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Critical role of cPLA$_2$ in Aβ oligomer-induced neurodegeneration and memory deficit

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Abstract

Soluble beta-amyloid (Aβ) oligomers are considered to putatively play a critical role in the early synapse loss and cognitive impairment observed in Alzheimer's disease. We previously demonstrated that Aβ oligomers activate cytosolic phospholipase A$_2$ (cPLA$_2$), which specifically releases arachidonic acid from membrane phospholipids. We here observed that cPLA$_2$ gene inactivation prevented the alterations of cognitive abilities and the reduction of hippocampal synaptic markers levels noticed upon a single intracerebroventricular injection of Aβ oligomers in wild type mice. We further demonstrated that the Aβ oligomer-induced sphingomyelinase activation was suppressed and that phosphorylation of Akt/protein kinase B (PKB) was preserved in neuronal cells isolated from cPLA$_2^{-/-}$ mice. Interestingly, expression of the Aβ precursor protein (APP) was reduced in hippocampus homogenates and neuronal cells from cPLA$_2^{-/-}$ mice, but the relationship with the resistance of these mice to the Aβ oligomer toxicity requires further investigation. These results therefore show that cPLA$_2$ plays a key role in the Aβ oligomer-associated neurodegeneration, and as such represents a potential therapeutic target for the treatment of Alzheimer's disease.

Keywords: Alzheimer's disease; Cytosolic phospholipase A$_2$; Soluble beta-amyloid oligomers; Memory; Apoptosis; Synaptotoxicity; Amyloid precursor protein

1. Introduction

Alzheimer's disease (AD) is the principal form of senile dementia and represents a major health problem in industrialized countries. Its prevalence is increasing and no effective treatment is currently available. Numerous studies on animal models and humans support the hypothesis that synaptic dysfunction and loss induced by the oligomers of the amyloid-beta peptide (Aβ) are the basis for the cognitive alterations observed in AD (for review, see Arendt, 2009). Indeed, the inhibition of long-term potentiation (LTP) and enhancement of long-term depression (LTD) are considered to be the first synaptic functional impairments induced by soluble Aβ oligomers (Lambert et al., 1998; Shankar et al., 2008; Wang et al., 2002). Alterations of several cellular metabolic pathways including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Gu et al., 2009) and N-methyl-D-aspartate (NMDA) recep-

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tor-mediated axonal transport (Decker et al., 2010a) follow this first event, leading to the reduction of synaptic plasticity and memory defects. Synapse degeneration results ultimately in the decrease of synaptic density up to 55%, which has been observed postmortem in the CA1 hippocampal region of mild AD patients (Arendt, 2009). The preservation of synaptic function and plasticity can therefore be considered to be critical during the early prodromal phase of AD and thus represents an important target for developing potential therapeutics.

The pathways and mechanisms by which Aβ oligomers induce the early synaptic disruptions are only partially known and involve several messenger molecules acting as intracellular and synaptic messengers. Arachidonic acid (AA; C20:4 ω6), a long-chain polyunsaturated *n*-6 fatty acid is one of these mediators. AA is involved in the regulation of long-term potentiation and in the retrograde signal generated by the activation of glutamate AMPA and NMDA receptors (Volterra et al., 1992; Williams et al., 1989). Furthermore, AA is converted by different enzymes into cannabinoids and eicosanoids, these derivatives being involved in synaptic signaling and neuroinflammation (Sheinin et al., 2008). However, AA also exerts its own effects, including the inhibition of presynaptic and postsynaptic channels as well as the formation and recycling of synaptic vesicles (Marza et al., 2008).

AA is released from the sn-2 position of membrane phospholipids by several phospholipases A_2 (PLA_2), a large protein family that includes secreted as well as cytosolic and membrane-associated enzymes (Burke and Dennis, 2009). Neuronal cells express at least 2 types of phospholipases A_2: (1) the calcium-independent membrane-associated enzyme ($iPLA_2$) that is thought to be involved in the metabolism of docosahexaenoic acid, the major *n*-3 long-chain polyunsaturated fatty acid in the brain; and (2) the calcium-dependent cytosolic enzyme ($cPLA_2$) that displays a strong selectivity for the AA-containing phospholipids (for review, see Sun et al., 2010). This latter 85-kDa enzyme, which is also referred to as GIVA-PLA_2 (Schaloske and Dennis, 2006), is activated and translocated to membranes after calcium influx and phosphorylation by mitogen-activated protein (MAP) kinases or other kinases (Clark et al., 1995). Activation of MAP kinases and perturbation of calcium homeostasis are well known to be involved in the deleterious effects of Aβ oligomers (Small, 2009; Wang et al., 2004). We previously reported that soluble Aβ oligomers caused early calcium-dependent release of AA associated with a transient relocalization of $cPLA_2$ to the plasma membrane, suggesting that activation of $cPLA_2$ and subsequent release of AA are critical steps for soluble Aβ oligomer-mediated apoptosis in cortical neurons. We confirmed this hypothesis by using antisense oligonucleotides and pharmacological inhibitors of $cPLA_2$ that abolished AA release and protected neurons against Aβ-induced apoptosis (Kriem et al., 2005). Furthermore, we demonstrated that activation of $cPLA_2$ by Aβ oligomers precedes that of sphingomyelinases (SMases) and subsequent production of ceramides associated with neuronal apoptosis (Malaplate-Armand et al., 2006). The critical role of $cPLA_2$ in AD is also supported by the results obtained by Sanchez-Mejia et al. (2008) on the J20 mice which express mutant human Aβ precursor protein (APP). These authors showed that breeding J20 with $cPLA_2^{-/-}$ mice improves the cognitive abilities of the resulting animals compared with the parent line. Contrary to the $iPLA_2^{-/-}$ mice whose gene deletion leads to severe motor dysfunctions (Shinzawa et al., 2008), $cPLA_2^{-/-}$ mice display no apparent deficiency of the nervous system and are resistant to the 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine neurotoxicity and to ischemic injury (Klivenyi et al., 1998; Tabuchi et al., 2003). Interestingly, inhibition of $cPLA_2$ in the developing visual system of zebra fish modifies the morphology of neuronal *tectum arbor* and induces faster branch dynamics, while addition of exogenous AA reverses these effects (Leu and Schmidt, 2008), suggesting that AA released by activated $cPLA_2$ can modulate synaptic plasticity.

We previously showed that intracerebroventricular (ICV) injections of nanomolar levels of $Aβ_{42}$ oligomers in wild type (WT) mice rapidly induce short- and long-term memory deficits, which mimic the early stages of AD (Garcia et al., 2010; Youssef et al., 2008). Because various interfering pathological mechanisms occur in AD transgenic murine models such as the one previously used by Sanchez-Mejia et al. (2008), we applied this model to $cPLA_2^{-/-}$ mice. In this study, we show that inactivation of $cPLA_2$ gene completely protects synaptic functions and cognitive abilities against Aβ oligomer toxicity. Furthermore, we provide evidence that in vivo resistance is mediated by maintenance of survival pathways in neuronal cells. Finally, we observed a reduction of APP expression level in synaptosome-enriched fractions from $cPLA_2^{-/-}$ mice, this effect being confirmed in vitro in $cPLA_2^{-/-}$ neurons cultures and in WT neuronal cells in which $cPLA_2$ expression was suppressed using pharmacological inhibitors or antisense oligonucleotides.

2. Methods

2.1. Materials

$Aβ_{42}$ peptides were obtained from Bachem (Weil am Rhein, Germany). To overcome problems of peptide solubility at high concentrations, fresh peptide stock solutions were prepared at 5 mg/mL in the disaggregating solvent hexafluoro-2-propanol, as previously described (Pillot et al., 1999) and kept frozen at −80 °C. For the incubation of peptides with neurons, aliquots of peptide stock solution were quickly dried under nitrogen and directly solubilized at the experimental concentrations into the culture medium. Peptide solutions were then applied onto the cells. Before the use on cell cultures, preparations of Aβ oligomers were

analyzed on 10% polyacrylamide gels. Coomassie-blue staining revealed Aβ oligomers to be essentially trimers and tetramers, as previously described (Berman et al., 2008; Youssef et al., 2008; Supplementary Fig. 1). All chemicals were purchased from Sigma (St Quentin Fallavier, France) unless otherwise specified. All media and growth supplements used for cell culture were obtained from Invitrogen (Illkirch, France). Oligonucleotides were purchased from Eurogentec (Angers, France).

2.2. Intracerebroventricular injection of soluble Aβ oligomers

Twelve-week-old male $cPLA_2^{-/-}$ mice (gift from Dr. J. Bonventre, Harvard Medical School) and $cPLA_2^{+/+}$ BALB/c mice (purchased from Janvier Breeding Laboratories, Le Genest St Isle, France) were housed 5 to 6 per cage with free access to food and water, and were kept in a constant environment (22 $\pm$ 2 °C temperature, 50 $\pm$ 5% humidity, 12-hour light cycle). Soluble $A\beta_{42}$ oligomers were prepared as described above as stock solutions at the concentration of 0.5 mM in sterile 0.1-M phosphate-buffered saline (PBS) (pH 7.4) and aliquots were stored at −20 °C until use. Soluble $A\beta_{42}$ oligomers (0.5 nmol in 1 μL) or vehicle (PBS) were injected into the right ventricle of anesthetized animals with stereotaxic coordinates from the bregma being in mm, anteroposterior: 0.22; lateral: 1.0; and dorsal: 2.5. Injections were made using a 10-μL Hamilton microsyringe fitted with a 26-gauge needle. Each group consisted of 12 animals. Learning and memory capacities were assessed using Y-maze and Morris water maze tests. The animal facilities and all animal procedures were approved by the Animal Care and Veterinary Committee of Meurthe-et-Moselle (Nancy, France).

2.3. Y-maze task

Immediate spatial working memory performance was assessed by recording spontaneous alternation behavior in a Y-maze as previously described (Sarter et al., 1988; Youssef et al., 2008). The Y-maze task was carried out on Day 4 after soluble Aβ oligomers administration. The maze was made of opaque plexiglas and arms were 40 cm long, 16 cm high, 9 cm wide, and positioned at equal angles. Mice were placed at the end of one arm and allowed to move freely through the maze during a 5-minute session. The series of arm entries were recorded visually and arm entry was considered to be completed when the hind paws of the mouse were completely placed in the arm. Alternation was defined as successive entries into the 3 arms on overlapping triplet sets. The percentage alternation was calculated as the ratio of actual (total alternations) to possible alternations (defined as the total number of arm entries minus 2), multiplied by 100.

2.4. Morris water maze task

The Morris water maze task was performed as previously described (Morris, 1984). The experimental apparatus consisted of a circular water tank (diameter = 80 cm; height = 50 cm) containing water at 22 °C, 25 cm deep and rendered opaque by adding an aqueous acrylic emulsion. A platform (diameter = 10 cm) was submerged 1 cm below the water surface and placed at the midpoint of 1 quadrant. The pool was placed in a test room homogenously illuminated with a light intensity of 100 lux and containing various prominent visual cues. The swimming paths of the animals were recorded using a video tracking system.

On Days 3 and 4 after injection, navigation to a visible platform was carried out before place-navigation in order to evaluate visual and motor abilities of the animals. Mice were submitted to 4 trials per day with 2 trials in the morning and 2 trials in the afternoon, and with an intertrial interval of at least 45 minutes. There was no extra maze cue in the room. The platform position and starting points were randomly distributed over all 4 quadrants of the pool. Mice that failed to find the platform after 60 seconds were manually guided to its location.

Memory-acquisition trials (training) were performed 4 times daily on Days 7–11 after injection of soluble Aβ oligomers to reach a steady state of escape latency. The mice were allowed to swim freely for 60 seconds, were left for an additional 30 seconds period on the hidden platform, and were then returned to the home cage during the intertrial interval. The intertrial intervals between 4 trials were 45 minutes. Start positions, set at each limit between quadrants, were randomly selected for each animal. In each trial, the time required to escape onto the hidden platform was recorded. Mice failing to find the platform within 60 seconds were manually placed on the platform for 10 seconds at the end of the trial.

Memory-retention tests (probe trials) were performed on Day 14, i.e., 3 days after the last training session. The platform was removed and each mouse was allowed to a free 60-second swim. The number of crossings over the previous location of the platform and the time spent in each of the 4 quadrants were measured by replay using a video recorder.

2.5. Cell cultures and treatments

Primary cultures of cortical neurons were prepared from mouse or rat fetuses dissected on embryonic Days 16–17 according to Bouillot et al. (1996) and Pillot et al. (1999). Briefly, dissociated cells were plated at 1.5×10^5 cells per cm^2 for mouse cells or at 5×10^4 cells per cm^2 for rat cells in plastic dishes precoated with polyornithine (1.5 mg/mL). Cells were cultured in serum free Dulbecco's modified Eagle's/F-12 medium and supplemented with hormones, proteins, salts and 0.1% (wt/vol) ovalbumin. Cultures were maintained at 35 °C in a humidified 6% CO_2 atmosphere.

Such culture conditions promote neuronal cell selection and prevent astroglial proliferation. After 6–7 days in vitro (DIV), the cellular population was determined to be at least 96% neuronal by immunostaining with neuron-specific markers. All experiments were performed on 6–7 DIV neurons. Cells were treated with 1 or 5 μM soluble oligomers of Aβ_{42} for the indicated times or with 1 or 2 μM of the cPLA$_2$ inhibitor methoxy arachidonyl fluorophosphonate (MAFP) for 24 hours. Two sets of cPLA$_2$ sense and antisense oligonucleotides were designed and used as previously described (Kriem et al., 2005).

2.6. Neuronal viability and monitoring of apoptosis

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Pillot et al., 1999; Sponne et al., 2004). Cell nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI) staining (Sponne et al., 2004). To evaluate the percentage of apoptotic cells, 10 independent fields of microscope were counted (approximately 400 cells) in 3 separate experiments with 3 determinations each (Kriem et al., 2005).

2.7. Measurement of sphingomyelinase activities

Cells (2×10^6) were washed twice with PBS, pH 7.4, and lysed in 0.1% Triton X-100 for 10 minutes at 4 °C. The lysates were sonicated for 30 seconds in ice-cold bath, and protein concentrations were determined by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Illkirch, France). Acidic and neutral sphingomyelinase (A-SMase and N-SMase) activities were measured at pH 4.5 and 7.4, respectively, with the fluorescent substrate 6-hexadecanoylamino-4-methylumbelliferyl phosphocholine (HMU-P-Chol; Moscerdam Substrates, Oegstgeest, the Netherlands) as previously described (Kilkus et al., 2003). Twenty-five microgram proteins, buffered either in 150 mM sodium acetate solution, containing 0.1% Triton X-100, 0.2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (pH 4.5) (A-SMase activity), or in PBS, 0.1% Triton X-100, 0.2% CHAPS, 0.5 mM $MgCl_2$ (pH 7.4) (N-SMase activity), were incubated for up to 10 hours with 0.6 mM HMU-P-Chol. The fluorescence was monitored at 370-nm excitation and 460-nm emission using a Fluostar microplate reader (BMG-Labtechnologies, Champigny s/Marne, France). SMases activities were calculated from the slope of the intrinsic fluorescence versus time graph, standardized per microgram of proteins and expressed as a percentage of control values.

2.8. Immunoblot analyses

Primary cultures of cortical neurons treated under different conditions as described in the figures legends were washed with ice-cold PBS. Cells were then solubilized in a 25 mM Tris-HCl (pH 7.4) lysis buffer containing 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) nonidet P-40, 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and protease inhibitors (Complete, Roche, Meylan, France). After homogenization, collected cells were then lysed by using 3 cycles of freezing and thawing and finally centrifuged at 10,000g at 4 °C for 10 minutes. Protein concentration in the supernatant was determined by BCA Protein Assay Kit (Pierce). Samples were mixed with an equal volume of 2× Laemmli buffer, and then denatured by heating the mixture at 100 °C for 5 minutes.

Synaptosomal membrane-enriched fractions were prepared as previously described (Keller et al., 1997). Briefly, brain regions were dissected and rapidly homogenized in ice-cold 10 mM Tris buffer (pH 7.4) containing 0.32 mM sucrose, 2 mM EDTA, 10 μg/mL sodium orthovanadate and protease inhibitors. The homogenate was spun at 800g for 15 minutes to remove nuclei and cell debris. The resulting supernatant was then centrifuged at 12,000g for 20 minutes and a second pellet was collected. This fraction, which is enriched in synaptosomes but also contains other components such as mitochondria, was used to evaluate the expression levels of the synaptic proteins.

Samples (cell extracts or synaptosome-enriched fractions) were then subjected to SDS polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The following antibodies were used: actin (1:1000 dilution; C-11 antibody; Santa Cruz, Heidelberg, Germany); Akt and P-Akt (S473) (1:1000 dilution; Cell Signaling, Saint Quentin en Yvelines, France); postsynaptic density protein 95 (PSD95; 1:500 dilution; Cell Signaling); β-tubulin (1:1000 dilution; Santa Cruz); synaptotagmin (1:1000 dilution; Calbiochem, Lyon, France); synaptophysin (1:500 dilution; Chemicon, Molsheim, France), glial fibrillary acidic protein (GFAP) (1:5000 dilution; Millipore, Molsheim, France), ionized calcium binding adapter molecule 1 (Iba1; 1:1000 dilution; Abcam, Paris, France), APP (1:1000 dilution; 22C11 antibody; Millipore). Immunoblots were probed with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution; Cell Signaling) and detected using the enhanced chemiluminescence protocol (ECL Kit; Amersham Biotech, Orsay, France). The Quantity One software (version 4.5), associated with the VersaDoc Imaging System (Model 1000; Bio-Rad, Marnes-la-Coquette, France), was used to quantify the protein levels.

2.9. Statistical analysis

StatView computer software (version 5.0, SAS Institute, Cary, NC) was used for the statistical analysis. Data were obtained from 3 to 5 separate experiments with 6 determinations each. Differences between control and treated groups were analyzed using Student t test. Multiple pairwise comparisons among the groups were performed using analysis of variance (ANOVA) followed by a Scheffe's post hoc test ($p < 0.05$ indicates significant differences between groups). For behavioral analysis, statistical comparisons were made by the Student t test and one-way analysis of

variance was carried out and followed by Fisher's post hoc test. All reported values are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. cPLA$_2^{-/-}$ mice are fully resistant to soluble Aβ oligomer-induced cognitive alterations

To determine whether cPLA$_2$ contributed to soluble Aβ oligomer-dependent cognitive impairment in vivo, we compared the memory and learning performances of cPLA$_2^{-/-}$ and cPLA$_2^{+/+}$ (WT) mice. WT mice or cPLA$_2^{-/-}$ mice were injected with 500 pmol soluble Aβ_{42} oligomers or vehicle (n = 12 per group) as previously described (Garcia et al., 2010; Youssef et al., 2008). The cognitive performances of mice were then monitored by Y-maze and Morris water-maze tests reflecting immediate spatial working memory performance, as well as learning and long-term memory capabilities, respectively.

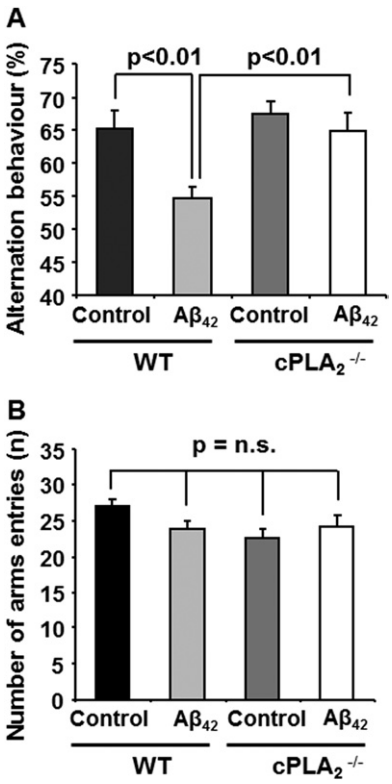

Fig. 1. cPLA$_2^{-/-}$ mice are resistant to soluble beta-amyloid (Aβ) oligomer-induced short-term memory impairment. The Y-maze tests were performed on Day 4 after intracerebroventricular (ICV) injection of soluble Aβ_{42} oligomers (500 pmol) or vehicle into cPLA$_2^{-/-}$ or wild type (WT) mice. Control mice were injected with vehicle (phosphate buffered saline [PBS]). (A) Spontaneous alteration behavior and the number of arm entries were measured during 5-minute sessions to evaluate spatial memory. (B) The number of arm entries did not differ among the different groups of mice. Locomotor, exploratory, visual, and motivational activities did not differ among groups. Data are presented as mean ± standard error of the mean (SEM) (n = 12).

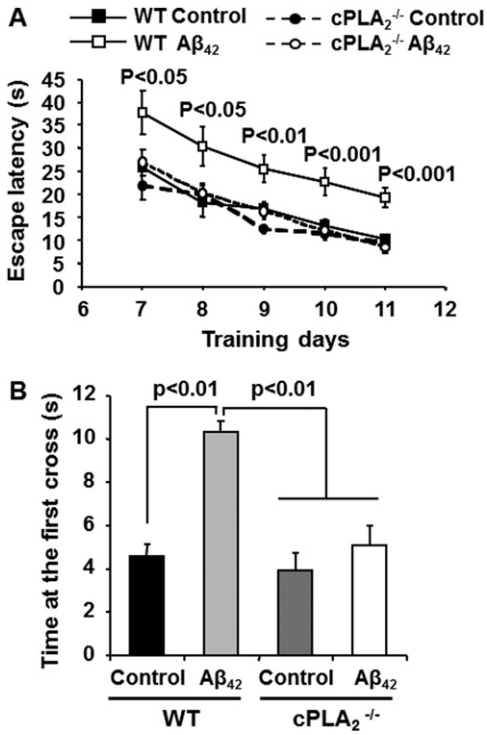

Fig. 2. cPLA$_2^{-/-}$ mice are resistant to soluble beta-amyloid (Aβ) oligomer-induced learning and long-term memory impairment. The Morris water-maze tests were performed from Day 7 to Day 14 after intracerebroventricular (ICV) injection of soluble Aβ_{42} oligomers (500 pmol) or vehicle into cPLA$_2^{-/-}$ or wild type (WT) mice. Control mice were injected with vehicle (phosphate buffered saline [PBS]). (A) The training trials were carried out on Days 7–11 after Aβ injection. Escape latency in training was measured and represented as the mean of a block of 4 trials per day. (B) The probe trial was carried out on Day 14 after Aβ_{42} injection. The time required for the first crossing over the platform site was recorded. All mice showed normal swimming performance (data not shown). Data are presented as mean ± standard error of the mean (SEM) (n = 12).

As expected, WT mice injected with soluble Aβ oligomers displayed significantly impaired spatial working memory (16% decrease in alternation behavior), while no alteration of short-term memory abilities were observed for the cPLA$_2^{-/-}$ mice after injection of Aβ oligomers (Fig. 1A). The number of arm entries did not change significantly among all the experimental groups (Fig. 1B), indicating that the observed changes were not due to exploratory, locomotor, visual, or motivational effects between WT and cPLA$_2^{-/-}$ mice.

In the Morris water maze, injection of Aβ oligomers did not suppress but dramatically reduced the learning abilities of the WT mice (12–20 seconds longer escape latencies along the 5 training days) (Fig. 2A). Such a partial loss—but not suppression—of learning abilities has been previously reported in other works using ICV injections of various forms of Aβ peptides (Aβ_{1-42}, Aβ_{1-40}, Aβ_{3-42}, Aβ_{25-35}) (Kim et al., 2010; Pan et al., 2010; Tsukuda et al., 2009; Youssef et al., 2008). The probe tests indicated that the long-term memory abilities of these mice were strongly

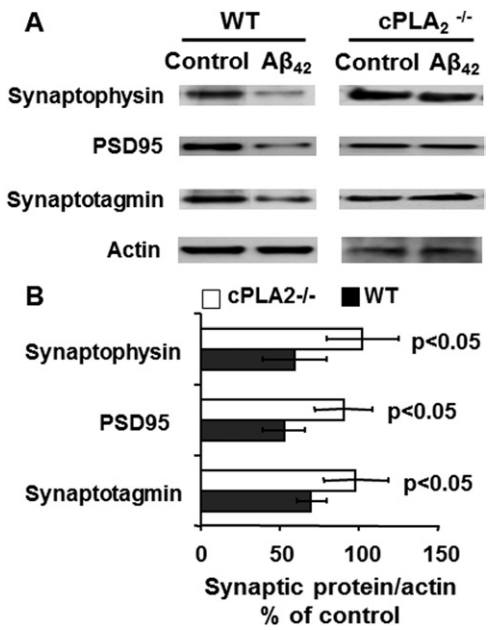

Fig. 3. cPLA$_2^{-/-}$ mice are resistant to soluble beta-amyloid (Aβ) oligomer-induced synaptic impairments. After the behavioral analysis was completed, mice were sacrificed at Day 14 after intracerebroventricular (ICV) injection and synaptosome-enriched fractions were prepared from hippocampus. (A) Representative immunoblots of synaptic proteins from cPLA$_2^{-/-}$ and wild type (WT) mice after Aβ or vehicle ICV injection are shown. (B) Densitometric analyses of synaptic proteins immunoblots from Aβ_{42}- and vehicle-exposed mice were normalized to actin. Data are expressed as % of control (vehicle injections) and are represented as mean ± standard error of the mean (SEM) of 3 separate immunoblots.

impaired (a 2.5-fold longer time at the first cross) (Fig. 2B). In contrast, in cPLA$_2^{-/-}$ mice, the Aβ-induced decrease in cognitive performance was not observed (Fig. 2A and B). Similar distances were achieved by the mice of the various experimental groups, indicating the absence of effect on locomotion, visual abilities, or motivation (data not shown). Therefore, the results of this cognitive test indicate that the inactivation of cPLA$_2$ gene rendered the mice fully resistant to Aβ-induced alterations of learning and long-term memory.

3.2. cPLA$_2^{-/-}$ mice are fully resistant to soluble Aβ oligomer-induced synaptic impairments

Increasing evidence suggests that soluble Aβ oligomers adversely affect synaptic function, which leads to cognitive failure associated with AD (Arendt, 2009; Gu et al., 2009). We next examined whether cPLA$_2$ influenced soluble Aβ oligomer-induced synaptic degeneration in vivo. Because the hippocampus plays a critical role in memory and is the first brain region affected in AD, we examined the expression of synaptic proteins in hippocampal neurosynaptosome-enriched fractions prepared from WT and cPLA$_2^{-/-}$ mice that were ICV-injected with Aβ_{42} oligomers or vehicle. In WT mice, both pre- and postsynaptic proteins were affected at Day 14 after Aβ oligomer injection. Synaptotagmin, synaptophysin, and PSD95 were dramatically decreased in WT mice upon Aβ oligomers exposure (Fig. 3A). This was confirmed by quantitative analyses of the immunoblots (Fig. 3B). In contrast, the levels of all synaptic proteins were maintained in hippocampal synaptosomal fractions obtained from Aβ-treated cPLA$_2^{-/-}$ mice (Fig. 3A and B). Thus, the preservation of cognitive functions in cPLA$_2^{-/-}$ mice after ICV administration of Aβ oligomers was accompanied by the absence of decline in hippocampal synaptic proteins levels, indicating that inactivation of cPLA$_2$ gene preserved synaptic integrity against AD-like stress.

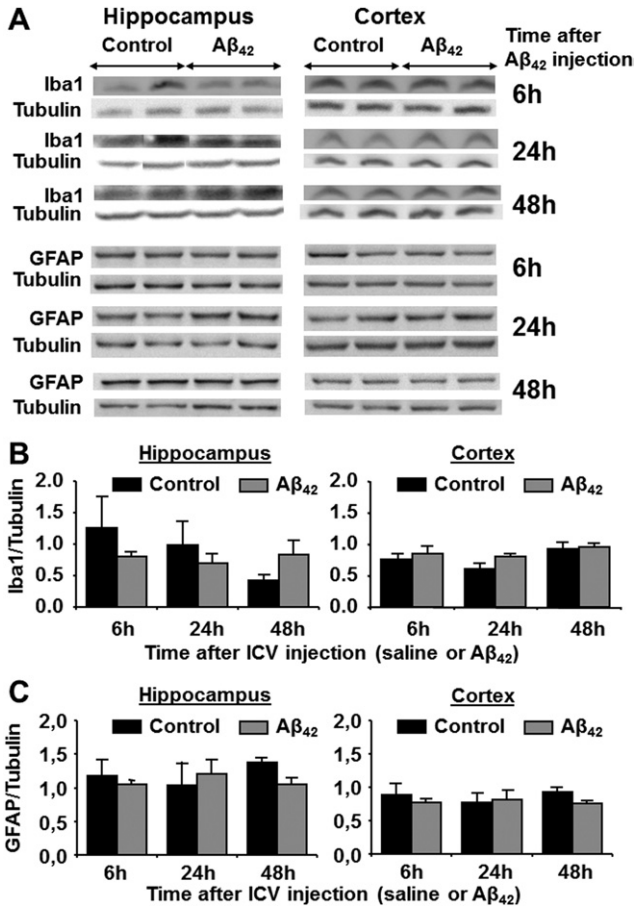

Fig. 4. Intracerebroventricular injection of beta-amyloid (Aβ) oligomers is not associated with neuroinflammation. Wild type (WT) mice were sacrificed 6, 24, and 48 hours after intracerebroventricular (ICV) injection of soluble Aβ_{42} oligomers (500 pmol) or vehicle and homogenates were prepared from cortex and hippocampus. (A) Immunoblots of the astroglial glial fibrillary acidic protein (GFAP) and microglial ionized calcium binding adapter molecule 1 (Iba1) markers are shown. (B) Densitometric analyses of Iba1 immunoblots from vehicle- and Aβ_{42}-exposed mice normalized to tubulin were carried out. Data are represented as mean ± standard error of the mean (SEM) of 2 separate immunoblots. (C) Densitometric analyses of GFAP immunoblots from vehicle- and Aβ_{42}-exposed mice after normalization to tubulin were performed. Data are represented as mean ± SEM of 2 separate immunoblots.

3.3. ICV injection of soluble Aβ oligomers is not associated with an increase in neuro-inflammation markers

AA released through the activation of $cPLA_2$ can be converted in a series of proinflammatory mediators which can lead to neuroinflammation and glial proliferation. To determine whether Aβ injection was followed by inflammatory processes and glial activation, we examined the expression levels of GFAP and Iba1 as astroglial and microglial markers, respectively (Romero-Sandoval et al., 2008) in Aβ- and vehicle-injected WT mice. We observed a weak and variable increase in hippocampal Iba1 levels 6 and 24 hours after vehicle ICV injection compared with the levels observed after Aβ administration (Fig. 4A and B). Iba1 expression was decreased in hippocampus 48 hours after vehicle injection compared with 6 and 24 hours. However, after Aβ ICV administration, it remained at the same level throughout the times studied (Fig. 4A and B). By contrast, no significant variation of Iba1 expression levels was observed in the cortex between 6 and 48 hours after vehicle or Aβ injections (Fig. 4A and B). Similarly, no change in GFAP levels was observed in both hippocampus and cortex in the same period of time in all groups studied excluding astroglial activation (Fig. 4A–C). These results suggest that inflammatory processes do not significantly contribute to Aβ-induced behavioral and synaptic impairments in our ICV injection model.

3.4. $cPLA_2^{-/-}$ neurons are resistant to soluble Aβ oligomer-induced neurotoxic and apoptotic effects

To determine whether the resistance of $cPLA_2^{-/-}$ mice to Aβ oligomer-induced cognitive and synaptic impairments could be associated with resistance of neuronal cells, we next investigated the effects of the inactivation of $cPLA_2$ gene on the soluble Aβ-induced neurotoxic and apoptotic effects in vitro. As expected from our previous reports (Florent et al., 2006; Garcia et al., 2010; Kriem et al., 2005; Malaplate-Armand et al., 2006), the incubation of cortical neurons of WT mice with 1 or 5 μM $A\beta_{42}$ oligomers for 24 hours resulted in a 40%–50% decrease in cell viability (Fig. 5A), whereas 1 μM $A\beta_{42}$ induced a 5-fold increase in apoptotic nuclei number, as compared with untreated WT neurons (Fig. 5B). Interestingly, $cPLA_2^{-/-}$ cortical neurons were fully resistant to these soluble Aβ effects (Fig. 5A and B). Moreover, cortical neurons of WT mice incubated with 1 μM $A\beta_{42}$ for 6 hours (i.e., prior to apoptosis measurement at 24-hour incubation time) exhibited a 65% reduction of the synaptic PSD95 protein expression level. In contrast, PSD95 level was preserved in $cPLA_2^{-/-}$ cortical neurons in the presence of 1 μM $A\beta_{42}$ oligomers (Fig. 5C and D).

We have previously identified SMases as potential downstream actors in the Aβ-activated $cPLA_2$ proapoptotic cascade (Malaplate-Armand et al., 2006). Here, the incubation of cortical WT neurons with 1 μM $A\beta_{42}$ oligomers for

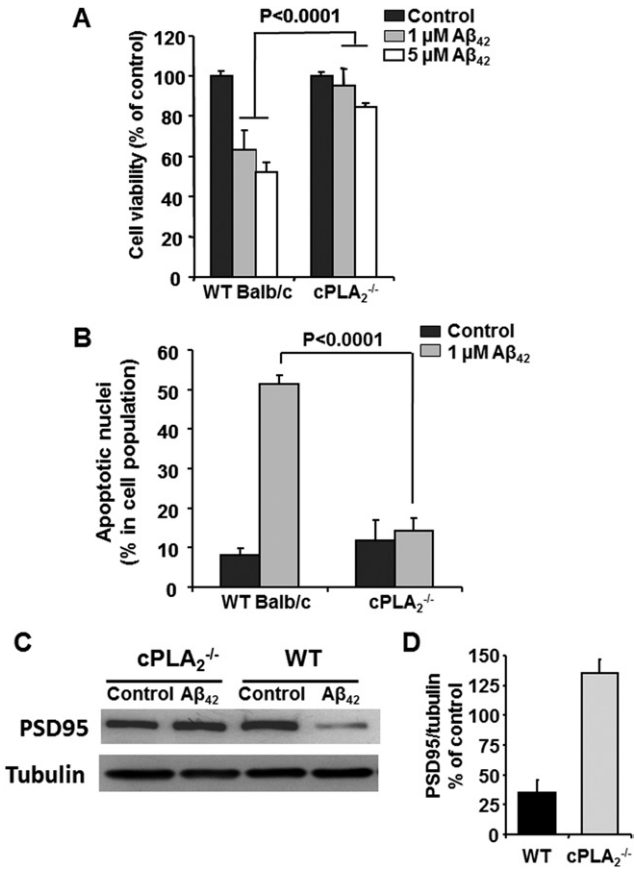

Fig. 5. Primary cortical neurons from $cPLA_2^{-/-}$ mice are resistant to soluble beta-amyloid (Aβ) oligomer-induced cytotoxic and apoptotic effects. Primary cultures of cortical neurons from wild type (WT) and $cPLA_2^{-/-}$ mice were incubated for 24 hours with soluble $A\beta_{42}$ oligomers. (A) Cell survival was monitored by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are normalized to the effect of vehicle, designated as 100%. (B) Apoptotic nuclei were visualized using 4,6-diamidino-2-phenylindole (DAPI) staining (data not shown) and quantified from 10 individual fields per coverslip. For each set of experiments, data are represented as the mean ± standard error of the mean (SEM) of 3 independent experiments with 4 determinations each. (C) Postsynaptic density protein 95 (PSD95) expression levels were measured in lysates of primary cultures of cortical neurons from WT and $cPLA_2^{-/-}$ mice which were incubated for 6 hours with 1 μM $A\beta_{42}$ oligomers or vehicle. A representative immunoblot is shown for 2 independent immunoblot analyses. (D) Densitometric analyses of PSD95 immunoblots on lysates of cortical neurons were performed. Data are expressed as % of the PSD95/tubulin ratio obtained in presence of $A\beta_{42}$ oligomers normalized to that of vehicle-treated cells (of the same genotype), and are represented as mean ± SEM of 2 separate immunoblots.

3 hours led to a marked increase in both A-SMase and N-SMase activities (3- and 2.5-fold, respectively). In $cPLA_2^{-/-}$ neurons, however, this activation of N- and A-SMases in response to $A\beta_{42}$ exposure was not observed (Fig. 6A and B).

Alterations of the prosurvival Akt signaling pathway have emerged as an important feature in AD-associated neuronal death (Lee et al., 2009). In WT cortical neurons, Akt phosphorylation was drastically reduced by 80%

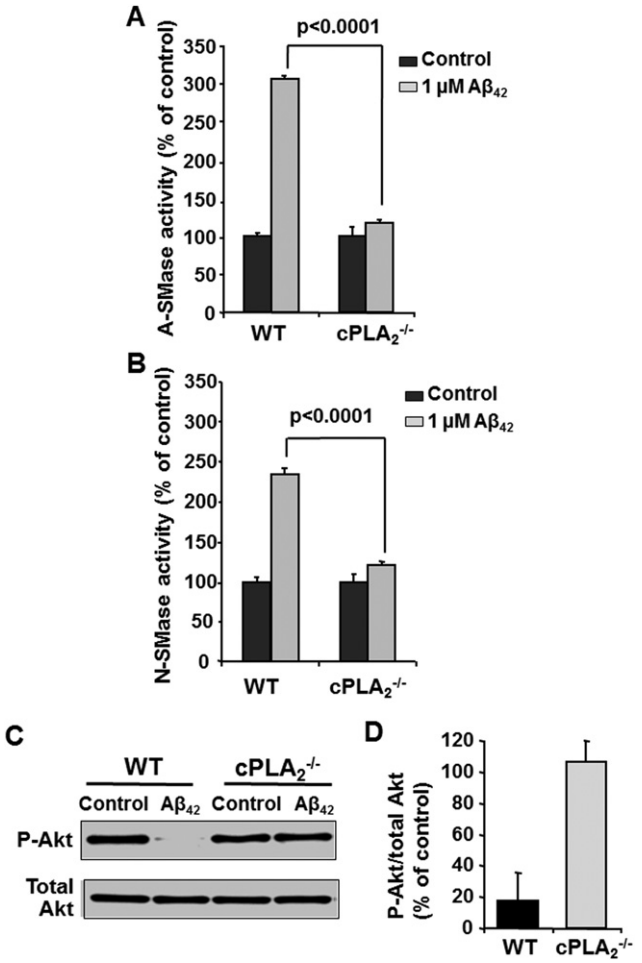

Fig. 6. Activation of sphingomyelinases (SMases) is suppressed and Akt pathway is preserved in $cPLA_2^{-/-}$ neurons in presence of soluble beta-amyloid (Aβ) oligomers. Treatment with 1 μM $A\beta_{42}$ oligomers for 6 hours was performed on primary cultures of cortical neurons. Acidic SMase (A) and Neutral SMase (B) activities were monitored by measuring the relative increase in fluorescence due to the cleavage of fluorogenic substrate in the presence of cell extracts. Data are represented as the mean ± standard error of the mean (SEM) of 3 independent experiments with 4 determinations each, normalized to the effect of vehicle, designated as 100%. (C) Analyses of the phosphorylation levels of Akt after $A\beta_{42}$ exposure were performed on immunoblots of protein extracts isolated from wild type (WT) or $cPLA_2^{-/-}$ neurons at 7 days in vitro (DIV). These neurons were exposed to 1 μM $A\beta_{42}$ soluble oligomers or vehicle for 3 hours. A representative immunoblot is shown for 3 independent immunoblot analyses. (D) Densitometric analyses of phospho-Akt (P-Akt) and total Akt immunoblots on lysates of primary cultures of neuronal cells were performed. Data are expressed as p-Akt/total Akt ratio obtained in presence of $A\beta_{42}$ oligomers normalized to vehicle-treated cells of the same genotype. Quantifications are shown as mean ± SEM of 3 separate immunoblots.

(average value) upon exposure to 1 μM soluble $A\beta_{42}$ oligomers during 3 hours, while it was not altered in the $cPLA_2^{-/-}$ cells compared with the untreated controls (Fig. 6C and D).

Taken together, these data indicate that $cPLA_2$ represents an important and crucial mediator of Aβ oligomer-induced apoptotic cascade and the suppression of its activity leads to a full resistance to the neurotoxic effects of Aβ oligomers.

3.5. Effects of gene inactivation and pharmacological inhibition of $cPLA_2$ on the level of neuronal APP expression

Because previous works reported that nonspecific inhibitors or activators of the AA/PLA_2 cascade modulate APP metabolism and hydrolysis (Emmerling et al., 1993; Kinouchi et al., 1995), we investigated the relationship between $cPLA_2$ expression and activity on one hand and APP expression on the other hand. We first examined the influence of $cPLA_2$ gene inactivation on APP expression and we

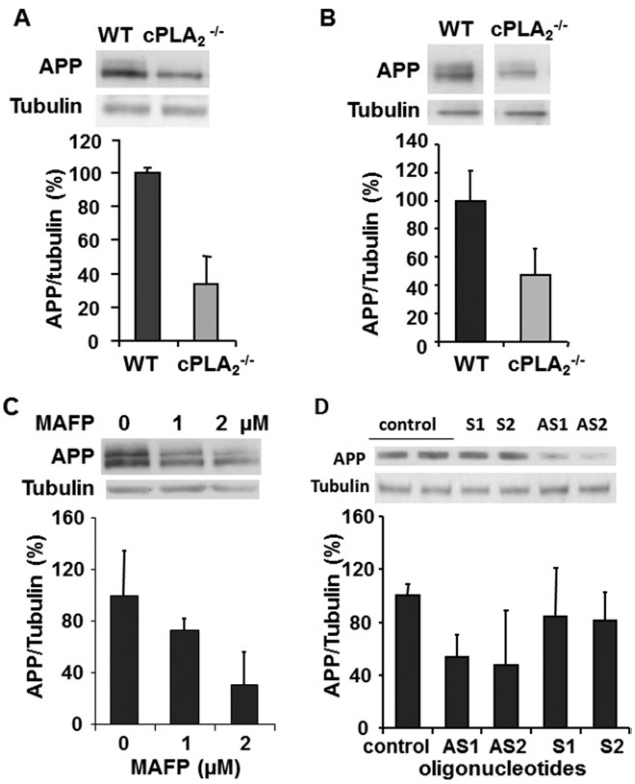

Fig. 7. Cytosolic phospholipase A_2 ($cPLA_2$) inhibition is associated with a lower level of neuronal beta-amyloid (Aβ) precursor protein (APP) full-length protein. Immunoblot analyses of full-length APP (A) in protein extracts from $cPLA_2^{-/-}$ and wild type (WT) mice primary cultures of cortical neurons, (B) in homogenates from $cPLA_2^{-/-}$ and WT mice hippocampus, (C) in protein extracts from rat primary cultures of cortical neurons treated for 24 hours with the specific pharmacological $cPLA_2$ inhibitor methoxy arachidonyl fluorophosphonate (MAFP), and (D) in protein extracts from rat primary cultures of cortical neurons treated for 48 hours with 4 μM antisense oligonucleotides against $cPLA_2$ (antisense oligonucleotide AS1 and AS2) and control sense oligonucleotides (S1 and S2). Representative densitometric analyses of immunoblots of APP normalized to tubulin were carried out. Data are shown as mean ± standard error of the mean (SEM) of 3 separate immunoblots.

observed that the levels of APP were 2-fold lower in extracts of neurons and hippocampal homogenates from $cPLA_2^{-/-}$ mice than in those from WT mice (Fig. 7A and B). We further observed that inhibition of $cPLA_2$ activity in rat cortical neurons by using 1 or 2 μM of the pharmacological $cPLA_2$ inhibitor MAFP for 24 hours decreased APP levels by 25% and 70%, respectively (Fig. 7C). We had previously demonstrated that the antisense oligonucleotides AS1 and AS2 targeting $cPLA_2$ protect neuronal cells against Aβ oligomer neurotoxicity (Kriem et al., 2005). Incubation of neuronal cells with 4 μM of AS1 and AS2 for 48 hours led to a 2-fold decrease in APP levels (Fig. 7D). Whether and how the reduction of APP levels and the resistance to Aβ oligomer neurotoxicity are mechanistically related, remains to be elucidated.

4. Discussion

In this study, we provide new evidence that $cPLA_2$ is an early and crucial mediator of Aβ oligomer-induced cognitive deficit associated with synaptic impairment. We found that inactivation of the $cPLA_2$ gene renders mice completely resistant to the behavioral alterations in learning as well as in short- and long-term memory that occur following a single ICV injection of soluble Aβ peptide. Accordingly, hippocampal synaptic integrity is maintained in $cPLA_2^{-/-}$ mice. In addition, the activation of the proapoptotic pathways induced by the Aβ oligomers is suppressed in neuronal cells of $cPLA_2^{-/-}$ mice. The influence of $cPLA_2$ in AD pathogenesis has been explored in chronic AD models such as transgenic mice models or in postmortem human brains. Indeed, its levels of messenger RNA (mRNA) and protein are increased in the hippocampal CA1 area of AD patients (Colangelo et al., 2002; Stephenson et al., 1996). $cPLA_2$ has also been proposed to be a key enzyme in several pathological processes in the nervous system such as ischemia/reperfusion injuries (Tabuchi et al., 2003) and experimental autoimmune encephalomyelitis, a model of multiple sclerosis (Marusic et al., 2005).

The studies on the AD mechanisms and on putative therapeutic strategies have largely been based on the use of transgenic mice expressing human APP and presenilin genes. In these models, large amounts of Aβ peptide are produced early, leading to its accumulation in amyloid plaques and generating complex pathological phenomena such as glial activation, inflammation, and oxidative stress. Such being the case, the respective contributions of the various forms of Aβ peptide, including the intraneuronal forms, extracellular monomers, oligomers, or amyloid fibrils are difficult to discriminate. For example, increased levels of proinflammatory mediators were measured in transgenic AD mice models. Sanchez-Mejia et al. (2008), in a study using transgenic hAPP J20 mice, suggested that $cPLA_2$ gene inactivation inhibits the AD neurodegenerative process by reducing the production of proinflammatory eicosanoids. In this study, we used a more relevant model mimicking the early stages of sporadic AD in which behavioral and synaptic impairments are induced upon acute exposure to small amounts of extracellular Aβ oligomers (Garcia et al., 2010; Youssef et al., 2008). The preparations of synthetic $A\beta_{42}$ oligomers used contained predominantly trimers and tetramers similar to those prepared from synthetic batches or produced in recombinant cell systems (Berman et al., 2008; Supplementary Fig. 1). This approach allows the determination of the neurotoxic transduction pathways induced by the Aβ oligomers in order to identify therapeutic targets aimed towards inhibiting or decreasing the sensitivity of neural cells to these oligomers. Evaluation of protein levels of astroglial or microglial markers indicated no significative neuroinflammation in this model. Dysfunction of synapses and reduction of plasticity are typical and early function-related events in the AD pathogenesis (Selkoe, 2002). Several papers reported that synaptic loss is initiated by alterations in glutamate receptors and other synaptic proteins involved in trafficking and endocytosis (Parameshwaran et al., 2008). It is worth noting here that a single injection of Aβ oligomers could induce drastic reductions of the levels of several synaptic markers, synaptotagmin, synaptophysin, and PSD95, and that this effect was abolished by $cPLA_2$ gene inactivation. Interestingly, Sanchez-Mejia et al. (2008) reported that 10 μM of $A\beta_{42}$ oligomers transiently increased surface levels of the AMPA receptor GluR1 and GluR2 subunits on cultured neurons, and the use of arachidonyl trifluoromethyl ketone (AACOCF3), another pharmacological inhibitor of $cPLA_2$ suppressed this effect. Despite that the concentration of $A\beta_{42}$ oligomers used was 10-fold higher than in our neuronal cultures, these results together support the notion that $cPLA_2$ plays a critical role in Aβ oligomer-induced neurosynaptotoxicity. The presynaptic proteins synaptophysin and synaptotagmin are involved in the formation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes with several other partners such as syntaxin-1 and Munc18. AA stabilizes interactions between Munc18, syntaxin-1 and synaptosomal-associated protein, 25 kDa (SNAP25), thus favoring exocytosis of neurotransmitters (Connell et al., 2007; Latham et al., 2007). Increase in free AA released by $cPLA_2$ in response to Aβ oligomers could therefore be involved in synaptic dysfunctions. Moreover, a fraction of $cPLA_2$ binds to Golgi apparatus in response to calcium and its activity is increased by Ser^{505} phosphorylation (Tucker et al., 2009). Previous reports showed that $cPLA_2$ activity modifies the transport of several proteins between Golgi vesicles and plasma membranes in nonneuronal cell types (Choukroun et al., 2000; Regan-Klapisz et al., 2009). Taking this into account along with the role of AA in Munc18-syntaxin interactions (Connell et al., 2007) and the influence of $cPLA_2$ on membrane organization and lipid vesicles formation (Gubern et al., 2008), it is tempting to speculate that Aβ-activated $cPLA_2$

could surrogate the transportation of synaptic proteins into various lipid compartments.

Besides synaptotoxicity, neuronal apoptosis is another hallmark of AD. Aβ oligomers generate a proapoptotic pathway through SMase activation and ceramide production (Alessenko et al., 2004; Malaplate-Armand et al., 2006). We previously demonstrated that $cPLA_2$ activation occurs upstream to that of SMases by using pharmacological inhibitors and antisense oligonucleotides (Malaplate-Armand et al., 2006). We confirm here the link between $cPLA_2$ and SMase activation in Aβ-induced neuronal apoptosis by showing that $cPLA_2$ gene inactivation completely precludes activation of both A- and N-SMases by Aβ oligomers. Furthermore, Akt/protein kinase B (PKB) survival pathway is also maintained in neuronal cells derived from $cPLA_2^{-/-}$ mice in the presence of Aβ oligomers, contrary to those derived from WT mice. Indeed, it has been demonstrated that AA released by $cPLA_2$ is a negative regulator of Akt/PKB activation by growth factors and insulin in striated muscle cells (Haq et al., 2003). We therefore show that the in vivo resistance to Aβ-induced alterations of cognitive abilities and synaptic protein levels is associated with the in vitro resistance of neuronal cells to the proapoptotic and anti-survival effects of Aβ oligomers.

Activation of Akt/PKB and PI3-kinase also modulates APP trafficking and lysosomal degradation (Shineman et al., 2009). Interestingly, we observed a reduction of APP levels in hippocampal homogenates and primary cultured neuronal cells from $cPLA_2^{-/-}$ mice. Moreover, this effect was mimicked in rat primary neuronal cultures in the presence of $cPLA_2$ inhibitors or antisense oligonucleotides. This is in contrast to the study of Sanchez-Meija et al. (2008), who did not observe any variation of the human APP level nor Aβ production in their transgenic J20 mice. Whether mouse endogenous APP expression levels were modified was not indicated in their study. Culicchia et al. (2008) recently described simultaneous overexpression of $cPLA_2$ and APP in several human glioblastoma cell lines, suggesting a link between these 2 proteins. After its maturation and transportation from endoplasmic reticulum and Golgi apparatus to synaptic membranes, APP is rapidly internalized and subsequently trafficked through endosomes back to the cell surface or degraded in the lysosomes (Thinakaran and Koo, 2008). Since $cPLA_2$ modifies the transport of several proteins between Golgi vesicles and plasma membranes in nonneuronal cells as we discussed above (Choukroun et al., 2000; Regan-Klapisz et al., 2009), $cPLA_2$ might regulate APP trafficking and metabolism similar to other synaptic proteins into various lipid compartments. Besides its role in Aβ peptide generation, APP protein is required for synaptic plasticity and other neuronal functions (Dawson et al., 1999; Priller et al., 2006). Moreover Aβ oligomers bind to the cognate Aβ extracellular domain of APP and generate a G protein-dependent intracellular signaling pathway through APP dimerization (Shaked et al., 2006, 2009). These data suggest that modifications of APP expression and/or processing could be involved in the $cPLA_2$ inhibition-mediated protection against Aβ oligomers neurotoxicity.

Recent studies have been devoted toward the design of antibodies (Hillen et al., 2010), peptides (Esteras-Chopo et al., 2008), flavonoids (Thapa et al., 2011), or other pharmaceutical compounds (Zhao et al., 2009) to block Aβ peptide oligomerization, but the in vivo efficiency of such a strategy requires extensive knowledge of the oligomerization process and the toxicity of the various oligomeric forms. Other studies have focused on the functional interactions between Aβ peptide and glutamate receptors (Decker et al., 2010b; Hu et al., 2009; Texidó et al., 2011; Zhao et al., 2010) or have attempted to reinforce neuronal defenses through the neurotrophic effects of growth factors or membrane lipids such as docosahexaenoic acid (Ma et al., 2009). Many NMDA receptor modulators have been characterized and are potential therapeutics for Alzheimer's disease. However, clinical assays are required to demonstrate their therapeutic efficiency. Neurotrophic compounds are interesting from a preventive point of view, and epidemiological studies should be used to determine the target populations. Other approaches have focused on the oxidative stress generated by Aβ peptide, but the specificity and efficiency are uncertain because oxidative stress is found in many diseases and is not the only pathological mechanism in AD. Finally, many studies identified intracellular mediators of the Aβ peptide neurotoxic effects. These intracellular targets include membrane-phospholipid remodeling enzymes such as synaptojanin and phospholipase D (Berman et al., 2008; Oliveira et al., 2010) or signaling proteins such as fyn kinase, glycogen synthase kinase-3β, cyclin-dependent kinase-5 (Crews and Masliah, 2010). Some inhibitors of these putative therapeutic targets have been proposed because of their potential specificity.

In conclusion, our study provides both in vitro and in vivo evidence for the crucial role that $cPLA_2$ plays in Aβ oligomer-induced neurodegeneration, synaptotoxicity and memory impairments at early stages of AD. The mechanisms by which $cPLA_2$ gene inactivation disrupts Aβ oligomers in vitro and in vivo neurotoxicity remain yet to be delineated, but our results led us to propose $cPLA_2$ as a potentially useful therapeutic target for developing disease modifying therapies for AD.

Disclosure statement

The authors have no actual or potential conflicts of interest.

The animal facilities and all animal procedures were approved by the Animal Care and Veterinary Committee of Meurthe-et-Moselle (Nancy, France).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2011.11.008.

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