours with constant shaking (300 rpm) to obtain oligomers. Incubation was continued for 22 hours (24 hours in total) at 37 °C with constant shaking (150 rpm) to obtain fibrils. At each time as well as prior to peptide incubation, aliquots of the different conformations were taken to be analyzed by transmission electron microscopy (TEM) and to be incubated with synaptosomes.

Characterization of amyloid $\beta_{1–40}$ sizes by Western blot analysis

$A\beta_{1-40}$ peptide preparations (0.1-1.5 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 8 % and/or 16 % acrylamide-bisacrylamide gels under nonreducing conditions. After transferred to Immobilon P membranes using a Mini Trans-Blot cell electro blotter (BIO-RAD Life Science Group, CA), membranes were blocked for 5 hours with 5 % BSA in Tris-buffered saline (20 mM Tris–HCl, 150mMNaCl) pH 7.5 containing 0.1 % Tween 20 (TTBS). Membranes were incubated with anti-Aβ 1/500 for at 4 °C overnight and, after washing with TTBS, incubation with m-IgGk BP-HRP 1/500 for 2 hours at room temperature was performed. Proteins were visualized by chemiluminescence with Pirce ECL Western Blotting substrate (Thermos Scientific) using standard X-ray film (Kodak X-Omat AR). To corroborate molecular weights, Precision Plus Protein Kaleidoscope Standards (Bio-Rad) was used as a molecular weight marker.

Evaluation of the amyloid $\beta_{1–40}$ and amyloid β_{1-42} peptide aggregation state by transmission electron microscopy

Aliquots (4 µl) from1 µM and/or 80 µM of monomers, oligomers, and fibrils peptide preparations were taken. To test the stability of the oligomer conformation during preincubation and enzymatic assays, other aliquots from this preparation were incubated at 37 °C during 10 and 30 minutes more. Aliquots were placed on either

carbon-coated or -uncoated grids and incubated for 60 seconds. 10 µl of glutaraldehyde (0.5 %) were subsequently added to each grid. The grids were incubated for 60 seconds more, washed with distilled water and dried. They were finally stained with 2 % uranyl acetate for 2 minutes, dried and examined with a Jeol 100 Cx II electron microscope.

Preincubation of synaptosomes with amyloid $\beta_{1\text{-}40}$

Aliquots of synaptosomes equivalent to 50 µg of protein were preincubated for 10 minutes with DMSO ($A\beta_{1\text{-}40}$ vehicle) or with $A\beta_{1\text{-}40}$ conformations: monomers/dimers, oligomers, and fibrils.

Once preincubation of synaptosomes was finished, samples were used to a) be analyzed by TEM, b) determine thiobarbituric acid reactive substances (TBARS) generation, c) determine LDH activity, d) determine MTT reduction, and e) assay the different enzymatic activities involved in 2-AG metabolism, except for LPAase activity assays in which synaptosomes were first preincubated for 30 minutes with KML29 (MAGL inhibitor) (Chang et al., 2012) and then with N-ethylmaleimide (NEM, to specifically assay NEM-insensitive LAPase activity) (Baker et al., 2000) and $A\beta_{1\text{-}40}$ preparation for the last 10 minutes of the preincubation time.

Evaluation of synaptosomal structure in the presence of amyloid $\beta_{1–40}$ peptide by transmission electron microscopy

Samples of synaptosomal fraction previously incubated with DMSO or with the different preparations of $A\beta_{1\text{-}40}$ (monomers/dimers, oligomers or fibrils) were fixed with glutaraldehyde at a final concentration of 2.5 %. The fixed material was then centrifuged at 33,000 g for 20 minutes at 4 °C, and the pellet was washed with PBS and subsequently post-fixed with 2 % OsO_4. Samples were again centrifuged and the pellet was washed, dehydrated through acetone and embedded in resin. Sections were cut on an ultramicrotome and examined with Joel 100 Cx II electron microscope.

MTT assay

Synaptosomal reducing capacity was determined spectrophotometrically by measuring MTT reduction to the insoluble intracellular formazan crystals. The methods used in the present study were similar to those previously used by Uranga and co-workers (Uranga et al., 2010).

TBARS determination

The spectrophotometrical thiobarbituric acid assay was used to measure lipid peroxidation, similarly to that described elsewhere (Adamczyk et al., 2006). Briefly, after preincubation, 0.2 ml of 30 % trichloroacetic acid (TCA), 0.02 ml of 5 N HCl and 0.2 ml of 0.75 % thiobarbituric acid (TBA) were added to 0.1 ml of synaptosomes. The mixtures were heated at 100 °C for 15 minutes and centrifuged at 1000 g for 10 minutes. The supernatant was collected and thiobarbituric acid reactive substances were measured at 535 nm.

Measurement of LDH release

The activity of the enzyme LDH released from synaptosomes was determined in the supernatant after centrifugation at 33,000 g for 20 minutes at 4 °C, using an LDH-P UV AA kit following the manufacturer's instructions (Uranga et al., 2010).

Preparation of radiolabeled substrates

1,2-diacyl-sn-glycerol (DAG) and [2-^{3}H]triacylglycerol (TAG) were synthesized by incubating bovine retinas with [2-^{3}H]glycerol as previously described (Pasquare de Garcia et al., 1986; Pascual et al., 2013). Monoacyl-sn-Glycerol (MAG) was obtained from [2-^{3}H]TAG by incubation with pancreatic lipase as specified elsewhere (Brockerhoff, 1969; Pascual et al., 2013). Lipids were extracted either with n-hexane:2-propanol (3:2 v/v) or in accordance with Folch (Folch et al., 1957) to avoid DAG and MAG isomerization (Pascual et al., 2013). They were subsequently separated by one-dimensional thin layer chromatography (TLC) (Giusto et al., 1979; Pascual et al., 2013).

DAGL, LPAase and MAGL activities assays

Assays were performed using radiolabeled substrates as specified elsewhere (Pascual et al., 2013; Pascual et al., 2014a). Prior to LPAase activity assay, synaptosomes were incubated with 1 µM KML29 (MAGL inhibitor) for 30 minutes and in the presence of 4.4 mM N-ethylmaleimide (NEM), to specifically assay NEM-insensitive LAPase activity and $A\beta_{1-40}$ preparations for the last 10 minutes.

DAGL and LPAase enzymatic reactions were stopped by adding chloroform:methanol (2:1, v/v), and MAGL activity assay was stopped by adding chloroform:methanol (1:1, v/v) and 100 µl of the corresponding buffer. Blank preparation was identical to each enzymatic assay except that proteins were absent. Lipid products derived from DAGL and LPAase activities were extracted as described by Folch (Folch et al., 1957).

Separation of enzymatic reaction products

DAGL and LPAase products were separated by TLC (Giusto et al., 1979; Pascual et al., 2014a), visualized by exposure to iodine vapors and scraped off for counting by liquid scintillation. Glycerol, MAGL product, was obtained from the aqueous phase, concentrated to dryness and counted by liquid scintillation. Radiolabeled samples were counted after the addition of 0.25 ml of water and 5 ml of 0.5 % Preblended Dry Fluor in toluene/Triton X-100 (4:1, v/v).

Other methods

Protein content was determined following Lowry and co-workers (Lowry et al., 1951).

Statistical analysis

Data were analyzed by two-way ANOVA. To determine differences among our experimental conditions, a post-test (Bonferroni test) was used. Statistical analyses were performed using GraphPad software (San Diego, CA, USA, www.graphpad.com) and corroborated using InfoStat software, 2009p version (FCA — *Universidad Nacional de Córdoba* — Argentina, www.infostat.com.ar). Three pools (two animals per pool) were prepared and each one was used to

assay three replicates per condition. Each pool was considered as an individual sample (minimal value of n= 3). Statistical significance was set at $p<0.05$, thus considering 0.05 global error (α). All figures are given as mean values ± standard error (SE).

Results

Amyloid β peptide aggregation state

TEM was used to visualize Aβ morphology in the different conformation states. $A\beta_{1-40}$ solution (1 µM) images are shown in Fig. 1 (A, B, C1 and C2). Before incubating $A\beta_{1-40}$, various monomers separately arranged and with no association among themselves were observed (Fig. 1A). When the peptide was incubated at 37 °C for 2 hours, it evidenced oligomer-like structures which were not aggregated into fibrils (Fig. 1B). Incubation at 37 °C for 24 hours showed $A\beta_{1-40}$ as fibrillar structures (Fig. 1 C1 and 1 C2). In order to confirm that fibrillar structures were similar to those described in the literature, a major concentration of $A\beta_{1-40}$ (80 µM) incubated at 37 °C for 24 hours was also analyzed by TEM (Fig. 1 C3 and C4). This allowed us to visualize an abundant amount of fibrils crisscrossed and covering the entire microscope field, thus confirming the presence of fibrillar peptide structures. With the purpose of evaluating oligomer stability during preincubation and enzymatic assays, the samples of this conformation obtained at 120 minutes of $A\beta_{1-40}$ incubation were incubated for 10 and 30 minutes more. At both times, these structures were found neither to exhibit significant changes in their conformation states nor to reveal a fibril-like structure (data not shown). This corroborates oligomer stability during the time of synaptosome preincubation and enzymatic assays.

Western Blot of the solubilized $A\beta_{1-40}$ which was not "aged" showed a band migrating below 10 KDa (Fig. 2A). After a 2-hour incubation, the peptides were observed in a band whose molecular weight was higher than 250 kDa and which remained in the stacking gel, either using a 8 % (Fig. 2B 1) or a 16% (Fig. 2B 2) SDS-PAGE. Finally, after a 24-hour incubation, the peptides also remained in the stacking gel (Fig. 2C).

When TEM was performed during $A\beta_{1-42}$ “aging”, not all of the images in the preparation were the same as those observed with $A\beta_{1-40}$. Structures similar to those of the oligomers were observed before $A\beta_{1-42}$ peptide incubation rather than

separately arranged monomers or dimers. When $A\beta_{1-42}$ was incubated at 37 °C for 2 hours, these structures did not exhibit any significant changes in their conformation compared to those obtained before “aging”. Incubation at 37 °C for 24 hours showed $A\beta_{1-42}$ as short fibrillar structures similar to protofibrils (data not shown).

Synaptosomal structure in the presence of different amyloid β_{1-40} peptide conformations

To determine if synaptosome morphology could be modified by different $A\beta_{1-40}$ peptide conformations, TEM of these subcellular structures in the presence of monomer/dimer, oligomer and fibril forms of the peptide was used. In the absence of $A\beta_{1-40}$ peptide and either with or without DMSO (Aβ vehicle), synaptosomes were observed as round structures surrounded by intact membranes (Fig. 3 A1 and 3 A2). Microphotography in the presence of 0.1 µM or 1 µM of the monomeric/dimeric conformation showed that whereas most synaptosomes were still whole, a few of them seemed to have impaired membranes (Fig. 3 B1 and B2). Occasionally, a larger conformation, similar to that of small oligomers, could be observed either close to or upon synaptosomes. These images were more often seen in the presence of the highest concentration of the peptide (Fig. 3 B2). When synaptosomes were exposed to 0.1 µM $A\beta_{1-40}$ peptide oligomeric conformations, many of these structures showed no unimpaired membrane but had indistinguishable borders instead (Fig. 3 C1). These structures were most frequently seen at 1 µM oligomer concentration and represented the majority of the synaptosomes observed in these preparations (Fig. 3 C2). The damaged synaptosomes in these fields coexisted with one or two oligomers upon them (Fig. 3 C). On the other hand, $A\beta_{1-40}$ peptide fibrils at a concentration of 0.1 µM evoked the aggregation of synaptosomes around them and, similarly to what occurred with oligomers, the synaptosomal surrounding membranes were extremely damaged (Fig. 3 D1). In these preparations, peptides with a conformation similar to that of oligomers were also observed (Fig. 3 D1 2). Similar results were observed with the

highest concentration of $A\beta_{1-40}$ peptide fibrils, being even more difficult to find fields with isolated synaptosomes (Fig. 3 D2).

Synaptosomal damage markers in the presence of different amyloid β_{1-40} and amyloid β_{1-42} peptide conformations

In order to evaluate synaptosomal damage by the presence of Aβ monomers/dimers, oligomers and fibrils, different parameters were analyzed. Mitochondrial function was determined by the reduction of MTT to formazan crystals and lipid peroxidation was determined by the formation of TBARS. None of these parameters changed when synaptosomes were preincubated with the different $A\beta_{1-40}$ structures (data not shown). Membrane integrity was determined by measuring the release of LDH from synaptosomes after treatment with Aβ conformations. Results with $A\beta_{1-40}$ showed that, while monomers/dimers did not modify LDH activity, both oligomers and fibrils at either 0.1 µM or 1 µM concentration increased this enzymatic activity. LDH activity in the presence of oligomeric $A\beta_{1-40}$ was similar at both concentrations used (12-13 %) and showed no differences compared to what occurred with 0.1 µM fibrils (11 %). However, the increase in LDH activity with 1 µM fibrils (7 %) was significantly lower than with 1 µM oligomers (Fig. 4A). The effects observed on the treatment with $A\beta_{1-40}$ oligomers appeared to be dose-independent. However, LDH activity assays after the incubation of synaptosomes with low $A\beta_{1-40}$ oligomer concentrations (0.02 µM - 1 µM) indicated that the impairment exerted by oligomers is dose-dependent, showing statistical significant membrane damage at concentrations either equal to or higher than 0.1 µM (Fig. 4B). In view of this, 0.1 µM of $A\beta_{1-40}$ was used to perform the enzymatic assays because it significantly evoked a synaptosomal membrane damage which is a specific effect of oligomeric conformations and is also consistent with Aβ concentration in AD patients´ cerebrospinal fluid (Grimmer et al., 2009). The highest concentration (1 µM) was used taking into account our previous work, where a concentration-effect curve within 0.1 and 1 µM concentrations of Aβ was assayed (Mulder et al., 2011). When LDH activity was assayed with the preparations of $A\beta_{1-42}$ (at 0, 2 and 24 hours of incubation), results

showed a 10-18 % increase in LDH release from the synaptosomes with no differences among each other at either 0.1 or 1 µM concentration (data not shown).

LPAase activity in synaptosomes incubated with different amyloid β_{1-40} peptide conformations

Synaptosomes preincubated for 10 minutes with 0.1 µM of the different $A\beta_{1-40}$ peptide conformations showed no differences in LPAase activity (Fig. 5A). When a similar assay was carried out at 1 µM of the different $A\beta_{1-40}$ peptide structures, only the fibrillar conformation of the peptide produced a significant increase (9 %) compared to that observed in the absence of the peptide (Fig. 5A).

DAGL activity in synaptosomes incubated with different amyloid β_{1-40} peptide conformations

Synaptosomes preincubated with either 0.1 µM or 1 µM $A\beta_{1-40}$ monomers/dimers for 10 minutes caused no changes in DAGL activity, as observed in the absence of $A\beta_{1-40}$ peptide (control condition) (Fig. 5B). On the other hand, oligomeric $A\beta_{1-40}$ produced a decrease in DAGL activity of 41 % and 49 % at 0.1 µM and 1 µM, respectively, with no significant differences between these concentrations (Fig. 5B). Conversely, whereas fibrillar conformation of the peptide at the highest concentration used (1 µM) increased DAGL activity (26 %) with respect to control, at the lowest concentration (0.1 µM) no significant differences were observed (Fig. 5B).

MAGL activity in synaptosomes incubated with different amyloid β_{1-40} peptide conformations

MAGL activity in synaptosomes exposed to $A\beta_{1-40}$ monomers/dimers in both assayed concentrations showed no changes with respect to the control condition (Fig. 5C). Furthermore, the oligomeric conformation of the peptide induced a decrease in MAGL activity (8-9 %) with no differences between 0.1 µM and 1 µM concentrations (Fig. 5C). In contrast to what was observed with oligomers, 1 µM of fibrillar $A\beta_{1-40}$ increased enzymatic activity (4 %) with respect to the control

condition whereas 0.1 μM of this conformation produced no changes in MAG hydrolysis (Fig. 5C).

Discussion

Amyloid beta accumulation in the brain is a hallmark of AD pathology. Aβ deposition occurs not only extra- but also intraneuronally (Takahashi et al., 2004; Li et al., 2007; Gouras et al., 2010). Intraneuronal Aβ is found in the brain of AD patients and individuals with mild cognitive impairment (Gouras et al., 2000). Therefore, intracellular conglomeration is the first signal of a neuronal alteration that correlates with cognitive decline. Observations in AD human brain have also been corroborated in animal models (Takahashi et al., 2002; Billings et al., 2005; Bayer et al., 2008).

Due to its high aggregation rate, $A\beta_{1-42}$ peptide is thought to be more toxic than $A\beta_{1-40}$ (Jarrett et al., 1993; Dahlgren et al., 2002). However, as the 17-21 fragment of the native protein is the nucleation site, using either $A\beta_{1-40}$ or $A\beta_{1-42}$ could be considered the same (Hilbich et al., 1991). Our results from TEM analysis with the different preparations (at 0, 2 and 24 hours of incubation) of both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides evidenced that, while the shortest peptide formed monomers or dimers, oligomers, and fibrils at each incubation period, the largest fragment formed oligomer structures rapidly, and protofibrils at a 24-hour incubation. Moreover, Western Blot analysis demonstrated i) that the immediately solubilized $A\beta_{1-40}$ peptide (not "aged") corresponded mainly to molecular structures below 10 kDa weight (monomers or dimers), ii) that after a 2-hour incubation, $A\beta_{1-40}$ corresponded to oligomeric molecular species larger than 250 kDa (more than 58 subunits), and iii) that after a 24-hour incubation, it corresponded to fibrils which remain in the stacking gel. In line with this, Bitan and co-workers (Bitan et al., 2003) demonstrated that $A\beta_{1-40}$ oligomerization is slower than that of $A\beta_{1-42}$, thus forming more easily monomer, dimer and tetramer structures while the larger peptide forms predominantly pentamers and hexamers.

LDH assays in synaptosomes incubated with $A\beta_{1-42}$ revealed that all the preparations exerted damage in the synaptosomal membrane at the same rate (data not shown). These results are in accordance with the above-mentioned TEM assays, as the structures obtained before and after "aging" $A\beta_{1-42}$ peptide for 2

hours not only had the same oligomeric structure but also evoked the same synaptosomal damage. Taking into account that the main purpose of our study was to analyze if 2-AG metabolism is modified by the presence of Aβ peptide in an oligomeric or fibrillar conformation, our results suggest that it is more convenient to use $A\beta_{1-40}$ than $A\beta_{1-42}$ because the former forms structures similar to monomers or dimers, oligomers, and fibrils more easily than $A\beta_{1-42}$.

Our results obtained by TEM in synaptosomes incubated in the presence of either $A\beta_{1-40}$ oligomers or fibrils showed an increase in the release of LDH, thus indicating that both $A\beta_{1-40}$ conformations alter synaptosomal membrane. In addition, fibrils generated the association of the synaptosomes which presented oligomers upon them, thus suggesting that oligomeric conformation could also be generated from Aβ fibrils. The fact that these oligomers were observed in those synaptosomes whose membranes were damaged suggests a localization of these $A\beta_{1-40}$ structures inside axon terminals, in agreement with results from previous studies (Kokubo et al., 2005; Pickett et al., 2016). It has also been reported that although oligomers are assembled from extracellular monomers, they are also released from intracellular pools (Walsh et al., 2000).

In the presence of $A\beta_{1-40}$ fibrils, especially at the highest concentration (1 μM), synaptosomes were found to be agglomerated, being very difficult to observe isolated synaptosomes. At 1 μM only a few of these synaptosomes showed oligomers on them. Although the presence of 1 μM $A\beta_{1-40}$ oligomers or fibrils favored LDH release from synaptosomes, this release was higher in the presence of oligomers. This suggests that the deleterious effect on the synaptosomal membrane is mainly produced by the oligomeric conformation of $A\beta_{1-40}$. Although membrane damage possibly implies alterations in its components, when lipid peroxidation was analyzed, no differences were observed with respect to $A\beta_{1-40}$ monomeric/dimeric form (data not shown). In spite of this, our results do not lead us to discard a change in membrane lipid composition. As to the mechanism of action of Aβ, whereas some studies favor the insertion of Aβ oligomeric form into the lipid bilayer and the disruption of membrane-generating pores, others support

its accumulation by binding to a ligand at particular synapses (Kayed et al., 2003; Arispe, 2004; Jang et al., 2013; Yates et al., 2013). In this respect, previous research showed an association of oligomers with surface membrane proteins in the early stages of AD. These proteins, in particular, have been reported to be enriched in isolated synaptosomes (Lacor et al., 2004). Regardless of the mechanism by which Aβ oligomers produce this deleterious effect, we corroborated that synaptic membrane disruption is a consequence of their action.

Results derived from MTT assay showed that $A\beta_{1-40}$ did not appear to affect mitochondrial viability (data not shown) although mitochondria in AD may be affected by Aβ (Pinho et al., 2014), which may, in turn, induce impairment of its function (Lin et al., 2006).

Maintaining adequate levels of endocannabinoids is helpful as it not only mitigates the harmful effects of Aβ but also facilitates its clearance (Bachmeier et al., 2013). In AD some changes have been reported in the levels and/or activity of the enzymes and receptors related to ECS. In line with this, previous research has shown controversial results depending on the AD stage and the nervous system region analyzed (Basavarajappa et al., 2017). Previous research found loss of CB1-positive neurons in areas of microglial activation in the frontal cortex of *postmortem* AD patient brains and decreased protein expression and G-protein coupling although CB1 radioligand binding was preserved (Ramirez et al., 2005). A decrease in CB1 protein expression in *postmortem* cortical brain tissue from AD patients staged at Braak V/VI was also reported by Solas and co-workers (Solas et al., 2013). Conversely, autoradiography with a CB1 radioligand on *postmortem* prefrontal cortex samples from AD patients from Braak stages I to VI indicated an up-regulation of CB1 receptor density in early AD stages followed by a reduction of this increase in later stages (Farkas et al., 2012). In addition, no differences in CB1 protein density or location were found in cortical neurons from AD brains by immunohistochemical studies (Benito et al., 2003) nor in hippocampus from Braak stage III, IV or VI AD patients by immunoblotting or immunohistochemical assays (Mulder et al., 2011). In agreement with this, immunoblotting and radioligand

binding assays in frontal cortex, anterior cingulate gyrus, hippocampus and caudate nucleus (Lee et al., 2010), and *in vivo* positron emission tomography (PET) demonstrated that CB1 receptor levels were unchanged in AD brains (Ahmad et al., 2014). As to CB2, whereas previous reports revealed an overexpression in AD brain cortex by immunoblotting (Solas et al., 2013), specifically in neuritic plaque-associated astrocytes and microglia (Benito et al., 2003), other studies showed either no changes in frontal cortex (Ramirez et al., 2005) or a decrease in CB2 brain availability by *in vivo* PET studies (Ahmad et al., 2016). Additionally, Ramirez and co-workers reported an enhanced CB1 and CB2 receptor protein nitration in frontal cortex AD brains (Ramirez et al., 2005). As to the enzymes involved in 2-AG metabolism, and in parallel with AD progression, immunoblotting showed a gradual increase in DAGLα and DAGLβ protein expression in human hippocampus and immunochemistry showed a redistribution of DAGLβ to activated microglia surrounding senile plaques in Braak stage VI AD brains (Mulder et al., 2011). In this report immunoblotting and immunohistochemistry techniques were also used to demonstrate changes in 2-AG hydrolyzing enzymes serine hydrolase α/β-hydrolase domain-containing 6 (ABHD6) and MAGL in human hippocampus during AD progression. The former, which was expressed in microglia (particularly in Braak stage VI), was found to decrease gradually, while the latter was reported to increase and to shift progressively from damaged neurons to activated glia (Mulder et al., 2011). 2-AG hydrolysis assays revealed a subcellular redistribution of MAGL from cytosol to membranes in frontal cortex *postmortem* AD brain (Mulder et al., 2011). Although FAAH up-regulation was identified by immunohistochemistry in AD neuritic plaque-associated glia together with an increase in its activity in entorhinal and parahippocampal areas (Benito et al., 2003), we have demonstrated that whereas FAAH activity decreases in the frontal cortical membranes from AD patients, it undergoes no changes at the protein level (Pascual et al., 2014b).

As discussed above, changes in ECS strongly depend not only on the brain area but also on the type of cells (neuron or glia) to be analyzed. In our study, synaptosomes (subcellular fraction containing exclusively synaptic terminals) were

used to assay the effect of different $A\beta_{1-40}$ peptide conformations on the ECS, particularly on 2-AG metabolism. However, astrocytes have an important role in ‘‘tripartite synapses’’ influencing neuronal function. Furthermore, endocannabinoids participate in this neuron-astrocyte communication. Bari and co-workers have identified differences and similarities in the ECS between synaptosomes and gliosomes (sub-cellular particles obtained from astrocytes) from rat brain cortex (Bari et al., 2011). In this respect, no differences were reported in 2-AG and AEA levels, AEA metabolizing enzymes activities and 2-AG hydrolysis activity. However, compared to synaptosomes, DAGL activity was found to be greater in gliosomes. Also, while in synaptosomes ionotropic transient receptor potential vanilloid 1 (TRPV1, which can bind AEA) was found to have no effect and both CB1 and CB2 receptors inhibited the stimulus-induced release of glutamate in gliosomes, CB1 receptor increased this neurotransmitter release but CB2 receptor and TRPV1 inhibited it (Bari et al., 2011).

In the presence of $A\beta_{1-40}$ oligomers, we observed a greater decrease in DAGL activity compared to that observed in MAGL activity, without changes in LPAase activity, thus leading to a decrease in 2-AG availability (Fig. 6A). This decrease in the neuroprotector 2-AG caused by this $A\beta_{1-40}$ conformation could contribute to the progression of AD. As the enzymes involved in endocannabinoid metabolism exert their activities when they are associated to the membrane, it is therefore feasible that the damage caused by oligomers in the synaptosomal membrane could modify either the environment of the enzymes or their structures, thus decreasing their activities. This was observed for DAGL and MAGL which could suggest that oligomers interfere particularly in lipase activity (Prescott et al., 1983; Dinh et al., 2004) since LPAase, whose mechanism of action is different (Nakane et al., 2002), is not affected.

On the other hand, in the presence of $A\beta_{1-40}$ fibrils, a higher increase in the synthetic activities with respect to the increase observed in the hydrolytic activity may lead to higher 2-AG availability (Fig. 6B). The effect exerted by fibrils could be a consequence of synaptosomal agglomeration produced by this $A\beta_{1-40}$

conformation, partially preventing the access of oligomers to the synaptic terminal, which could minimize the damage in the synaptosomal membrane. This is supported by the fact that when a higher concentration of fibrils was used, synaptosomes were much more agglomerated and membrane damage was lower than with oligomers. This is consistent with the above-mentioned hypothesis, according to which fibril deposits could constitute a reservoir of neurotoxic oligomers (Lacor et al., 2004), thus preventing their deleterious action.

We have previously reported that the net balance between 2-AG synthesis and hydrolysis reveals low availability of this endocannabinoid in synaptic terminals during aging (Pascual et al., 2013). The results collected in the presence of $A\beta_{1-40}$ oligomers are consistent with those observed in aging, thus suggesting in both cases a decrease in the neuroprotection exerted by 2-AG.

Conclusions

Summing up, our results lead us to conclude that 2-AG availability is differently modulated by $A\beta_{1-40}$ conformations through the enzymatic activities responsible for its metabolism. Thus, oligomeric $A\beta_{1-40}$ conformation, which has been recently proposed to be the neurotoxic agent in AD, decreases neuroprotector 2-AG availability. In this respect, the enzymes involved in 2-AG synthesis and hydrolysis could, therefore, be promising therapeutic targets in AD.

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Conflict of interest

The authors declare no conflicts of interest including any financial, personal or other relationships with other people or organizations.

Author contribution

SJP and ACP conceived and designed the experiments and analyzed the data. NMG helped with the design of the experiments. ACP and VLG performed the experiments. SJP wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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Figure legends

Figure 1. TEM of amyloid β_{1-40} peptide preparation during the different incubation periods: A: 0 hours, B: 2 hours and C: 24 hours. Aliquots from 1 µM (A, B, C1, and C2) or 80 µM $A\beta_{1-40}$ (C3 and C4) were taken before (A) and after "aging" the peptide at different incubation periods (B and C). Aliquots were placed on carbon-coated (A, B1, C1 and C2) or -uncoated (B2, C3 and C4) grids, fixed with 0.5 % glutaraldehyde, stained with 2 % uranyl acetate and examined with an electron microscope. Figures correspond to: C3 and B1 40,000x, C1 80,000x, A1, B2, C2 and C4 140,000x, A2 200,000x.

Figure 2. Immunoblot analysis of amyloid β_{1-40} peptide preparation during different incubation periods: A: 0 hours, B: 2 hours and C: 24 hours. Aliquots (0.1-1.5 µg protein) were taken before (A) and after "aging" the peptide in different incubation periods (B and C), and they were separated by SDS-PAGE in 8 % (B1 and C) and/or 16 % (A and B2) acrylamide-bisacrylamide gels under nonreducing conditions. Transference to membranes, blocking, incubation with primary and secondary antibodies and chemiluminescence detection were performed as specified in the Experimental procedure section. Numbers on the right indicate molecular weights.

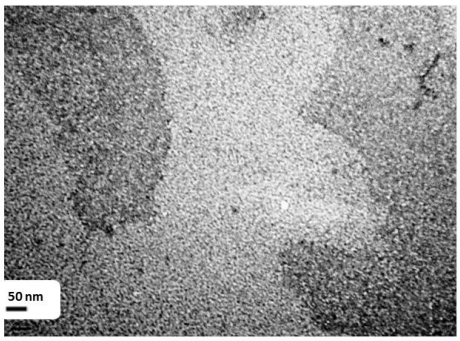
Figure 3. TEM of adult rat CC synaptosomes incubated with A: DMSO, B: $A\beta_{1-40}$ monomers/dimers, C: $A\beta_{1-40}$ oligomers and D: $A\beta_{1-40}$ fibrils. Aliquots of synaptosomes equivalent to 50 µg of protein were incubated for 10 minutes with DMSO ($A\beta_{1-40}$ vehicle) or with 0.1 µM and 1 µM of the different $A\beta_{1-40}$ conformations: monomers/dimers, oligomers, and fibrils. After incubation, samples were fixed with glutaraldehyde in a final concentration of 2.5 %, post-fixed with 2 % OsO_4, dehydrated through acetone and embedded in resin. Sections were cut on an ultramicrotome and examined with an electron microscope. Figures correspond to: D1 1 40,000x, A1, B1 1, B2 1, C1 1, C1 2, C2 1, D2 1 and D2 2 80,000x; A2, B1 2, B2 2, C1 3, C1 4, C2 2, D1 2, D2 3 and D2 4 140,000x. Black arrows indicate synaptosomes with impaired membrane while white arrows indicate the presence of $A\beta_{1-40}$ oligomers.

Figure 4. Activity of LDH released from adult rat CC synaptosomes after treatments with $A\beta_{1-40}$ peptide. Synaptosomes (50 µg of protein) were preincubated for 10 minutes with $A\beta_{1-40}$ monomers/dimers, oligomers or fibrils at 0.1 µM and 1 µM concentrations (A) or $A\beta_{1-40}$ oligomers at 0.02, 0.05, 0.07, 0.1 µM and 1 µM concentrations (B), precipitated by centrifugation and LDH activity was measured in the supernatant by spectrophotometry using the LDH-P UV AA kit. Results are expressed as a percentage of corresponding control values (100 %) and represent the mean ± SE of a minimum of three individual samples. ***$p<0.001$, **$p<0.01$ and *$p<0.05$ with respect to control condition; $^{++}p<0.01$ with respect to 1 µM oligomers.

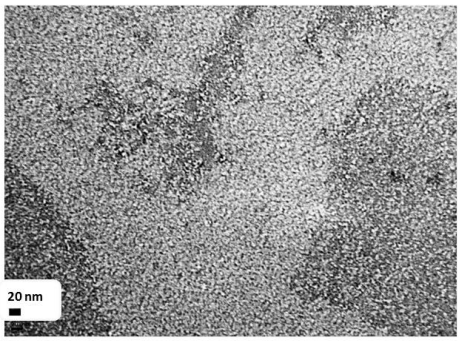
Figure 5. Enzymatic activities involved in 2-AG metabolism. LPAase (A), DAGL (B) and MAGL (C) activities in adult rat CC synaptosomes after treatments with $A\beta_{1-40}$. Synaptosomes (50 µg of protein) were preincubated with 0.1 µM or 1 µM of $A\beta_{1-40}$ monomers/dimers, oligomers or fibrils and subsequently incubated with the radiolabeled substrate as specified in Experimental procedure section. Results are expressed as a percentage of corresponding control values (100 %) and represent the mean ± SE of a minimum of three individual samples. ***$p<0.001$ and **$p<0.01$ with respect to control condition.

Figure 6. Modulation of 2-AG metabolism by $A\beta_{1-40}$ peptide. A: $A\beta_{1-40}$ oligomers decreased 2-AG availability in adult rat CC synaptosomes by inhibiting its synthesis (DAGL) in a higher percentage than its hydrolysis. B: $A\beta_{1-40}$ fibrils increased 2-AG availability in adult rat CC synaptosomes by stimulating its synthesis (DAGL and LPAase) in a higher percentage than its hydrolysis. LPA: 1-oleoyllysophosphatidicacid, DAG: diacylglycerol, 2-AG: 2-arachydonoylglycerol, LPAase: lysophosphatidic acid phosphohydrolase, DAGL: diacylglycerol lipase, MAGL: monoacylglycerol lipase.

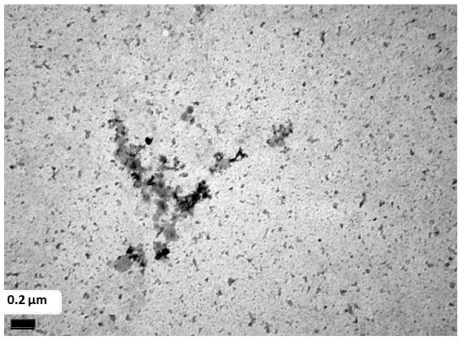
A1 Time= 0 hs [Aβ]= 1 µM



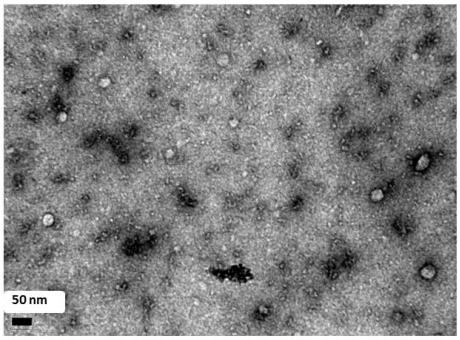
A2 Time= 0 hs [Aβ]= 1 µM



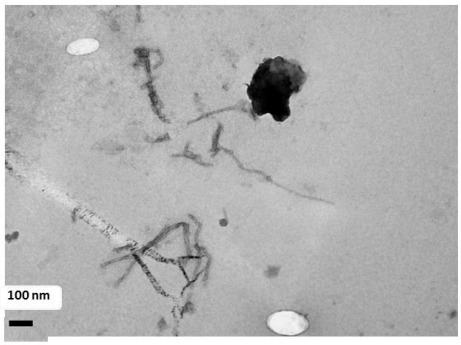
B1 Time= 2 hs [Aβ]= 1 µM



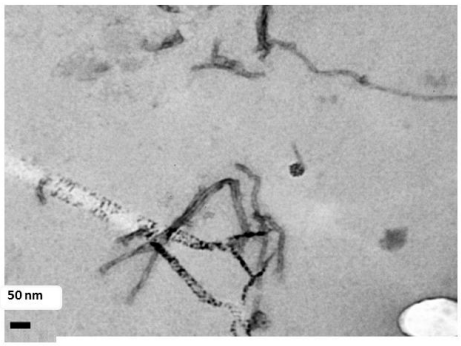
B2 Time= 2 hs [Aβ]= 1 µM



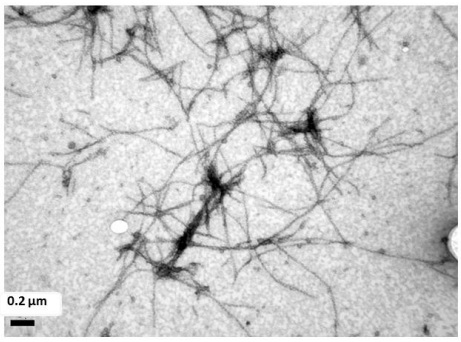
C1 Time= 24 hs [Aβ]= 1 µM



C2 Time= 24 hs [Aβ]= 1 µM



C3 Time= 24 hs [Aβ]= 80 µM



C4 Time= 24 hs [Aβ]= 80 µM

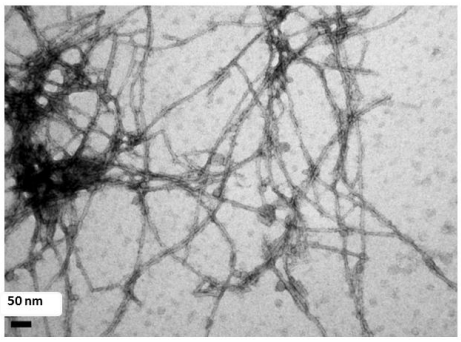


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Figure 1

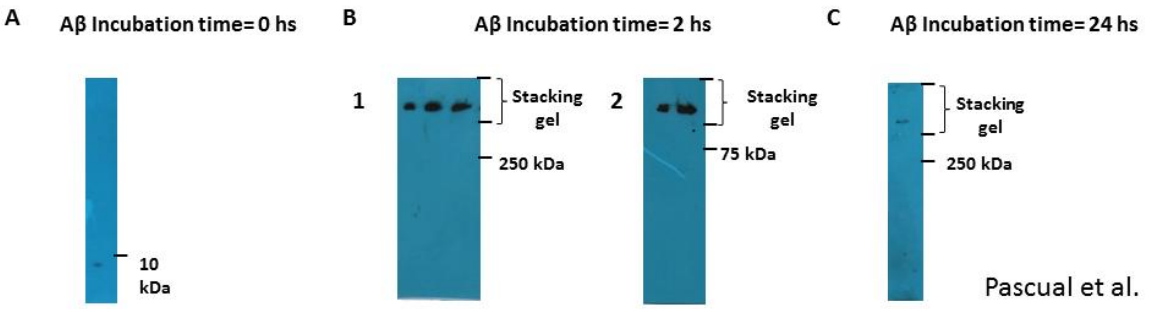

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Figure 2

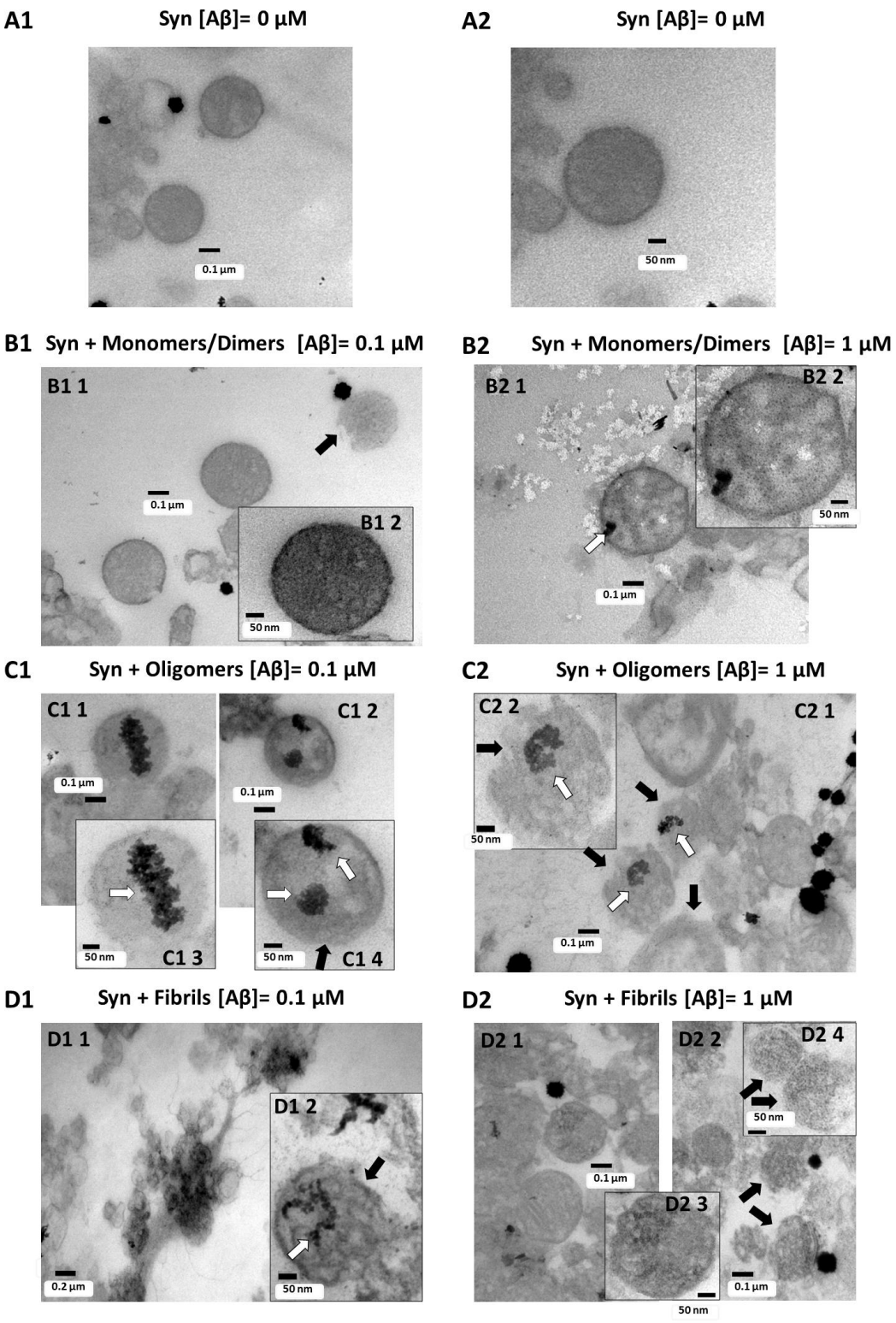

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Figure 3

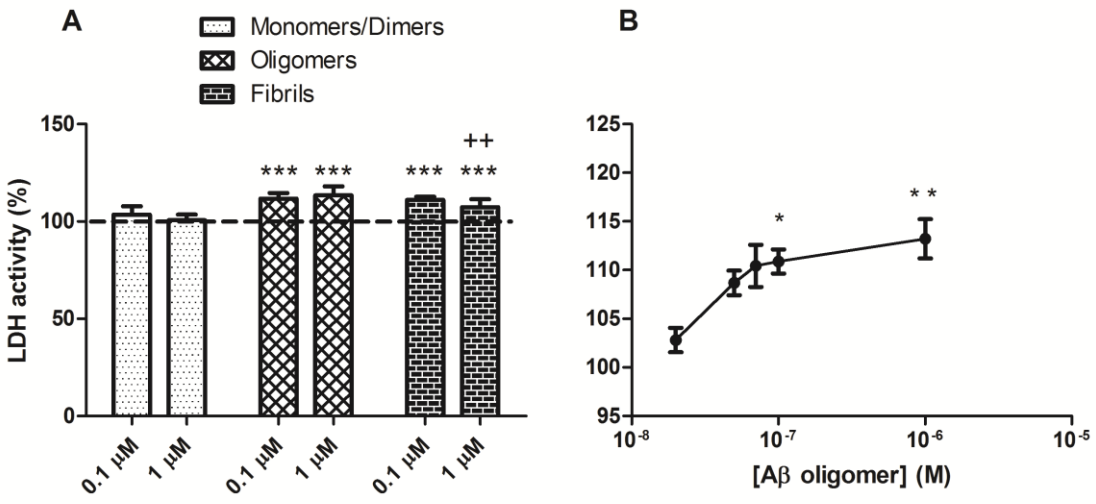

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Figure 4

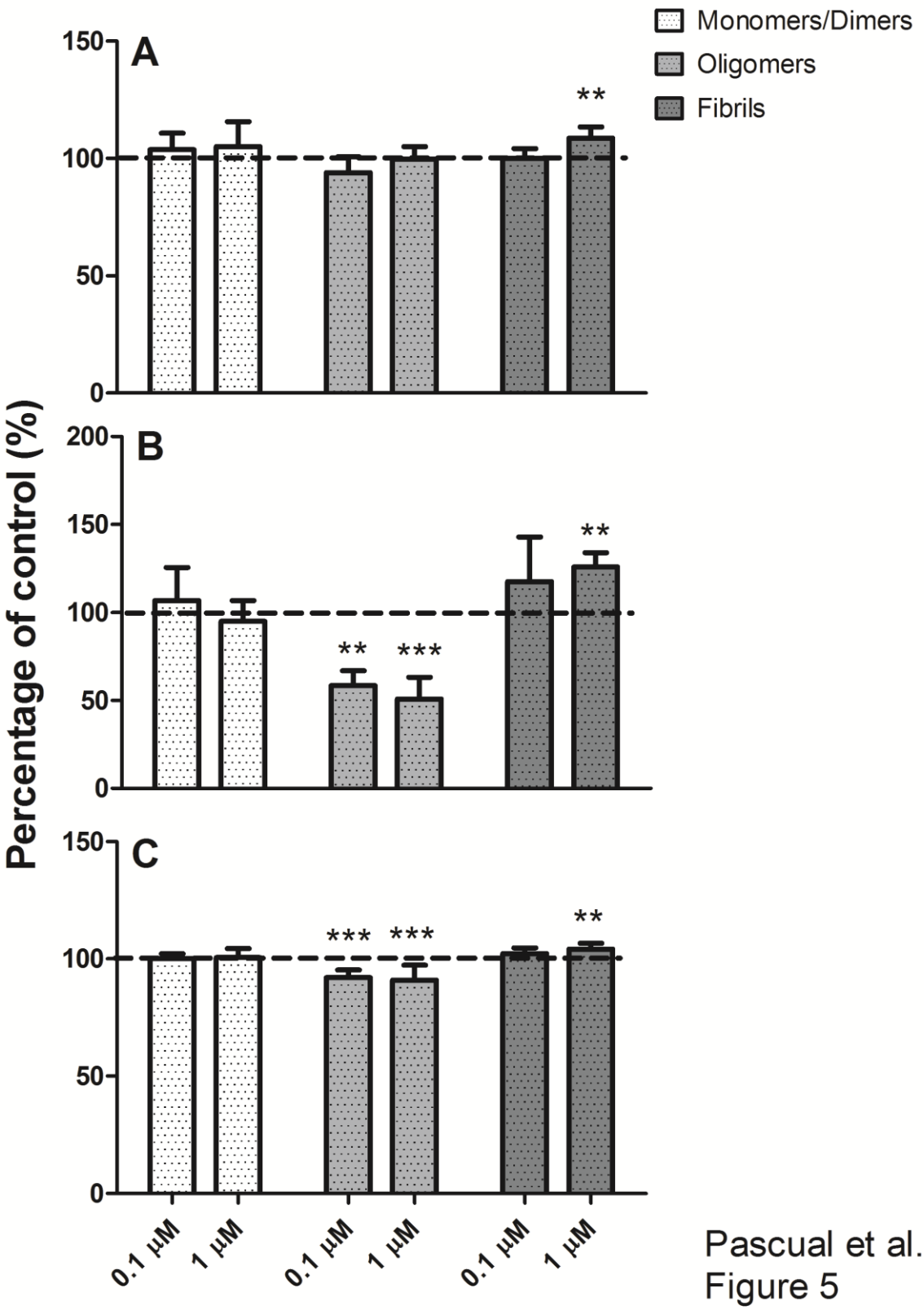

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Figure 5

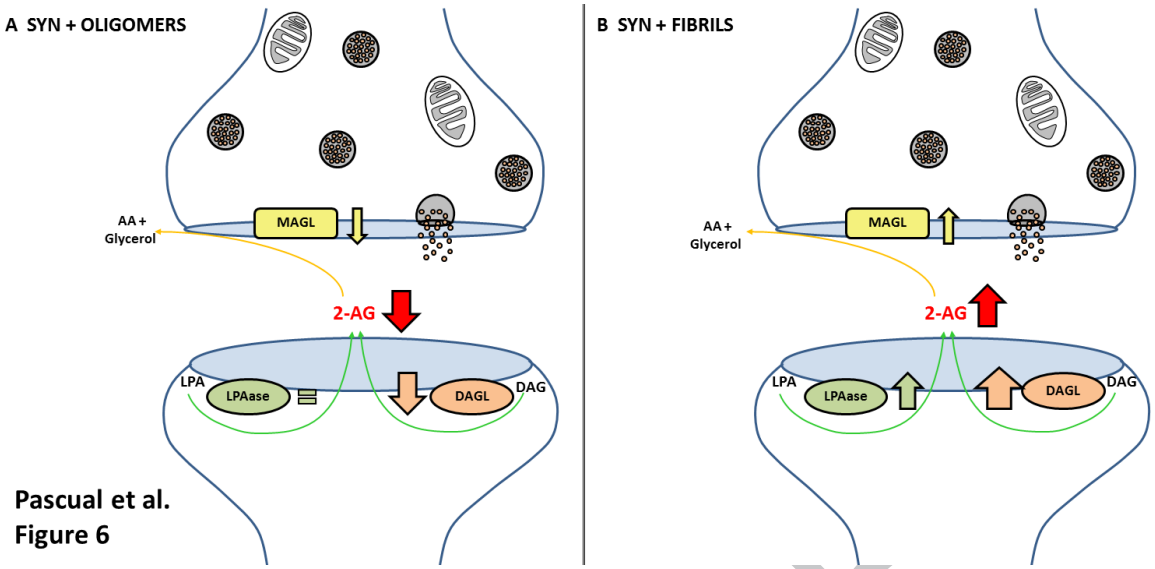

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Figure 6

Highlights

- Aβ oligomers disrupt synaptosomal membrane and impair lipase activities
- 2-AG synaptosomal availability is diminished in the presence of Aβ oligomers
- Aβ fibrils trigger synaptosomal aggregation
- 2-AG synaptosomal availability increases by stimulus of its synthesis in the presence of Aβ fibrils

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cc: yuri Bozzi
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Title: 2-arachidonoylglycerol metabolism is differently modulated by oligomeric and fibrillar conformations of amyloid beta in synaptic terminals
Section: Cellular and Molecular Neuroscience

Dear Dr. Pasquare,

We are pleased to inform you that your manuscript referenced above has been accepted for publication in Neuroscience. We hope that the review has been a positive experience and that your manuscript has been improved by the process.

We are currently planning to publish your paper in the Cellular and Molecular Neuroscience section, based on the Section you chose when you submitted your paper. Please notify us if you would prefer to have your paper published in a different section. The sections are:

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