Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1773 (2007) 254–263
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamcr

Neuroprotective effect of TNFα against the β-amyloid neurotoxicity mediated by CDK5 kinase

Daniel I. Orellana [a], Rodrigo A. Quintanilla [a], Ricardo B. Maccioni [a,b,*]

[a] *Laboratory of Cellular, Molecular Biology and Neurosciences, Faculty of Sciences, Universidad de Chile; Las Encinas 3370, Ñuñoa, Santiago, Chile*
[b] *Department of Neurological Sciences, Faculty of Medicine, Universidad de Chile, Av. Salvador 486, Providencia, Santiago, Chile*

Received 15 June 2006; received in revised form 2 October 2006; accepted 16 October 2006
Available online 21 October 2006

Abstract

The tumor necrosis factor alpha (TNFα) plays a dual role in producing either neurodegeneration or neuroprotection in the central nervous system. Despite that TNFα was initially described as a cell death inductor, neuroprotective effects against cell death induced by several neurotoxic insults have been reported. Tau hyperphosphorylation and neuronal death found in Alzheimer disease is mediated by deregulation of the cdk5/p35 complex induced by Aβ treatments. Since TNFα affects cdk5 activity, we investigated its possible protective role against the Aβ-induced neurodegeneration, as mediated by cdk5. TNFα pretreatments significantly reduced the hippocampal neuronal cell death induced by the effects of $A\beta_{42}$ peptide. In addition, this pretreatment reduced the increase in the activity of cdk5 induced by $A\beta_{42}$ in primary neurons. Next, we investigated the Alzheimer type phosphorylation of tau protein induced by $A\beta_{42}$. We observed that the pretreatment of neurons with TNFα reduces tau hyperphosphorylation. Taken together, these results define a novel neuroprotective effect of TNFα in preventing neuronal cell death and cdk5-dependent tau hyperphosphorylation. This phenomenon, taken together with other previous findings, suggests that the inflammatory response due to Aβ peptide plays a key role in the development of Alzheimer etiopathogenesis.

Keywords: TNFα; Cdk5; Aβ; Tau; Neurodegeneration; Alzheimer disease

1. Introduction

TNFα is a pro-inflammatory cytokine produced by many cell types. At the level of the central nervous system (CNS), TNFα can be synthesized and released by astrocytes, microglia, and some neuronal cell types [1,2]. TNFα also plays a key role in modulating normal brain physiology [3]. In addition, it has been shown that TNFα is necessary for the synaptic plasticity in cortical neurons [4,5]. Considerable studies have been made in understanding the molecular mechanism that mediates TNFα duality as mediator of neurodegeneration or neuroprotection. TNFα exerts its biological functions through the action of two main receptors, TNF-R1 and TNF-R2, both of them induces NF-κB pathway activation [6]. The activation of NF-κB by TNFα is necessary for neuronal survival [7,8]. Additionally, a model mouse that lack of TNFα receptors is more susceptible than wild-type animals to brain injury and neuronal damage [9,10,11]. Binding of TNFα to its receptor and the activation of NF-κB pathway seems to be critical in the dual role of this cytokine [12,6].

Currently, it is well accepted that inflammatory mechanism is involved in the pathogenesis of Alzheimer disease (AD). In AD cytokines such as IL-6, IL-1β and TNFα are secreted by microglial cells, astrocytes and/or neuronal cells, and can induce the synthesis of amyloid precursor protein (APP) [13]. On the other hand, APP or the amyloid beta peptide (Aβ) can induce the expression of IL-6, IL-1β or TNFα in astrocytes and microglial cells in culture [14,15].

The cdk5/p35 complex plays a pivotal role in the development of nervous system, as it has been shown through engineered targeted mutations of *cdk5*, *p35*, and *p39* genes producing cortical migration defects [16,17]. The function of this enzyme, a member of the proline-directed protein kinases (PDPKs) family that also includes the glycogen synthase kinase

* Corresponding author. Laboratory of Cellular, Molecular Biology and Neurosciences, Faculty of Sciences, Universidad de Chile; Las Encinas 3370, Ñuñoa, Santiago, Chile.
E-mail address: rmaccion@uchile.cl (R.B. Maccioni).

0167-4889/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2006.10.010

3β (gsk3β), is regulated by the neurospecific activators p35 [18,19] and p39 [20]. Overactivation of PDPKs, such as cdk5 and gsk3β, has been implicated in the aberrant phosphorylation of tau at proline-directed sites implicated in the ethiopathogenesis of Alzheimer disease [21,22].

It is known that an increase in the cdk5 activity is an important factor in neurodegeneration [23,24]. We have previously demonstrated that both Aβ and IL-6 treatments of hippocampal neurons induce an increase in the cdk5 activity [25,26]. The cdk5-mediated neurotoxicity is, at least in part, due to an increase in tau hyperphosphorylation [25]. Additional studies have proposed that cdk5 inhibits the neuroprotective effect mediated by the transcription factor MEF2 [27,28]. Moreover, evidence suggests that TNFα modulates the expression of MEF2 in mouse *soleus* muscle [29]. These observations indicate a possible common activation pathway.

Moreover, pretreatment of primary neurons with TNFα protected neurons against Aβ toxicity by suppressing accumulation of ROS and cytosolic Ca^{2+} increases [12]. Until now, the neuroprotective effect of TNFα and the possible role of cdk5 in this event are unknown. In the present study, we show that pretreatment of hippocampal neurons with TNFα prevents the neurodegeneration induced by $A\beta_{42}$ by inhibiting the cdk5 activity and decreasing their protein levels in hippocampal neurons. Additionally, the pretreatment of TNFα prevented tau hyperphosphorylation induced by $A\beta_{42}$, and decreased the cortical neurons aggregation, both processes governed by the cdk5. Even though p35, the cdk5 activator, is involved in cdk5 function, no significant changes in protein expression were evidenced. Based on these findings, we suggest that in hippocampal neurons, TNFα protects cells against Aβ neurotoxicity, by modulating cdk5 activity. These events may contribute to the whole neuroprotective effect exerted by TNFα in AD.

2. Materials and methods

2.1. Materials

Human recombinant TNFα was obtained from US Biologicals. $A\beta_{42}$ was purchased from Global Peptide Services. Cdk5 monoclonal antibody (mAb) J3 and polyclonal antibody C8; p35 mAb N20 were obtained from Santa Cruz Biotechnology; AT8, mAb was from Innogenetics (Belgium); Tau-1 mAb and Tau5 mAb were donated by Dr. Lester Binder. Dr. Peter Davis generously donated PHF1 mAb. MitotoCapture™ apoptosis detection kit and roscovitine were purchased from Calbiochem (San Diego, La Joya). Tissue culture reagents were obtained from Gibco BRL.

2.2. Primary rat cultures of hippocampal neurons

Primary rat hippocampal neuronal cultures were established from the hippocampus of 18-day-old Sprague–Dawley rat fetuses according with methods described previously [26]. Neurons were seeded onto 0.1% polylysine-coated 60-mm plastic Petri dishes (1.0×10^6cell/60-mm culture dish) for biochemical experiments. TNFα at a concentration of 5 ng/ml was added to hippocampal neurons culture at 4 days in vitro (4-DIV), which continued in culture for additional 48 h. Sister cultures that did not receive TNFα treatment were used as controls.

2.3. Amyloid-β preparation

Synthetic $A\beta_{42}$ peptide solutions (>70% HPLC) were prepared as described previously [21]. Briefly, stock solutions were prepared by dissolving lyophilized aliquots of Aβ peptide in dimethylsulfoxide (DMSO) at 15 mg/ml (3.5 mM). Aliquots of peptide stock solution (70 nmol in 20 μl DMSO) were added to sterilize MilliQ H_2O. The solution was incubated for 3 days, after which the Aβ fibrils were concentrated by centrifugation (14 000 rpm for 30 min) to separate the soluble supernatant containing oligomers of the peptide from the insoluble fibrillary (pellet) form of the peptide. Soluble oligomeric forms of the peptide were resuspended in Neurobasal/B27 media and stored at −20 °C. This soluble $A\beta_{42}$ was added to the culture medium at a final concentration of 10 μM by 24 h, a concentration that has been shown to cause early events of neurotoxicity in cultured hippocampal neurons [21]. These conditions of a non-detergent-containing extraction buffer allow the formation of a soluble Aβ peptide fraction containing many oligomeric species, which is more toxic than fibrillary Aβ [30].

2.4. Neuronal aggregation assay

Dissociated rat embryonic cortical neurons (E18) were washed with Hank 1× and resuspended in plating media. The neurons were plating in media with 1 mM EGTA, 1 mM $CaCl_2$, and TNFα (5 ng/ml). Then, the neuronal aggregation assay was carried out as described in [31] with minor modifications. Briefly, the cells were incubated at 80 rpm in a 37 °C floor shaker. After 15 min at 37 °C, aggregates were fixed with 4% paraformaldehyde (10 min) and permeabilized with PBS containing 0.2% Triton X-100 (10 min). Coverslips were rinsed with PBS followed by H_2O. Cresyl violet stain was applied for 5 min and the coverslips mounted with PBS containing 50% glycerol. The surface of 10 viable aggregates (5 or more cells) for each condition was measured using the software Axiovision 3.5 (Carl Zeiss, Germany).

2.5. Cell viability

Cell viability was evaluated using the MTT assay previously described at [26]. Briefly, neurons were seeded in polylysine-coated 96-well plates at 2.0×10^4 cells/100 μl per well in Neurobasal/B27 medium without phenol red. Then, neurons were treated with TNFα 5 ng/ml, $A\beta_{42}$ 10 μM, or both. The results were represented in the graphs as a percent of control.

2.6. Mitochondrial viability

MitoCapture™ Apoptosis Detection Kit was used in order to distinguish between healthy and apoptotic cells by detecting the changes in the mitochondrial membrane potential. In healthy cells, MitoCapture™ reagent accumulates and aggregates in the mitochondria, emitting a bright red fluorescence. In apoptotic cells, this reagent cannot aggregate in the mitochondria due to the altered mitochondrial membrane potential, and thus it remains in the cytoplasm in its monomeric form and emits a green fluorescence. Fluorescence changes and images were acquired and analyzed with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany). The filters used were BP 505–530 and HFT 488 for green, and LP 560 and HFT488/543 for red. Laser excitations were 488 nm 5% and 543 nm 80%. Quantification of 20 cells per condition was measured using the software LSM 510 META. The results were represented in the graphs as a fluorescence ratio between red/green channels, respectively.

2.7. Immunodetection assays

The immunological reactivity of hippocampal cell extracts treated with cytokines and the untreated controls was assayed by Western blots techniques, as previously described by [26] using antibodies that recognize Alzheimer phosphoepitopes on tau (AT8, PHF-1), cdk5 protein, p35 protein, and with the antibodies Tau1 and Tau5. Quantification of blots was carried out by scanning the photographic films of nitrocellulose membranes, by using the Kodak digital Science densitometry program from Kodak. Molecular size markers were from Bio-Rad Laboratories.

2.8. Immunoprecipitation and cdk5 activity assays

Primary hippocampal cultured neurons were plated at 1×10^6 cells/cm^2 on polylysine-coated 60-mm dishes. After treatments, cells were lysed in RIPA

buffer plus protease inhibitors. 200 μg of total protein was used for immunoprecipitation with an anti-cdk5 J3 monoclonal antibody used at a final dilution of 1:50. Then, the antigen–antibody complex was captured with either agarose–proteins A or G. Then, *in vitro* kinase assay was carried out as described by [26]. Quantification was autoradiography with a Molecular Imager FX (Bio-Rad).

2.9. RT-PCR assays

Total RNA was extracted from the control, as well as from either, TNFα, $A\beta_{42}$ or Aβ+TNFα-treated hippocampal cell cultures using the TRIZOL (Gibco BRL) reagent. Then RT-PCR was carried out as described [26]. PCR amplification of samples was performed with specific primers against cdk5 and actin. Cdk5 primers were forward: 5′ GCATTGAGTTTGGGCACGACA 3′, reverse: 5′AAAACCGGGAAACCCATGAGA 3′; β-actin, forward: 5′ TCTACAATGAGCTGCGTGTG 3′, reverse:5′TACATGGCTGGGGTGTT-GAA 3′.

2.10. Statistical analyses

One-tailed Student's *t* test for regression analyses was performed with the SigmaPlot software (Jandel Scientific, Corte Madera, CA). Data are presented as means±SEM (*n*). *n*=number of experiments. Statistical significance was considered with *p* values equal or lower than 0.05 ($p \leq 0.05$).

3. Results

3.1. TNFα does not affect the cell viability of rat hippocampal neurons and protects them from the neuronal cell death induced by $A\beta_{42}$

Our first goal was to define the effect of relatively low concentrations of TNFα on the viability of cell cultures. Thus,

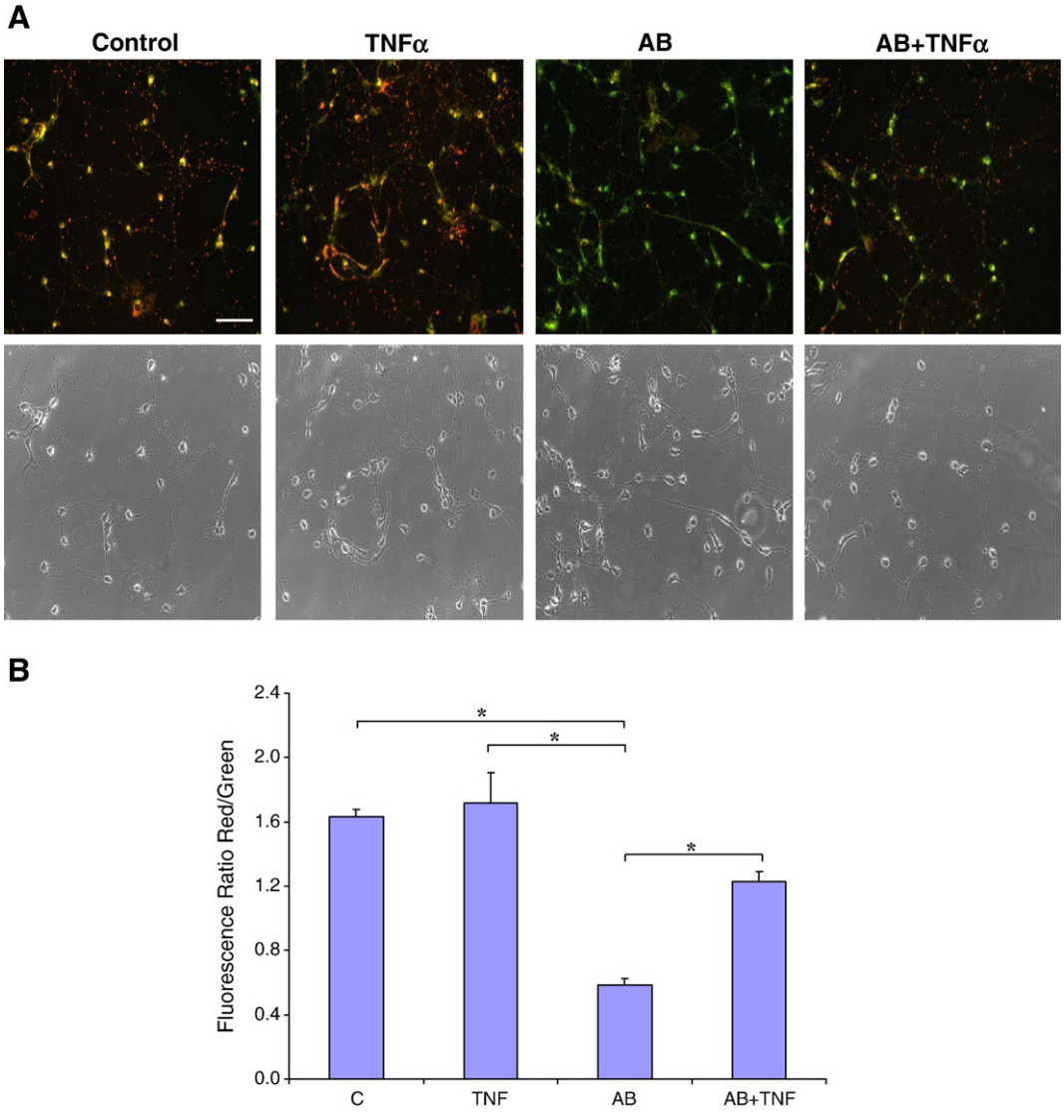

Fig. 1. TNFα protects from the mitochondrial viability loss induced by Aβ peptide in hippocampal neurons. (A) Cultures of hippocampal neurons were treated with TNFα (5 ng/ml), $A\beta_{42}$ (10 μM) or $A\beta_{42}$ plus TNFα pretreatment. Fluorescence changes were measured by using MitoCapture kit (see Materials and methods). Neither fluorescence variations nor morphological change was observed between control cells and cells treated with TNFα. (B) Quantification of the fluorescence changes measured with MitoCapture™ kit (20 neuronal cells, $n=4$). No significant differences in the mitochondrial viability after treatment with TNFα were evident, between control neurons and treated with TNFα. Nevertheless, a significant reduction in the mitochondrial viability was observed in the cultures treated with $A\beta_{42}$ which was reverted by pretreatment with TNF. The asterisk denotes statistical differences ($p<0.05$).

hippocampal neurons were treated with TNFα (5 ng/ml by 48 h). The direct administration of TNFα did not affect cell viability as determined by MitoCapture apoptosis kit and MTT reduction assays (see Figs. 1 and 2). No fluorescence variations neither morphological change were observed, when control cells were compared with hippocampal neurons treated with TNFα (Fig. 1A). Quantification of fluorescence, expressed as a ratio between red and green fluorescence, demonstrated that TNFα did not induce mitochondrial potential loss [control: 1.633 ±0.044; TNFα: 1.719±0.16; $n=3$] (Fig. 1B). Consistently, MTT reduction assays also indicated that TNFα treatments had no effect on neuronal cell death (Fig. 2A) [control: 99.75 ±0.88%; TNFα: 88.7±1.4%; $n=5$]. Although TNFα alone is apparently toxic as observed in the MTT data in Fig. 2A, these results are agreement with previous observations [12], which showed that a bigger TNFα concentration (100 ng/ml) induced a similar neuronal cell death.

On the basis of these results, it was important to determine the protective role of TNFα in hippocampal neurons that were exposed to $A\beta_{42}$ peptide. Thus, we carried out similar MTT and mitochondrial viability studies. In this study, hippocampal neurons were pretreated with TNFα (5 ng/ml by 48 h) and then treated with $A\beta_{42}$ (10 μM) for additional 24 h. The effect on cell viability by $A\beta_{42}$ alone was also measured. A severe decrease in the red fluorescence and an increase in the green fluorescence can be observed in the neurons treated with $A\beta_{42}$, indicating that $A\beta_{42}$ is able to disrupt the mitochondrial potential in hippocampal neurons (Fig. 1A). However, pretreatments with TNFα were able to reverse the decrease in the red fluorescence induced by $A\beta_{42}$ and remains mitochondrial potential unaltered (Fig. 1A and B). Quantification of fluorescence showed that $A\beta_{42}$ induced a decrease of 2.6 times in the fluorescence ratio regarding control cells, and the TNFα treatment was able to reverse cell death induced by $A\beta_{42}$ treatment (Fig. 1B) [Aβ: 0.588±0.03; Aβ+TNFα: 1.229±0.06; $n=3$, $p<0.001$]. Consistently, MTT assays show that $A\beta_{42}$ treatments reduce cell viability (Fig. 2A) [control: 99.75±0.88; Aβ: 62.7±3.3%; $n=5$], while pretreatment with TNFα reverted the neurodegenerative effect of Aβ on hippocampal neurons (Fig. 2A) [Aβ: 62.7±3.3%; Aβ+TNFα: 95.8±1.4%; $n=5$]. As a control, hippocampal neurons treated with 10 μM roscovitine (a cdk5 inhibitor) did not show neuronal cell death. Then, the cdk5 inhibition by roscovitine, applied in the presence of Aβ, showed to protect from cell death induced by the amyloid peptide (Fig. 2D, Aβ+R). Interestingly, roscovitine protects from Aβ-induced neuronal death, in the same fashion that TNFα treatment does (Fig. 2A). Moreover, when hippocampal neurons were preincubated under both conditions (TNFα and roscovitine), the total protection reach out equivalent percentage (Fig. 2A). These observations indicated that TNFα and roscovitine could induce protection against neuronal death by using a common pathway.

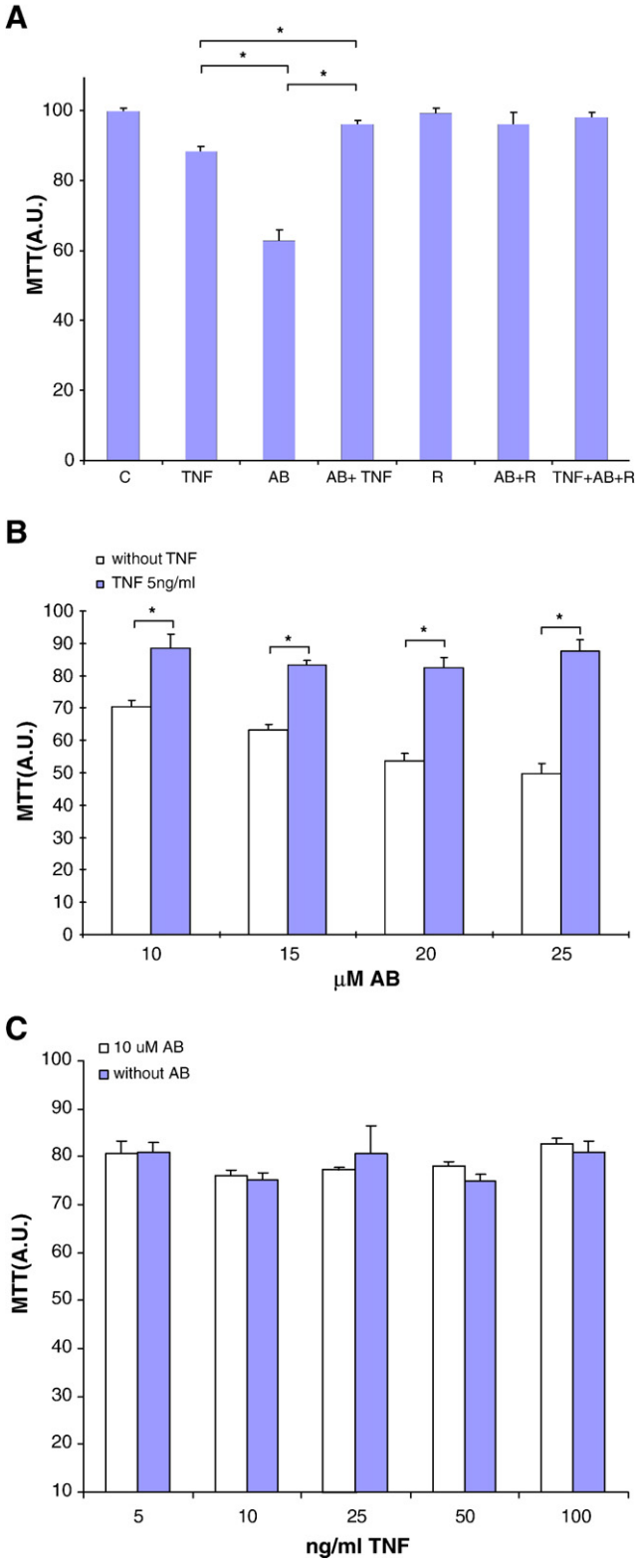

Fig. 2. TNFα effects on cells viability of rat hippocampal neurons. Cell viability was evaluated using the MTT reduction assay. (A) MTT assay ($n=5$) shows that TNFα does not affect the hippocampal neurons viability and the neuronal cell death induced by Aβ was prevented in cells pretreated with this cytokine. Roscovitine (10 μM), a cdk5 inhibitor, did not show neuronal cell death, and protects from the cell death induced by $A\beta_{42}$, as well as TNFα. The asterisk denotes statistical differences ($p<0.05$). (B) Constant concentration of TNFα (5 ng/ml) still showed a protective effect against increasing $A\beta_{42}$ concentrations. (C) Increasing concentrations of TNFα did not induce an increase in the hippocampal viability and still protects against $A\beta_{42}$ neurotoxicity.

In order to determinate whether TNFα still has a protective effect against increasing concentrations of $A\beta_{42}$, we varied the peptide concentration from 10 to 20 μM maintaining stable the TNFα concentration (5 ng/ml). Under these conditions, TNFα still showed a protective effect against the highest $A\beta_{42}$ concentrations (Fig. 2B). In addition, we varied the concentration of TNFα from 5 to 100 ng/ml but maintaining stable the $A\beta_{42}$ concentration (10 μM). Increasing concentrations of TNFα were not able to induce an increase in the hippocampal viability, and these bigger concentrations still showed a protective effect against $A\beta_{42}$ (Fig. 2C). These results suggest that TNFα prevents neuronal cell death as induced by Aβ in rat hippocampal neurons. The following experiments were carried out to explore the molecular mechanisms involved in these cellular events.

3.2. TNFα induces a decrease in the activity of cdk5 in rat hippocampal neurons

Although, it is known that pretreatment of hippocampal neurons with TNFα prevents neuronal cell death in Aβ-treated cells [12], the role of cdk5/p35 complex in the neuroprotection induced by TNFα is a novel event. With the aim of exploring this issue, we studied the levels of cdk5 protein in hippocampal neurons that were treated with TNFα alone or pretreated with TNFα and then exposed to $A\beta_{42}$ peptide. A significant reduction of the cdk5 protein levels was observed in hippocampal neurons after TNFα pretreatments (Fig. 3A) [control: 106.72±6.63; TNFα: 69.13±12.22; $p<0.05$]. Those hippocampal neurons pretreated with TNFα, in the presence of $A\beta_{42}$ also showed a significant reduction in the cdk5 proteins levels (Fig. 3A) [Aβ: 115.48±17.54; Aβ+TNFα: 73.77±16.09; $p<0.05$]. Additionally, the $A\beta_{42}$ treatment did not induce any particular changes in the cdk5 protein levels regarding the control (Fig. 3A) [control: 106.72±6.63; Aβ: 115.48±17.54; $p=0.236$]. Due to the decrease in the cdk5 protein levels, we decide to study whether there are changes in the cdk5 mRNA levels in response to TNFα treatment. We did not observe significant changes in the cdk5 mRNA levels (Fig. 3B). This result suggests that TNFα treatment decrease the cdk5 protein levels, and that this deregulation could involve some post-translational modifications.

Since, we found a reduction in the cdk5 protein levels, the next step was to study the cdk5 activity. Interestingly, TNFα pretreatment significantly reduced the pathological increase of cdk5 activity by the effects of $A\beta_{42}$ (Fig. 4A) [Aβ: 150.63±10.18; Aβ+TNFα: 112.55±9.73; $p<0.01$]. $A\beta_{42}$ induced an increase in the cdk5 activity with respect to control cells [control: 121.07±6.63; Aβ: 150.63±10.18; $p=0.02$], in agreement with our previous observations [25]. Significant reduction of the cdk5 activity was also observed in TNFα-treated neurons without Aβ [control: 106.72±6.63; TNFα: 97.93±2.88; $p=0.02$]. Roscovitine was used as a control to inhibit the cdk5 activity increased by $A\beta_{42}$ treatment, and prevents Aβ-induced cdk5 activity, as well as TNFα treatment (data not shown).

Consistent with the fact that the cdk5 activity depends of its specific activators p35 and p39 [32,33], we have observed that

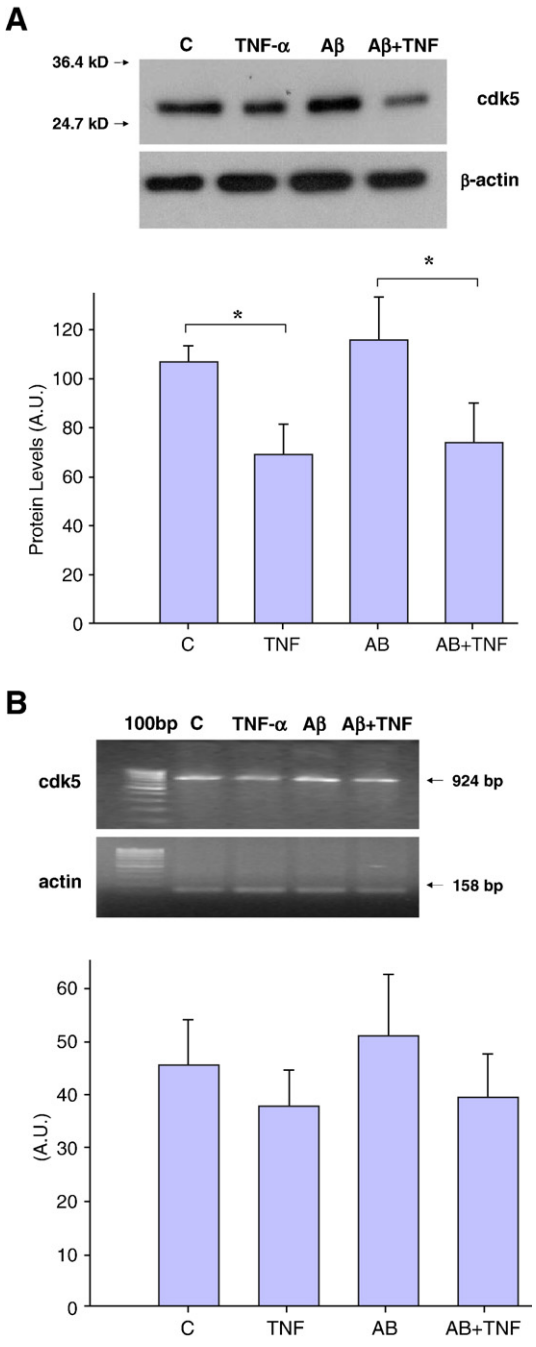

Fig. 3. TNFα induces a decrease in the cdk5 protein levels in rat hippocampal neurons. (A) Western blot assay ($n=5$) of hippocampal neurons untreated and treated with TNFα (5 ng/ml by 48 h), showing that TNFα induces a decrease in the cdk5 protein levels, analyzed with a mAb against cdk5 (J3) ($n=5$). Protein levels were normalized with the endogenous actin levels. (B) RT-PCR of hippocampal neurons shows that TNFα did not induce significant changes in the DNA fragment of 924 bp corresponding to cdk5 as compared with untreated control. RT-PCR amplification was normalized with endogenous β-actin transcript and not significant differences was observed for mRNA ($n=3$).

IL-6 induces deregulation of the cdk5 activity increased the p35 levels [26]. Thus, we investigated the effect of TNFα in the p35 protein levels. In these studies, although p35 was present, TNFα did not induce significant changes on the p35 levels between control and TNFα-treated neurons (Fig. 4B) [control: 89.58±

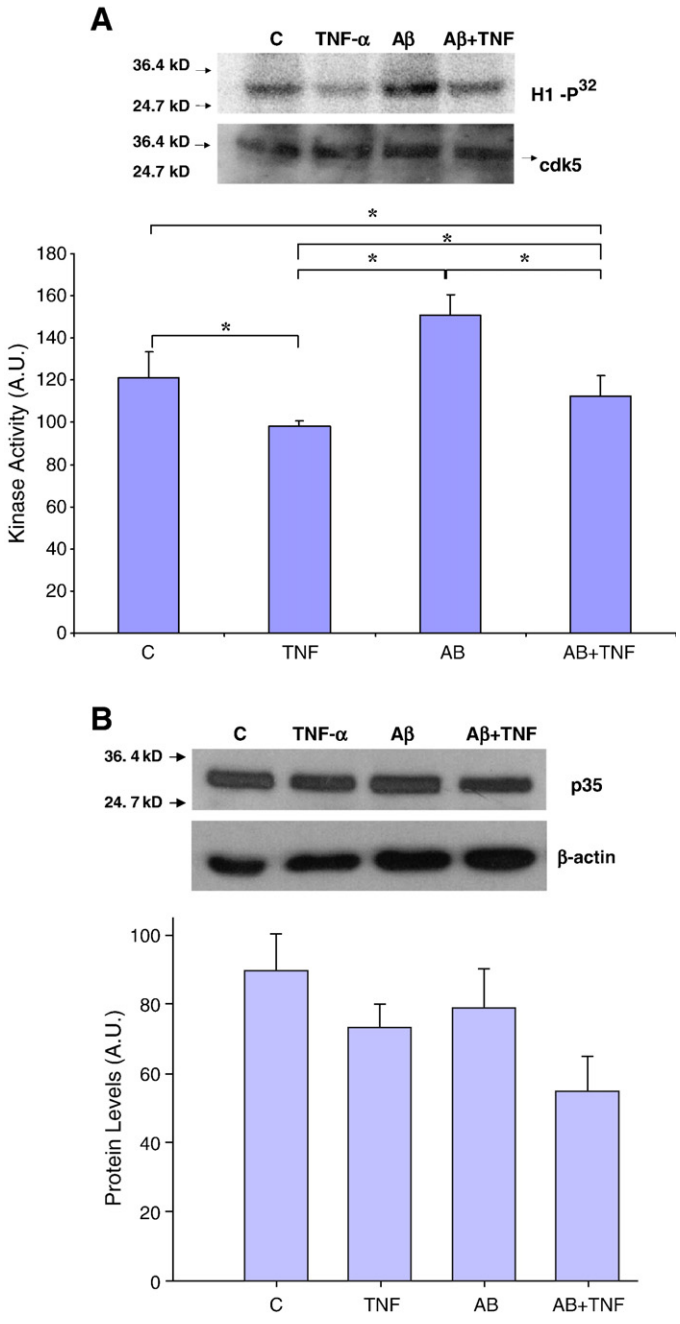

Fig. 4. TNFα induces a decrease in cdk5 activity in rat hippocampal neurons. (A) In vitro kinase assay shows that TNFα induces a significant reduction in the cdk5 activity. Pretreatments with TNFα were able to reduce the increase in the cdk5 activity induced by $A\beta_{42}$ peptide. In the lower panel we show a densitometric analysis from 4 different experiments. (B) Western blot analysis of hippocampal neurons shows no differences in the p35 levels, analyzed with a polyclonal antibody against p35 (C20) ($n=4$) between control and the different treatments. The p35 protein levels were normalized with the endogenous actin levels.

10.68; TNFα: 73.25±6.65; $p=0.09$] in neurons exposed to Aβ, and Aβ+TNFα (Fig. 4B) [Aβ: 78.69±11.47; Aβ+TNFα: 54.93±9.84; $p=0.06$]. These observations discard a direct role of p35 in the regulation of cdk5 activity by TNFα.

According to Kwon et al., one of the functions of cdk5 is to regulate the adhesion process in cortical neurons [31]. In order to evaluate whether TNFα modulating cdk5 activity was able to affect this process, neuronal aggregation assays were carried out. This assay showed that viable aggregates were formed in the presence of TNFα. Besides, it was observed that 1 mM $CaCl_2$ used as positive control favored aggregates, as opposed by negative effects in the presence of EGTA (Fig. 5A). Quantification of two-dimensional surface area of individual aggregates revealed that TNFα led to an increased aggregation of rat cortical neurons (Fig. 5B) [control: 61.53±9.16; TNFα: 544.57±66.28; $p<0.01$]. However, the effect in the aggregation phenomenon did not observed in presence of TNFα plus EGTA. This suggests that TNFα itself does not induce a calcium-stimulated neuronal aggregation suggesting another mechanism of action. These results are in agreement with Kwon et al. [31], indicating an indirect role of TNFα facilitating the neuronal aggregation, perhaps modifying the cdk5 activity.

3.3. TNFα decrease the $A\beta_{42}$ induced Alzheimer type phosphorylation of tau in rat hippocampal neurons

In order to evaluate whether the reduction of cdk5 activity induced by TNFα had and effect on the tau phosphorylation patterns, we used antibodies that recognize hyperphosphorylated tau epitopes (Fig. 6). A significant reduction on the hyperphosphorylated levels of tau recognized by AT8 antibody was observed in the hippocampal neurons preincubated with TNFα. Interestingly, pretreatments with TNFα also reduced the AT8-tagged hyperphosphorylated tau that it was increased by Aβ (Fig. 6) [control: 38.19±4.4; TNFα: 29.32±1.9; $p\leq0.05$; and Aβ: 59.2±9.8; Aβ+TNFα: 23.64±10; $p<0.05$]. Consistently, by using PHF1 antibody, TNFα also reduces the PHF1-tagged hyperphosphorylated tau (Fig. 6) [control: 67.2±10.95; TNFα: 43.62±7.3; $p\leq0.05$; and Aβ: 78.52±8.82; Aβ+TNFα: 52.97±5.94; $p<0.05$]. On the other hand, the levels of unphosphorylated tau, detected with the Tau1 antibody, were increased in TNFα treatments (Fig. 6) [control: 44.73±1.49; TNFα: 52.49±2.65; $p<0.05$; and Aβ: 43.95±1.43; Aβ+TNFα: 50.24±2.68; $p<0.05$]. As a control, no differences were observed in the Tau5 levels (Fig. 6). These results indicate that TNFα prevents Aβ-induced tau hyperphosphorylation, an event that could be explained for an apparent decrease in cdk5 activity induced by TNFα. Additionally, TNFα regulating cdk5 activity can prevent Aβ-induced neurotoxicity in primary hippocampal neurons, showing a novel neuroprotective role of this cytokine in Alzheimer disease.

4. Discussion

In this study, we demonstrate that TNFα regulates the cdk5 activity, modulating the cdk5 protein levels. In addition, TNFα pretreatment induced a significant reduction in the cdk5 activity increased by $A\beta_{42}$. Survival of hippocampal neurons treated with TNFα was not compromised, thus allowing us to carry out most of our studies under conditions that assure high cell viability. Despite that MTT assay has shown a decrease in the cell viability of 10% upon TNFα treatments, the percentage of cell death is acceptable and allowed us to carry out all our experiments under low conditions of cell damage. This result is

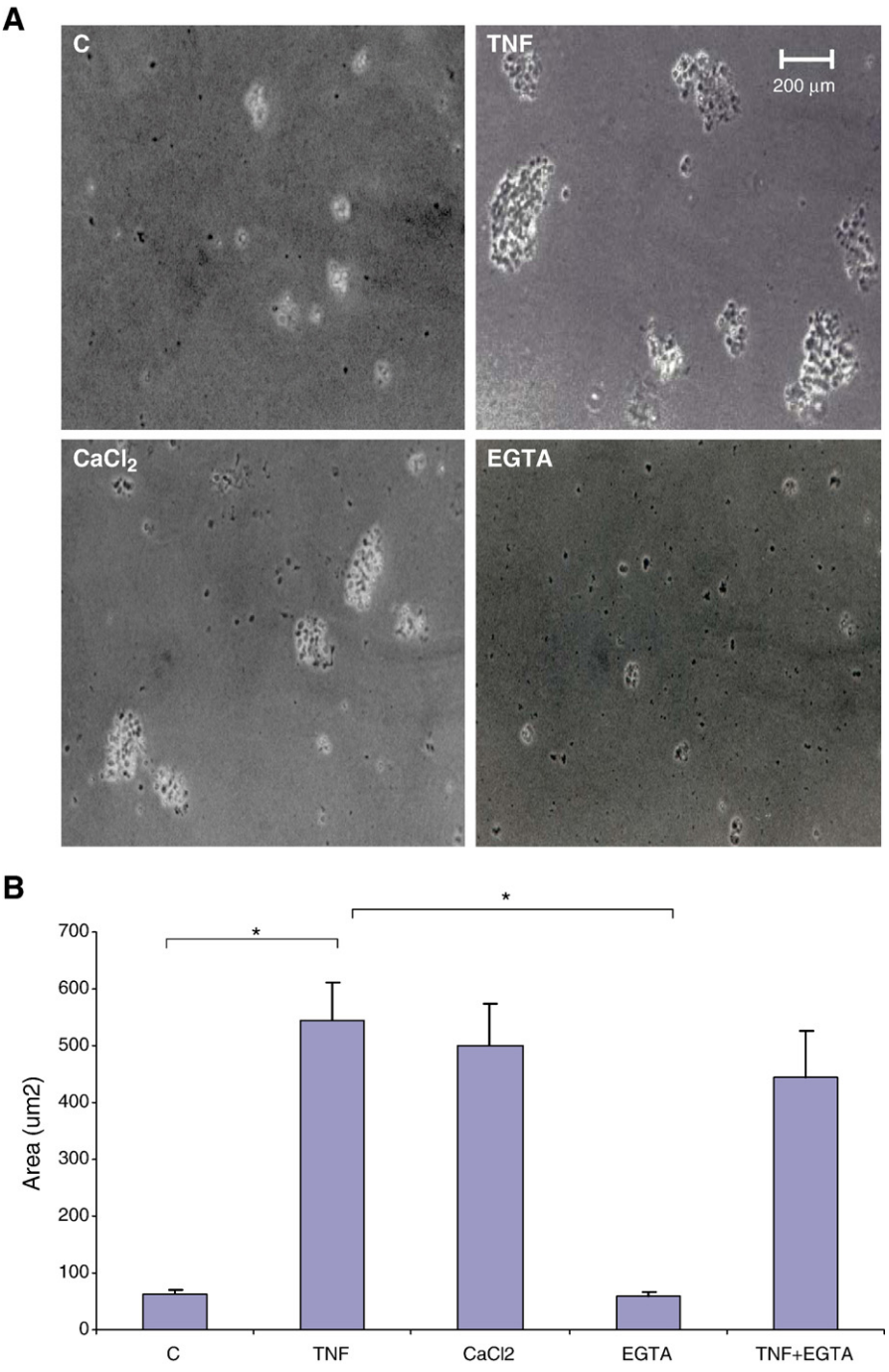

Fig. 5. TNF induces aggregation of rat embryonic cortical neurons. (A) Dissociated embryonic cortical neurons were aggregated in the presence of 1 mM EGTA, 1 mM $CaCl_2$, TNFα (5 ng/ml) or 1 mM $CaCl_2$ with TNFα. Aggregates formed in the presence of 1 mM $CaCl_2$ but not in 1 mM EGTA. In the presence of 1 mM $CaCl_2$ and TNFα, viable aggregates formed were larger. (B) Quantification of two-dimensional surface area of 10 aggregates ($n=3$) is shown. The asterisk denotes statistical differences ($p<0.05$).

in agreement with previous data where TNFα protected hippocampal neurons from $A\beta_{40}$ toxicity [12]. Thus, TNFα showed a potent neuronal death protection in Aβ-treated neurons. Nevertheless, in our studies we used soluble $A\beta_{42}$ which is much more susceptible to aggregation than $A\beta_{40}$, and is much more toxic than the other forms of Aβ peptide [30,34].

It has been shown that Aβ induces an increase in the cdk5 activity, and it induces phosphorylations of tau protein at Alzheimer epitopes [21,35]. The use of inhibitors against cdk5 reduces the increase in the cdk5 activity and the tau hyperphosphorylation and apoptosis in neurons exposed to Aβ peptide [21,36]. In regard with possible mechanisms, our results suggest that TNFα could be acting by inhibiting cdk5, because it significantly reduces the rise in cdk5 activity promoted by Aβ. In addition, TNFα decreases tau hyperphosphorylation and neuronal death.

It is known that inhibition of cdk5 kinase activity by using the specific inhibitor roscovitine leads to the formation of larger

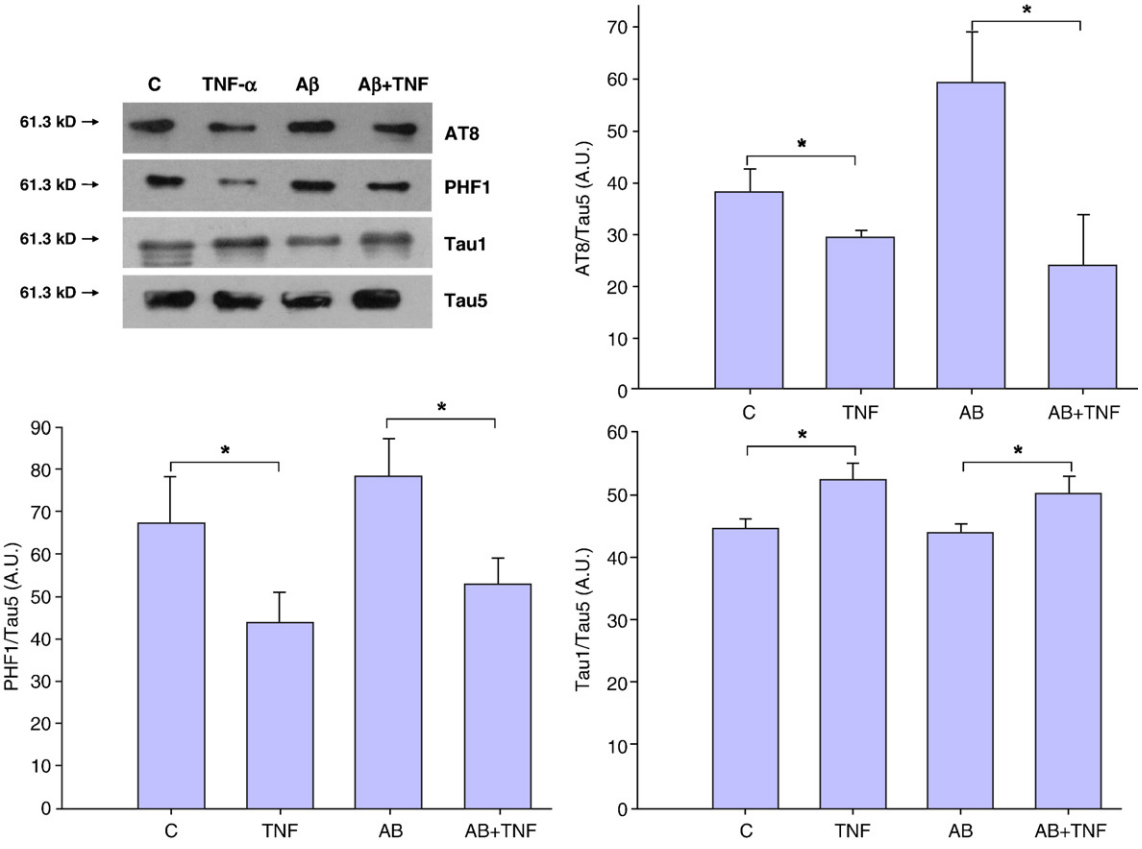

Fig. 6. TNFα prevents tau hyperphosphorylation induced by Aβ in hippocampal neurons. Western blot assay ($n=3$) of hippocampal neurons untreated and treated with TNFα (5 ng/ml by 48 h) or Aβ42 (10 μM by 24 h) with TNFα, showing that TNFα induces a decrease in the tau hyperphosphorylated levels, analyzed with a mAb against AT8, PHF1. On the other hand, TNFα induces an increase in unphosphorylated Tau1 levels. As control the levels of tau were measured with Tau5. AT8 recognizes hyperphosphorylated tau epitopes in Ser^{202} and Thr^{205}. PHF1 recognizes hyperphosphorylated tau epitopes in Ser^{396} and Thr^{404}. Tau1 recognizes unphosphorylated tau epitopes in Ser^{202} and Thr^{205}. Tau5 recognizes a conformational epitope on tau in a phosphorylation-independent manner. Protein levels were normalized with the Tau5 levels. The asterisk denotes statistical differences ($p<0.05$).

aggregates of embryonic cortical neurons [31]. Here, we reported that TNFα induces an equivalent effect on cortical neurons aggregation. This result reinforces the inhibitory effect of TNFα on the reduction of the cdk5 kinase activity as measured by *in vitro* kinase assay.

Even though these studies confirm previous results supporting TNFα protective role [12], our findings support a novel effect of TNFα in promoting a neuroprotective action against Aβ peptide, and particularly on the soluble set of oligomeric forms of the amyloid peptide. In this paper, we describe that TNFα treatment reduces cdk5 activity affecting protein levels and activity. However, TNFα can also activate other signaling pathways involved in neuroprotection and tau hyperphosphorylation, such as: MAPK, p38, and gsk3β [28,36]. This last enzyme does not show any particular changes in their protein levels and activation in hippocampal neurons exposed to TNFα (data not shown). For this reason, and gathering previous evidence [21,25,28,36], we postulate that TNFα could protect hippocampal neurons by reducing the cdk5 activity and preventing tau hyperphosphorylation and neuronal death.

Even though the activator p35 is present, no significant changes in its protein expression were evidenced. Despite the fact the cdk5 activity is also regulated by its activator p35 [32], and that we were able to detect p35 levels present in our system, TNFα pretreatment did not produce any significant changes in the p35 protein levels, thus discarding its role in the TNFα-mediated regulation of cdk5 activity. Previous data have reported changes in the cdk5 activity in colon cancer cells with a decrease in cdk5 protein expression and kinase activity without significant changes in the p35 protein levels [37]. Additionally, there is evidence that shows that the promoter region of cdk5 displays specific sites for the interaction with the transcriptional factor AP-1 [38]. Moreover, TNFα can induce their effects through AP-1 activation [6], so it is possible that TNFα regulates the cdk5 protein levels and its activity by acting on cdk5 post-translational modifications. In addition, some studies have documented that cdk5 inhibits the neuroprotective effect mediated by MEF2 [27, 28] and phosphorylates STAT3 regulating its transcriptional activity [39]. Consistently, it has been shown that TNFα modulates the expression of MEF2 in the mouse *soleus* muscle [29], and STAT3 in hepatic cells [40]. Therefore, it is also possible that TNFα could also modify MEF2 and STAT3 through a cdk5-dependent mechanism. However, further studies are required to inquire on this hypothesis.

On the other hand, the use of specific antibodies against hyperphosphorylated tau epitopes such as AT8 and PHF-1, and

antibodies that recognized the unphosphorylated tau isoforms such as Tau1 allows us to visualize tau phosphorylating patterns by proline-directed protein kinases (PDPKs), making cdk5 a reasonable candidate for such an effect. Nevertheless, it is known the role of phosphatases such as PP1, PP2A and PP2B in the regulation of tau phosphorylation patterns [41]. It has been demonstrated that TNFα induces the activation of protein phosphatases [42]. Therefore, in our system, TNFα might also be activating phosphatases and inducing tau dephosphorylations, an aspect that remains to be investigated in depth.

There are some studies which have shown TNFα neurotoxicity [43,44]. Apparently, in these cases the toxicity was mediated by activated microglia cells from cortical cultures, which tend to have a larger number of glial cells. The hippocampal cultures used in our study are completely depleted of glia, since these cultures were treated in the presence of Ara-C (10 mM). These suggest that the neuroprotective effect of TNFα is not mediated by glia.

In summary, our data suggest that TNFα induces a neuroprotective effect in hippocampal neurons against $A\beta_{42}$ neurotoxicity. These phenomena suggest that the inflammatory response due to Aβ effects could play a key role in Alzheimer disease etiopathogenesis. Taking together, the present data provide a novel molecular mechanism involved in these neuroprotective events of TNFα in preventing neuronal cell death and tau hyperphosphorylation dependent of cdk5.

Acknowledgement

This research was supported by grants from FONDECYT project 1050198 (to R.B.M.), and by support from the International Center for Biomedicine (ICC). D.O. was a predoctoral fellow of the biotechnology program, from the Millennium Institute CBB.

References

[1] B. Cheng, S. Christakos, M.P. Mattson, Tumor necrosis factors protect neurons against metabolic–excitotoxic insults and promote maintenance of calcium homeostasis, Neuron 12 (1994) 139–153.
[2] R.T. Perry, J.S. Collins, H. Wiener, R. Acton, R.C. Go, The role of TNF and its receptors in Alzheimer's disease, Neurobiol. Aging 22 (2001) 873–883.
[3] L. Vitkovic, J. Bockaert, C. Jacque, "Inflammatory" cytokines: neuromodulators in normal brain? J. Neurochem. 74 (2000) 457–471.
[4] E.C. Beattie, D. Stellwagen, W. Morishita, J.C. Bresnahan, B.K. Ha, M. Von Zastrow, M.S. Beattie, R.C. Malenka, Control of synaptic strength by glial TNFalpha, Science 295 (2002) 2282–2285.
[5] D. Stellwagen, R.C. Malenka, Synaptic scaling mediated by glial TNF-alpha, Nature 440 (2006) 1054–1059.
[6] V. Baud, M. Karin, Signal transduction by tumor necrosis factor and its relatives, Trends Cell Biol. 11 (2001) 372–377.
[7] B. Kaltschmidt, M. Uherek, H. Wellmann, B. Volk, C. Kaltschmidt, Inhibition of NF-kappaB potentiates amyloid beta-mediated neuronal apoptosis, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 9409–9414.
[8] P. Fernyhough, D.R. Smith, J. Schapansky, R. Van Der Ploeg, N.J. Gardiner, C.W. Tweed, A. Kontos, L. Freeman, T.D. Purves-Tyson, GW. Glazner, Activation of nuclear factor-kappaB via endogenous tumor necrosis factor alpha regulates survival of axotomized adult sensory neurons, J. Neurosci. 25 (2005) 1682–1690.
[9] A.J. Bruce, W. Boling, M.S. Kindy, J. Peschon, P.J. Kraemer, M.K. Carpenter, F.W. Holtsberg, M.P. Mattson, Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors, Nat. Med. 2 (1996) 788–794.
[10] P.G. Sullivan, A.J. Bruce-Keller, A.G Rabchevsky, S. Christakos, D.K. Clair, M.P. Mattson, S.W. Scheff, Exacerbation of damage and altered NF-kappaB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury, J. Neurosci. 19 (1999) 6248–6256.
[11] C. Thompson, D. Gary, M. Mattson, A. Mackenzie, G.S. Robertson, Kainic acid-induced naip expression in the hippocampus is blocked in mice lacking TNF receptors, Brain Res. Mol. Brain Res. 123 (2004) 126–131.
[12] S.W. Barger, D. Horster, K. Furukawa, Y. Goodman, J. Krieglstein, M.P. Mattson, Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca^{2+} accumulation, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 9328–9332.
[13] T. Wyss-Coray, L. Mucke, Inflammation in neurodegenerative disease—A double-edged sword, Neuron 35 (2002) 419–432.
[14] B.D. Gitter, L.M. Cox, R.E. Rydel, P.C. May, Amyloid beta peptide potentiates cytokine secretion by interleukin-1 beta-activated human astrocytoma cells, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 10738–10741.
[15] I. Blasko, M. Stampfer-Kountchev, P. Robatscher, R. Veerhuis, P. Eikelenboom, B. Grubeck-Loebenstein, How chronic inflammation can affect the brain and support the development of Alzheimer's disease in old age: the role of microglia and astrocytes, Aging Cell 3 (2004) 169–176.
[16] T. Ohshima, J.M. Ward, C.G. Huh, G. Longenecker, H.C. Pant, R.O. Brady, L.J. Martin, Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11173–11178.
[17] T. Chae, Y.T. Kwon, R. Bronson, P. Dikkes, E. Li, Mice lacking p35, a neuronal specific activator of cdk5, display cortical lamination defects, seizures, and adult lethality, Neuron 18 (1997) 29–42.
[18] L.H. Tsai, I. Delalle, V.S. Caviness, T. Chae, p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5, Nature 371 (1994) 419–423.
[19] G. Alvarez, J.R. Munoz-Montano, J. Satrustegui, J. Avila, E. Bogonez, J. Diaz-Nido, Lithium protects cultured neurons against beta-amyloid-induced neurodegeneration, FEBS Lett. 453 (1999) 260–264.
[20] S. Humbert, R. Dhavan, L.H. Tsai, p39 activates cdk5 in neurons, and is associated with the actin cytoskeleton, J. Cell Sci. 113 (Pt 6) (2000) 975–983.
[21] A. Alvarez, R. Toro, A. Caceres, R.B. Maccioni, Inhibition of tau phosphorylating protein kinase cdk5 prevents beta-amyloid-induced neuronal death, FEBS Lett. 459 (1999) 421–426.
[22] J. Lucas, F. Hernandez, P. Gomez-Ramos, M.A. Moran, R. Hen, J. Avila, Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice, EMBO J. 20 (2001) 27–39.
[23] G.N. Patrick, L. Zukerberg, M. Nikolic, S. de la Monte, P. Dikkes, L.H. Tsai, Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration, Nature 402 (1999) 615–622.
[24] M.S. Lee, Y.T. Kwon, M. Li, J. Peng, R.M. Friedlander, L.H. Tsai, Neurotoxicity induces cleavage of p35 to p25 by calpain, Nature 405 (2000) 360–364.
[25] A. Alvarez, J.P. Munoz, R.B. Maccioni, A Cdk5–p35 stable complex is involved in the beta-amyloid-induced deregulation of Cdk5 activity in hippocampal neurons, Exp. Cell Res. 264 (2001) 266–274.
[26] R.A. Quintanilla, D.I. Orellana, C. Gonzalez-Billault, R.B. Maccioni, Interleukin-6 induces Alzheimer-type phosphorylation of tau protein by deregulating the cdk5/p35 pathway, Exp. Cell Res. 295 (2004) 245–257.
[27] Z. Mao, A. Bonni, F. Xia, M. Nadal-Vicens, M.E. Greenberg, Neuronal activity-dependent cell survival mediated by transcription factor MEF2, Science 286 (1999) 785–790.
[28] X. Gong, X. Tang, M. Wiedmann, X. Wang, J. Peng, D. Zheng, L.A. Blair, J. Marshall, Z. Mao, Cdk5-mediated inhibition of the protective effects of transcription factor MEF2 in neurotoxicity-induced apoptosis, Neuron 38 (2003) 33–46.

[29] S.E. Chen, E. Gerken, Y. Zhang, M. Zhan, R.K. Mohan, A.S. Li, M.B. Reid, Y.P. Li, Role of TNF-{alpha} signaling in regeneration of cardiotoxin-injured muscle, Am. J. Physiol.: Cell Physiol. 289 (2005) C1179–C1187.
[30] S. Gandy, The role of cerebral amyloid beta accumulation in common forms of Alzheimer disease, J. Clin. Invest. 115 (2005) 1121–1129.
[31] Y.T. Kwon, A. Gupta, Y. Zhou, M. Nikolic, L.H. Tsai, Regulation of N-cadherin-mediated adhesion by the p35-Cdk5 kinase, Curr. Biol. 10 (2000) 363–372.
[32] L.H. Tsai, I. Delalle, V.S. Caviness Jr., T. Chae, E. Harlow, p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5, Nature 371 (1994) 419–423.
[33] S. Humbert, R. Dhavan, L. Tsai, p39 activates cdk5 in neurons, and is associated with the actin cytoskeleton, J. Cell Sci. 113 (Pt 6) (2000) 975–983.
[34] E.M. Snyder, Y. Nong, C.G. Almeida, S. Paul, T. Moran, E.Y. Choi, A.C. Nairn, M.W. Salter, P.J. Lombroso, G.K. Gouras, P. Greengard, Regulation of NMDA receptor trafficking by amyloid-beta, Nat. Neurosci. 8 (2005) 1051–1058.
[35] J. Busciglio, A. Lorenzo, J. Yeh, B.A. Yankner, Beta-amyloid fibrils induce tau phosphorylation and loss of microtubule binding, Neuron 14 (1995) 879–888.
[36] Y.L. Zheng, S. Kesavapany, M. Gravell, R.S. Hamilton, M. Schubert, N. Amin, W. Albers, P. Grant, H.C. Pant, A Cdk5 inhibitory peptide reduces tau hyperphosphorylation and apoptosis in neurons, EMBO J. 24 (2005) 209–220.
[37] E. Kim, F. Chen, C.C. Wang, L.E. Harrison, CDK5 is a novel regulatory protein in PPARgamma ligand-induced antiproliferation, Int. J. Oncol. 28 (2006) 191–194.
[38] T. Ishizuka, H. Ino, K. Sawa, N. Suzuki, M. Tatibana, Promoter region of the mouse cyclin-dependent kinase 5-encoding gene, Gene 166 (1995) 267–271.
[39] A.K. Fu, W.Y. Fu, A.K. Ng, W.W. Chien, Y.P. Ng, J.H. Wang, N.Y. Ip, Cyclin-dependent kinase 5 phosphorylates signal transducer and activator of transcription 3 and regulates its transcriptional activity, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6728–6733.
[40] Y. Yamada, I. Kirillova, J.J. Peschon, N. Fausto, Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 1441–1446.
[41] F. Liu, I. Grundke-Iqbal, K. Iqbal, C.X. Gong, Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation, Eur. J. Neurosci. 22 (2005) 1942–1950.
[42] G. Schett, C.W. Steiner, Q. Xu, J.S. Smolen, G. Steiner, TNFalpha mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response, Cell Death Differ. 10 (2003) 1126–1136.
[43] C.C. Chao, T.W. Molitor, S. Hu, Neuroprotective role of IL-4 against activated microglia, J. Immunol. 151 (1993) 1473–1481.
[44] H. Takeuchi, S. Jin, J. Wang, G. Zhang, J. Kawanokuchi, R. Kuno, Y. Sonobe, T. Mizuno, A. Suzumura, Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner, J. Biol. Chem. 281 (2006) 21362–21368.