Tissue plasminogen activator mediates amyloid-induced neurotoxicity via Erk1/2 activation

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Tissue plasminogen activator (tPA) is the main activator of plasminogen into plasmin in the brain where it may have beneficial roles but also neurotoxic effects that could be plasmin dependent or not. Little is known about the substrates and pathways that mediate plasmin-independent tPA neurotoxicity. Here we show in primary hippocampal neurons that tPA promotes a catalytic-independent activation of the extracellular regulated kinase (Erk)1/2 signal transduction pathway through the N-methyl-D-aspartate receptor, G-proteins and protein kinase C. This results in GSK3 activation in a process that requires de novo synthesis of proteins, and leads to tau aberrant phosphorylation, microtubule destabilization and apoptosis. Similar effects are produced by amyloid aggregates in a pathological treatments and in wt and tPA−/− mice neurons. Consistently, in Alzheimer’s disease (AD) patients’ brains, high levels of tPA colocalize with amyloid-rich areas, activated Erk1/2 and phosphorylated tau. This is the first demonstration of an intracellular pathway by which tPA triggers kinase activation, tau phosphorylation and neurotoxicity, suggesting a key role for this molecule in AD pathology.

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Introduction

Tissue plasminogen activator (tPA) is a serine protease well known for its role in the blood coagulation system degrading fibrin clots (Collen, 1999). This function involves the activation of plasminogen into the protease plasmin. In addition to their established fibrinolytic role in blood vessels, tPA and plasminogen/plasmin have also been implicated in both normal and pathological situations in the central nervous system (Strickland, 2001). Thus, plasminogen activation by tPA has a protective role in certain neurological situations such as stroke, where plasmin accelerates the clearance of fibrin deposits. In fact, tPA is the only drug approved to treat ischaemic stroke. Another pathological situation where the tPA–plasmin system has been suggested to play a role is in Alzheimer’s disease (AD) (Selkoe, 2001). In vitro experiments have shown that amyloid-β (Aβ) peptide, the main component of senile plaques, can stimulate tPA activity and mRNA expression (Kingston et al., 1995; Wnendt et al., 1997; Tucker et al., 2000a), resulting in plasmin generation and subsequent Aβ degradation (Ledesma et al., 2000; Tucker et al., 2000a, b). Recently, the contribution of the tPA–plasmin system in the clearance of Aβ has also been shown in vivo (Melchor et al., 2003). These data suggest that tPA, through plasmin generation, could have a beneficial role for AD treatment.

However, tPA could also exert detrimental effects. In stroke models without clot formation or during a permanent occlusion, tPA induces neuronal death (Wang et al., 1998; Nagai et al., 1999a) and its inhibition protects neurons against damage (Yepes et al., 2000). Neurotoxic effects of tPA have also been described during excitotoxin-induced neuronal degeneration and seizure (Tsirka et al., 1997; Pawlak and Strickland, 2002; Yepes et al., 2002). These effects raise concerns about the therapeutical benefits of tPA.

Understanding the molecular mechanisms leading to tPA neurotoxicity is necessary to evaluate the potential of this protease for the treatment of neurological diseases. In some situations, the neurotoxic effects of tPA can be explained by excessive plasmin generation and subsequent laminin degradation which causes neuronal death (Chen and Strickland, 1997; Nagai et al., 1999b). On the other hand, the use of tPA−/− and plasminogen−/− mice has led to accumulating evidence for tPA-induced neurotoxicity independent of plasminogen (Nagai et al., 1999a; Nicole et al., 2001; Yepes et al., 2002). The identification of the substrates for tPA that mediate its function in the absence of plasminogen is under intense scrutiny. Moreover, the intracellular signalling pathways triggered by tPA and how they correlate with tPA plasmin-independent neurotoxic effects are unknown. In the present work, using rodent hippocampal neurons, we demonstrate that tPA, independent of plasmin generation, induces the activation of extracellular regulated kinase (Erk)1/2 signal transduction pathway that involves the N-methyl-D-aspartate (NMDA) receptor, monomeric G-proteins and protein kinase C (PKC). The activation of this signalling pathway results in GSK3 activation, tau hyperphosphorylation, microtubule destabilization and neuronal apoptosis. We also analysed this molecular mechanism in the context of AD pathogenesis. Our results show that tPA mediates amyloid toxicity in vitro and is overexpressed in amyloid-rich areas and senile plaques of AD human brains colocalizing with activated Erk1/2 and phosphorylated tau (phospho-tau).
Results

tPA induces Erk1/2 activation in neurons, independently of plasmin

Among the mitogen-activated kinase (MAPK) family, a role for the Erk1/2 signalling pathway in neuronal death has been proposed in seizure and ischaemia (Murray et al., 1998; Namura et al., 2001). Since an involvement for tPA has been suggested in these situations, we investigated whether tPA neurotoxic effects could be mediated by this pathway. We first analysed Erk1/2 activation after tPA treatment in a neuroblastoma cell line (N2a) and also in rat hippocampal neurons in primary cultures, a cellular system that closely resembles the in vivo situation. Figure 1Aa shows that exposure of N2a cells to tPA led to a sustained Erk1/2 activation (detected by phosphorylated Erk1/2 (phospho-Erk1/2) Western blot analysis) that reached its maximal level at 30 min and started to decrease after 12 h. To confirm the correlation between phosphorylation of the kinases and increased catalytic activity, we assayed Erk1/2 activity in immunoprecipitates from N2a differentiated cells treated with tPA. Recombinant transcription factor ELK-1 was used as substrate, and the phosphorylation was monitored by Western blot. Figure 1Ab shows that tPA treatment increased Erk1/2 activity in agreement with the phosphorylation data shown in Figure 1Aa. Similar activation of Erk1/2 pathway was detected in tPA-treated rat hippocampal neurons: an increase in Erk1/2 phosphorylation was observed at 30 min, sustained up to 12 h and decreased to basal levels after 24 h of treatment (Figure 1Ba). Erk1/2 activation by tPA was dose-dependent. Thus, while it was already detectable when hippocampal neurons were incubated with low concentrations of tPA (2–5 μg/ml), it reached maximal levels at 10–20 μg/ml (see Supplementary data, Figure S1). Erk1/2 activation was reduced to control levels when tPA treatment was performed in the presence of U0126, an inhibitor of MAPK/Erk kinase (MEK)1/2, the kinase upstream in Erk1/2 signalling (Figure 1Bb, upper panel), indicating the specificity of the pathway. The effects observed cannot be attributed to overall upregulation of Erk1/2 because no significant variations were found in the total levels of the protein (Figure 1A and B, bottom panels).

To determine whether Erk1/2 activation by tPA is plasmin-dependent, we exposed hippocampal neurons to a catalytically inactive mutant of tPA (tPAS478A). This resulted in Erk1/2 activation similarly to the wild-type protein (Figure 1C). Furthermore, treatment with plasmin alone did not modify the levels of phospho-Erk1/2 (Figure 1C). These results indicate that plasmin formation is not required for tPA-induced Erk1/2 activation. On the other hand, Erk1/2 activation upon tPA addition was abolished in the presence of Pefabloc/tPA (Figure 1C), a synthetic inhibitor that shows...
much higher affinity for tPA ($K_i = 0.035 \mu M$) than for urokinase plasminogen activator (uPA) ($K_i = 3.4 \mu M$) (Renatus et al., 1998). It is important to note that the effect of this inhibitor on proteases such as factor Xa, trypsin or thrombin is quite unlikely in our experimental system given the low activity of these molecules in CNS neurons cultured in serum-free medium (Sinnreich et al., 2004). Although Pefabloc/tPA has been described to bind the catalytic domain of tPA inhibiting its enzymatic activity (Renatus et al., 1997), our results suggest that this interaction also affects tPA signalling (see Discussion). This result confirms the direct relationship between tPA and Erk1/2 activation.

Given all the above, this first series of results imply that extracellular tPA is an efficient plasmin-independent activator of the MEK–Erk1/2 pathway in neurons.

Activation of Erk1/2 pathway by tPA is mediated by PKC/G-protein and involves NMDA receptor

The fact that tPA is an extracellular protease implies that the activation of signalling pathways requires its interaction with cell surface proteins which would be responsible for the signal transduction into the cell. Several receptors have been shown to bind tPA on the surface of neuronal cells. In particular, it has been shown that tPA can bind to annexin A2 (Hajjar et al., 1998; Jacovina et al., 2001) and NMDA receptor (Nicole et al., 2001; Liu et al., 2004). Hence, we analysed whether or not these molecules are involved in the tPA-induced Erk1/2 activation in hippocampal neurons. Treatment with tPA in the presence of homocysteine, an amino acid that inhibits the binding of tPA to annexin A2 (Hajjar et al., 1998), had no effect on Erk1/2 activation (Figure 2A). In contrast, incubation with MK-801, a specific inhibitor of NMDA receptor, abolished Erk1/2 phosphorylation induced by tPA (Figure 2A). These results indicate that NMDA receptor can mediate the activation of Erk1/2 induced by tPA at the cell surface.

We next investigated the molecular linkage between tPA–NMDA receptor and MEK-Erk1/2 activation. Since monomeric G-proteins and PKC have been involved in NMDA receptor function (Skeberdis et al., 2001; Benquet et al.,

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**Figure 2** tPA-induced Erk1/2 activation involves NMDA receptor, G-proteins and PKC. Equal amounts of protein from hippocampal neurons were analysed by Western blot using antibodies against activated or total Erk1/2. (A) Cells were treated for 2 h with neuronal medium (Control), with 20 μg/ml tPA (tPA) or with 20 μg/ml tPA in the presence of: 50 μM homocysteine (tPA + HC), 10 μM MK801 (tPA + MK). HC, treatment with homocysteine alone; MK, treatment with MK801 alone. (B) Treatment of hippocampal neurons for 2 h with tPA (20 μg/ml) in the presence of 50 ng/ml PTX (tPA + PTX50) or 100 ng/ml (tPA + PTX100) results in a significant decrease in Erk1/2 activation in comparison to cells treated only with 20 μg/ml tPA (tPA). Control, treatment with medium alone; PTX50, treatment with 50 ng/ml of PTX alone; PTX100, treatment with 100 ng/ml of PTX alone. (C) Western blot analysis of cells treated for 2 h with neuronal medium (Control), with 20 μg/ml tPA (tPA) or with 20 μg/ml tPA in the presence of the following inhibitors: 100 nM staurosporine (tPA + St), 2 μM G06976 (tPA + G0), 1 μM wortmannin (tPA + Wt) or 50 μM LXR94002 (tPA + LY). Graphs on the right panels correspond to the quantification of Erk1/2 activation with respect to control levels normalized to the total amount of Erk1/2 (mean value from three independent experiments). *P<0.005 compared to control conditions.
In vitro (detectable after 24 h) suggests the existence of intermediate activation (significant during 12 h) and tau phosphorylation 3Ab). Yet, the different time at which tPA induces Erk1/2 activation is not PI3K-dependent. Altogether, these results indicate that tPA triggers Erk1/2 activation in hippocampal primary neurons through a mechanism that involves NMDA receptor, PTX-sensitive G-proteins and PKC.

**tPA treatments induce GSK3 activation, tau hyperphosphorylation, microtubule destabilization and apoptosis in hippocampal neurons**

We moved next to analyse the downstream effectors of tPA-induced Erk1/2 activation. In the context of neuronal cell death, a common event described in several neurodegenerative processes is the aberrant phosphorylation of the microtubule-stabilizing protein tau. Among other kinases, Erk1/2 has been shown to phosphorylate tau both in vitro (Drewes et al, 1992; Mandelkow et al, 1993) and in vivo (Ferreira et al, 1997; Rapoport and Ferreira, 2000). Therefore, we reasoned that tau would be a downstream effector of tPA that could lead to neurotoxicity. To test this premise, hippocampal neurons were treated with exogenous tPA and the level of tau phosphorylation was monitored after various lengths of time using antibodies that recognize both unphosphorylated (Tau-1) and phosphorylated (AT180) epitopes. Short incubation times (3–12 h) did not change the phosphorylation state of tau as determined by unaltered Tau-1 staining and lack of recognition by AT180 (Figure 3Aa). By contrast, a progressive loss in Tau-1 signal indicative of the phosphorylation of Tau-1 epitope could be detected at longer times (24–48 h) (Figure 3Ab). The concomitant increase in AT180 recognition, which relies on an epitope different from Tau-1, indicates that tPA-induced tau phosphorylation occurs in several sites of the molecule and rules out the possibility of loss of Tau-1 signal due to protein degradation. This was also confirmed by the unaltered signal obtained with the antibody 7.51 that recognizes tau molecule independently of its phosphorylation state (Figure 3Ab). These effects were significantly reversed when tPA treatments were carried out in the presence of the U0126 inhibitor, demonstrating the requirement of tPA-triggered MEK-Erk1/2 kinase activity for tau phosphorylation (Figure 3Ab). Yet, the different time at which tPA induces Erk1/2 activation (significant during 12 h) and tau phosphorylation (detectable after 24 h) suggests the existence of intermediate molecules. In vitro and in vivo evidence points to a key role for GSK3 in directly promoting tau hyperphosphorylation. We therefore investigated whether tPA addition could alter GSK3 activity. Figure 3Ac shows that tPA treatment of rat hippocampal neurons induced GSK3 activation determined by the decrease in Ser phosphorylation (Ser21 of GSK3α and Ser9 of GSK3β), which correlated with tau phosphorylation in the Tau-1 and AT180 epitopes at 24 or 48 h. The decrease in Ser phosphorylation was observed for α and β GSK3 isoforms, suggesting that both isoforms are involved in tau phosphorylation after tPA treatment. We also analysed the effects of tPA in Tyr phosphorylation of GSK3, another way of GSK3 activation independent of Ser dephosphorylation. As shown in Figure 3Ac, analysis of the phosphorylation in Tyr of GSK3 did not reveal significant changes upon tPA addition, indicating that tPA-induced GSK3 activation relies on Ser dephosphorylation. Addition of SB415286, a potent and selective inhibitor of GSK3 that competes with ATP, inhibited tPA-induced tau phosphorylation. The similar kinetics observed for tau phosphorylation (Figure 3Ab and c) and GSK3 activation together with inhibition by SB415286 strongly suggest that GSK3 is directly responsible for tau hyperphosphorylation at least in the epitopes analysed. To test whether tPA-induced GSK3 activation depends on Erk1/2 or not, we tested the phosphorylation level of GSK3 upon tPA addition in the presence of the MEK-Erk1/2 inhibitor U0126. Both GSK3 activation and tau phosphorylation were abolished in such conditions (Figure 3Ac). These results indicate that both MEK-Erk1/2 pathway and GSK3 are required for the tPA-induced aberrant phosphorylation of tau and that the Erk1/2 signal triggered by tPA is upstream of GSK3 activation. Still, the fact that Erk1/2 activation reaches maximal levels at 0.5–2 h than being sustained until 12 h, and GSK3 activation/tau phosphorylation is only detected after 24 h raises the question of whether the initial activation of Erk1/2 or that sustained for 12 h is responsible for promoting the cascade. Figure 3Ad shows that addition of U0126 inhibitor 2 h after the tPA treatment failed to prevent GSK3 activation/tau phosphorylation, in contrast to the effect of simultaneous addition of tPA and U0126. This result indicates that the early activation of Erk1/2 by tPA (for 2 h or less) is sufficient to promote the neurotoxic cascade. In addition, to gain further insight into the reasons for the time gap between Erk1/2 activation and GSK3 activation/tau phosphorylation, we tested whether de novo protein synthesis is required. Hence, neurons were treated with tPA in the presence of the protein synthesis inhibitor cycloheximide. In these conditions, tPA-Erk1/2-induced GSK3 activation and tau phosphorylation were abolished (Figure 3Ae), pointing to the participation of additional intermediate molecules in the tPA-induced neurotoxic cascade.

To further investigate the neurotoxic effects of tPA-induced tau hyperphosphorylation, we analysed the stability of microtubules, since tau hyperphosphorylation can result in microtubule disassembly (Billingsley and Kincaid, 1997). Hippocampal neurons exposed to tPA presented a reduction in polymerized tubulin after 48 h (51 ±14%) of treatment that was maximal (82 ±2.8%) after 72 h (Figure 3Ba). These effects were due to tPA-induced Erk1/2 activity and not to secondary effects of tPA long-term incubation because the simultaneous addition of tPA and U0126 inhibitor brought the levels of polymerised tubulin to values similar to untreated neurons (Figure 3Ba). An antibody against the membrane...
Figure 3 tPA exposure promotes GSK3 activation, tau aberrant phosphorylation, microtubule destabilization and apoptosis in hippocampal neurons. (A) (a, b) Western blots using the antibodies Tau-1 and AT180 that recognize tau dephospho and phospho epitopes, respectively. Equal amount of protein (10 µg) was loaded in each lane. Primary hippocampal neurons were incubated for the indicated time (h) as follows: with neuronal medium (Control), 20 µg/ml tPA (tPA) or 20 µg/ml tPA plus 10 µM U0126 (tPA + U0). The 7.S1 antibody was used as a control to rule out tau degradation. (c-e) GSK3 activation (detected as decrease in Ser phosphorylation) and Tau phosphorylation (detected by Tau-1 and AT180) reverted both GSK3 activation and tau phosphorylation. However, U0126 added 2 h later than tPA failed to revert GSK3 activation/tau phosphorylation (d, tPA2h + U0). Tau phosphorylation is reverted in the presence of SB415286 (c, tPA + SB), a selective inhibitor of GSK3. U0, U0126 alone; CHX, cycloheximide alone. (B) Effects of tPA in microtubule stability. Western blot of microtubule pellets (a) and immunofluorescence of cells (b-d) using an antibody against tubulin. Neurons were incubated with medium alone (Control), 20 µg/ml tPA for 72 h (tPA) or 20 µg/ml tPA in the presence of 15 µM U0 inhibitor for 72 h (tPA + U0). Transferrin receptor (TfR) was used as loading control. The graph shows quantification of blots corresponding to three independent experiments. Bars = 10 µm. (C) Apoptosis analysis in neurons treated for 72 h with medium alone (a, d), 20 µg/ml tPA (b, e) or 20 µg/ml tPA plus 10 µM U0126 (c, f). (a–c) Labelling with propidium iodide to detect total nuclei; (d–f) fluorescent TUNEL to detect DNA fragmentation. Bars = 10 µm. (g) The graph represents the mean percentage of apoptotic cells with standard deviations from three independent experiments. *P<0.005 in comparison to control (untreated) cells.
protein transferrin receptor was used as a control to demonstrate that the effects were specific for tubulin. Loss of microtubule stability and the rescue with U0126 inhibitor were also evident by immunofluorescence microscopy (Figure 3Bb–d). As microtubule disruption may lead to cell death (Billingsley and Kincaid, 1997), we assessed the effects of tPA long-term treatments in neuronal viability quantifying the levels of apoptosis by TUNEL. Figure 3C shows that chronic tPA treatment resulted in a higher rate of apoptosis. Under control conditions, only 14.4 ± 4.7% (P < 0.005) of the neurons were apoptotic. Contrarily, after 72 h of tPA exposure, the percentage of apoptotic neurons raised to 43.2 ± 10.9% (P < 0.005). As with microtubule stability, addition of U0126 inhibitor and tPA simultaneously rescued the deleterious effect, reducing apoptosis to control-like values 22.5 ± 3.5% (P < 0.005) and ruling out possible deleterious effects of long-term incubations with U0126. We extended our analysis on cell viability upon tPA treatments by assessing Trypan blue uptake. Incorporation of this dye into the cells reflects alterations in membrane permeability that have been related to necrosis (Perry et al., 1997). Although less numerous than the apoptotic cells, the results revealed a significant increase in the number of Trypan blue-positive tPA-treated cells at 48 h (12.2 ± 3.1%, P < 0.005) and 72 h (26.5 ± 7.3%, P < 0.005) with respect to control cells (in average 4.3 ± 0.5%, P < 0.005) (see Supplementary data, Figure S3), indicating that the neurotoxic effects of tPA could lead to both apoptosis and necrosis.

**tPA mediates Erk1/2 activation, tau phosphorylation and apoptosis induced by Aβ**

It has been recently described (Rapoport and Ferreira, 2000) that Aβ aggregates can induce sustained activation of Erk1/2 signalling pathway, that leads to abnormal phosphorylation of tau and neuritic degeneration (see also Figure 4Ab). Given that we observed equivalent consequences upon tPA incubation and that amyloid aggregates enhance tPA expression (Wnendt et al., 1997; Tucker et al., 2000a), we hypothesized that Aβ neurotoxic effects could be mediated by the induction of tPA. To address this question, hippocampal neurons in culture were exposed to amyloid peptide solution (Aβ1–40) (Lorenzo and Yankner, 1994; Ferreira et al., 1997), resulting in increased tPA production, sustained activation of Erk1/2 and aberrant phosphorylation of tau (Figure 4B). It is important to note that the Aβ solution promoted a sustained increase in tPA levels and activity during the time period analysed comparable to those promoted by the addition of exogenous tPA (see Supplementary data, Figure S2). Notably, when the inhibitor Pefabloc/tPA (Renatus et al., 1997, 1998) (see Figure 1Ca), was added concomitantly with Aβ, Erk1/2 activation was prevented and tau aberrant phosphorylation was significantly suppressed (Figure 4B). The contribution of tPA to Aβ-induced neurotoxicity was further investigated in hippocampal neurons derived from tPA−/− mice and wild-type mice. Addition of the amyloid peptide solution to the mice neuronal cultures resulted in tau hyperphosphorylation in the Tau-1 and AT180 epitopes (Figure 4Ca), microtubule disruption (Figure 4Cb) and increased apoptosis (Figure 4Cc) in the wild-type neurons, but not in the tPA−/− neurons. Altogether, these data show that, at least in vitro, one pathway by which amyloid promotes neurotoxicity is via tPA-induced Erk1/2 activation and tau hyperphosphorylation.

**High levels of tPA correlate with amyloid-rich areas, activated Erk1/2 and phospho-tau in the brain of AD patients**

To gain further insight into how relevant the findings reported could be for the human disease, we analysed tPA expression in control and AD human brains by immunohistochemistry. Serial sections from frontal cortex (Figure 5A and B, patient 1) and hippocampi (Figure 5A, patient 2) were fixed and stained for Aβ deposition using Congo red (Figure 5Aa, c and e) and for tPA using a polyclonal antibody to human tPA (Figure 5Ab, d and f). Increased levels of tPA were found in all AD brains analysed (n = 21) (see Supplementary Table S1, Supplementary data). High tPA signal was observed near Congo red-positive areas (Figure 5Ad and f) (in 122 out of 140 senile plaques analysed), but also as small spots not associated to them (Figure 5Ad, arrowheads). Interestingly, tPA staining in the Congo-red negative sites correlated with increased signal with the antibody 6E10 that recognizes an epitope in the APP protein that is included in Aβ sequence (Figure 5Bb and c), suggesting that tPA increased expression is an early response to the aggregation event (see also double immunohistochemistry for tPA and 6E10, Supplementary data, Figure S4a–c). tPA signal was not above background levels in age-matched, control individuals (n = 5) when the same regions were evaluated (Figure 5Ab). The absence of staining in the non-affected individuals cannot be attributed to failure in the detection procedure because tPA signal was observed in blood vessels, most likely corresponding to endothelial cells that produce tPA (Figure 5Ab).

To further test the relevance in vivo of the Aβ-induced tPA-mediated neurotoxic cascade we observed in vitro, double immunostainings for tPA and activated Erk1/2 or tPA and phospho-tau were performed in the hippocampus and cortex of AD patients. High tPA signal correlated with increased phospho-Erk1/2 staining (Figure 5Ca). Furthermore, high levels of tPA were also detected in phospho-tau (using AT8 antibody) positive areas (Figure 5Cb and Supplementary data, Figure S4, d–i). Quantitative analysis of the in situ data revealed that 92% of tPA accumulations colocalize with increased levels of activated Erk1/2 and 64% with aberrantly phospho-Tau. This high correlation supports the existence of a link between high tPA levels and Erk1/2 activation and tau phosphorylation also in vivo.

**Discussion**

The findings reported here constitute the first evidence for a tPA-triggered signalling cascade in neuronal cells that could explain the neurotoxic effects of this molecule (see Introduction). The pathway implicates MEK-Erk1/2 kinases and is plasminogen/plasmin independent. Activation of Erk1/2 by tPA is mediated through the NMDA receptor and involves PTX-sensitive G-protein/s and PKC. We show that tPA-induced Erk1/2 activation results in GSK3 activation in a process that requires de novo protein synthesis. GSK3 activation leads to tau hyperphosphorylation, microtubule destabilization and apoptosis in cultured primary neurons.

Our work explored the possibility that tPA exerts these deleterious effects in AD pathogenesis. This is indeed supported by the results obtained in neurons from wild type and tPA−/− mice and by pharmacological inhibition of the molecule, which indicate that tPA mediates similar
neurotoxic effects produced by Aβ aggregates. That this may be relevant in the disease is suggested by the abundant levels of tPA specifically found in amyloid-rich areas of AD brains that correlate with higher levels of Erk1/2 activation and hyperphosphorylated tau. However, recent data have suggested that the tPA-plasminogen system plays a protective role in AD by clearance of Aβ peptide (Ledesma et al., 2000; Tucker et al., 2000a, b; Melchor et al., 2003). Given that these observations are focused in the catalytic activity of tPA while the effects we described are plasmin-independent, we propose the following scenario to explain the apparent contradictory effects of tPA in AD. Under physiological conditions, aggregation of Aβ upregulates tPA expression (Kingston et al., 1995; Wnendt et al., 1997), resulting in plasmin production.
and subsequent Aβ degradation. During AD, Aβ deposits could trigger a similar mechanism, but tPA induction does not lead to efficient plasmin generation and Aβ clearance. In this regard, a decrease in tPA enzymatic activity has been reported in the serum of AD patients (Aoyagi et al., 1992) and in brains of AD mouse models (Melchor et al., 2003), and reduced plasmin levels have been found in brains from AD patients (Ledesma et al., 2000). Lack of tPA catalytic activity could be explained by different reasons. Melchor et al. (2003) suggested that it is caused by upregulation of PAI-1 levels that efficiently inhibit plasmin generation by tPA. On the other hand, evidence from our laboratory points to alterations in membrane cholesterol-rich microdomains in AD patients that impair plasminogen binding and activation (Ledesma et al., 2003). Alternatively, tPA itself could be unable or less efficient to generate plasmin due to excessive levels or due to genetic mutations although analysis of tPA polymorphisms did not show any significant difference between control and AD patients (Clarimon et al., 2003). In any case, paucity of plasmin could facilitate Aβ accumulation, being a continuous stimulus for tPA upregulation. This local increase of tPA would produce a plasmin-independent Erk1/2 and GSK3β activation, tau hyperphosphorylation and neuronal death.

An important issue in AD research is that of the possible link between the two hallmarks of the disease: amyloid plaques and deposits of hyperphosphorylated tau. In this regard, several groups have reported that Aβ induces the activation of different kinases leading to tau hyperphosphorylation and progressive neuronal degeneration (Takashima et al., 1993; Busciglio et al., 1995; Ferreira et al., 1997). In this work, we present data supporting a role for tPA in the phosphorylation of tau induced by Aβ. Among the kinases with the ability to phosphorylate tau, GSK-3β (Takashima et al., 1996; Ferreira et al., 1997) and Erk1/2 (Ferreira et al., 1997; Rapoport and Ferreira, 2000) are the best characterized to phosphorylate the protein in response to Aβ. Here, we show that tPA-induced tau phosphorylation takes place by the sequential and sustained activation of Erk1/2 first and GSK3 later. The fact that the MEK-Erk1/2 inhibitor U0126 precluded both GSK3 activation and tau phosphorylation indicates that this event is initially triggered by MEK-Erk1/2. On the other hand, the kinetic data and the prevention of tau phosphorylation by a specific inhibitor of GSK3 suggest that this kinase is directly responsible. Further experiments will be necessary to clearly establish the connection between Erk1/2 and GSK3 in tau phosphorylation induced by tPA. A recent report (Takahashi-Yanaga et al., 2004) has described that MEK1 can promote the activation of GSK3β by direct phosphorylation. Additionally, it has been shown that many substrates of GSK3β, including tau, must be primed—that is, prephosphorylated—by other kinases before GSK3β action (Harwood, 2001; Jope and Johnson, 2004). Thus, it is also possible that MEK-Erk1/2 activation would be necessary to generate primed tau that can be then more efficiently phosphorylated by GSK3. Alternatively, protein synthesis of intermediate molecules might be required, given the capacity of activated Erk1/2 to enter the nucleus and trigger gene transcription (Cobb, 1999). Our results showing the dependency on de novo protein synthesis of the tPA-induced signalling cascade strongly support this view.

Our work also unveils several steps of the molecular mechanism by which the soluble extracellular protease tPA activates the cytosolic kinase Erk1/2. We show that, among the molecules with capacity to bind tPA at the neuronal surface, the NMDA receptor plays a crucial role in the transduction of signalling triggered by tPA. It has been proposed that tPA potentiates NMDA-induced excitotoxicity by direct interaction with the NMDA receptor and cleavage of the NR1 subunit (Nicole et al., 2001). However, recent reports suggest that the catalytic activity of tPA is not involved in NMDA receptor-mediated neurotoxicity (Matys and Strickland, 2003; Liu et al., 2004). Our data showing that tPA-triggered Erk1/2 activation is catalytic-independent yet involves the NMDA receptor supports a scenario in which tPA signal transduction requires the binding but not the proteolytic processing of the NMDA receptor by tPA. We propose that this binding would be precluded by the interaction of tPA with the inhibitor Pefabloc/tPA. We further demonstrate that
PTX-sensitive G-protein/s and PKC participate in the intermediate stages that link tPA-NMDA receptor binding and Erk1/2 activation. This is consistent with the reported role of G proteins and PKC in NMDA signalling pathway (Skeberdis et al., 2001; Benquet et al., 2002).

Finally, tPA is the only FDA-approved treatment for acute stroke due to its thrombolytic properties. However, the excitotoxic neuronal damage produced in animal models (Tsirka, 1997; Wang et al., 1998) questions the validity of this approach as a convening therapy. Our results that high tPA is detrimental to neurons through a catalytic independent Erk1/2 pathway activation adds further support to such concerns. This information should be taken into account also for the treatment of neurological disorders such as AD, where the plasminogen system has been seen as a possible target.

Materials and methods

Cell culture
Mouse neuroblastoma N2a cells were grown in DME medium containing 10% fetal bovine serum (Invitrogen). Primary cultures of rat or mice hippocampal neurons (glial contamination <5%) were prepared from embryos as described (Goslin and Banker, 1991). tPA−/− hippocampal neurons were obtained by the same procedure using tPA−/− mice (a kind gift of Dr P Carmeliet, Katholieke Universiteit Leuven, Belgium) (Carmeliet et al., 1994).

Drug treatments
N2a were serum deprived for 8 days or more. tPA (Actilyse, Boehringer Ingelheim) was added to the culture medium at 20 μg/ml, for different incubation times. U0126 (10 μM), LY294002 (50 μM), wortmannin (1 μM), staurosporine (100 nM), G06976 (2 μM) and MK801 (10 μM) (Sigma) were added 15 min prior to tPA treatment, unless otherwise indicated. Homocysteine (50 μM) and PTX (50 and 100 ng/ml) (Sigma) were added 20 h before tPA addition. SB415286 [3-[3-(chloro-4-hydroxyphenyl)]ami-no]-4-(2-nitrophenyl)-1H-pyrrrole-2,5-dione (25 μM) (Tocris Cookson Ltd) and cycloheximide (3 μg/ml) (Sigma) were added 24 h after tPA addition. The catalytic inactive mutant tPA, S478A tPA, (generous gift from Genentech Inc.) or human plasmin (American Diagnostica) were added at 20 or 5 μg/ml, respectively, for 1 h. The tPA chemical inhibitor Pefabloc/tPA (2,7-bis-[4-aminobenzylidene]-cycloheptanone-[1] dihydrochloride) (Pentapharm) (Renatus et al., 1997, 1998) was used at 15 μM and it was mixed with tPA 1 h prior to addition to the cell medium.

Western blot
Neuronal cultures were extracted in buffer A: 1% Triton X-100, 25 mM MES pH 7.00, 2 mM EDTA, 5 mM DTT, CLAP (25 μg/ml each of chymostatin, leupeptin, antipain and pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium fluoride). Protein concentration was determined by BCA method (Pierce). The same amount of protein (10 μg) was loaded in each lane. Primary and secondary antibodies are described in Supplementary data. ECL method (Amersham) was used and quantification was carried out on scanned autoradiographies under conditions of non-saturated signal using the NIH program.

References
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