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Sub-lethal levels of amyloid β-peptide oligomers decrease non-transferrin-bound iron uptake and do not potentiate iron toxicity in primary hippocampal neurons

C. D. SanMartín · A. C. Paula-Lima · C. Hidalgo · M. T. Núñez

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Abstract Two major lesions are pathological hallmarks in Alzheimer's disease (AD): the presence of neurofibrillary tangles formed by intracellular aggregates of the hyperphosphorylated form of the cytoskeletal tau protein, and of senile plaques composed of extracellular aggregates of amyloid beta (Aβ) peptide. Current hypotheses regard soluble amyloid beta oligomers (AβOs) as pathological causative agents in AD. These aggregates cause significant calcium deregulation and mediate neurotoxicity by disrupting synaptic activity. Additionally, the presence of high concentrations of metal ions such as copper, zinc, aluminum and iron in neurofibrillary tangles and senile plaques, plus the fact that they accelerate the rate of formation of Aβ fibrils and AβOs in vitro, suggests that accumulation of these metals in the brain is relevant to AD pathology. A common cellular response to AβOs and transition metals such as copper and iron is the generation of oxidative stress, with the ensuing damage to cellular components. Using hippocampal neurons in primary culture, we report here the effects of treatment with AβOs on the (+)IRE and (−)IRE mRNA levels of the divalent metal transporter DMT1. We found that non-lethal AβOs concentrations decreased DMT1 (−)IRE

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C. D. SanMartín · A. C. Paula-Lima · C. Hidalgo
Facultad de Medicina, Center for Molecular Studies of the Cell, Universidad de Chile, Santiago, Chile

A. C. Paula-Lima
Department of Basic and Communitarian Sciences, Facultad de Odontología, Universidad de Chile, Santiago, Chile

C. Hidalgo
Physiology and Biophysics Program, Facultad de Medicina, Institute of Biomedical Sciences and Biomedical Neuroscience Institute, Universidad de Chile, Santiago, Chile

C. Hidalgo (✉)
Facultad de Medicina, ICBM, Universidad de Chile, Independencia 1027, Santiago 838-0453, Chile
e-mail: chidalgo@med.uchile.cl

M. T. Núñez
Biotechnology and Department of Biology, Facultad de Ciencias, Institute for Cell Dynamics, Universidad de Chile, Santiago, Chile

M. T. Núñez (✉)
Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Santiago 780-0024, Chile
e-mail: mnunez@uchile.cl

without affecting DMT1 (+)IRE mRNA levels, and inhibited non-transferrin bound iron uptake. In addition, since both iron and AβOs induce oxidative damage, we studied whether their neurotoxic effects are synergistic. In the range of concentrations and times used in this study, AβOs did not potentiate iron-induced cell death while iron chelation did not decrease AβOs-induced cell death. The lack of synergism between iron and AβOs suggests that these two neurotoxic agents converge in a common target, which initiates signaling processes that promote neurodegeneration.

Keywords Alzheimer's disease · Amyloid-beta neurotoxicity · DMT1 · Iron neurotoxicity

Introduction

Alzheimer's disease (AD), the leading cause of dementia worldwide, is an age-related neurodegenerative disorder characterized by progressive memory loss. Accumulation of the amyloid beta (Aβ) peptide in the brain correlates directly with AD pathogenesis (Ferreira and Klein 2011; Glenner and Wong 1984; Masters et al. 1985). Increased Aβ peptide levels favor self-association and generation of different Aβ aggregates, which include insoluble Aβ fibrils and soluble Aβ oligomers that are toxic to neurons and other brain cell types (Louzada et al. 2004; Paula-Lima et al. 2011; Paula-Lima et al. 2005; Paula-Lima et al. 2009). Particularly, soluble Aβ oligomers (AβOs) accumulate specifically in AD human brain and cerebrospinal fluid and act as potent and diffusible neurotoxins, which associate with synapses (Lacor et al. 2007) and interfere with synaptic plasticity inhibiting long-term potentiation (LTP), a classic paradigm of memory-associated synaptic mechanisms (Lambert et al. 1998; Walsh et al. 2002). At the cellular level, AβOs induce oxidative stress (De Felice et al. 2007), promote excitotoxicity (Alberdi et al. 2010) and cause a swift and prolonged increase in intracellular Ca^{2+} concentration (Paula-Lima et al. 2011; SanMartin et al. 2012).

A progressive accumulation of systemic iron—accompanied by increased oxidative stress—occurs during ageing (Zecca et al. 2004). The highest iron accumulation occurs in the hippocampus and cortex, the most affected regions in AD, and in the *substantia nigra*, which is the affected brain area in Parkinson′s disease (Smith et al. 1997; Stankiewicz et al. 2007). In particular, iron accumulates in amyloid plaques and neurofibrillary tangles, the histopathological features of AD (Smith et al. 1997). Moreover, iron and other heavy metals such as aluminum, copper and zinc, accelerate the rate of formation of Aβ fibrils and AβOs in vitro (Ryu et al. 2008; Smith et al. 1997; Zecca et al. 2004). The Aβ aggregates formed in the presence of iron are more toxic that those formed in its absence (Liu et al. 2011), whereas iron scavengers reduce the formation of Aβ fibrils aggregated in the presence of iron (House et al. 2004).

Iron is a known inductor of oxidative stress in hippocampal cell cultures (Núñez-Millacura et al. 2002). Through the Fenton reaction iron triggers the production of the highly reactive hydroxyl radical (Núñez et al. 2012), which eludes the cellular antioxidant defenses and rapidly reacts with lipids, proteins and DNA generating irreversible oxidative damage (Gutteridge and Halliwell 2000). Neuronal iron uptake occurs via transferrin-dependent and transferrin-independent pathways. Because of the presence of non-transferrin-bound iron (NTBI) in cerebrospinal fluid (Moos and Morgan 1998), NTBI uptake is likely to occur in neurons that express DMT1, such as hippocampal pyramidal and granule cells, cerebellar granule cells, pyramidal cells of the piriform cortex, substantia nigra, and the ventral portion of the anterior olfactory nucleus (Gunshin et al. 1997). The high expression levels of the DMT1 in these regions suggest that DMT1-mediated iron uptake is necessary for their function.

The mammalian DMT1 gene undergoes alternative splicing originating the 1A and 1B mRNA DMT1 variants from the 5′-end, and the (+)IRE or (−)IRE variants from the 3′-end (Hubert and Hentze 2002), These mRNA variants give rise to four DMT1 protein isoforms, all of them active in Fe^{2+} transport. The DMT1-1B isoforms are ubiquitous whereas the DMT1-1A isoforms are found predominantly in the apical membranes of the duodenum (Hubert and Hentze 2002). Hippocampal neurons possess DMT1-1B mRNA with undetectable levels of DMT1-1A mRNA (Haeger et al. 2010). A recent report showed that hippocampal neurons contain primarily the DMT1-1B/(+)IRE protein isoform, which shows cytoplasmic distribution, colocalization with late endosome/lysosome markers and iron regulation, as expected from the presence of the iron responsive element in its sequence (Pelizzoni et al. 2012).

The generation of oxidative stress and damage is a common toxicological event for both AβOs and transition metals such as iron, raising the question whether the neurotoxic effects of AβOs and iron are additive. Using hippocampal neurons in primary culture, here we studied the effects of AβOs treatment on DMT1 (+)IRE and (−)IRE mRNA levels. We also investigated using optical techniques if AβOs affected NTBI uptake. In addition, we studied whether their effects on cell viability are synergistic. Our results indicate that treatment with AβOs decreased the levels of DMT1 (−)IRE mRNA and inhibited NTBI uptake, but did not potentiate iron-induced toxicity.

Materials and methods

Materials

Aβ peptide (Aβ1-42) was purchased from Bachem Inc. (Torrance, CA), Hanks-glucose solution, DMEM medium, Neurobasal medium, B-27 were from Gibco (Carlsbad, CA), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) from Sigma Chemical Corp (St Louis, MO). The DNA binding dye SYBR green (Platinum SYBR Green qPCR SuperMix UDG) and trizol reagent were from Invitrogen (Carlsbad, CA). The ImProm-II™ Reverse Transcriptase kit was from Promega (Madison, WI), the DNA-free™ Kit was from Ambion (Austin, TX), and Calcein-AM was from Molecular Probes (Grand Island, NY).

Primary hippocampal cultures

Cultures were prepared from 18-day old embryos obtained from pregnant Sprague–Dawley rats as previously described (Paula-Lima et al. 2011). Briefly, brains were removed and placed in a dish containing Hanks-glucose solution. Hippocampi were dissected and, after stripping away meninge membranes, cells were gently dissociated in Hanks-glucose solution, centrifuged and resuspended in DMEM medium supplemented with 10 % horse serum. Dissociated hippocampal neurons were plated on polylysine-coated plates and after 1 h DMEM was replaced by Neurobasal medium supplemented with B-27. Cells were incubated for 18–21 days in vitro (DIV) at 37 °C in a humidified 5 % CO_2 atmosphere prior to experimental manipulations.

Cell viability assay

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT to formazan, using the assay procedure described elsewhere (Paula-Lima et al. 2011). The extent of formazan production was quantified in triplicates by measuring optical density at 540 nm in a microplate reader (Synergy 2, BioTek).

Preparation of AβOs

The $A\beta_{1-42}$ peptide, prepared as a dried hexafluore-2-propanol film as described previously (De Felice et al. 2007; Paula-Lima et al. 2011), was stored at −80 °C for up to 4 months. Prior to use, this peptide film was dissolved in sterile DMSO (5 mM stock solution). To prepare AβOs using standard methods (Lacor et al. 2007), the 5 mM $A\beta_{1-42}$ peptide solution was subsequently diluted to 100 µM with cold phosphate buffered saline (PBS), aged overnight at 4 °C and centrifuged at 14,000×g for 10 min at 4 °C to remove insoluble aggregates (protofibrils and fibrils). The supernatant containing soluble AβOs was transferred to clean tubes and stored at 4 °C. The oligomeric state of AβO preparations was confirmed by electron microscopy (Paula-Lima et al. 2011). Only fresh AβO preparations (1 day old) were used in all experiments.

RNA isolation and qRT-PCR

To extract RNA cells were lysed as described in previous work (Haeger et al. 2010). Total RNA was isolated using Trizol reagent. To remove any contaminating genomic DNA, a DNAase digestion step was included. RNA purity was assessed by the 260/280 absorbance ratio and RNA integrity by gel electrophoresis. cDNA was synthesized from total RNA (0.5 µg) using a commercial reverse transcriptase kit. Twenty-five ng of cDNA was used in 20 µl final volume for PCR amplification (Applied Biosystem Thermal cycler). Amplification was performed using the primers and conditions detailed in Supplementary Table 1. Real-time quantitative PCR (qRT-PCR) was performed in an amplification system (MX3000P, Stratagene, La Jolla, CA) using the DNA binding dye SYBR green. Levels of DMT1 (+)IRE and (−)IRE mRNA were calculated by the relative $2^{-\Delta\Delta Ct}$ method

(Pfaffl 2001), and normalized respect to levels of β-actin mRNA. Dissociation curves were analyzed to verify purity of products. All samples were run at least in triplicate.

Determination of neuronal iron uptake

Iron uptake was determined with the fluorescence probe Calcein-AM as described previously (Muñoz et al. 2011). In brief, cultured hippocampal neurons grown in glass covers were incubated for 15 min at 37 °C in Hanks-glucose solution containing the lipophilic acetoxymethyl ester of calcein (Calcein-AM, 1 μM). Calcein fluorescence was determined as a function of time by confocal microscopy. After baseline fluorescence collection, the medium was supplemented with Fe as the complex $FeCl_3$-sodium nitrilotriacetate (Fe–NTA, 1:2.2, mol:mol) (Núñez et al. 2004) and the fluorescence intensity was recorded for an additional period of at least 200 s. The decrease in calcein fluorescence was taken as an indication of increased iron uptake. Calcein signals are presented as F/F_o values, were F correspond to the experimental fluorescence and F_o to the basal fluorescence.

Statistical analysis

Results are expressed as Mean ± SEM. Statistical significance was evaluated as indicated in the legend to each figure, using InStat, GraphPad Prism Software. $p < 0.05$ were considered significant.

Results

Effects of AβOs on DMT1 mRNA levels and iron uptake

The mRNA levels of the DMT1 (+)IRE and (−)IRE transcripts were evaluated by RT-PCR in mature primary hippocampal neurons (21 DIV) treated with AβOs for 24 h. Incubation with AβOs significantly decreased DMT1 (−)IRE mRNA levels ($p < 0.05$) but did not produce a significant change in DMT1 (+)IRE ($p = 0.23$) relative to the untreated controls (Fig. 1).

To test if the decrease in DMT1 (−)IRE mRNA levels produced by incubation with AβOs affected iron uptake, we determined the rate of iron entrance into cells by calcein fluorescence quenching. Previous studies have determined that calcein fluorescence decreases following iron binding to calcein with a Kd of 0.22 μM (Epsztejn et al. 1997). Moreover, the decrease of calcein fluorescence with time is a suitable tool to detect iron uptake in primary hippocampal neurons (Muñoz et al. 2011). Here, neurons were pre-incubated for 24 h with 0.5 μM AβOs (a non-lethal concentration) prior to loading with calcein-AM. The iron-dependent decrease of calcein fluorescence was detected by time-lapse confocal microscopy after the addition of 30 μM Fe–NTA. It is important to point out that the incubation media had no added transferrin, so changes in calcein fluorescence were the result of NTBI uptake. Calcein fluorescence in neurons loaded with calcein-AM was clearly visible both in soma and neurites (Fig. 2a, b). To detect the fluorescence decrease induced by iron addition in neurites, the sites of most synaptic inputs, fluorescence signals were acquired with initial conditions near saturation. Addition of 30 μM Fe–NTA caused a significant decrease in calcein fluorescence in controls (Fig. 2c, d) and in neurons treated with AβOs (Fig. 2e, f). Both in AβOs-treated and control cells, iron addition produced an immediate decrease of calcein fluorescence that followed a single exponential decay function (Fig. 2g). Experiments in which we used less-saturated images confirmed that addition of 30 μM Fe–NTA to neuronal cultures previously

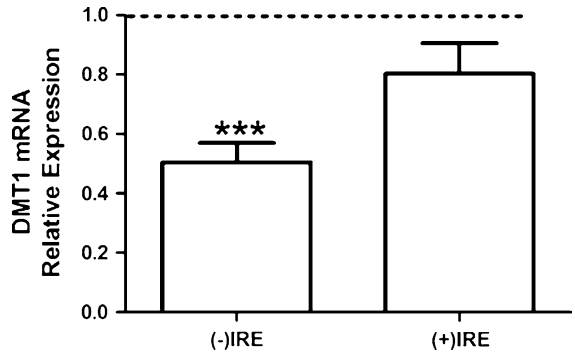


Fig. 1 AβOs decrease DMT1 (−)IRE mRNA levels. The relative mRNA levels for DMT1 (−)IRE and (+)IRE were determined by qRT-PCR in neurons treated with 0.5 μM AβOs for 24 h. All values were normalized to β-actin mRNA levels and expressed as fold of control. Values represent mean ± SEM of three different experiments, performed in triplicates. The error between controls in each experiment was <0.25. Statistical significance was analyzed by one-way ANOVA followed by Tukey post hoc test; $^*P < 0.0005$. $N = 3$

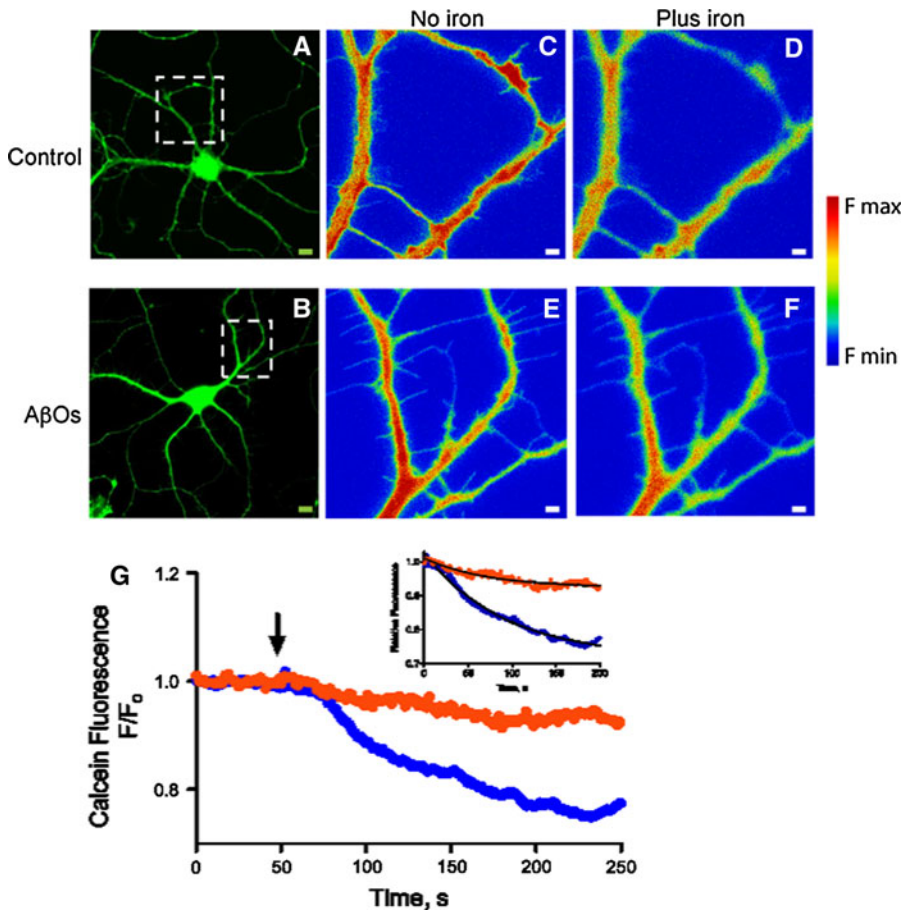


Fig. 2 AβOs affect neuronal iron uptake. Representative confocal image of (**a**) a control hippocampal neuron loaded with calcein-AM; (**b**) a neuron pre-incubated for 24 h with 0.5 μM AβOs prior to loading with calcein-AM. The *insets* in *a* and *b* correspond to the magnified pseudocolor images representing the fluorescence intensity of calcein (**c**) before and (**d**) after the addition of 30 μM Fe–NTA to the extracellular medium. (**e**) Pseudocolor confocal image showing the fluorescence intensity of calcein of a neuron treated with 0.5 μM AβOs for 24 h (**e**) before and (**f**) after the addition of 30 Fe–NTA to the extracellular medium. In the pseudo color rainbow scale (right) "warmer" colors correspond to higher fluorescence. (**g**) Calcein fluorescence recorded as a function of time after the addition of 30 μM Fe–NTA. Fluorescence time course data correspond to a representative experiment from 22 registers showing control neurons (*blue circles*) and neurons treated with AβOs (*red circles*). The *inset* in (**g**) corresponds to best fit of the single exponential decay function. (Color figure online)

treated with AβOs produced a lower decrease in calcein fluorescence than in control neurons, with similar exponential decay time constants as those calculated from the experiments illustrated in Fig. 2. Basal calcein fluorescence was in all cases significantly higher in control neurons compared to neurons incubated with AβOs (Fig. 3a) even though the conditions of incubation with calcein were identical in both cases. Likewise, initial rates of iron-induced calcein fluorescence decay (Fig. 3b) and total calcein fluorescence decrease (Fig. 3c) were significantly higher in control neurons than in neurons treated with AβOs. These results indicate that primary hippocampal neurons rapidly take up iron from the extracellular medium presumably via DMT1, since transferrin-dependent iron uptake was not operational. They also reveal that AβOs treatment decreased basal calcein fluorescence, suggesting that incubation with AβOs decreased the intracellular labile iron pool (LIP) and inhibited significantly the kinetics of NTBI uptake.

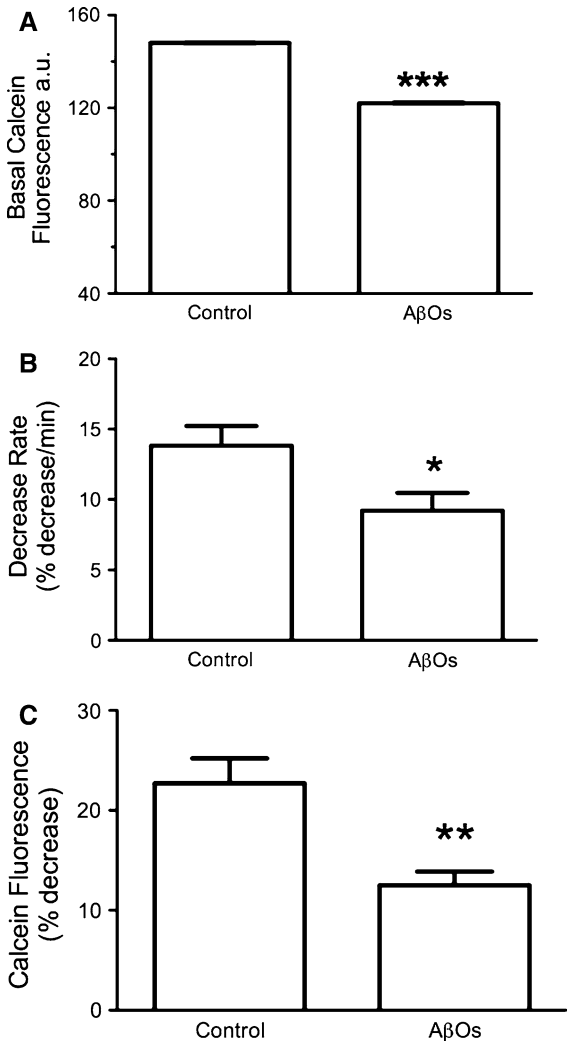


Fig. 3 AβOs decrease the intracellular labile iron pool (LIP) and inhibited significantly the kinetics of NTBI uptake. (**a**) Mean ± SEM values of the basal calcein fluorescence taken between 0 and 50 s in control neurons and in neurons incubated with AβOs for 24 h. (**b**) Initial rates of iron-induced calcein fluorescence decay expressed as Mean ± SEM values of the slopes, taken between 50 s and 200 s and expressed as % decrease/min calculated from the data shown in Fig. 2g. (**c**) Total calcein fluorescence decrease

Toxicity of AβOs in neurons with different intracellular iron content

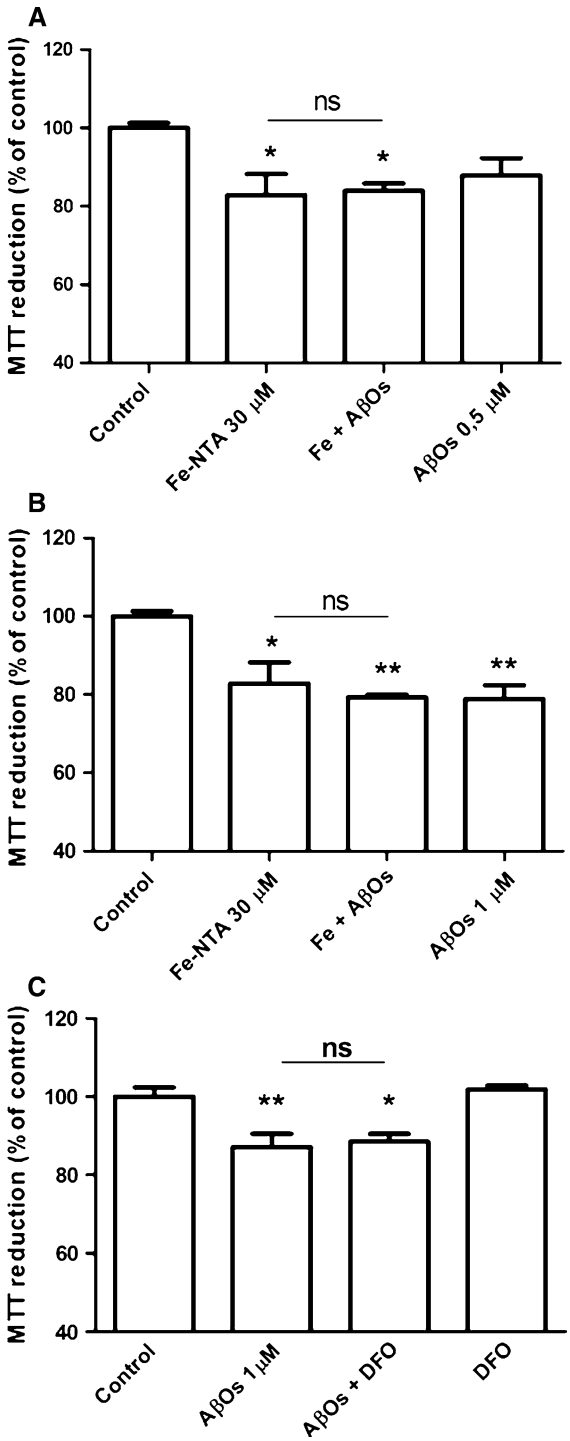
As described above, both AβOs and iron exert concentration-dependent harmful effects that may result in neuronal death, but whether their neurotoxic effects are synergistic remains an open question. Accordingly, we investigated here if prolonged incubation of primary hippocampal neurons with iron potentiates the neurotoxicity of AβOs. To this aim, hippocampal cultures were incubated with Fe–NTA for 48 h, and AβOs (0.5 μM or 1 μM) were added to the cultures during the last 24 h of incubation. Cell viability is not affected by 24 h incubation with 0.5 μM AβOs, while a higher concentration of AβOs (1 μM) decreases cell viability to 78.9 ± 7.1 % (Paula-Lima et al. 2011). Incubation with 30 μM Fe–NTA for 48 h produced a significant decrease in neuronal viability, to 82.9 ± 5.4 % of control (Fig. 4a). The combined presence of Fe–NTA and 0.5 μM AβOs did not potentiate the cell death induced by Fe–NTA alone (Fig. 4a). Likewise, addition of 1 μM AβOs for the last 24 h to cultures incubated with Fe–NTA for 48 h did not potentiate the decrease in cell viability induced by iron, which is comparable to the decrease in cell viability produced by 1 μM AβOs alone (Fig. 4b).

To test if iron chelation protects neurons from the neurotoxic effects of AβOs, primary hippocampal neurons were treated with the iron chelator desferrioxamine (3 μM) 1 h before and during incubation with AβOs. We found that preincubation with desferrioxamine did not prevent the decrease in cell viability produced by incubation for 24 h with 1 μM AβOs (Fig. 4c). The cell viability of neurons treated for 25 h only with desferrioxamine (101.8 ± 2.2 %) was similar to that of control neurons (100 ± 5.2 %).

Discussion

The multi-factor origin of AD may compromise diverse pathways engaged in different cell signaling mechanisms. The neurotoxic effects of AβOs are well described but the molecular mechanisms affected by AβOs are not known in detail. In this work, we studied the effects of AβOs on iron transport and neurotoxicity. Our results show that AβOs (added at a non-lethal concentration) did not potentiate the neurotoxicity of iron but decreased mRNA levels of the DMT1 (−)IRE transcript and inhibited NTBI uptake. In addition, iron chelation did not protect from the cell viability decrease produced by a higher AβOs concentration.

Our results show that incubation with a sub-lethal concentration of AβOs reduced the levels of DMT1 (−)IRE mRNA without affecting significantly DMT1



◀**Fig. 4** Aβ*O*s do not modify iron toxicity. MTT reduction effected by neurons incubated for 48 h with 30 μM Fe–NTA or by neurons incubated with 30 μM Fe–NTA for 48 h with 0.5 μM (**a**) or 1 μM (**b**) Aβ*O*s added during the last 24 h. (**c**) MTT reduction effected by neurons pre-incubated with 3 μM desferrioxamine (DFO) for 1 h and treated with 1 μM Aβ*O*s for 24 h. Results are expressed as Mean ± SEM of four independent experiments performed in quadruplicates. Data was analyzed for statistical significance by one way ANOVA, followed by Tukey post hoc test. *$p < 0.05$; **$p < 0.005$

(+)IRE mRNA levels. Knowledge on the regulation of DMT1 (−IRE) mRNA levels through its 3′ untranslated region (UTR) is scarce. A recent report described a decrease of DMT1(-IRE) mRNA levels by microRNA Let-7d in erythroid cells (Andolfo et al. 2010). Micro RNAs are short (22 nucleotides) single-stranded RNAs that post-transcriptionally regulate gene expression by binding to imperfect complementary sites within the 3′-UTRs of their mRNA targets (Van Wynsberghe et al. 2011). In view of our results, it would be worthwhile to explore in future studies if Aβ*O*s treatment increases microRNA Let-7d levels in primary hippocampal neurons. Moreover, since hippocampal neurons lack the DMT1 1A transcript (Haeger et al. 2010), our findings suggest that Aβ*O*s might reduce the expression of the DMT1 1B (−)IRE isoform. Of note, a recent study (Garrick et al. 2012) describes that the ubiquitin E3 ligase parkin decreases the content of the DMT1 1B (−)IRE protein isoform by stimulation of proteosomal degradation. Accordingly, a future projection of our results will be to determine if Aβ*O*s enhance the degradation of the DMT1 1B (−)IRE protein isoform by the proteosome.

In addition, our results show that non-lethal levels of Aβ*O*s reduced neuronal NTBI uptake, and suggest that incubation with Aβ*O*s decrease the basal LIP levels.

Neurons also incorporate iron through endocytosis of iron-loaded transferrin. Iron dissociates from transferrin in the acid endosomal compartment, from where DMT1 transports the free endosomal iron to the cytoplasm. Studies in transfected kidney cells showed higher surface expression of the DMT1 (+)IRE isoform, that was internalized from the plasma membrane with slower kinetics than the DMT1(−)IRE isoform, which is efficiently sorted to recycling endosomes (Lam-Yuk-Tseung and Gros 2006). Our findings implicate the DMT1 (−)IRE isoform in neuronal NTBI uptake. In addition, primary hippocampal neurons displayed very fast NTBI uptake, which was significantly reduced by Aβ*O*s suggesting that the DMT1 1B (-IRE) isoform is present in the plasma membrane. Future studies should address if the significant decrease in DMT1 (−)IRE mRNA levels

produced by AβOs results in decreased expression of the DMT1 1B (-IRE) protein isoform and hinders transferrin-bound iron uptake.

At the times used in this work, incubation with a non-lethal concentration of AβOs did not enhance the neurotoxic effects of iron on cell viability, while iron chelation did not protect from the neurotoxic effects of AβOs. The lack of synergy of the effects of AβOs and Fe under the conditions used in our work suggests that AβOs and Fe induce cellular signaling pathways that converge on a common target. We have shown in previous work that calcium release via the ryanodine receptor/calcium release channel is activated by the increased oxidative tone produced either by AβOs (Paula-Lima et al. 2011) or by iron (Muñoz et al. 2011). We propose that under our conditions, AβOs plus Fe do not increase ryanodine receptor activity over the levels induced solely by Fe or AβOs. Yet, at longer times AβOs and Fe may act in a synergistic manner to generate oxidative stress and abnormally high calcium signals, or iron may induce the formation of different AβOs aggregates with higher toxicity (Bolognin et al. 2011; Liu et al. 2011). Both conditions are likely to favor cell death, as occurs progressively in the brain of AD patients where the accumulation of iron and AβOs begins many years before the diagnosis of the pathological cognitive deficit.

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