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Aging Enables Ca^{2+} Overload and Apoptosis Induced by Amyloid-β Oligomers in Rat Hippocampal Neurons: Neuroprotection by Non-Steroidal Anti-Inflammatory Drugs and R-Flurbiprofen in Aging Neurons

María Calvo-Rodríguez[a], Mónica García-Durillo[a], Carlos Villalobos[a,*] and Lucía Núñez[b]
[a]*Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid and Consejo Superior de Investigaciones Científicas (CSIC), Valladolid, Spain*
[b]*Departamento de Bioquímica y Biología Molecular y Fisiología, Universidad de Valladolid, Spain*

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Abstract. The most important risk factor for Alzheimer's disease (AD) is aging. Neurotoxicity in AD has been linked to dyshomeostasis of intracellular Ca^{2+} induced by small aggregates of the amyloid-β peptide 1-42 ($A\beta_{42}$ oligomers). However, how aging influences susceptibility to neurotoxicity induced by $A\beta_{42}$ oligomers is unknown. In this study, we used long-term cultures of rat hippocampal neurons, a model of neuronal *in vitro* aging, to investigate the contribution of aging to Ca^{2+} dishomeostasis and neuron cell death induced by $A\beta_{42}$ oligomers. In addition, we tested whether non-steroidal anti-inflammatory drugs (NSAIDs) and R-flurbiprofen prevent apoptosis acting on subcellular Ca^{2+} in aged neurons. We found that $A\beta_{42}$ oligomers have no effect on young hippocampal neurons cultured for 2 days *in vitro* (2 DIV). However, they promoted apoptosis modestly in mature neurons (8 DIV) and these effects increased dramatically after 13 DIV, when neurons display many hallmarks of *in vivo* aging. Consistently, cytosolic and mitochondrial Ca^{2+} responses induced by $A\beta_{42}$ oligomers increased dramatically with culture age. At low concentrations, NSAIDs and the enantiomer R-flurbiprofen lacking anti-inflammatory activity prevent Ca^{2+} overload and neuron cell death induced by $A\beta_{42}$ oligomers in aged neurons. However, at high concentrations R-flurbiprofen induces apoptosis. Thus, $A\beta_{42}$ oligomers promote Ca^{2+} overload and neuron cell death only in aged rat hippocampal neurons. These effects are prevented by low concentrations of NSAIDs and R-flurbiprofen acting on mitochondrial Ca^{2+} overload.

Keywords: $A\beta_{42}$ oligomers, aging, Alzheimer's disease, calcium, hippocampal neurons, mitochondria, non-steroidal anti-inflammatory drugs, NSAIDs, R-flurbiprofen

INTRODUCTION

Aging is the most important risk factor for Alzheimer's disease (AD). The etiology of AD and the role of aging remain largely unknown. Unfortunately, efficient therapies are lacking and concern

*Correspondence to: Dr. Carlos Villalobos, Instituto de Biología y Genética Molecular (IBGM), c/Sanz y Forés 3, 47003 Valladolid, Spain. Tel.: +34 983 184821; Fax: +34 983 184800; E-mail: carlosv@ibgm.uva.es.

is rising on the failure of costly clinical trials with promising compounds. A critical example is Tarenflurbil® (R-flurbiprofen), an enantiomer of flurbiprofen without anti-inflammatory activity selected for a clinical trial because of its ability to modulate γ-secretase [1]. The reasons for failure are controversial, but researchers claim that protection efficiency may vary according to stage of the disease and/or age [2].

Increasing evidence indicates that small soluble aggregates or oligomers of $A\beta_{42,}$ rather than monomers of fibrils, are the most likely neurotoxin in AD [3]. We have shown that $A\beta_{42}$ oligomers, but not fibrils, induce Ca^{2+} entry, mitochondrial Ca^{2+} overload and apoptosis in cerebellar granule cells [4]. In addition, we reported that several NSAIDs and R-flurbiprofen protected cerebellar neurons against apoptosis by depolarizing mitochondria partially and preventing mitochondrial Ca^{2+} overload [4, 5]. These results, together with results from other labs using mouse models of familial AD, have contributed to put forward the so-called Ca^{2+} hypothesis of AD [6–9]. The death target and mechanism of Ca^{2+} entry induced by $A\beta_{42}$ oligomers remain controversial [10, 11]. In addition, the role of aging in the susceptibility of neurons to $A\beta_{42}$ oligomers neurotoxicity and the effects on intracellular Ca^{2+} are unclear at present.

Multiple sources of evidence suggest that long-term cultures of rat hippocampal neurons display many of the hallmarks of aging *in vivo*, including accumulation of reactive oxygen species, lipofuscin granules, heterochromatic foci, activation of the Jun N-terminal protein kinase and p53/p21 pathways, gradual loss of cholesterol, and changes in Ca^{2+} channel density and NMDA receptor expression [12–17]. Therefore, long-term cultures of hippocampal neurons may provide a suitable model for investigating Ca^{2+} remodeling in aged hippocampal neurons.

Here we have used rat hippocampal neurons cultured for different days *in vitro* (DIV) to study the effects of *in vitro* aging on Ca^{2+} overload and susceptibility to neuron cell death induced by $A\beta_{42}$ oligomers. In addition, we have tested the effects of very low concentrations of NSAIDs and R-flurbiprofen on Ca^{2+} overload and neuron cell death induced by $A\beta_{42}$ oligomers in aged neurons. Our results show that $A\beta_{42}$ oligomers fail to increase cytosolic and mitochondrial Ca^{2+} concentrations in young neurons. Consistently, they also fail to induce cytochrome c release and apoptosis in young neurons. However, sensitivity to $A\beta_{42}$ oligomers is acquired during *in vitro* aging when $A\beta_{42}$ oligomers promote massive Ca^{2+} influx and mitochondrial Ca^{2+} overload leading to cytochrome c release and apoptosis. We conclude that aging enables Ca^{2+} overload and neuron cell death induced by $A\beta_{42}$ oligomers in hippocampal neurons. In addition, we also found that NSAIDs and R-flurbiprofen prevent mitochondrial Ca^{2+} overload and neuron cell death in aged neurons only at low concentrations while at larger concentrations they promote apoptosis.

MATERIALS AND METHODS

Reagents

Wistar rat pups (newborn P0 - 1) are from the Valladolid University animal facility. Fura2/AM, cytochrome c antibody (MA5-11283), wt coelenterazine and lipofectamine® 2000 are from Invitrogen (Barcelona, Spain). Fetal bovine serum (FBS) is from Lonza (Barcelona, Spain). Horse serum, Neurobasal medium, HBSS medium, MEM medium, B27 and L-glutamine are from Gibco (Barcelona, Spain). Papain solution is from Worthington (Lakewood, NJ, USA). Coelenterazine n is from Biotium (Hayward, California, USA). NSAIDs and R-Flurbiprofen are from Cayman Chemical Company (Madrid, Spain). $A\beta_{42}$ peptides are from Bachem AG (Bubendorf, Switzerland). Poly-D-lysine and Annexin V are from BD (Madrid, Spain). DNase I is from Sigma (Madrid, Spain). The mitGAmut plasmid was kindly donated by P. Brulet (CNRS, Gif-sur-Yvette, France). Other reagents and chemicals are either from Sigma or Merck.

Primary hippocampal neuron culture

Hippocampal neurons are obtained from Wistar rat pups under sterile conditions as reported by Brewer et al. [18] with further modifications introduced by Pérez-Otaño et al. [19]. Briefly, rat pups are decapitated and, after brain removal, meninges are discarded and the hippocampus is separated from cortex. Hippocampal tissue is then cut in small pieces, transferred to papain solution (20 μg/ml) and incubated at 37°C for 30 min with occasional gentle shaking. After 15 min, DNase I (50 μg/ml) and tissue pieces are washed with Neurobasal Medium. A cell suspension is obtained using a fire-polished pipette in Neurobasal supplemented with 10% FBS. Cell suspension is then centrifuged at 160 *g* for 5 min and the cell pellet finally suspended in Neurobasal

medium. Hippocampal cells are plated onto poly-D-lysine-coated, 12 mm diameter glass coverslips at 30×10^3 cells/dish, and cultured in the same medium supplemented with L-glutamine (2 mM), gentamicin (1 μg/ml), 2% B27, and 10% FBS. Cells are maintained in a humidified 37°C incubator with 5% CO_2 without further exchange of the media. Cells are cultured for 2, 8, or >13 DIV before experiments. This procedure has been described in detail elsewhere [10].

Preparation of $A\beta_{42}$ oligomers

$A\beta_{42}$ oligomers are prepared as reported recently by a new procedure [10]. Briefly, $A\beta_{42}$ is initially solved at 1 mM in ice cold hexafluoroisopropanol (HFIP), and separated into aliquots in sterile microcentrifuge tubes. The solution is then incubated for 2 h at room temperature (RT) to allow monomerization. HFIP is removed under vacuum in a speed vac. (800 g × 10 min at RT), and the peptide film is stored desiccated at –20°C. For aggregation, the peptide is first suspended in dry dimethyl sulfoxide to a concentration of 5 mM. For complete suspension of the peptide, it is subjected to ultrasounds for 10 min, distributed in propylene non-siliconized tubes, and stored at –20°C. Medium (MEM) supplemented with 0.5 mg/ml Fe^{2+}, 0.5 mg/ml Cu^{2+}, and 0.5 mg/ml Zn^{2+} is added to bring the peptide to a concentration of 80 μM and is incubated at 37ºC for 24 h. For experiments, $A\beta_{42}$ is solved in medium to a final concentration of 2 μM.

Fluorescence imaging of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and in situ immunofluorescence

Coverslips containing cultured hippocampal neurons for different DIV are incubated in standard external medium (SEM) containing (in mM) NaCl 145, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10, and Hepes 10 (pH 7.42). Then, cells are incubated with fura2/AM dye (4 μM) for 60 min at RT in the dark. Coverslips are placed on the perfusion chamber of a Zeiss Axiovert 100 TV microscope and perfused continuously with the same pre-warmed (37ºC) SEM. For imaging, cells are epi-illuminated alternately at 340 and 380 nm lights with a filter wheel, and light emitted at 520 nm is recorded with a Hamamatsu ER camera (Hamamatsu Photonics, France) every 5 s. Pixel by pixel ratios of consecutive frames are captured, and $[Ca^{2+}]_{cyt}$ of regions of interest (ROIs) corresponding to individual neurons are expressed as the ratio of fluorescence emission at 520 nm following excitation at 340 and 380 nm (Ratio F340/F380), as reported in detail previously [13]. For calculations, responses were averaged from responsive neurons easily selected by their morphology different from glial cells. Responsive cells were considered all those showing a change in the slope of the Ca^{2+} recording after stimulation. In some experiments, cells were identified assessing the single cell content of β-tubulin III and glial fibrillary acidic protein (GFAP) by indirect immunofluorescence in the same cells used for calcium imaging as reported previously [4]. Briefly, after calcium imaging, cells were fixed with p-formaldehyde and incubated with anti-β-tubulin III (1:400) and anti GFAP (1:200) for 1 h at 37°C. Then, cells were washed and incubated with 1:100 labeled anti IgG antibodies. Nuclei were stained by incubation with DAPI 0.2 mg/ml for 5 min.

Bioluminescence imaging of mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mit}$)

Hippocampal neurons cultured for different DIV are transfected with the mitGAmut plasmid using lipofectamine® 2000. The mitGAmut plasmid contains a mutated, low affinity aequorin targeted to mitochondria and a GFP sequence to help selecting transfected neurons [20]. After 24 h, cells are incubated for 2 h with 4 μM n or wt coelenterazine at RT in the dark, washed with SEM, and placed into a perfusion chamber thermostated to 37ºC under a Zeiss Axiovert S100 TV microscope. Then cells are perfused continuously (5–10 ml/min) with test solutions based on the SEM described above prewarmed at 37ºC. Bioluminescence images are taken with a Hamamatsu VIM photon counting camera handled with an Argus-20 image processor. Photonic emissions are integrated for 10 s periods. At the end of each experiment, cells are permeabilized with SEM containing 0.1 mM digitonin and 10 mM $CaCl_2$, added here to release all the residual aequorin photonic emissions [21]. Photons were quantified using the Hamamatsu Aquacosmos software and converted into mitochondria free Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) values as reported previously [22, 23]. In experiments with permeabilized cells, perfusion was performed in a standard internal medium (SIM) containing (in mM) 130 KCl, 5 NaCl, 2 $MgCl_2$, 5 Succinate, 2 KH_2PO_4, 1 ATP, 20 HEPES/KOH, pH 7.0. Cells were permeabilized by perfusing them with SIM containing 50 μM digitonin for 1 min. Then,

cells were incubated with SIM containing 200 nM Ca^{2+} that resembles resting intracellular Ca^{2+} concentration (buffered with EGTA), in the presence or absence of NSAIDs for 5–7 min. After that, perfusion is switched to SIM containing 10 μM Ca^{2+}, in the presence or absence of the corresponding NSAID, to induce mitochondrial Ca^{2+} uptake. Media with low concentrations of Ca^{2+} were prepared with different concentrations of $CaCl_2$ and the Ca^{2+} buffers H-EDTA and tris-EGTA are prepared according to the computer program MaxQuelator (Chris Patton, Stanford University). Further details have been reported previously [23].

Apoptosis measurements

Hippocampal neurons cultured for different DIV are incubated for 1 h with vehicle or 2 μM $A\beta_{42}$ oligomers in the same SEM described above and in the presence or the absence of different NSAIDs. After $A\beta_{42}$ treatment, cells are washed once and returned to the original Neurobasal medium for an additional 24 h period. After that, cells are washed with phosphate buffered saline (PBS) once and apoptosis is evaluated using Annexin V (1:20, 10 min) using annexing binding buffer 1x containing (in mM) NaCl 140; $CaCl_2$ 2.5; Hepes 10 (pH 7.4) and assessed by fluorescence microscopy using a Nikon Eclipse TS100 microscope (objective 40x) as reported previously [12].

Measurements of cytochrome c release

Cytochrome c release from mitochondria is tested by immunofluorescence as reported previously [4, 12]. Hippocampal neurons cultured for several DIV are treated for 1 h with vehicle or $A\beta_{42}$ oligomers, washed and cultured for 24 h. Then, cells are fixed with p-formaldehyde and cytochrome c was tested by indirect immunofluorescence using a specific antibody against cytochrome c (1:300; Mouse anti-cytochrome c antibody MA5-11283 purchased from Invitrogen, Barcelona, Spain). Immunopositive cells are revealed using Alexafluor 488-tagged antibodies (1:300). Nuclei are identified by DAPI staining. Optical density in individual neurons is carried out to quantify cytochrome c release using Image J software (National Institute of Mental Health, Bethesda, MA, USA).

Statistics

Changes in fluorescence ratio are expressed as $\Delta[Ca^{2+}]_{cyt}$ (ΔRatio F340/F380) using Origin Lab 7.0. Data are presented as mean ± SEM. When only two means are compared, Student's *t* test is used. For more than two groups, statistical significance of the data is assessed by one-way or two-way ANOVA and compared using Bonferroni's multiple comparison tests using Origin Lab 7.0 software. Differences are considered significant at $p < 0.05$. Power analysis was carried out using GraphPad StatMate 2 software.

RESULTS

Apoptosis induced by $A\beta_{42}$ oligomers increases dramatically with culture age

The effects of $A\beta_{42}$ oligomers on apoptosis were investigated in hippocampal neurons cultured for 2, 8, and >13 DIV. Cultures were treated with 2 μM $A\beta_{42}$ oligomers for 1 h and then cells were incubated in Neurobasal Medium for 24 h before measuring apoptosis. Although this oligomer concentration is higher than the physiological concentration, it has been widely used in the literature for reporting quick effects of oligomers. Apoptosis was estimated by monitoring everted phosphatidylserine using Annexin V staining. Representative bright field (transmission) and immunofluorescence images (Anx V) are shown in Fig. 1A. The relative abundance (percent) of apoptotic neurons in cell cultures at different DIV treated or not treated with $A\beta_{42}$ oligomers are shown in Fig. 1B. $A\beta_{42}$ oligomers did not induce apoptosis in young neurons (2 DIV). However, in mature neurons (8 DIV), $A\beta_{42}$ oligomers modestly promoted apoptosis, and this effect increased rather dramatically in aged neurons (>13 DIV). Therefore, neuronal apoptosis induced by $A\beta_{42}$ oligomers depends critically on the age of the cultures.

Cytosolic Ca^{2+} responses to $A\beta_{42}$ oligomers are increased in aged neurons

The effects of $A\beta_{42}$ oligomers on $[Ca^{2+}]_{cyt}$ were studied in young, mature, and aged neurons *in vitro*. Hippocampal neurons cultured for different time periods (2, 8, and >13 DIV) were incubated with fura2/AM and subjected to fluorescence Ca^{2+} imaging before and after stimulation with $A\beta_{42}$ oligomers and N-methyl D-aspartate (NMDA). Representative Ca^{2+} images coded in pseudocolor and recordings

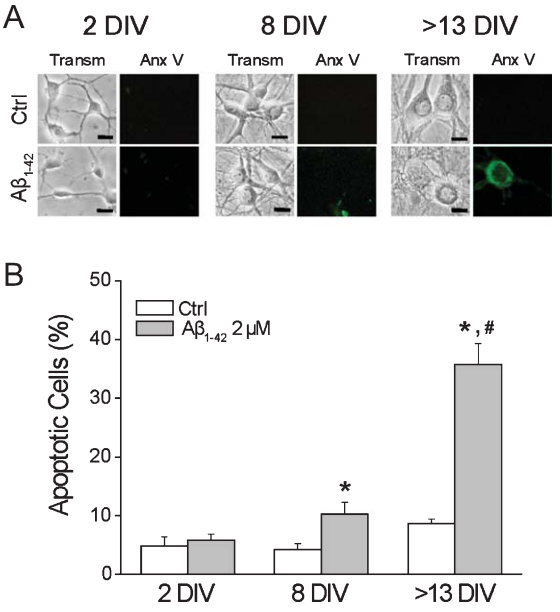

Fig. 1. $A\beta_{42}$ oligomers induce apoptosis in mature and aged hippocampal neurons. Primary hippocampal neurons cultured for 2, 8, and >13 DIV (13–19 DIV) were treated for 1 h in absence (Control) or presence of $A\beta_{42}$ oligomers (2 μM) and apoptosis was assessed 24 h later by staining with Annexin V. A) Representative bright field (transm) and Annexin V immunofluorescence (Anx V) microphotographs of cultured neurons. Bars represent 10 μm. B) Percent of apoptotic cells in Control cells and in cells treated with 2 μM $A\beta_{42}$ oligomers. Values represent mean ± SEM of 5, 6, and 10 independent experiments corresponding to 521, 685, and 1053 cells. $^{*}p < 0.05$ compared to Control group; $^{\#}p < 0.05$ compared to 2 and 8 DIV (two-way ANOVA analysis). Standard power analysis yielded 90% power for significant comparison means.

are shown in Fig. 2A. We found that $[Ca^{2+}]_{cyt}$ rises induced by $A\beta_{42}$ oligomers depended strongly on culture time. At 2 DIV, only less than 40% of the neurons responded to oligomers (Fig. 2B). In addition, the size of the $[Ca^{2+}]_{cyt}$ rises in responsive cells was very small. The same cells responded to NMDA 100 μM. In contrast, nearly all mature (8 DIV) and aged (>13 DIV) neurons responded clearly to $A\beta_{42}$ oligomers (Fig. 2B). In addition, $[Ca^{2+}]_{cyt}$ rises induced by $A\beta_{42}$ oligomers were much larger in mature and aged neurons than in young cells. Figure 2C compares the average responses to $A\beta_{42}$ oligomers at different culture periods. Rises in $[Ca^{2+}]_{cyt}$ increased significantly in mature (8 DIV) relative to young (2 DIV) neurons, and effects increased further in aged neurons (>13 DIV). Thus, Ca^{2+} responses to $A\beta_{42}$ oligomers depend strongly on the age of the cultures. Consistent with previous reports [12], Ca^{2+} responses induced by NMDA increased with culture time as well (Fig. 2A). Finally, it is also noteworthy that, as reported previously [12], resting $[Ca^{2+}]_{cyt}$ levels were larger in aged neurons compared with young neurons.

To confirm that the effects of $A\beta_{42}$ oligomers were restricted to neurons, two-fold immunofluorescence was carried out in the same cells used for calcium imaging. Figure 3 shows that identified neurons displayed large changes in cytosolic Ca^{2+} while identified glial cells in the same microscopic fields did not respond to oligomers.

Mitochondrial Ca^{2+} responses to $A\beta_{42}$ oligomers are increased in aged neurons

We tested next the effects of $A\beta_{42}$ oligomers on mitochondrial Ca^{2+} ($[Ca^{2+}]_{mit}$) in rat hippocampal neurons cultured for different DIV. For this end, we carried out bioluminescence imaging of neurons transfected with a plasmid expressing a low-affinity aequorin targeted to mitochondria. This probe also contains GFP for easy selection of transfected neurons for bioluminescence imaging (mitGAmut) [20]. Figure 4A shows typical GFP fluorescence (GFP, top) and AEQ bioluminescence (aequorin, bottom) images of transfected neurons stimulated with 2 μM $A\beta_{42}$ oligomers. It also shows the acute effects of 2 μM $A\beta_{42}$ oligomers on $[Ca^{2+}]_{mit}$ in neurons at different DIV. $A\beta_{42}$ oligomers failed to increase $[Ca^{2+}]_{mit}$ in young neurons (2 DIV). However, $A\beta_{42}$ oligomers raised $[Ca^{2+}]_{mit}$ in mature neurons (8 DIV) and these effects increased further in aged (>13 DIV) neurons (Fig. 4A). Average data shows that $A\beta_{42}$ oligomers have no effect on $[Ca^{2+}]_{mit}$ in 2 DIV neurons while promoting mitochondrial Ca^{2+} uptake in mature and aged cultures, since the effects are significantly larger in aged neurons (Fig. 4B). Therefore, effects of $A\beta_{42}$ oligomers on mitochondrial Ca^{2+} uptake in intact cells also strongly depends on the age of the cultures.

Mitochondrial Ca^{2+} uptake contributes to apoptosis induced by $A\beta_{42}$ oligomers in aged neurons

To evaluate the contribution of mitochondrial Ca^{2+} overload to the neurotoxicity induced by $A\beta_{42}$ oligomers, we studied whether inhibition of mitochondrial Ca^{2+} uptake affects apoptosis in aged neurons. To this end, we tested whether mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) prevented specifically mitochondrial Ca^{2+} uptake.

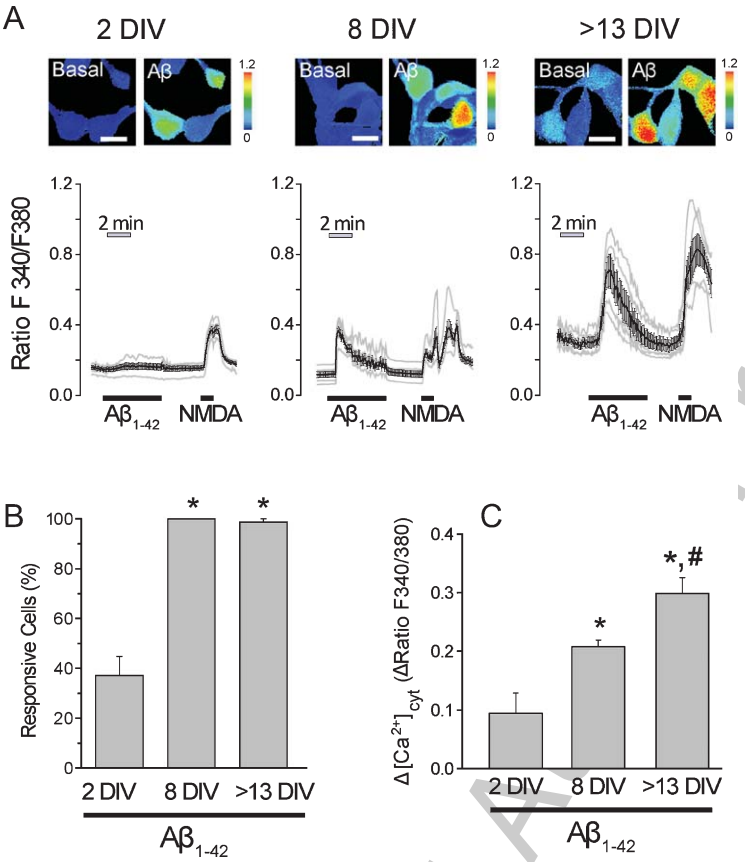

Fig. 2. Effects of $A\beta_{42}$ oligomers and NMDA on cytosolic Ca^{2+} increase with age in culture. Hippocampal neurons cultured for different DIV were loaded with fura2 and subjected to Ca^{2+} imaging. A) Pictures show pseudocolor images of $[Ca^{2+}]_{cyt}$ (Ratio F340/F380) before and after $A\beta_{42}$ oligomers perfusion in neurons cultured for 2, 8, and >13 DIV (13–19 DIV). Pseudocolor scale is shown at right. Traces show representative single-cell $[Ca^{2+}]_{cyt}$ responses to 2 μM $A\beta_{42}$ oligomers and 100 μM NMDA in hippocampal neurons at 2, 8, and >13 DIV. B) Bars represent average percent (mean ± SEM) of cells responsive to 2 μM $A\beta_{42}$ oligomers. Cells were considered responsive when clear rises in $[Ca^{2+}]_{cyt}$ over resting values were recorded. C) Bars represent average rises in $[Ca^{2+}]_{cyt}$ as ΔRatio F340/F380 (the maximum Ratio at peak response minus the resting ratio level) in response to $A\beta_{42}$ oligomers for neurons at different DIV. For B and C, values represent mean ± SEM of 10, 24 and 59 cells studied in 2, 6, and 9 experiments. $^{*}p < 0.05$ compared to 2 DIV group. $^{\#}p < 0.05$ compared to 2 and 8 DIV (One way ANOVA analysis). Standard power analysis yielded 95% power for significant comparison means.

Permeabilized neurons were perfused with intracellular medium containing 10 μM Ca^{2+} in the absence and the presence of FCCP (see Materials and Methods). Figure 5A illustrates a typical recording of $[Ca^{2+}]_{mit}$ in permeabilized neurons transfected with mitochondria-targeted aequorin and stimulated with 10 μM Ca^{2+}. FCCP prevented the rise in $[Ca^{2+}]_{mit}$ induced by 10 μM Ca^{2+}. This effect is specific for mitochondria as it does not affect the rise in $[Ca^{2+}]_{cyt}$ induced by $A\beta_{42}$ oligomers (Fig. 5B).

Once we established a method to inhibit specifically mitochondrial Ca^{2+} uptake, we studied the effects of FCCP on $A\beta_{42}$-induced apoptosis in aged neurons. Figure 5C shows that FCCP significantly inhibits apoptosis induced by $A\beta_{42}$ oligomers in aged neurons (>13 DIV). To confirm the role played by mitochondria in apoptosis induced by $A\beta_{42}$ oligomers in aged neurons, the effects of $A\beta_{42}$ oligomers on cytochrome c release were tested at different DIV. We found that $A\beta_{42}$ oligomers promoted release of cytochrome c in aged cultures (>13 DIV) but not in the young neurons as shown by indirect immunofluorescence against cytochrome c (Fig. 6). Thus, inhibition of mitochondrial Ca^{2+} overload prevents cytochrome c release and apoptosis induced by $A\beta_{42}$ oligomers in aged neurons.

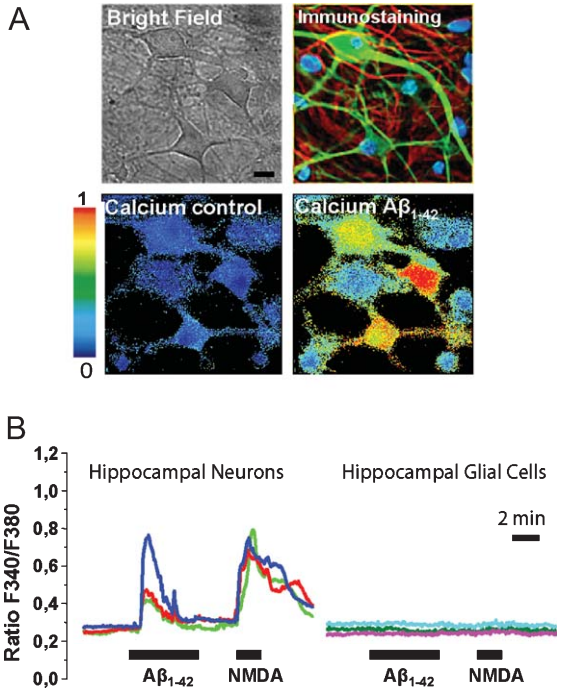

Fig. 3. $A\beta_{42}$ oligomers increased cytosolic Ca^{2+} only in hippocampal neurons but not in glial cells. Hippocampal neurons were cultured for 22 DIV, loaded with fura2 and subjected to Ca^{2+} imaging. Then, cells in the same microscopic field were fixed and subjected to two-fold immunofluorescence for identification of neurons and glial cells. A) Top pictures show a bright field image and immunostaining of the cells. Glial cells are coded in red and neurons are coded in green. Nuclei are coded in blue. Bottom pictures are calcium images coded in pseudocolor before (Left) and after (Right) treatment with $A\beta_{42}$ oligomers. B) Representative cytosolic Ca^{2+} recordings of individual neurons (Left) and glial cells (Right) stimulated with $A\beta_{42}$ oligomers and NMDA. Data representative of 32 cells studied in 4 independent experiments

NSAIDs and R-flurbiprofen inhibit mitochondrial Ca^{2+} uptake induced by $A\beta_{42}$ oligomers in aged hippocampal neurons

Once we established the importance of mitochondrial Ca^{2+} uptake for apoptosis induced by $A\beta_{42}$ oligomers in aged neurons, we investigated whether low concentrations of salicylate and other NSAIDs, including R-flurbiprofen that lacks anti-inflammatory activity, may prevent mitochondrial Ca^{2+} overload and apoptosis induced by $A\beta_{42}$ oligomers in aged hippocampal neurons. These compounds are considered mild mitochondrial uncouplers that dissipate partially the mitochondrial potential ($\Delta\psi$), the driving force for mitochondrial Ca^{2+} uptake as reported previously [4]. Figure 7 shows typical single-cell recordings of $[Ca^{2+}]_{mit}$ in permeabilized neurons challenged with 10 μM Ca^{2+} in the absence and the presence of low concentrations of NSAIDs and R-flurbiprofen. We found that 10 μM Ca^{2+} increased $[Ca^{2+}]_{mit}$ in permeabilized hippocampal neurons (Fig. 7A). This effect was inhibited by 100 μM salicylate, 1 μM sulindac sulphide, and 1 μM R-flurbiprofen as shown by representative recordings (Fig. 7B–D). All three compounds tested significantly inhibited mitochondrial Ca^{2+} uptake in permeabilized neurons (Fig. 7E).

Effects of NSAIDs and R-flurbiprofen are specific for mitochondria since none of the above compounds decreased the rise in $[Ca^{2+}]_{cyt}$ induced by $A\beta_{42}$ oligomers (Fig. 8). However, a deepened, more sophisticated quantification of calcium imaging results could provide new insights on cytosolic Ca^{2+} responses in cultured hippocampal neurons. Thus, at fairly low concentrations, NSAIDs inhibit mitochondrial Ca^{2+} uptake without affecting the rise in cytosolic Ca^{2+}. Thus, NSAIDs inhibit specifically mitochondrial Ca^{2+} uptake at very low concentrations.

NSAIDs and R-flurbiprofen prevent apoptosis induced by $A\beta_{42}$ oligomers in aged hippocampal neurons

The effects of NSAIDs and R-flurbiprofen on apoptosis induced by $A\beta_{42}$ oligomers were tested in aged neurons (>13 DIV). NSAIDs were added 30 min prior, during, and 30 additional min after treatment with $A\beta_{42}$ oligomers. Figure 9A shows representative bright-field images of hippocampal neurons treated with vehicle (Control) and $A\beta_{42}$ oligomers in the presence and the absence of 100 μM salicylate. Apoptosis was estimated in similar cultures using annexin V staining. All compounds tested including 100 μM salicylate (Fig. 9B), 0.5 μM sulindac sulphide (Fig. 9C), and 0.5 μM R-flurbiprofen (Fig. 9D) significantly prevented apoptosis induced by $A\beta_{42}$ oligomers. We noticed that neuroprotection afforded by R-flurbiprofen decreased as we increased its concentration. Accordingly, we tested the dose-dependent effects of R-flurbiprofen alone on apoptosis and compared them with the effects of $A\beta_{42}$ oligomers. Figure 9E shows that R-flurbiprofen has no effect on apoptosis at 0.1 μM. However, at 1 μM R-flurbiprofen, apoptosis becomes apparent, and at larger concentrations (>10 μM), R-flurbiprofen induced apoptosis significantly and to an extent quite similar to the effects of $A\beta_{42}$ oligomers. Thus, R-flurbiprofen protects neurons against $A\beta_{42}$ oligomers

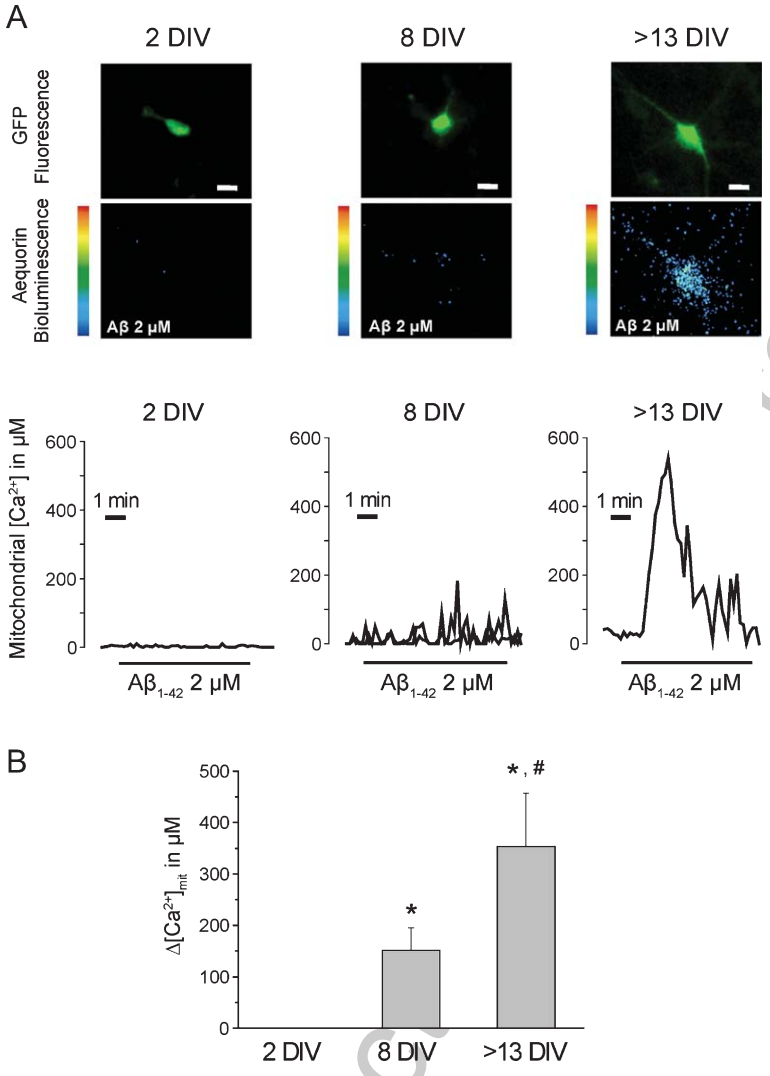

Fig. 4. Effects of $A\beta_{42}$ oligomers on mitochondrial Ca^{2+} increase with age in culture. Hippocampal neurons cultured for 2, 8, and >13 DIV (13–15 DIV) were transfected with mitGAmut plasmid expressing GFP-aequorin targeted to mitochondria and subjected to bioluminescence imaging for monitoring mitochondrial Ca^{2+}. A) Pictures show GFP (top) and accumulated photonic emissions/aequorin bioluminescence (bottom) images of representative microscopic fields of cultured hippocampal neurons at different DIV. Pseudocolor bar shown at left. Bars represent 10 μm. Traces show representative, single-cell recordings of mitochondrial Ca^{2+} responses to 2 μM $A\beta_{42}$ oligomers at 2, 8, and >13 DIV. B) Average values (mean ± SEM) of rises in $[Ca^{2+}]_{mit}$ induced by $A\beta_{42}$ oligomers. Values represent mean ± SEM of 5, 7, and 18 cells studied in 3, 4, and 9 experiments. $^{*}p < 0.05$ compared to 2 DIV; $^{\#}p < 0.05$ compared to 8 DIV (One way ANOVA analysis). Standard power analysis yielded 85%, 90%, and 75% power for significant comparison means comparing 2/8 DIV, 2/13 DIV, and 8/13 DIV, respectively.

at low concentrations while promoting apoptosis at large concentration.

DISCUSSION

Aging is the most important risk factor for AD. Aβ is the main component of amyloid plaques and small aggregates of $A\beta_{42}$ are involved critically in AD. However, how aging influences susceptibility to neurotoxicity induced by $A\beta_{42}$ oligomers is largely unknown. In this study we have investigated the acute effects of $A\beta_{42}$ oligomers on cell death and subcellular Ca^{2+} in an *in vitro* model of neuronal aging: the long-term culture of rat hippocampal neurons. We found that $A\beta_{42}$ oligomers at μM concentrations do not induce apoptosis in young neurons. In contrast, $A\beta_{42}$ oligomers promote apoptosis in mature neurons, and this effect increases further

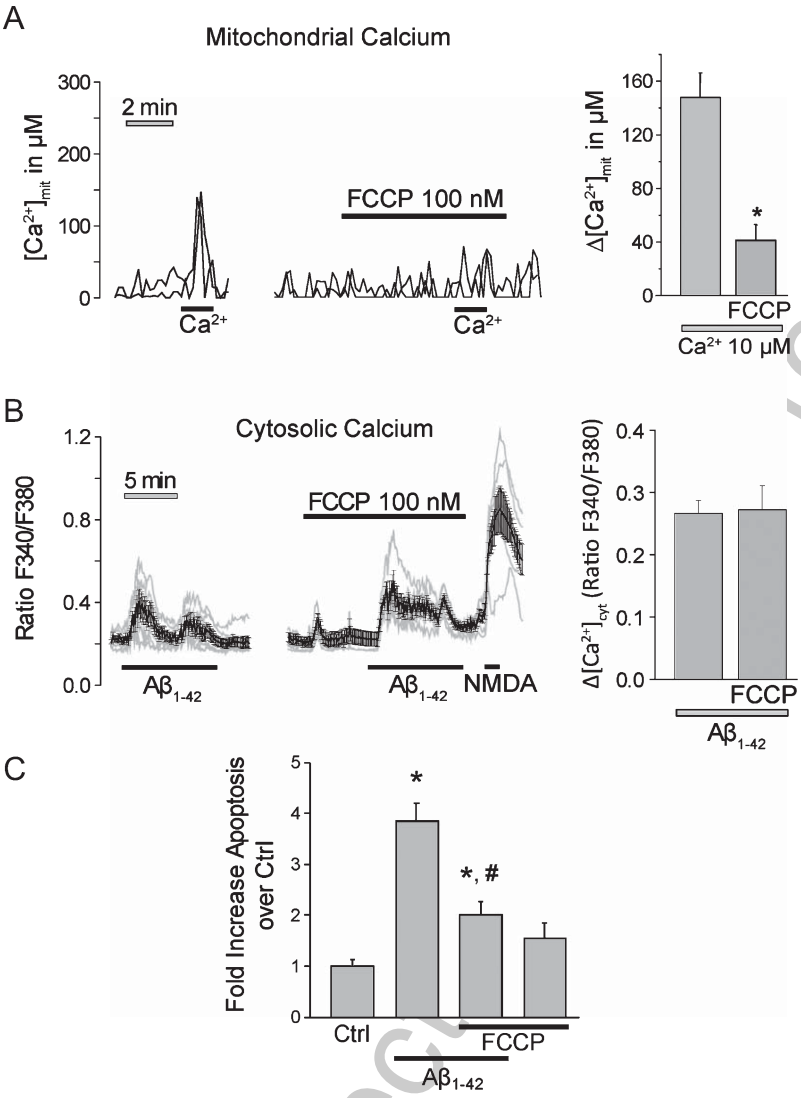

Fig. 5. Mitochondrial Ca^{2+} uptake contributes to Aβ-induced neurotoxicity in aged neurons. A) The effects of the mitochondrial uncoupler FCCP on mitochondrial Ca^{2+} uptake induced by $A\beta_{42}$ oligomers were tested in hippocampal neurons by bioluminescence imaging. Hippocampal neurons cultured for 8–12 DIV were transfected with the mitGAmut plasmid expressing GFP-aequorin targeted to mitochondria and subjected to bioluminescence imaging for monitoring $[Ca^{2+}]_{mit}$. Hippocampal cells expressing mitGAmut were permeabilized in intracellular medium containing 200 nM Ca^{2+} (see methods) and treated with FCCP 100 nM before adding intracellular medium containing 10 µM Ca^{2+}. Traces are representative recordings of $[Ca^{2+}]_{mit}$ in individual cells before and after Ca^{2+} 10 µM perfusion in absence (left) or presence (right) of FCCP. Bars are averages from 7 and 6 cells respectively from at least three independent cultures. $^{*}p < 0.05$ compared to Ca^{2+} 10 µM alone. Power 95% for significant comparison means. B) The effect of FCCP on the rise in cytosolic Ca^{2+} induced by $A\beta_{42}$ oligomers was tested in aged neurons (>13 DIV). Traces are representative, single-cell $[Ca^{2+}]_{cyt}$ responses to $A\beta_{42}$ oligomers (2 µM) before and after FCCP. Bars show average data ($\Delta[Ca^{2+}]_{cyt}$ Ratio F340/F380; means ± SEM of 59 and 23 individual neurons from 9 and 3 independent cultures respectively). Differences between Control and FCCP treated groups were not statistically significant ($p > 0.05$). C) The effects of FCCP on Aβ -induced apoptosis were tested in aged (>13 DIV) hippocampal neurons. Bars show fold increase apoptosis relative to Control (mean ± SEM, $n = 7$ independent experiments). Data are from 798 cells (Control), 737 cells ($A\beta_{42}$), 832 cells ($A\beta_{42}$ + FCCP) and 813 cells (FCCP alone). $^{*}p < 0.05$ versus Control; $^{\#}p < 0.05$ versus group treated only with $A\beta_{42}$. Power analysis revealed 95% power for comparison between Control and $A\beta_{42}$, 95% power for comparison between while $A\beta_{42}$ and $A\beta_{42}$ plus FCCP, 80% power for comparison between Control and $A\beta_{42}$ plus FCCP.

in long-term cultures of rat hippocampal neurons. These results open the question as to how aged cells acquire increased sensitivity to $A\beta_{42}$ oligomers. Consistently, we show that cytosolic and mitochondrial Ca^{2+} responses to $A\beta_{42}$ oligomers are missing in young neurons (2 DIV), becoming significant

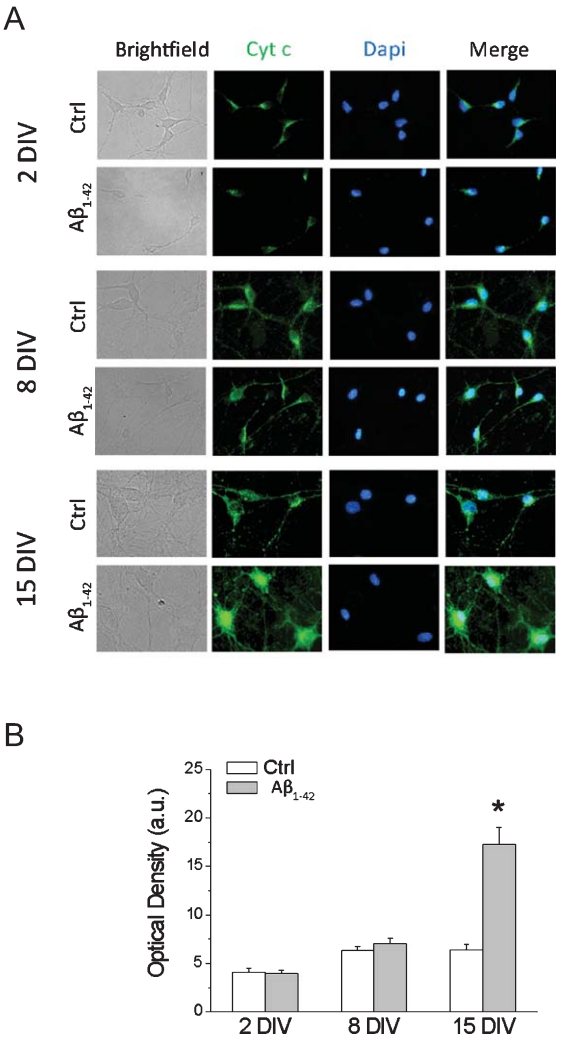

Fig. 6. Effects of $A\beta_{42}$ oligomers on cytochrome c release increase with age in culture. Indirect immunofluorescence against cytochrome c in hippocampal neurons at different DIV, pretreated with vehicle or $A\beta_{42}$ oligomers (2 μM). A) Representative immunofluorescence of released cytochrome c in cultured hippocampal neurons at 2, 8, and 15 DIV. Green colors show location of cytochrome c. Nuclei are stained blue with DAPI. Bars represent 10 μm. Merging pictures are also shown at right. B) Quantitative analysis of immunofluorescence intensity levels (Optical Density in arbitrary units) for cytochrome c. Bars represent mean ± SEM of 3 independent experiments, derived from 64, 83, and 42 Control cells (for 2, 8, and >13 DIV respectively) and 47, 67, and 57 treated cells (for 2, 8, and >13 DIV respectively for $A\beta_{42}$ oligomers treatment). $^{*}p < 0.05$ versus Control group (two-way ANOVA). Standard power analysis revealed 85% power for significant comparison means.

in mature neurons (8 DIV) and increasing dramatically in aged neurons (>13 DIV). These results strongly suggest that enhanced cytosolic and mitochondrial Ca^{2+} responses of aging neurons contribute to explain the increased susceptibility to neuron cell damage induced by $A\beta_{42}$ oligomers in aged neurons.

Importantly, the apoptotic response of aged neurons to Aβ oligomers depends on the mitochondrial Ca^{2+} overload rather than the cytosolic one. This view is supported by the fact that specific inhibition of mitochondrial Ca^{2+} uptake with mitochondrial uncouplers (FCCP) prevents apoptosis without preventing rises in cytosolic Ca^{2+}. Consistently with the key role of mitochondrial Ca^{2+} overload, we show that $A\beta_{42}$ oligomers promote cytochrome c release only in aged neurons. Therefore, aged neurons are much more sensitive to $A\beta_{42}$ oligomers than young neurons because they underlie much larger Ca^{2+} loads in response to $A\beta_{42}$ oligomers than their young counterparts. We have reported recently that Ca^{2+} responses to NMDA are also increased in aged neurons *in vitro* and this effect is mediated by changes in NMDA receptor subunit expression similar to those found *in vivo* [12, 16]. Consistently, Ca^{2+} responses induced by NMDA also increased along culture time. Accordingly, age-associated changes in NMDA receptor subunit expression may contribute to the enhanced sensitivity to $A\beta_{42}$ oligomers and increased risk of AD with aging. Nevertheless, changes in expression of other death targets related to intracellular Ca^{2+} may also be involved in the enhanced sensitivity to Aβ oligomers and increased risk of AD with aging. For example changes in the activity and/or expression of the neuron specific isoform of Na,K ATPase α3 are also consisting with our results [24]. Triggering mechanisms and pathways for Ca^{2+} entry induced by $A\beta_{42}$ oligomers may include activation of NMDA receptors [25], α7 nicotinic acetylcholine receptors [26], metabotropic glutamate receptor 5 [27], and the above mentioned neuron specific Na,K ATPase α3 [24]. Interestingly, changes in expression of some of these receptors could be strongly influenced by aging *in vivo* and *in vitro* [12, 24], thus contributing to age-related changes in susceptibility to $A\beta_{42}$ oligomers and AD.

Regardless of the Ca^{2+} entry pathway activated by $A\beta_{42}$ oligomers in aged neurons, our results point to mitochondrial Ca^{2+} overload as key player in neuron cell death induced by $A\beta_{42}$ oligomers. As stated above, this view is supported by the effects of low concentrations of FCCP that prevent mitochondrial Ca^{2+} overload without affecting the rise in $[Ca^{2+}]_{cyt}$ and protect largely against apoptosis induced by $A\beta_{42}$ oligomers. Therefore, any compound that limits mitochondrial Ca^{2+} uptake may potentially protect against $A\beta_{42}$ oligomers. Compelling evidence

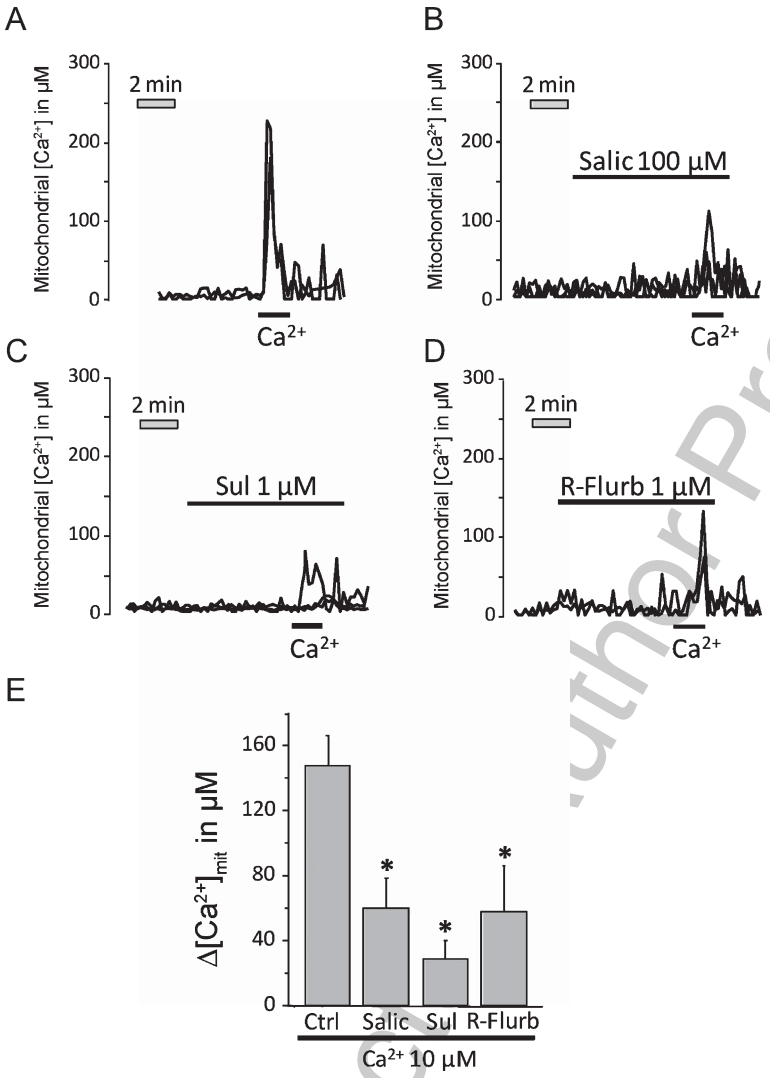

Fig. 7. NSAIDs and R-flurbiprofen inhibit mitochondrial Ca^{2+} uptake in aged neurons. A–D) Hippocampal neurons cultured for 8–12 DIV were transfected with mitGAmut plasmid expressing GFP-aequorin targeted to mitochondria and were subjected to bioluminescence imaging for monitoring $[Ca^{2+}]_{mit}$. Hippocampal cells expressing mitGAmut were permeabilized in intracellular medium containing 200 nM Ca^{2+} (see methods) and were treated with 100 µM Salicylate (B), 1 µM Sulindac sulfide (C) or 1 µM R-flurbiprofen (D) before being stimulated with the same intracellular medium containing 10 µM Ca^{2+}, in order to stimulate mitochondrial Ca^{2+} uptake. Traces show representative recordings of the effects of the NSAIDs on mitochondrial Ca^{2+} rises induced by 10 µM Ca^{2+} in hippocampal neurons. E) Bars represent data from 7, 7, 3, and 7 cells, respectively, studied in at least three independent experiments. $^{*}p < 0.05$ versus Control group (one-way ANOVA). Standard power analysis yielded 85% power for significant comparison means.

indicates that different NSAIDs may protect against AD [28, 29]. Multiple mechanisms have been proposed for neuroprotection afforded by NSAIDs including inhibition of pro-inflammatory activity of surrounding glia, modulation of γ-secretase activity involved in Aβ processing and inhibition of mitochondrial Ca^{2+} overload [4, 5, 29]. In fact, we showed that NSAIDs and R-flurbiprofen inhibit mitochondrial Ca^{2+} overload and neuron cell death induced by $A\beta_{42}$ oligomers in cerebellar granule cells [4] and by NMDA in aged rat hippocampal neurons [12]. Unfortunately, a large phase III clinical trial using Tarenflurbil (R-flurbiprofen) did not slow cognitive decline or the loss of activities of daily living in patients with mild AD [1]. It has been argued that this failure may be related to the fact that anti Aβ activity could be decreased in aged individuals with well-developed AD. We show that NSAIDs including salicylate, sulindac sulphide, and R-flurbiprofen prevent mitochondrial Ca^{2+} overload induced by Ca^{2+}

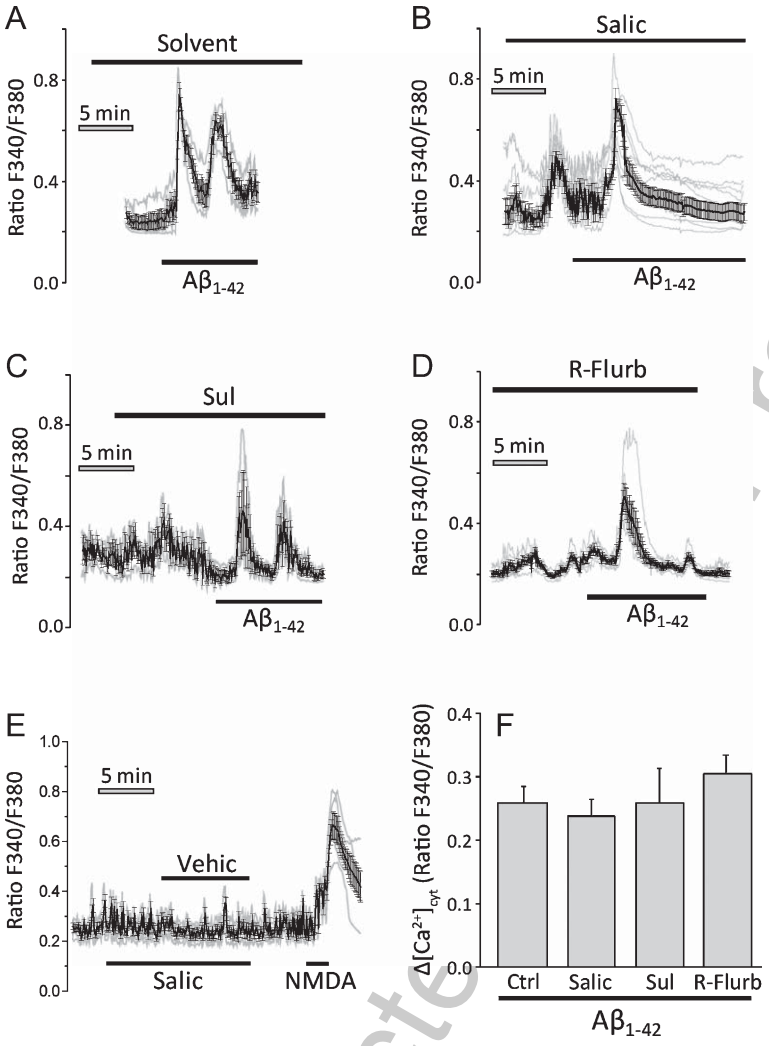

Fig. 8. NSAIDs and R-flurbiprofen do not prevent $[Ca^{2+}]_{cyt}$ rises induced by $A\beta_{42}$ oligomers. Aged cultured hippocampal neurons were loaded with fura2 and subjected to Ca^{2+} imaging to assess $[Ca^{2+}]_{cyt}$. A–D) Representative recordings of the effects of 100 μM salicylate (B), 1 μM sulindac sulfide (C), or 1 μM R-flurbiprofen (D) on $[Ca^{2+}]_{cyt}$ increases induced by $A\beta_{42}$ oligomers in aged neurons (>13 DIV). E) Lack of effects of long-term treatment salicylate and vehicle on $[Ca^{2+}]_{cyt}$. F) Average (mean ± SEM) data of cytosolic Ca^{2+} rises induced by $A\beta_{42}$ oligomers in the absence and presence of 100 μM salicylate, 1 μM sulindac, or 1 μM R-flurbiprofen is shown (Data is from 68 cells, 10 experiments for Control; 41 cells, 5 experiments for salicylate; 38 cells, 5 experiments for Sulindac; and 31 cells, 6 experiments for R-flurbiprofen). Differences among treated cells and Control were not statistically significant ($p > 0.05$).

in permeabilized neurons and apoptosis induced by $A\beta_{42}$ oligomers, strongly suggesting that NSAIDs and R-flurbiprofen may protect against AD by preventing mitochondrial Ca^{2+} overload induced by $A\beta_{42}$ oligomers in aging neurons.

NSAIDs and R-flurbiprofen are considered mild mitochondrial uncouplers. Thus, at low concentrations, they depolarize partially mitochondria [4]. However, at large concentrations, such as those required for anti-inflammatory activity or for modulating γ secretase, they collapse the mitochondrial potential [4], thus compromising energy supply. This may be particularly true for aged neurons, including aged neurons *in vitro* that show a significant loss of mitochondrial potential compared to young neurons [12, 30]. Consistently, we found that large concentrations of R-flurbiprofen that may collapse the mitochondrial potential promoted apoptosis to almost the same extent than $A\beta_{42}$ oligomers. It is difficult to extent these results to the *in vivo* situation. However, while low concentrations (<1 μM) of NSAIDs and R-flurbiprofen protect efficiently against mitochondrial calcium overload acting as partial mitochondrial uncouplers, at high concentrations

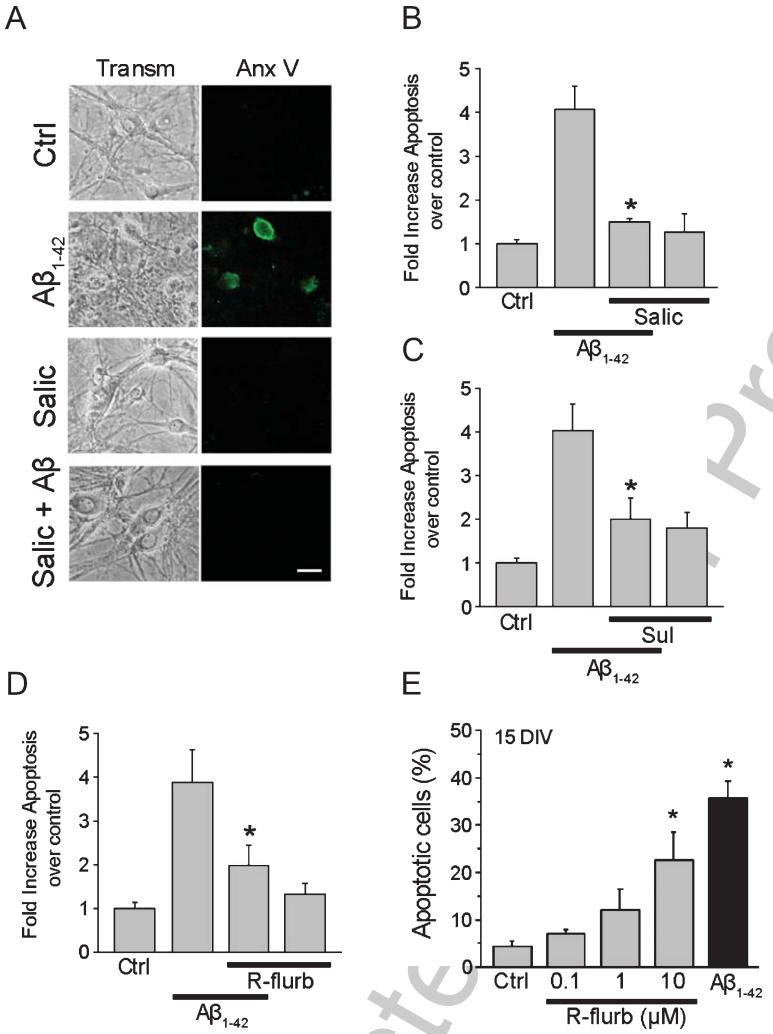

Fig. 9. NSAIDs and R-flurbiprofen prevent apoptosis induced by $A\beta_{42}$ oligomers in aged neurons at low concentrations. Hippocampal neurons (>13 DIV) were treated for 1 h with $A\beta_{42}$ oligomers (2 μM) with solvent, NSAID or R-flurbiprofen and apoptosis was assessed 24 h later. A) Representative bright field pictures of aged neurons treated with $A\beta_{42}$ oligomers in presence or absence of 100 μM salicylate. Bars represent 20 μm. B) Effects of salicylate 100 μM on apoptosis induced by $A\beta_{42}$ oligomers. Bars show number of apoptotic cells (mean ± SEM) relative to Control. Data are from 324, 353, 381, and 325 cells for Control, $A\beta_{42}$, salicylate + $A\beta_{42}$, and salicylate alone respectively studied in 4 experiments. $^{*}p < 0.05$ versus $A\beta_{42}$. 90% power for significant comparison mean. C) Effects of sulindac sulphide 0.5 μM on apoptosis induced by $A\beta_{42}$ oligomers. Bars show mean ± SEM apoptosis relative to Control. Data are from 686, 682, 699, and 646 for Control, $A\beta_{42}$, sulindac + $A\beta_{42}$ and sulindac, respectively, studied in 6 experiments. $^{*}p < 0.05$ versus $A\beta_{42}$. 80% power for significant comparison mean. D) Effects of R-flurbiprofe 0.5 μM on apoptosis induced by $A\beta_{42}$ oligomers. Bars show mean ± SEM apoptosis relative to Control. Data are from 875, 864, 903, and 846 for Control, $A\beta_{42}$, R-Flurbiprofen + $A\beta_{42}$ and R-Flurbiprofen alone, respectively, studied in 6 experiments. $^{*}p < 0.05$ versus $A\beta_{42}$. 80% power for significant comparison mean. E) Percent of apoptotic cells in Control cells and cells treated with increasing concentrations of R-flurbiprofen. Data are from 376, 341, 392, 357, and 1088 for Control, 0.1, 1, and 10 μM R-Flurbiprofen and $A\beta_{42}$ respectively, studied in 3 independent experiments. $^{*}p < 0.05$ compared to Control group. Power analysis revealed a power of 85% for significant comparison means.

(>10 μM) NSAIDs and R-flurbiprofen may become toxic, particularly in the context of aging, where mitochondrial potential of neurons is compromised.

In summary, we show here that young neurons are permissive to $A\beta_{42}$ oligomers. However, when neurons age, they become sensitive to cell damage induced by $A\beta_{42}$ oligomers that promote Ca^{2+} entry, mitochondrial Ca^{2+} overload, cytochrome c release and neuronal apoptosis. Changes in Ca^{2+} responses could be triggered by age-associated changes in the expression of $A\beta_{42}$ oligomer death targets including probably NMDA receptors and neuron specific

Na,K ATPase 3α. In addition, changes in subcellular Ca^{2+} handling related to aging may also contribute to enhanced sensitivity to oligomers. For instance, it has been reported that the loss of calcium buffering may contribute to selective neuronal vulnerability in AD [31]. Further research is required to ascertain more precisely the remodeling of subcellular Ca^{2+} in aging and its contribution to enhanced sensitivity to $A\beta_{42}$ oligomers and AD.

ACKNOWLEDGMENTS

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