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Amyloid-β_{42} Activates the Expression of BACE1 Through the JNK Pathway

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Abstract. The sequential endoproteolytic cleavages operated by the γ-secretase and the β-secretase (BACE1) on the amyloid-β protein precursor (AβPP) result in the production of the amyloid-β (Aβ) species, with two C-terminal variants, at residue 40 or at residue 42. Accumulation in brain tissue of small, soluble aggregates of $A\beta_{42}$ is the major pathogenic event of Alzheimer's disease (AD). However, the physiologic activity of Aβ peptides is still elusive. Here, we show that expression of BACE1 is regulated by $A\beta_{42}$, which augments BACE1 gene transcription through the JNK/c-jun signaling pathway. Of note, $A\beta_{40}$ has much less effect on BACE1 expression. These findings unveil a positive feedback loop in which γ-secretase cleavage of AβPP releases a functionally-active peptide, $A\beta_{42}$, that promotes BACE1 transcription. Thus, gene expression induced by $A\beta_{42}$ may have implications in the neuronal dysfunction and degeneration that occurs in AD.

Keywords: Alzheimer's disease, amyloid-β_{42}, BACE1, γ-secretase, JNK/c-jun pathway

INTRODUCTION

Accumulation in the brain of aggregates of amyloid-β peptides (Aβ) is the major pathologic event of Alzheimer's disease (AD) [1]. Aβ results from two sequential endoproteolytic cleavages operated on the amyloid-β protein precursor (AβPP). The β-secretase (BACE1) cleaves the ectodomain of AβPP, producing an AβPP C-terminal fragment that is further cleaved within the transmembrane domain by the γ-secretase, resulting in the release of Aβ peptides, with two C-terminal variant, at residue 40 ($A\beta_{40}$) or at residue 42 ($A\beta_{42}$) [1]. The altered activity of both BACE1 and the γ-secretase are involved in the pathogenesis of AD.

AD is classified into two forms: sporadic late-onset AD, which is related to aging, and familial early-onset AD (FAD), caused by gene mutations. Mutations of presenilin 1 (PS1), the catalytic subunit of γ-secretase, are the most common genetic defect of early-onset familial AD and lead to an increased production of $A\beta_{42}$ [2, 3].

Recently we reported that PS1 mutations expressed in cells activates the expression of BACE1 [4]. The expression and the activity of PS1 and BACE1 is augmented in the brain of late-onset sporadic AD [5, 6].

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The cause of the altered AβPP processing and $A\beta_{42}$ overproduction in sporadic cases of AD is unclear, but is likely to include oxidative stress.

We and other research groups have shown that oxidative stress upregulates the expression of BACE1 through the activity of γ-secretase [7, 8]. Moreover we showed that the presence of presenilins is necessary to increase BACE1 expression under stress conditions [8, 9]. Now we investigated the pathway that mediates the expression of BACE1 under oxidative stress.

MATERIALS AND METHODS

Cell culture, treatments, and transfection

SK-N-BE neuroblastoma cells were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium containing 2 mM glutamine and supplemented with 100 mL/L fetal bovine serum, 10 mL/L non-essential amino acids, and 10 mL/L antibiotic mixture (penicillin-streptomycin amphotericin), in a humidified atmosphere at 37°C with 5% CO_2. For differentiation, 2×10^6 cells were plated in 75 cm^2 culture flasks (Costar, Lowell, MA, USA) and exposed to 10 μM retinoic acid for 10 days.

Mouse embryonic fibroblast (MEF) wt, PS1-/-, PS2 -/-, PSdko (provided by Dr. Bart De Strooper, Center for Human Genetics, Leuven, Belgium) [10], AβPP+/APLP2-, AβPP-/APLP2+, AβPP/APLP2 dko (provided by Dr. Ulrike Mueller, Max Plank Institute for Brain Research, Frankfurt, Germany) [11, 12], JNK1/2dko (provided by Dr. Roger Davis, University of Massachusetts, Worcester, Ma, USA) were cultured in low glucose Dulbecco's modified Eagle's medium containing 2 mM glutamine and supplemented with 100 mL/L fetal bovine serum, 10 mL/L non-essential amino acids, and 10 mL/L antibiotic mixture (penicillin-streptomycin amphotericin), in a humidified atmosphere at 37°C with 5% CO_2.

Cells were incubated with 1 μM Aβ peptides (40, 42 scramble, and 42, Anaspec, San Jose, CA, USA) up to 6 h. The lyophilized commercial peptides were dissolved as a stock solution in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma Chemical Company, St Louis, MO, USA) and stored at −80°C in aliquots. The desired amount was left under the cell culture hood to allow for 1,1,1,3,3,3-hexafluoro-2-propanol to evaporate, brought to 1 μM with sterile double distilled water and immediately added to the cells to avoid aggregation. Twenty microliters of Aβ preparations, prepared as described in material and methods section, were added to a carbon-coated Formvar grid for 10 min, dried, and stained with 2% fosfotungstic acid. Grids were examined by transmission electron microscopy.

Cell permeable, JNK inhibitory peptide (Phoenix Pharmaceuticals, Inc), at 2 μM, was added to MEFs wild type cells at the time of $A\beta_{42}$ peptides [13].

AβPP reconstitution in MEF cells was performed by transfecting a human AβPP 695 construct together with pcDNA3.1, PS1 wt, PS2 wt, JNKwt and JNK dominant negative (DN) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Transgenic mice

One-month-old male CRND8 mice, containing a double mutation in the human AβPP gene (K670M/N671 L and V717F) and C57bl/6 wild type littermate were used. Genotyping of CRND8 mice was performed by PCR, as described previously [14]. All animal procedures were conducted according to the ethical guidelines for treatment of laboratory animals of Albert Einstein College of Medicine, Bronx, NY, USA.

Tissue and cell extracts

Cytosolic and nuclear extracts of animal tissues were prepared as described previously [15]. Briefly, cerebral tissues were homogenized at 10% (w/v) in a Potter Elvehjem homogenizer (Wheaton, Millville, NJ, USA) using a homogenization buffer containing 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM EDTA, 1% nonylphenyl-polyethylene glycol, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, and 2.5 mg/mL leupeptin. Homogenates were centrifuged at 1000 g for 5 min at 4°C.

Supernatants were removed and centrifuged at 15,000 g at 4°C for 40 min to obtain the cytosolic fraction. The pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/mL aprotinin, and 2.5 mg/mL leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000 g for 20 min at 4°C. The resulting supernatants containing nuclear proteins were carefully removed and protein content

was measured using a commercially available assay (Bio-Rad, Segrate, Italy). Preparation of cell lysates and nuclear extracts were performed as described previously [16, 17].

Antibodies and immunoblot analysis

The following antibodies were used: polyclonal anti-BACE1 antibody (Chemicon, Temecula, CA, USA); monoclonal β actin antibody and polyclonal lamin A (Sigma Chemical Company); polyclonal anti PS1, anti PS2, anti pAsk, anti Ask, anti pJNKK, anti JNKK, anti pJNK and anti JNK, anti pc-jun, anti c-jun antibodies (Cell Signaling Technology, Beverly, MA, USA). Lysates and nuclear fractions extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 9.3% acrylamide gels using the mini-PROTEAN II electrophoresis cell (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Hybond-C extra Amersham Life Science, Arlington Heights, IL, USA). Non-specific binding was blocked with 50 g/L non-fat dry milk in 50 mM Tris–HCl, pH 7.4, containing 200 mM NaCl and 0.5 mM Tween-20 (Tris-buffered saline Tween). The blots were incubated with different primary antibodies, followed by incubation with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline Tween containing 20 g/L non-fat dry milk. Reactions were developed with an enhanced chemiluminescence system according to the manufacturer's protocol (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy).

Analysis of gene expression

For the quantitative SYBR Green (2x iQ YBR Green PCR Super Mix; Bio-Rad Laboratories) real-time PCR, 40 ng of cDNA was used per reaction. RNA extraction was performed with the RNeasy kits, according to the manufacturer's protocol, for cell lines and brain tissue (Qiagen, CA, USA); cDNA was obtained with the SuperScript® III CellsDirect cDNA Synthesis Kit (Invitrogen, CA, USA). Primer sequences, designed with PRIMER 3 software (Applied Biosystems, Monza, Italy) were:
Human BACE1 : 5′-CATTGGAGGTATCGACCA CTCGCT-3′ and 5′CCACAGTCTTCCATGTCCAAG GTG-3′.
Human β actin: 5′-GGCACTCTTCCAGCCTTC CTTC-3′ and 5′-GCGGATGTCCACGTCACACTT CA-3′.
Murine BACE1 : 5′-GCATGATCATTGGTGGTA TC-3′ and 5′-CCATCTTGAGATCTTGACCA-3′.
Murine β actin: 5′-AGCTATGAGCTGCCTGACG GC-3′ and 5′-CATGGATGCCACAGGATTCCA-3′.

Quantitative PCR was performed on a real-time iCycler sequence detector instrument (Bio-Rad Laboratories). After 3 min of initial denaturation, the amplification profile included 30 s denaturation at 95°C and extension at 72°C. Primer annealing was carried out for 30 s at 60°C. The results were obtained with the comparative Ct method using the arithmetic formula $2^{-\Delta\Delta Ct}$. Samples obtained from at least three independent experiments were used to calculate the mean and SD.

Run-on assay

MEFs as well as SK-N-BE differentiated neuroblastoma cells (6×10^6) were lysed in 200 μl lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4, 0.5% Nonidet-P40) and briefly centrifuged. Nuclei were then stored at −80°C in a storage buffer consisting of 40% glycerol, 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, and 0.1 mM EDTA. Pipetting should be done with a wide-bore pipet tip to avoid breaking nuclei obtained.

To 50 μl of nuclei, 50 μl of reaction buffer containing radiolabeled nucleotide (50 Mm HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 150 mM KCl, 10% glycerol, 2.8 mM ATP, 2.8 mM GTP, 2.8 mM CTP, 3.2 mM UTP, 40 μCi of $\{^{32}P\}$UTP (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy)) were added and the samples were incubated at 30°C for 10 min with shaking. RNA was then extracted from the nuclear run-on reaction using Trizol (Invitrogen) according to manufacturer's protocol.

Transcripts obtained were quantified by hybridization to nitrocellulose strips, containing BACE1 cDNA (1 μg each) immobilized using a dot blot apparatus, at 42°C for 72 h. Before hybridization, the membranes were prehybridized using a hybridization buffer consisting of 50% formamide, 6 × SSC, 10X Denhardt's solution and 0.2% SDS for at least 6 h at 42°C. After hybridization strips were washed once with 6xSSC and 0.2% SDS at room temperature for 10 min, then twice with 2x SSC and 0.2% SDS, followed by two washes in 0.2x SSC and 0.2% SDS, all at 65°C for 10–30 min each wash. The strips were then exposed to Kodak hyperfilms (Amersham-Pharmacia Biotech Italia, Cologno Monzese, Italy) at −80°C for 4 days. Band intensity was determined by scanning densitometry and the level of each BACE1 mRNA was

normalized to the levels of RNA obtained by nuclei supernatants (saved for RNA isolation).

Enzyme-linked immunosorbent assay

The levels of $A\beta_{x-40}$ and $A\beta_{x-42}$ were measured by sandwich ELISA method following the manufacturer's instructions (IBL, Gunma, Japan). Samples were analyzed following the manufacturer's instructions. The Aβ concentration was detected using a Benchmark Microplate Reader and evaluated by 'microplate manager' v. 5.1 software (Bio-Rad). ELISA analysis of all samples was performed in two different experiments.

Activator protein 1 (AP-1) transcription factor determination

The activity of AP-1 was determined by a commercially available kit (Active Motif), designed specifically to detect and quantify AP-1 activation. The kit contains a 96-well plate to which oligonucleotide containing a TPA-responsive element (TRE) has been immobilized. AP-1 dimers contained in nuclear extracts bind specifically to this oligonucleotide and are detected through use of an antibody direct against c-fos, fosB, fra-1, fra-2, c-jun, junB, junD. Addition of a secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry.

RNA interference

The mouse retroviral shRNA c-jun and the non-silencing shRNA sequence, that was expressed under the control of the U6 promoter and contains no homology to known mammalian genes, were purchased by Open Biosystems. The cells were harvested 48 h after shRNA transfection before samples preparation.

Statistical analysis

Data in bar graphs represent mean ± SEM, and were obtained from average data of at least three independent experiments. Morphological images are representative of at least three experiments with similar results. Statistical analysis was performed by Student's *t*-test or ANOVA, followed by the Bonferroni post test, when appropriate ($p < 0.05$ was considered significant).

RESULTS

$A\beta_{42}$ activates the transcription of BACE1 in different experimental models

We have recently shown that the activity of the γ-secretase are required to obtain the upregulation of BACE1 mediated by oxidative stress [8]. Moreover, we found that PS1 mutations augment the expression of BACE1 by altering the activity of the γ-secretase [4].

The AβPP derivatives that results from the γ-secretase cleavage are the Aβ peptides and the AβPP intracellular domain (AICD). We investigated which derivative is responsible for BACE1 upregulation. We first analyzed the effect of the AICD fragments 57 and 59, the AβPP derivatives resulting from the γ cleavage of AβPP, as well as AICD 50 and 51 that are the ε cleavage derivatives [18–20]. Transfection of different cell lines, such as SK-N-BE, SH-SY5Y, and HEK-293 with the corresponding AICD constructs determined no changes of BACE1 expression (data not shown) [see 4]. The above experiments ruled out the role of AICD in the overexpression of BACE1 and pointed to the other derivative of the γ-cleavage, the Aβ peptides. We have previously observed that $A\beta_{42}$ increase BACE1 expression in primary neuronal cultures [4]. The data of the present study confirm and extend our results using different experimental models. We first studied whether Aβ peptides may function in gene expression by using a run on assay. We used MEFs knock out cells for PS1 and PS2 [10] and for AβPP or AβPP-like protein 2 (APLP2) (AβPP+/APLP2-) (AβPP-/APLP2+), as well as MEFs AβPP/APLP2 dko [21, 22]. We incubated MEFs PSdko and AβPP/APLP2 dko with $A\beta_{40}$ and $A\beta_{42}$ synthetic peptides, at a concentration of 1 μM, fresh preparation. The aggregation state of synthetic Aβ preparations was examined with electron microscopy and no fibrillar or oligomeric structures were detected (data not shown).

MEFs PS dko and AβPP/APLP2 dko were incubated with $A\beta_{42}$ and $A\beta_{40}$, and the amount of nascent RNA was serially evaluated from 5 min until 1 h. In both MEFs dko cells treated with $A\beta_{42}$, there was a time-dependent increase in BACE1 nascent RNA, reaching a peak of 5 fold increase at 1 h, compared to non treated cells (Fig. 1A–D). Incubation with $A\beta_{40}$ did not change the amount of BACE1 nascent RNA (Fig. 1A–D). Incubation of MEFs PS dko and AβPP/APLP2 dko with $A\beta_{42}$ was followed by a significant increase (approx. + 100%) of BACE1 protein levels after 12 h of incubation (Fig. 1E–H).

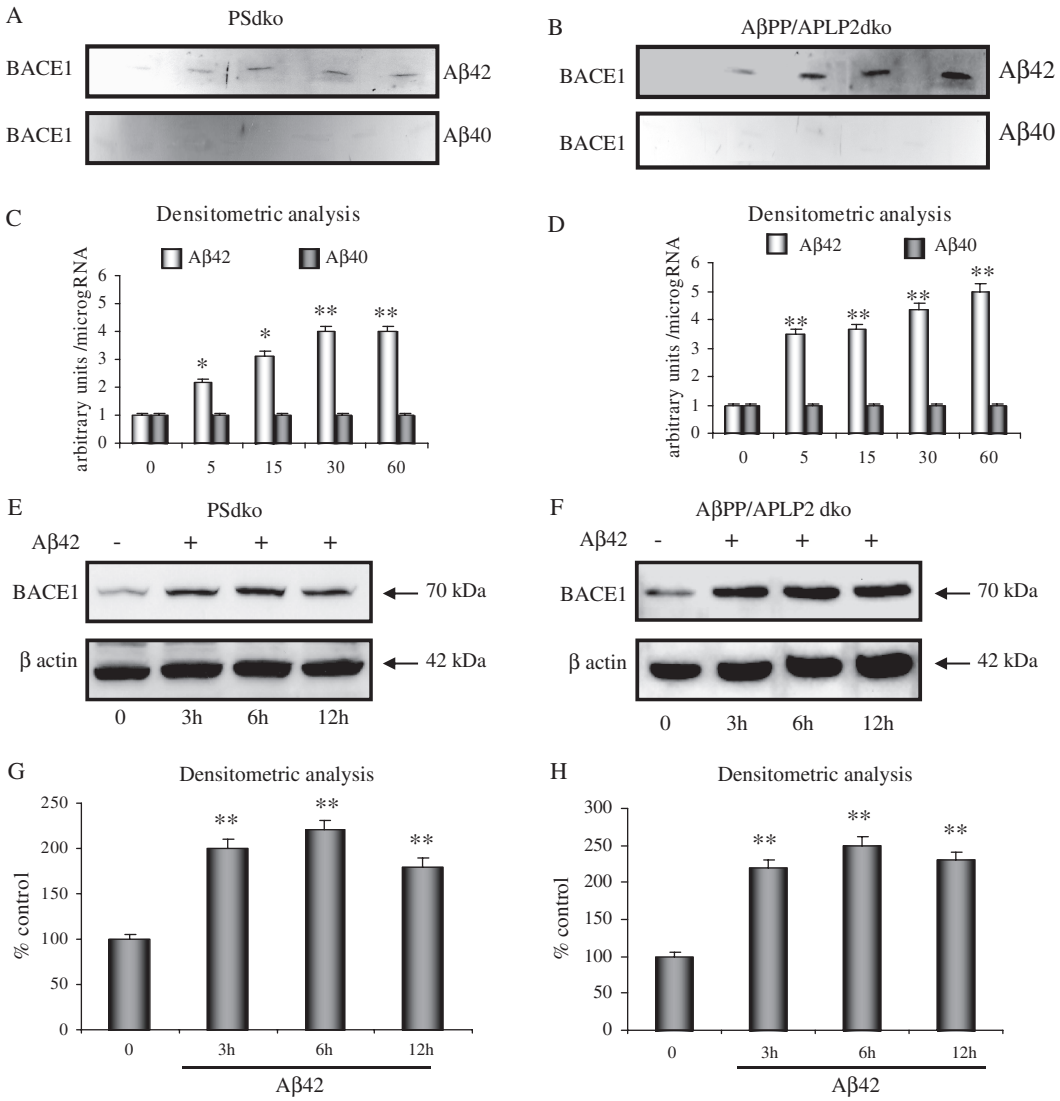

Fig. 1. Effect of Aβ peptides on BACE1 transcription and expression in MEF cells. A–D) Run on assay. Nascent RNA of BACE1 increased by 3.5–4 fold in MEFs PSdko (A, C) and in MEFs AβPP/APLP2 dko (B, D) when incubated with 1 μM $A\beta_{42}$ peptide. Treatment with $A\beta_{40}$ did not change BACE1 nascent RNA. E–H) Treatment of MEFs PSdko (E, G) or MEFs AβPP/APLP2 dko (F, H) with 1 μM $A\beta_{42}$ peptide was followed by a significant increase in BACE1 protein levels after 3 hours up to 12 h. The error bars represent standard deviations. Experiments were conducted in triplicate. *significantly different from controls ($p < 0.05$). **significantly different from controls ($p < 0.02$).

Then, we confirmed the role of Aβ peptides on the upregulation of BACE1 in differentiated SK-N-BE neuroblastoma cells. We treated differentiated neuroblastoma cells with $A\beta_{42}$ scramble, $A\beta_{40}$, $A\beta_{42}$, or medium only, up to 6 h. $A\beta_{42}$ determined an increase in BACE1 mRNA production (3 fold increase) after 1 h of incubation (Fig. 2A) and in BACE1 protein levels (+120%) after 6 h of incubation (Fig. 2B, C). Incubation with the scramble peptide or $A\beta_{40}$ did not change neither the amount of BACE1 mRNA nor BACE1 protein levels (Fig. 2A–C). Furthermore, we performed a run on assay to evaluate the function on gene expression of Aβ peptides in this cellular model. As expected, cells treated with $A\beta_{42}$ showed a significant increase in BACE1 nascent RNA (2.3 fold increase) after 1 h of incubation, compared to non treated cells (Fig. 2D, E). Incubation with the scramble peptide or $A\beta_{40}$ did not change the amount of BACE1 nascent RNA (Fig. 2D, E).

Then we ascertained the effect of $A\beta_{42}$ on the expression of BACE1 *in vivo*, using double mutant AβPP transgenic mice (KM670/671NL + V717F) [14]

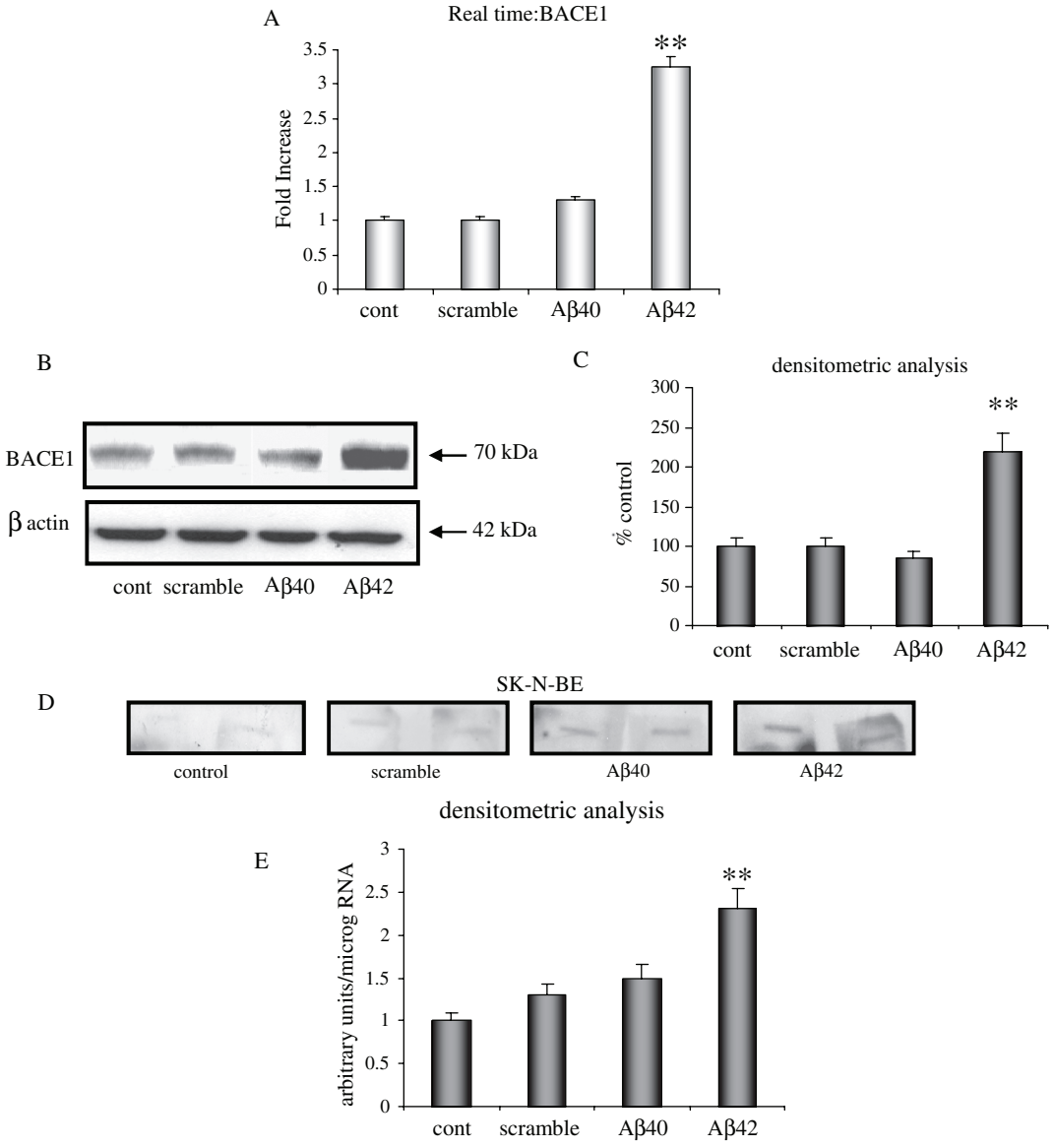

Fig. 2. Effect of Aβ peptides on BACE1 transcription and expression in differentiated SK-N-BE neuroblastoma cells. A) Treatment of differentiated neuroblastoma cells with 1 μM $A\beta_{42}$ peptide for 1 h induced a 3 fold increase in BACE1 mRNA, (B, C) as well as a 100% increase in BACE1 protein levels. Treatment with $A\beta_{42}$ scramble or $A\beta_{40}$ did not change BACE1 mRNA or protein levels (B, C). D, E) Run on assay. Nascent RNA of BACE1 increased by 2, 3 fold in SK-N-BE differentiated cells when incubated with 1 μM $A\beta_{42}$ peptide. Treatment with $A\beta_{42}$ scramble or $A\beta_{40}$ did not change BACE1 nascent RNA. The error bars represent standard deviations. Experiments were conducted in triplicate. **significantly different from controls ($p < 0.02$).

as model of early $A\beta_{42}$ overproduction and accumulation. As reported in Fig. 3A, 1-month-old CRND8 transgenic mice presented significantly higher cerebral levels of total $A\beta_{42}$ (+65%) than non transgenic littermate. Of note, levels of total $A\beta_{40}$ are very similar in both transgenic and non transgenic mice (Fig. 3A). The CRND8 1-month-old transgenic mice also presented a 3 fold higher expression of BACE1 (Fig. 3B) as well as a significant increase (+100%) in BACE1 protein levels (Fig. 3C, D) with respect to non transgenic littermates.

The JNK/c-jun pathway mediates the transcriptional activity of $A\beta_{42}$

We investigated the mechanism of the transcriptional activity operated by $A\beta_{42}$. The c-jun N-terminal

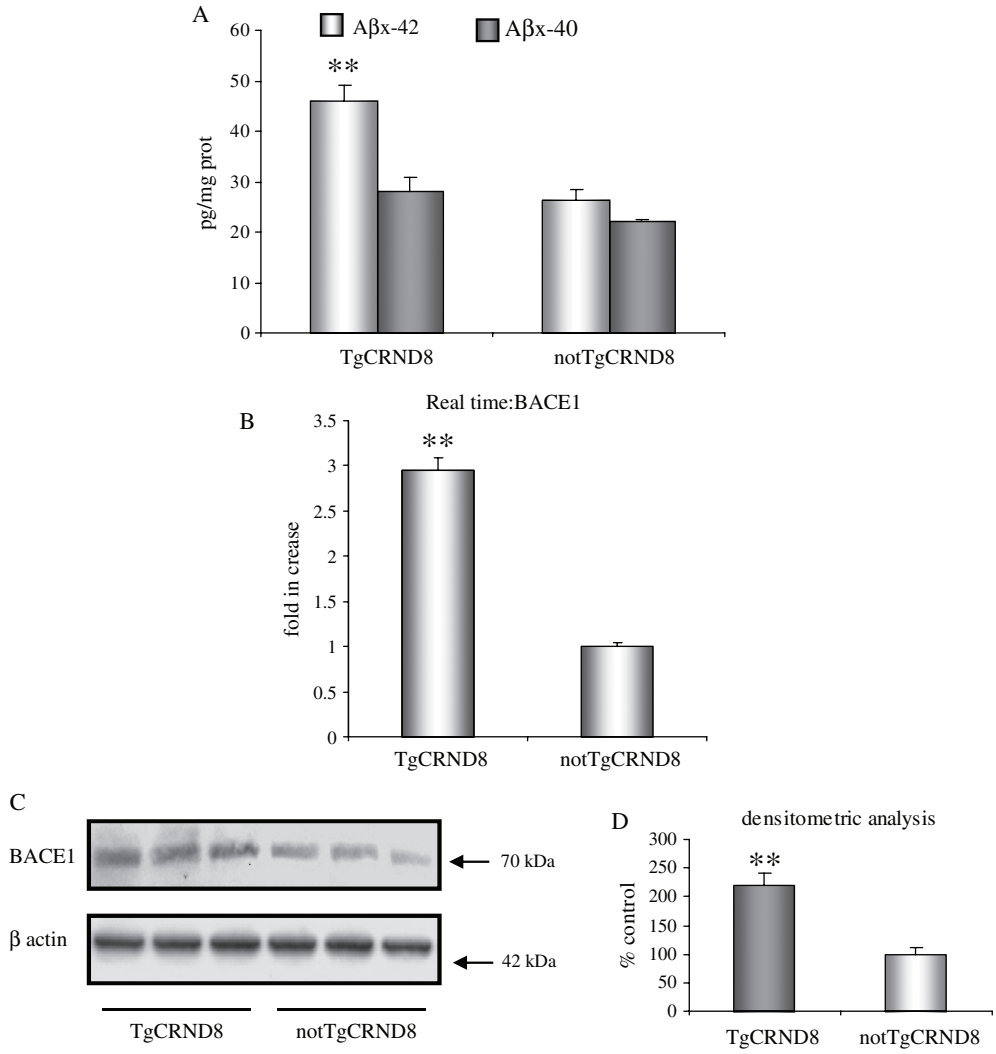

Fig. 3. BACE1expression in CRDN8 transgenic mice. A) Content of $A\beta_{42}$, but not of $A\beta_{40}$, was higher in 1-month-old CRND8 transgenic mice as compared to non transgenic littermates. B) CRND8 transgenic mice presented higher BACE1 mRNA, as well as (C and D) BACE1 protein levels, as compared to non transgenic littermates. The error bars represent standard deviations. Experiments were conducted in triplicate. **significantly different from controls ($p < 0.02$).

kinase (JNK) signaling pathway is a potential player of this role, since it is activated by Aβ peptides [23–26]. MEFs wild type incubated with $A\beta_{42}$ showed the activation of JNK, and of the kinases that belong to the pathway of JNK signaling, the apoptosis signal-regulating kinase 1 (ASK-1), the jun kinase kinase (JNKK), and c-jun (Fig. 4A). Then, we tested if the JNK pathway was activated in SK-N-BE differentiated neuroblastoma cells. As reported in Fig. 4B, incubation of cells with $A\beta_{42}$ was followed by a robust activation of JNK and c-jun, as shown by the increased levels of phospho-JNK and phospho c-jun in SK-N-BE nuclear fractions (Fig. 4B).

Figure 4C shows the nuclear protein levels of JNK and c-jun phosphorylated forms in transgenic or not transgenic CRDN8 mice. The elevation of both JNK and c-jun isoforms was observed in transgenic mice as compared to control mice.

To further ascertain the role of JNK in the activation of BACE1, we incubated MEFs JNK1/2 dko with $A\beta_{42}$. Of note, in the MEFs JNK ko the basal expression of BACE1 is much lower than in wild type cells

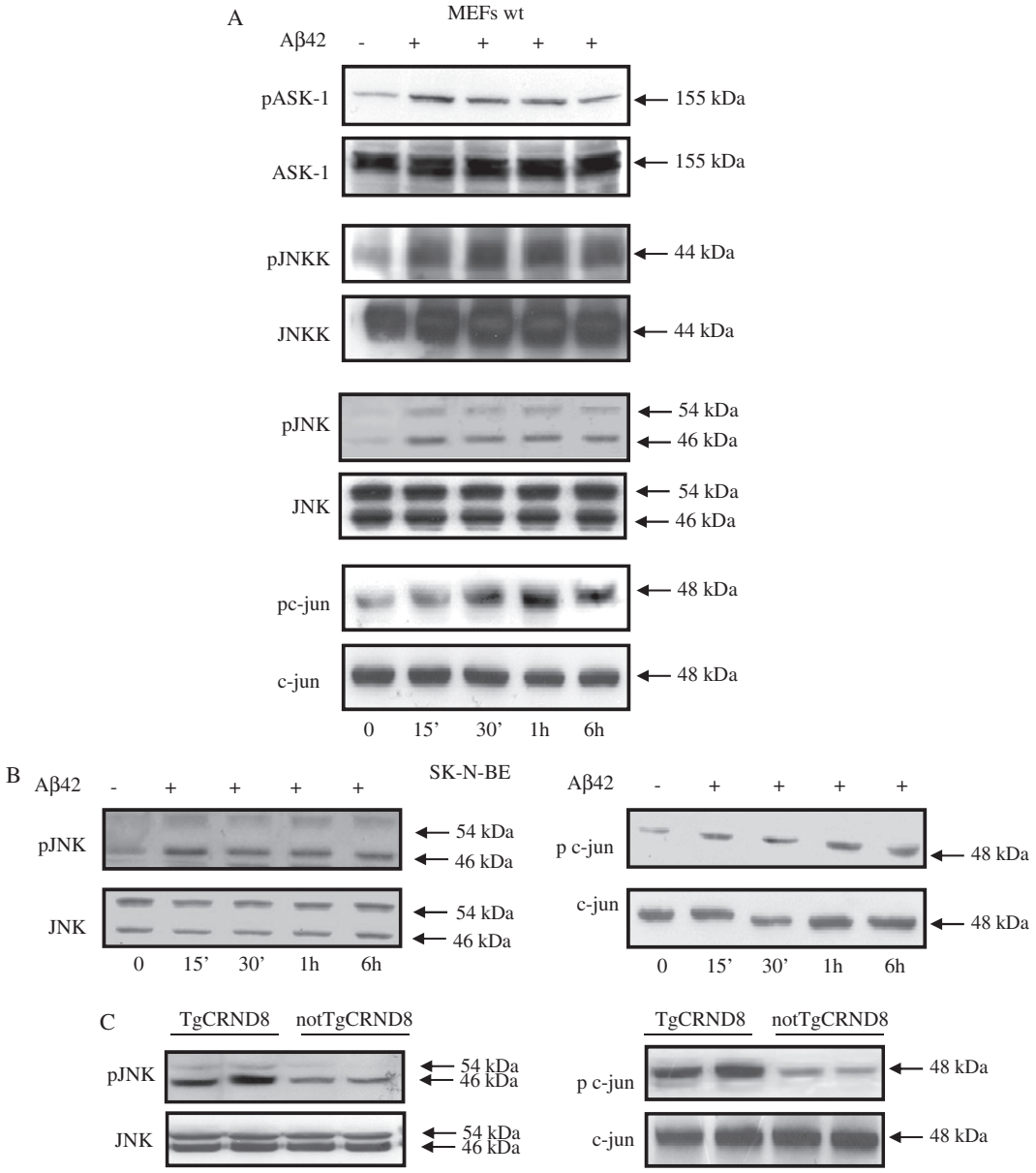

Fig. 4. JNK pathway activation in our experimental models mediated by $A\beta_{42}$ peptide. A) $A\beta_{42}$ activates the JNK pathway in MEFs wild type up to 6 h. B) Significant increase in phosphorylation of JNK and c-jun after treatment of SK-N-BE differentiated cells with 1 μM $A\beta_{42}$ peptide. C) JNK and c-jun phosphorylated isoforms are higher in 1-month-old CRND8 transgenic mice respect to non transgenic littermates.

(Fig. 5A, B) and incubation with $A\beta_{42}$ did not change the expression of BACE1. Remarkably, transfection with JNK wild type, but not with a JNK dominant negative construct (Fig. 5C, D), reconstituted the effect of $A\beta_{42}$ on BACE1 expression.

Finally we tested the role of the transcription factor activator protein 1 (AP-1), the transcriptional element of the JNK signaling pathway. AP-1 is formed by proteins of the jun, fos and ATF families that become active upon homo- and heterodimerization. JNK activates AP-1 by phosphorylating c-jun [27]. In Fig. 5E is reported that $A\beta_{42}$ treatment resulted in a significant increase in c-jun and c-fos DNA binding activity.

To inhibit the activation, we silenced c-jun with the RNA interference technique. The inhibition of c-jun blocked the increase of BACE1 protein levels induced by $A\beta_{42}$ (Fig. 6A, B).

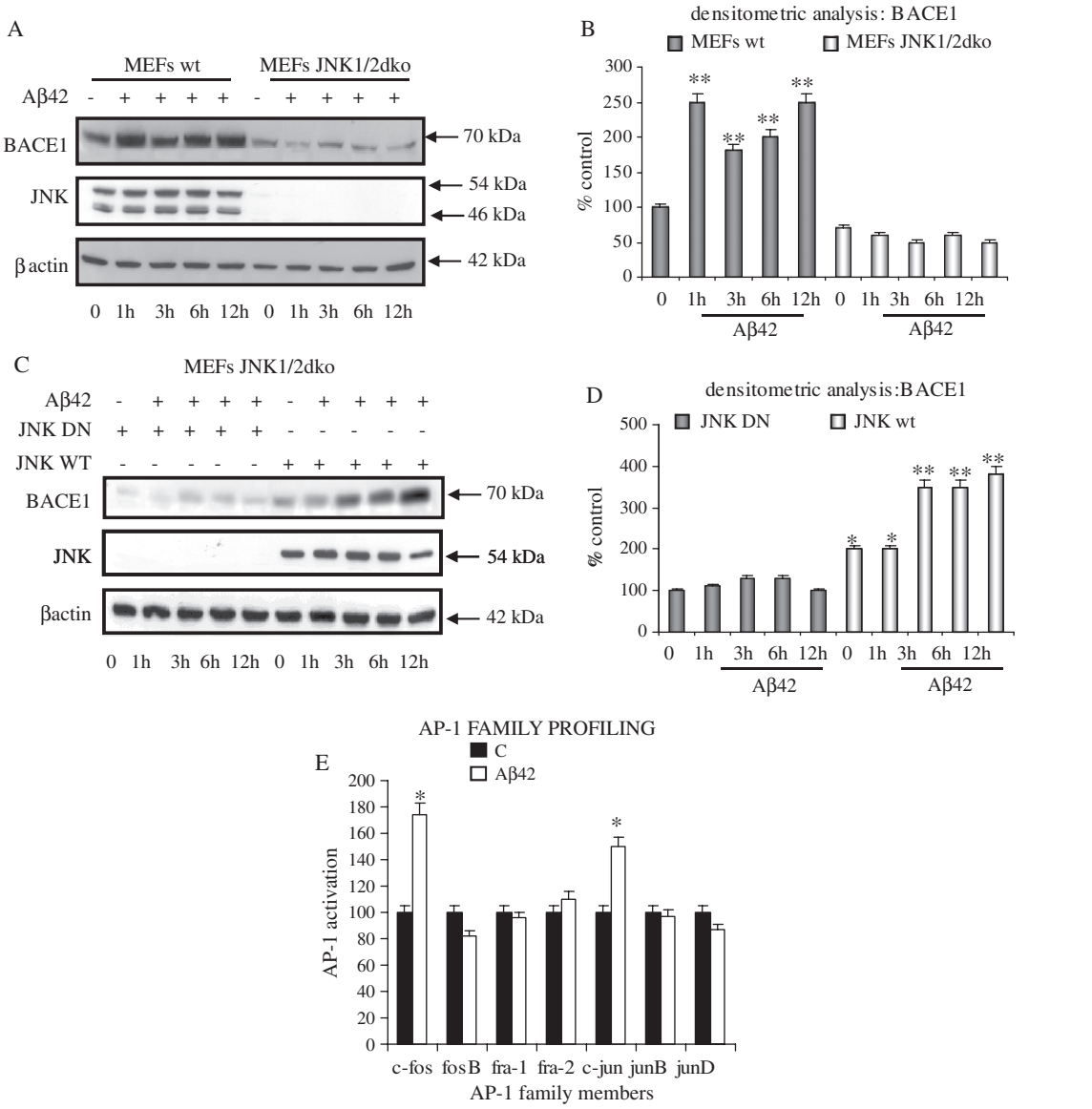

Fig. 5. Role of JNK signalling pathway on the transcription of BACE1. A, B) MEFs JNK1/2 dko had low basal levels of BACE 1 and were resistant to the effect of $A\beta_{42}$ on BACE1 expression. C, D) Transfection of MEFs JNK ko with JNK, but not with JNK dominant negative (DN), restores the upregulation of BACE1 protein levels induced by $A\beta_{42}$. E) $A\beta_{42}$ treatment induce the activation of AP-1 transcription factor, showed by a significant increase of cfos and c-jun DNA binding activity. The error bars represent standard deviations. Experiments were conducted in triplicate. *significantly different from controls ($p < 0.05$). **significantly different from controls ($p < 0.02$).

Then, we treated MEFs wild type with a cell permeable, selective JNK inhibitory peptide (13). JNK inhibition prevented the up-regulation of BACE1 expression by $A\beta_{42}$ (Fig. 6C, D).

DISCUSSION

Our study reveals a novel effect function of Aβ peptides. We demonstrated that $A\beta_{42}$ is the player of a positive feedback loop from the γ-secretase cleavage on the β-secretase cleavage of AβPP. Thereby, Aβ might play a role not just as a toxic molecule, but as a functional signaling intermediate. Previous studies have proposed different physiological functions of Aβ [28, 29]. The more convincing clue finding came from the study of Kamenetz et al. [30], indicating Aβ as the player of a negative feedback loop that regulates the synaptic activity. Accordingly, Aβ production is

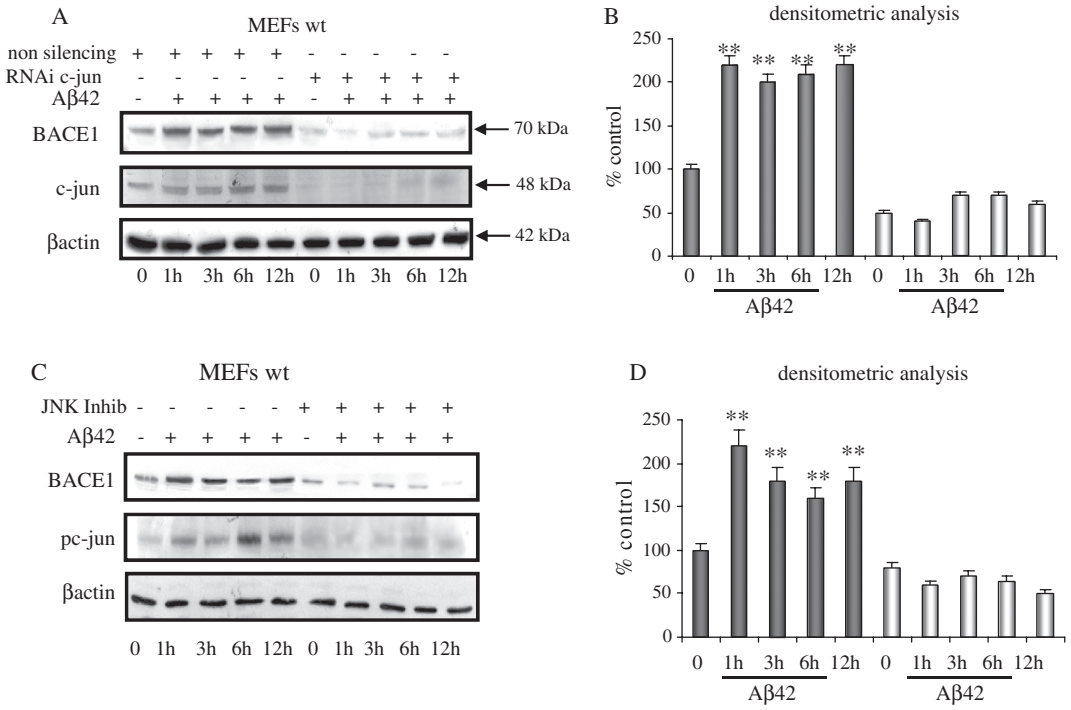

Fig. 6. Inhibition of JNK blocked the increase of BACE1 protein levels induced by $A\beta_{42}$. A, B) JNK inhibition of MEFs wild type with the selective peptide abolishes the effect of $A\beta_{42}$ on BACE1 protein levels. C, D) RNAi silencing of c-jun reduces the basal levels of BACE1 and eliminates the effect of $A\beta_{42}$. The error bars represent standard deviations. Experiments were conducted in triplicate. **significantly different from controls ($p < 0.02$).

proportional to the level of brain activity, as shown in the recovery from severe cranial trauma [31]. Taken together these data argue that Aβ is closely related with synaptic activity and may provide a physiological control on synaptic activity, protecting against excessive glutamate release. On the basis of our results, it should be investigated if the regulation of synaptic function operated by Aβ depends on its transcriptional activity.

Recently Gatta and coworkers [32] performed a microarray assay showing that exposure of neuroblastoma cells to an $A\beta_{42}$-Alluminum complex is followed by a selective changes in gene expression. This approach revealed that the activated genes are involved in the modulation of calcium homeostasis, glutamatergic transmission, oxidative stress, inflammation and neuronal apoptosis [32].

It has been also reported that Aβ peptides, in the fibrillar form, exert their toxicity through the binding to cell-surface holo-AβPP in cortical neurons [33]. In our experiments, $A\beta_{42}$ upregulates BACE1 in AβPP ko MEFs exactly as in wt or in PSdko MEFs. These findings rule out AβPP as putative receptor for Aβ, at least in this conformation (soluble, not fibrillar) as transducer for the modulation of BACE1. We have also shown that the transcriptional activity promoted by $A\beta_{42}$ on BACE1 is transmitted by the activation of the JNK/c-jun pathway.

This pathway links all the pathological hallmarks of AD: JNK activation has been reported to regulate the phosphorylation of AβPP, leading to modulation of Aβ levels [34, 35], as well as to mediate the phosphorylation of tau *in vitro* [36]. Furthermore, the JNK pathway is activated in preclinical models of AD, including Tg2576 and Tg2576/PS1$^{P264\,L}$ transgenic mice [37, 38], as well as in brains of AD cases [39–41].

Indeed, we have previously found a significant activation of JNK/AP1 in oxidative stress *in vitro* as well as *in vivo* models, and the upregulation of BACE1 in these models was not seen when JNK pathway had been genetically or pharmacologically abolished [8]. It remains to be determined how $A\beta_{42}$ does activate JNK pathway. Aβ is known to alter intracellular calcium homeostasis [42], and JNK could be activated by the calcium/calmodulin dependent protein kinase II (CAMKII) [43] or by a PI3K inducing signal, mediated by calcium release [44]. Furthermore, it has been

observed that Aβ-induced increase in intracellular calcium concentration stimulates BACE1 expression, resulting in accelerated Aβ generation, and that this process is mediated by the calcineurin-NFAT1 signaling pathway. NFAT1 is normally dephosphorylated by calcium dependent manner by calcineurin, while it is phosphorylated and inactivated by JNK [45].

Thereby, the interaction of $A\beta_{42}$ with multiple receptors is likely to produce the activation of the JNK/c-jun pathway. Different proteins, such as AβPP itself, TrkA, p75NTR, NMDA, and AMPA receptors, have been shown to interact directly or indirectly with Aβ peptides [46]. Moreover, LRP family of receptors and apolipoprotein E, of which the ε4 allele has a strong linkage with AD, could represent a way for Aβ to enter into the cells [47, 48] and to activate the signaling pathway that leads to BACE1 upregulation.

Being able to know the genes activated by $A\beta_{42}$ may be determinant to understanding the precise series of events that cause dysfunction and degeneration of neurons in AD. Moreover, to understand how $A\beta_{42}$ induces its own production by upregulating BACE1 expression would lead to new tools to interrupt the amyloid vicious cycle, with potential therapeutic consequences.

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