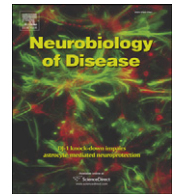




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JNK regulates APP cleavage and degradation in a model of Alzheimer's disease

Alessio Colombo, Antonio Bastone, Cristina Ploia, Alessandra Scip, Mario Salmona, Gianluigi Forloni, Tiziana Borsello*

Istituto di Ricerche Farmacologiche "Mario Negri", Via La Masa 19, 20156 Milano, Italy

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ABSTRACT

Secretion of Amyloid-beta peptide ($A\beta$) circulating oligomers and their aggregate forms derived by processing of beta-amyloid precursor protein (APP) are a key event in Alzheimer's disease (AD).

We show that phosphorylation of APP on threonine 668 may play a role in APP metabolism in H4-APP^{sw} cell line, a degenerative AD model. We proved that JNK plays a fundamental role in this phosphorylation since its specific inhibition, with the JNK inhibitor peptide (D-JNK1), induced APP degradation and prevented APP phosphorylation at T668. This results in a significant drop of β APPs, $A\beta$ fragments and $A\beta$ circulating oligomers. Moreover the D-JNK1 treatment produced a switch in the APP metabolism, since the peptide reduced the rate of the amyloidogenic processing in favour of the non-amyloidogenic one. All together our results suggest an important link between APP metabolism and the JNK pathway and contribute to shed light on the molecular signalling pathway of this disease indicating JNK as an innovative target for AD therapy.

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Introduction

One important pathological feature of Alzheimer's disease (AD) is the formation of circulating oligomers (Lesne et al., 2006; Shankar et al., 2008) by peptides called beta-amyloid ($A\beta$) that are derived from amyloid precursor protein (APP) after beta/gamma secretase cleavage. Because of its critical role in the pathogenesis of AD, understanding the cellular and molecular events underlying APP/ $A\beta$ metabolism has been and remains fundamental for AD.

Proteolysis of APP is regulated not only by the secretase enzymes, but also by an intricate signal cascade (Brown et al., 2000). It has recently emerged that the C-terminal intracellular region (AICD) of APP plays an important functional role in regulating APP metabolism (Slomnicki and Lesniak, 2008). In particular, in AICD region of APP the highly conserved 682-YENPTY-687 motif is recognized by proteins containing phosphotyrosine interaction domains (PID). The proteins that link AICD are many, including c-Jun N-terminal kinase (JNK)-interacting protein-1b (JIP-1b) (Cao and Sudhof, 2001; Taru et al., 2002). JIP-1 is a scaffold protein for JNKs (Whitmarsh et al., 2001) and thus couples APP to the JNK mitogen-activated protein (MAP) kinase pathway.

The AICD region of APP contains eight potential phosphorylation sites, seven of which (Y653, S655, T668, S675, Y682, T686 and Y687) were found phosphorylated in AD brains (Lee et al., 2003). These phosphorylation sites may interfere with protein binding, and in turn, with different intracellular pathways, altering APP function. In particular, T668-P-APP is not just increased in AD brains but also colocalizes with the enzyme responsible for the $A\beta$ production (BACE1). Notably, in the presence of the T668A mutation the $A\beta$ generation is strongly reduced (Lee et al., 2003). Furthermore T668 is important for defining the conformational state of APP and consequently its intracellular homeostasis (Ramelot and Nicholson, 2001; Pastorino and Lu, 2006).

It has been shown that T668 phosphorylation of the AICD involves several kinases including GSK3 β , JNK3, Cdc2 and Cdk5. However the contribution of the individual kinases to this phosphorylation depends on the type of cell as well as the conditions (physiological vs pathological). In addition all these kinases are strongly involved in neurotoxicity and implicated in many neurodegenerative pathways (Suzuki et al., 1994; Iijima et al., 2000; Aplin et al., 1996; Standen et al., 2001).

We previously demonstrated that JNK plays a key role in the phosphorylation of T668 in cortical neurons by using a specific cell permeable JNK inhibitor peptide (D-JNK1). D-JNK1 reduced P-APP (T668) levels and this correlated with a $A\beta$ fragments drop (Colombo et al., 2007).

In this study we investigated the mechanisms of $A\beta$ generation by using the H4-APP^{sw} cell line over-expressing the human APP gene carrying the double Swedish mutation and focused on the role of JNK signalling in APP phosphorylation. This cell line mimics the hallmark

* Corresponding author. Neuronal Death and Neuroprotection Unit, Neuroscience Department, Istituto di ricerche Farmacologiche: "Mario Negri", Via La Masa 19, 20157 Milano, Italy. Fax: +39 02 3546277.

E-mail address: borsello@marionegri.it (T. Borsello).

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of AD pathological condition, since it is characterized by an over-production of A β fragments.

We show that JNK plays a key role in the phosphorylation of APP-T668 also in this AD model and that D-JNK11, by inhibiting this phosphorylation, induces APP degradation and produces a switch on the APP cleavage, reducing the rate of amyloidogenic processing in favour of the non-amyloidogenic one.

Materials and methods

H4-APP^{sw} cell cultures

H4 cells, a neuroglioma cell line expressing the double Swedish mutation (K595N/M596L) of human APP (APP^{sw}), were seeded onto 96-well plates ($\sim 7 \times 10^4$ cells/well) and on 35 mm culture dishes ($\sim 7 \times 10^5$ cells/dish) and allowed to grow to 80% confluence in OPTI-MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco, 15140-122), 200 μ g/ml Hygromycin B (Sigma, H3274) and 2.5 μ g/ml Blasticidin S (Invitrogen, 46-1120), 5% CO₂, 95% air in a humidified atmosphere. Cells were treated with D-JNK11 (2, 20, 40, 80 μ M, Xigen SA, Lausanne, Switzerland) for 24 h before cell lysis. In this cell line, and in contrast to cortical neurons, higher doses of D-JNK11 were not excitotoxic. To investigate protein degradation, cultures were also treated with MG132 (5 μ M, Alexis) 1 h before D-JNK11 treatment (80 μ M).

H4-APP^{sw} cells survival assayed by MTT

Cell survival was assayed by measuring the conversion of the yellow, water-soluble thiazolyl blue tetrazolium bromide (Sigma Aldrich, #M5655) to the blue, water-insoluble formazan. MTT assays were performed in 96-well plates incubating cells with MTT solution for 4 h at 37 °C. Data are presented as the percentage of survival relative to untreated control cultures. All MTT assays were performed in triplicates.

Cellular lysis

Total protein extracts were obtained by scraping cells in lysis buffer (Bonny et al., 2001). Cells were washed twice in ice-cold PBS and lysed for 10 min at 4 °C in 1% Triton x-100 lysis buffer supplemented with proteases (1 \times CPIK, Roche, 10634200) and phosphatases (1 μ M 4-NPP, Roche, 10030536) inhibitors.

Media proteins precipitation

For each dish, 400 μ l of medium were incubated over night at 4 °C with 100 μ l of TCA 50%. After centrifugation at 14,000 rpm for 30 min, 4 °C, the pellets were washed twice with 500 μ l acetone and reconstituted in 50 μ l Urea 4 M.

Western blot analysis

Proteins were separated by 10–14% SDS polyacrylamide gel and transferred to a PVDF membrane. The membranes were blocked in Tris-buffered saline containing 5% no fat milk powder and 0.1% Tween 20 for 1 h at room temperature. Primary antibodies were diluted in the same buffer and membranes were incubated over night at 4 °C using: 1:2000 anti APP clone 22C11 (APPs) (Chemicon, MAB348), 1:500 anti P-APP T668 (a generous gift from Prof. P. Davis, Albert Einstein College of Medicine of Yeshiva University, NY, USA), anti-BACE (Prosci Inc., 2253). Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system. All the blots were normalized using a α -tubulin antibody (Santa Cruz Biotechnology, SC-8035). Quantification of Western blots was carried out using ImageQuant TL software (Amersham Biosciences) and was based on at least six independent experiments.

C-83/C-99 fragments analysis

Proteins of cellular lysates were separated using the NuPAGE Novex Bis-Tris Mini Gels System (Invitrogen, NP0321). The membranes were blocked in Tris-buffered saline containing 5% no fat milk powder, BSA 1% and 0.05% Tween 20 for 1 h at room temperature. Primary antibodies were diluted in the same buffer and membranes were incubated over night at 4 °C using 1:2000 Anti Amyloid Precursor Protein (APP) C-Terminal (Sigma Aldrich, A8717) (Kern et al., 2006).

Dot blot analysis for A β circulating oligomers

Equal protein amounts of total lysate were spotted onto nitrocellulose membrane for each tested condition. Tris-buffered saline (20 mM Tris, 0.8% NaCl, pH 7.4) containing 0.001% Tween-20 (TBST) was used for washing. The membrane was blocked for 1 h with 5% non-fat dried milk in TBST and incubated over night in primary anti-oligomer antibody (A11, 1:1000, Biosource, AHB0052) in 5% non-fat dried milk/TBST. The membrane was developed using horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system. All the blots were normalized using a α -tubulin antibody (Santa Cruz Biotechnology, SC-8035). Quantification of dot blots was carried out using ImageQuant TL software (Amersham Biosciences) and was based on at least three independent experiments. A control membrane with primary antibody omitted was simultaneously processed.

Immunocytochemistry

H4 cells plated on coverslips were fixed in 4% paraformaldehyde, 5% sucrose-PBS for 30 min on ice. Cells were incubated for 1 h with 10% serum in PBS-Triton 0.3% at room temperature. Primary antibodies specific to N-terminal of APP (Clone 22C11, Chemicon, MAB348) (1:150), anti P-APP T668 (a generous gift from Prof. P. Davis, Albert Einstein College of Medicine of Yeshiva University, NY, USA) (1:150), anti beta amyloid (clone 4G8, Signet Covance, 39200) (1:200) and anti sAPP β (IBL, 18957) (1:250) were diluted in PBS-Triton 0.1%–1% serum and incubation was over night at 4 °C. After washing in PBS, cells were incubated for 1 h at room temperature with secondary Alexa546 and Alexa488 antibodies (Invitrogen, Molecular Probe). To stain the nuclei, cells were incubated with Hoechst reagent (Invitrogen) 1:500 in PBS for 7 min at room temperature. Coverslips were mounted in Fluorsave mounting medium (Calbiochem, 345789). Staining was acquired with a Olympus microscope equipped with a Olympus confocal scan unit (microscope BX61 and confocal system FV500) managed by AnalySIS Fluoview software with 3 lasers line, UV-diode laser (405 nm), Ar–Kr (488 nm), He–Ne green (546 nm), respectively used to detect Hoechst staining and secondary antibody conjugated to Alexa 488 and Alexa 546. Double staining was revealed with a scanning sequential mode to eliminate possible bleed-through effect. The images were acquired at 100 \times magnification, NA 0.85 at room temperature and the imaging medium was air.

ELISA assay

Concentration of β -peptides in culture media was measured using a specific sandwich-type enzyme-linked immunoabsorbent assay (ELISA) (Human Amyloid β 1–40 Assay Kit, IBL, 27714; Human Amyloid β 1–42 Assay Kit, IBL, 27711) according to the manufacturer's protocols. Intra assay variability was less than 5% and inter-assay variability less than 10%. All samples were tested at least three times independently to verify reproducibility.

BACE activity assay

BACE enzyme activity was measured using a TruPoint Beta-Secretase Assay Kit (Perkin Elmer inc., AD0258) based on SignalClimb

technology used in a time-resolved fluorescence assay (TRF). TruPoint BACE substrate is a ten amino acid long peptide, with a fluorescent europium chelate coupled to one end and a quencher of europium fluorescence to the other end via lysine. If the sample contains BACE activity the europium chelate and the quencher will be separated as the substrate is cleaved. The europium signal increases and it can be measured in a detection instrument having TRF-option like the VICTOR platform (Perkin Elmer Inc.). Cells were lysed in lysis buffer with proteases and phosphatases inhibitors and for each measurement 20 μ g of total cellular extract were tested. Results were expressed as S/B ratio (mean signal of sample/mean signal of background) and each sample was tested at least three times independently to verify reproducibility.

Statistical analysis

All experiments were repeated at least three times using independent culture preparations. Quantitative data were statistically analyzed by paired a *T*-test with two-tailed distribution. A *p* value of <0.05 was considered significant.

Results

D-JNK11 effect on APP and P-APP in H4-APP^{SW} cells line

H4-APP^{SW} line is stably transfected with human APP^{SW}-double Swedish mutation (K595N/M596L) and is characterized by a 15 folds increase in A β fragments production. To assess potential toxic effects of the D-JNK11 inhibitor, H4-APP^{SW} cells were incubated with increasing doses (2, 20, 40, 80 μ M) for 24 h; no significant cell death was found (data not shown). The effect of D-JNK11 on APP level and phosphorylation state of the T668 was studied by analyzing the ratio APP/Tubulin and P-APP/APP following peptide treatment and in control conditions.

We first measured the ratio APP/Tubulin to study the effect of D-JNK11 (80 μ M) on APP protein level. APP/Tubulin was measured from cell lysates by western blot to monitor the intracellular APP and corresponding media were analyzed for secreted APPs. As shown in Fig. 1, D-JNK11 decreased APP levels in the lysates (35%, *p*<0.05). In parallel, APPs levels in the media dropped by 35% (*p*<0.05) (Figs. 1A, B). These results confirmed an effect of D-JNK11 on APP homeostasis in a

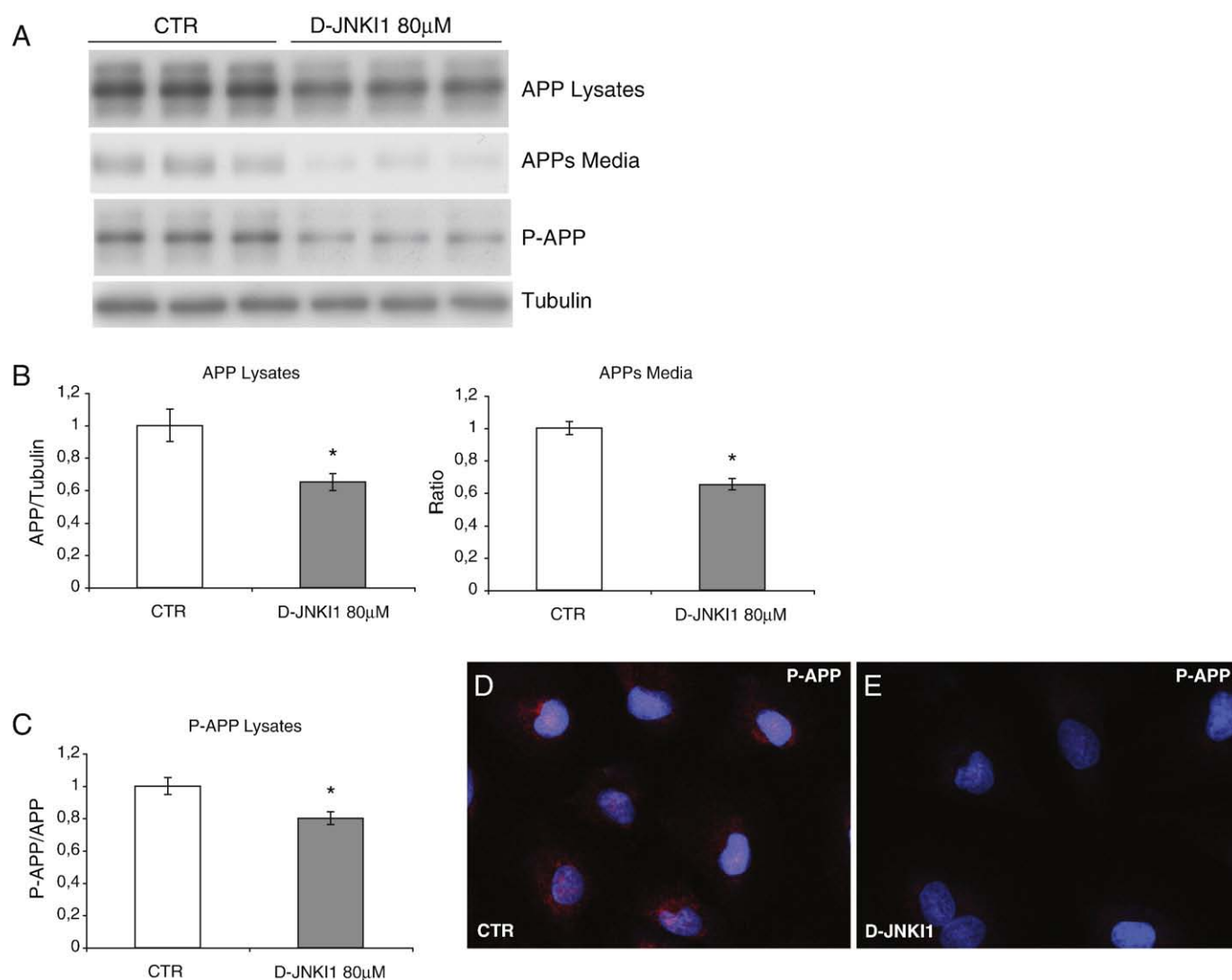


Fig. 1. D-JNK11 effect on APP and P-APP. H4-APP^{SW} cells were treated with 80 μ M D-JNK11 as indicated. (A) H4-APP^{SW} cells were treated with 80 μ M D-JNK11 for 24 h and cellular lysates were blotted for total APP (22C11 antibody) and for P-APP (T668). Proteins precipitated from culture media were blotted for total APPs. Loading control = tubulin. (B) Densitometry analysis clearly shows that the peptide reduces total APP and APPs. APP drops of 35% in both media and lysates of treated cells. (C) D-JNK11 inhibits the P-T668-APP and in fact the densitometry of the ratio P-APP/APP was reduced by 20%. Quantifications were from eight independent experiments (\pm SEM), **p*<0.05. (D, E) Immunofluorescence staining for P-T668-APP in control and D-JNK11 treated cells. Cells treated with 80 μ M D-JNK11 (E) show a sensible decrease of the staining compared to control condition (D).

pathological AD model as previously described in cortical neurons (Colombo et al., 2007).

In order to investigate the effect of D-JNK11 on APP phosphorylation, we used an antibody that recognizes specifically the P-T668 site (Colombo et al., 2007). Results were normalized to total APP.

As described before we measured the P-APP/APP ratio in control and D-JNK11 treated cells. The D-JNK11 (80 μ M) treatment reduced the ratio by 20% compared to control conditions (Figs. 1A, C).

To better assess the P-APP change in the presence of D-JNK11 and its sub-cellular localization we performed immunofluorescence analysis with P-APP-T668 antibody in control and treated cells. In control cells P-APP was localized mainly in the cytosol (even if some staining was present in the nuclei). D-JNK11 treatment resulted

in weaker P-APP labelling, but did not affect cellular distribution. (Figs. 1D, E).

We concluded that D-JNK11 is able to inhibit T668 phosphorylation in a degenerative model, where there is an over-expression of the pathological mutated APP (APP^{SW}). The effect of D-JNK11 on APP degradation and cleavage was subsequently investigated.

D-JNK11 effect on APP degradation in H4-APP^{SW} cells

We previously demonstrated that D-JNK11, by preventing APP phosphorylation, destabilizes the protein and induces its degradation via the proteasome (Colombo et al., 2007; Muresan and Muresan, 2007). To test this hypothesis in our model, we applied MG132, a

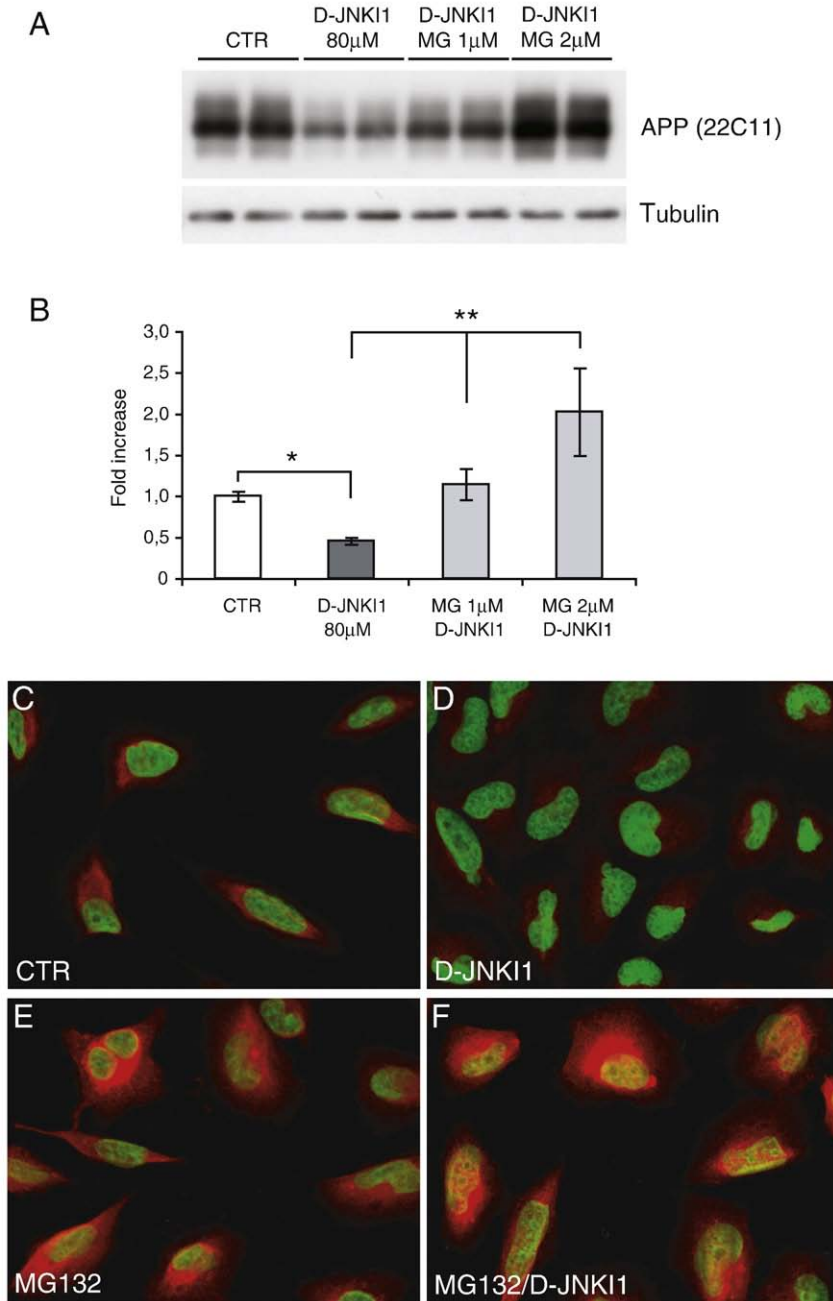


Fig. 2. D-JNK11 induces APP^{SW} degradation. (A, B) Western blot analysis in cellular lysates and quantification demonstrates that APP level drops in the presence of D-JNK11, while the co-treatment with MG132/D-JNK11 rescue the APP decrease. The inhibition of proteasome by MG132 is able to rescue the drop of APP level given by D-JNK11 treatment. Loading control = tubulin. Quantifications were from six independent experiments (\pm SEM), * p < 0.05, *** p < 0.01. (C–F) Immunostaining for total APP in H4-APP^{SW} cells. Co-treatment of cells with MG132 2 μ M and D-JNK11 80 μ M (F) rescue the decrease of APP level in comparison with D-JNK11 alone (D). The MG132 on control cells (E) by preventing APP degradation increases APP labelling level as expected in comparison to control condition (C).

proteasome inhibitor against the 26S complex, in a co-treatment with D-JNK11 for 24 h.

Cellular APP levels were assessed in D-JNK11 alone, MG132/D-JNK11 co-treated and control cells. As shown in the representative western blot (Fig. 2A), D-JNK11 induced a decrease of APP levels (see Figs. 2A, B). Normalisation with tubulin and subsequent quantification confirmed that co-treatment with 1 μ M MG132 and D-JNK11 restored APP levels, while higher doses of MG132 (2 μ M) dramatically increased APP levels (2 fold in comparison to controls) (Figs. 2A, B). D-JNK11 treatment may present some inter-experimental variability.

Data were also confirmed with immunofluorescence: APP staining was reduced in D-JNK11 treated cells (Fig. 2D) compared to controls (Fig. 2C) while the treatment with MG132 alone (Fig. 2E) or in combination with D-JNK11 (Fig. 2F) blocked APP degradation and increased APP staining in the cells.

These findings indicated that D-JNK11 induces APP degradation via the proteasome.

D-JNK11 treatment interferes with the APP cleavage of H4-APP^{sw} cells

To determine if D-JNK11 has an effect on the amyloidogenic processing of APP, we used two different antibodies (see Scheme 1): 1- β APPs and 2-4G8 antibody. These two antibodies identify different portions of APP: β APPs recognizes the COOH portion of the soluble fragment produced only after the β -secretase cleavage, while the 4G8 identifies the NH2 portion just of the beta amyloid sequence.

Immunofluorescence analysis underlined a powerful reduction of β APPs labelling in D-JNK11 treated compared to control cells (Fig. 3). In control conditions β APPs was sparse (Fig. 3A), but D-JNK11 treatment caused a massive reduction in β APPs labelling. The D-JNK11 inhibitor had no impact on β APPs distribution inside the cell (Fig. 3B).

Similar to the β APPs, 4G8 APP staining was peri-nuclear and weakly diffused in the cytosol, this labelling was reduced in treated (Fig. 3D) compared to control cells (Fig. 3C) but it did not vary the localization as seen for β APPs.

Taken together these results suggested that D-JNK11 might reduce APP β -cleavage.

To further investigate its effect we also examined the media of cells with ELISA kits for the A β fragments 1–40 and 1–42. D-JNK11 treatment

produced a powerful reduction of A β fragments in the media, correlating with the drop observed with immunofluorescence. In fact, quantification revealed a drop of 60% of A β 1–40 and 1–42 (Fig. 3E).

D-JNK11 moves the ratio C-83/C-99 in favour of non-amyloidogenic pathway

APP processing releases a soluble N-terminal fragment and leaves a membrane anchored C-terminal fragment. The length of this fragment differs depending on which secretase makes the first cut on the protein. The alpha and the beta secretases cut the APP in different sites and this produces respectively 83 or 99 amino acid long fragments (C-83 and C-99). We here examined the effect of D-JNK11 on the C-83/C-99 ratio.

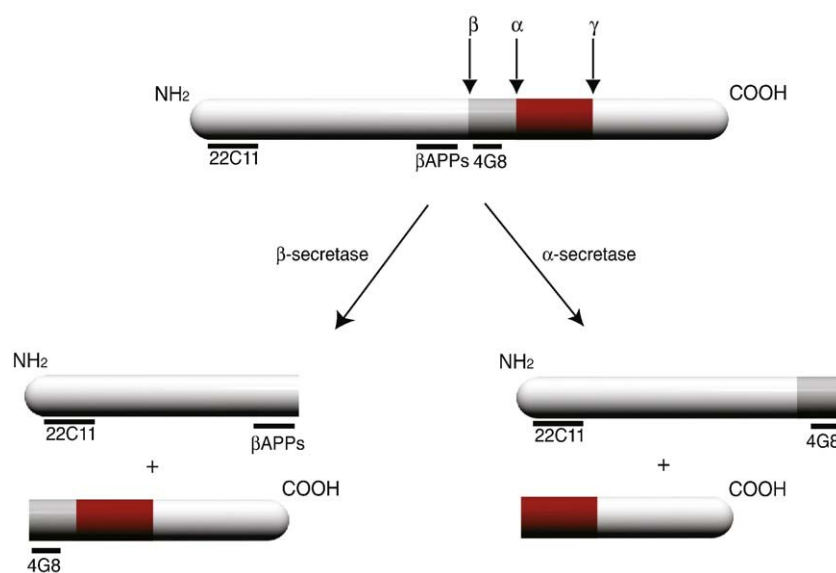
Cell lysates were examined by Western blot analysis with an antibody to the carboxy-terminal region of APP and immunoreactive bands were quantified by densitometry. The ratio of C-83/C-99 in control and treated cells at the two highest concentrations (40 and 80 μ M) of D-JNK11 was compared. D-JNK11 treatment decreased the C-99 fragment level produced by the β -cleavage of APP at variance with the C-83 fragment level produced by the α -cleavage (Fig. 4A). The C-99/C-83 ratio was decreased by 60% with 40 μ M D-JNK11 and 90% with 80 μ M (Fig. 4B). D-JNK11 caused a remarkable shift of the C-83/C-99 ratio in favour of the C83 fragment, promoting the non-amyloidogenic processing of APP.

D-JNK11 reduces soluble oligomers formation in H4-APP^{sw} cells

Because D-JNK11 strongly reduced extracellular A β fragments we analyzed its effect on A β oligomers production in total cellular extracts in comparison to untreated cells. D-JNK11 treatment lowered the amount of oligomeric species in comparison to control cells. In fact D-JNK11 decreased in a dose dependent manner the soluble A β oligomers by 40% with 40 μ M and by 60% with 80 μ M as shown in the dot blot analysis and its densitometry quantification (Figs. 4C, D).

D-JNK11 effect on BACE1 expression/activity

Due to the strong effect of D-JNK11 on the APP cleavage we then analyzed the possibility that it could interfere with the degradation



Scheme 1. APP antibodies. Scheme of the APP cleavage processing with the α , β and γ secretase cutting points (see arrows). The diagram shows the position of APP epitopes recognized by the different antibodies. The 22C11 epitope is in the NH₂ region and identifies the APP full length (both mature and immature), but also β APPs and α APPs. The β APPs antibody is specific for β secretase cleavage, because the epitope is just before the β secretase cut point and detects the β APPs. The 4G8 antibody recognizes the first 17 amino acids of the beta amyloid sequence.

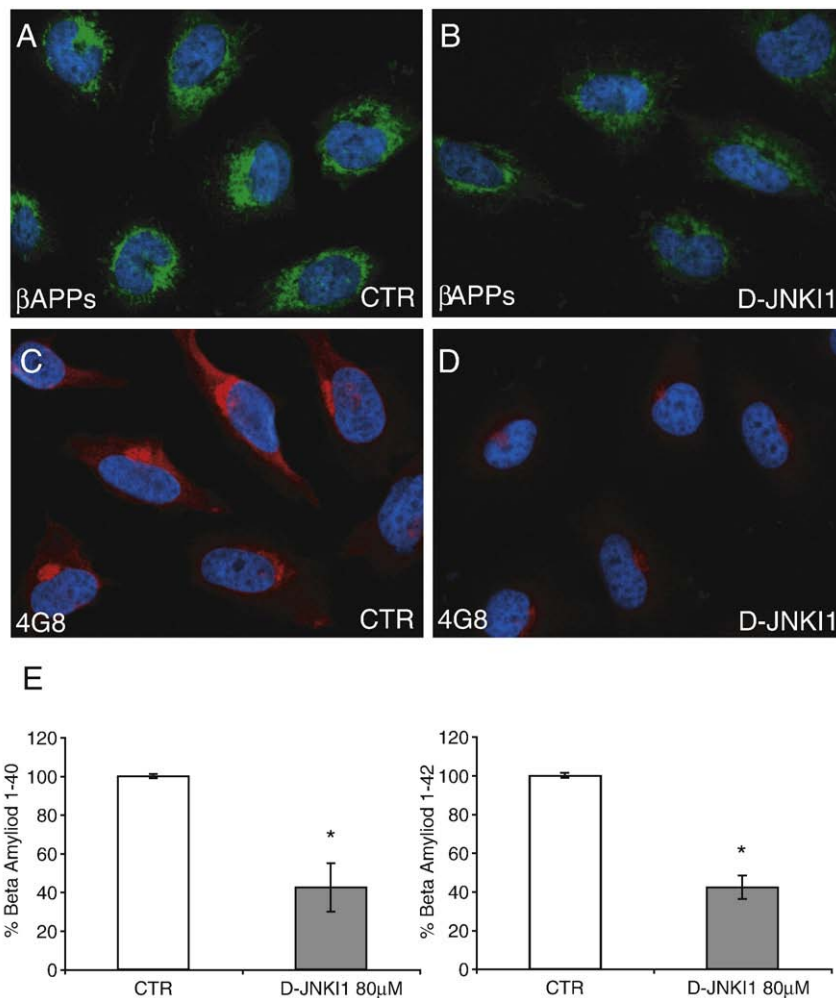


Fig. 3. D-JNK1 effect on beta amyloid production (A, B) Immunostaining for intracellular β APPs. H4-APP^{sw} cells treated with D-JNK1 80 μ M for 24 h (B) shows decrease of β APPs staining in comparison with untreated cells (A). The labelling localization of β APPs did not change but was only weaker compared to control condition. (C, D) Immunostaining for intracellular A β fragments (4G8) in control and D-JNK1 treated cells. D-JNK1 treated cells (D) shows a sensible decrease of the staining for beta amyloid in contrast to untreated cells (C), this localization did not change within the cells. (E) Quantitative determination of beta amyloid fragments (1–40 and 1–42) in culture media by ELISA assay. The D-JNK1 treatment had the same effect on A β 1–40 and A β 1–42 with a reduction of the fragments level of 60% in treated cells compared to control cells. Data were expressed as mean of three independent experiments (\pm SEM), * p < 0.05.

and/or activity of the β -secretase (BACE1), an endo-protease responsible for the production of β amyloid (A β) peptides. Notably, BACE1 colocalizes with P-APP in AD brains, suggesting a possible link between the two. Western blot analysis revealed no changes in BACE1 cellular levels following D-JNK1 treatment (Figs. 5A, B).

We then analyzed the activity of the enzyme after D-JNK1 treatment by using a specific BACE1 activity assay kit (Holsinger et al., 2004) and no differences were found between control and treated cells (Fig. 5C). These results suggest that although D-JNK1 leads to A β levels reduction, it has no impact on either BACE1 expression or activity levels.

Discussion

APP is phosphorylated at multiple sites in the C-terminal cytoplasmic domain (Suzuki et al., 1994) but the phosphorylation of T668 is particularly intriguing since it induces conformational changes that affect APP function and metabolism (Ramelot and Nicholson, 2001; Muresan and Muresan, 2005; Muresan and Muresan, 2007). Lee et al. (2003) reported that phosphorylated APP (T668) accumulated at high levels in human AD brains and raised the likelihood that this specific phosphorylation is correlated to A β generation (Akiyama et al., 2005; Ando et al., 2001; Lee et al., 2003). Thus, T668 phosphorylation is highly relevant to AD pathology.

However, until today, the mechanisms of APP phosphorylation are not completely characterized. This is because JNK (Colombo et al., 2007; Standen et al., 2001), GSK3- β (Aplin et al., 1996) and Cdk2/5 (Iijima et al., 2000; Muresan and Muresan, 2005) can phosphorylate the same site (T668) and their roles vary depending on the conditions and the type of cell (Muresan and Muresan, 2007).

We have previously described that in cortical neurons (physiological conditions) the principal kinase involved in the phosphorylation of APP-T668 is JNK and in fact its specific inhibition, by D-JNK1, highly reduces APP processing/A β production (Colombo et al., 2007).

The aim of this study was to clarify JNK action on APP in a pathological AD model, the “H4-APP^{sw} cell line”. This neuroglioma cell line is stably transfected with human APP gene carrying the double Swedish mutation (K595N/M596L), and characterized by a 15 fold increased A β production reproducing a “degenerative state” that mimics AD pathological condition.

We found an important effect of D-JNK1 on APP degradation: APP level decreased by 35% in both cell lysates and in the corresponding media in the presence of the peptide. The D-JNK1-induced degradation could be rescued with MG132 (proteasome inhibitor) suggesting that the phosphorylation in T668 is important for the APP^{sw} stability as previous reported in another model. It could be speculated that in a cellular system with an APP over-expression as this, degradation could

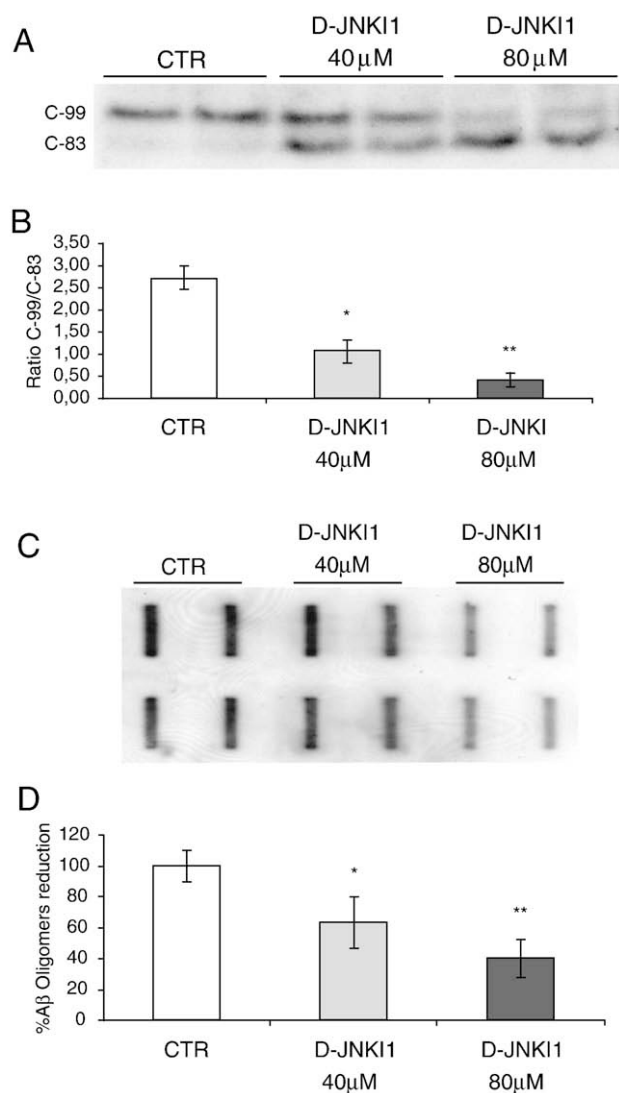


Fig. 4. C-83/C-99 APP ratio and soluble A β oligomers production. (A) Western blot analysis specific for C-terminal fragment of APP after α -secretase cleavage (C-83) and β -secretase cleavage (C-99). (B) Western blot densitometry expressed as ratio between C-99 fragment and C-83 fragment. D-JNK11 treatment reduces the level of C-99 and increase C-83 in a dose dependent way, with a drop of the ratio C-99/C-83 of 90% with 80 μ M. Quantifications were from three independent experiments (\pm SEM), * p <0.05, ** p <0.01. (C) Dot blot analysis specific for A β oligomeric species in total cellular lysates. (D) Dot blot densitometry. D-JNK11 treatment reduces in a dose dependent way the A β oligomers production with a maximum reduction of 60% at higher used concentration. Quantifications were from three independent experiments (\pm SEM), * p <0.05, ** p <0.01.

represent a mechanism for eliminating excess APP. At the same time these findings are in line with our previous work in neurons (Colombo et al., 2007), and others proved that T668 phosphorylation protects APP from degradation (Muresan and Muresan, 2007).

Another important finding of our study is the key action of JNK on the A β fragment production.

In the H4-APP^{SW} cells treated with D-JNK11, P-APP was significantly decreased and this correlated with a powerful reduction of β APPs, A β fragments and soluble oligomers. We then looked for and succeeded in finding a correlation between P-APP and the decrease of A β fragments in the media of treated cells. The extracellular A β fragments were strongly reduced (60%) with the JNK inhibitor treatment. The intracellular soluble A β oligomers were also powerfully reduced (60%) as the extracellular counterpart.

This result is particularly significant because soluble A β oligomers are indicated as the first causative agents of AD pathology.

These decreases are significant and are the result of the two described phenomena:

1. increased degradation of APP
2. inhibitory effect on JNK mediated phosphorylation of APP at T668.

Finally, D-JNK11 treatment increased C-83 level and reduced C-99 level producing a switch between amyloidogenic versus non amyloidogenic pathway, indicating a remarkable shift towards the α secretase cleavage. The effect of the JNK inhibitor was dose-dependent and the higher dose used (80 μ M) provoked a reduction of 90% of the C-99/C-83 ratio.

Our results proved that in a pathological model, characterized by a hyper-production of A β fragments, JNK regulates APP^{SW} phosphorylation at the T668 and its specific inhibition prevents A β production and promotes the α -cleavage as a substitute of the β -cleavage.

We examined if D-JNK11 might have an indirect effect, that we could not exclude "a priori", on the beta-Amyloid precursor protein cleavage enzyme 1 (Lee et al., 2003). BACE1 has been identified as a major neuronal beta-secretase, critical for the formation of beta-amyloid peptides and co-localizes with P-APP in human AD brains. We proved that D-JNK11 treatment did not interfere either with BACE1 expression-level or with its activity.

In summary this study, for the first time, provides evidence that the MAP-kinase JNK, promotes the amyloidogenic/beta-APP-cleavage versus non amyloidogenic/alpha-APP-cleavage, by phosphorylating APP, thus indicating JNK as a novel molecular signalling pathway of the disease.

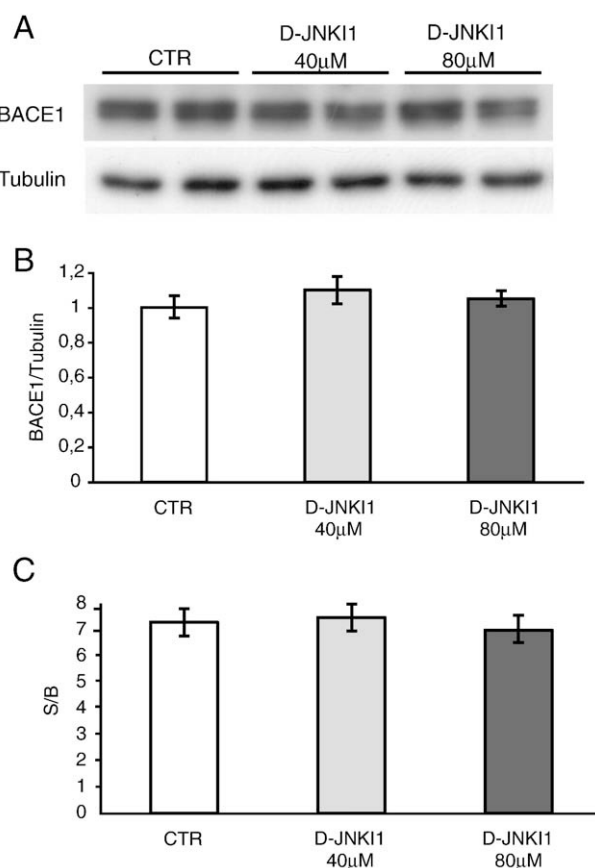


Fig. 5. BACE1 expression/activity. (A, B) Western blot analysis of the BACE1 protein level in cellular lysates after 24 h D-JNK11 treatment (A) and its quantification by densitometry (B). Data show that the protein level is not influenced by the treatment. Quantifications were from six independent experiments (\pm SEM). (C) Enzymatic activity assay for BACE1. Data are expressed as ratio between the mean of sample and the mean of blank (S/B). The BACE1 activity is unchanged after D-JNK11 treatment. Quantifications were from three independent experiments (\pm SEM).

Inhibition of c-Jun N-terminal kinase may indeed provide a means of suppressing more pathological mechanisms in AD pathology in fact JNK is involved in APP and Tau hyper-phosphorylation.

The possibility that D-JNK11 might prevent APP phosphorylation, amyloidogenic cleavage, reducing also soluble A β oligomer species, possibly the Tau hyper-phosphorylation and that as the same time it acts as a neuroprotectant suggests possible therapeutic usefulness.

Because the neuroprotective action of D-JNK11 has been tested in several experimental models of disease (Borsello et al., 2003; Hirt et al., 2004; Wang et al., 2003; Suckfuell et al., 2007; Zhuang et al., 2006; Beckham et al., 2007) we propose JNK inhibition as an innovative strategy against Alzheimer's disease.

Our in vitro findings indicate the potential value of JNK inhibition against AD, however further studies are needed to investigate in vivo therapeutic effect of the D-JNK11 as well as its inhibition on Tau.

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