

Tissue plasminogen activator induces microglial inflammation via a non-catalytic molecular mechanism involving activation of MAPKs and AKT signalling pathways and AnnexinA2 and Galectin1 receptors.

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ABSTRACT

Inflammatory responses mediated by glial cells play a critical role in many pathological situations related to neurodegeneration such as Alzheimer's disease. Tissue plasminogen activator (tPA) is a serine protease which best-known function is fibrinolysis, but it is also involved in many other physiological and pathological events as microglial activation. Here we found that tPA is required for A β -mediated microglial inflammatory response and TNF α release. We further investigated the molecular mechanism responsible for tPA-mediated microglial activation. We found that tPA induces a catalytic-independent rapid and sustained activation of ERK1/2, JNK, AKT and p38 signalling pathways. Inhibition of ERK1/2 and JNK resulted in a strong inhibition of microglial activation, whereas AKT inhibition led to increased inflammatory response, suggesting specific functions for each signalling pathway in the regulation of microglial activation. Furthermore, we demonstrated that AnnexinA2 and Galectin1 receptors are involved in tPA signalling and inflammatory response in glial cells. The present study provides new evidences supporting that tPA plays a cytokine-like role in glial activation by triggering receptor-mediated intracellular signalling circuits and opens new therapeutic strategies for the treatment of neurological disorders in which neuroinflammation plays a pathogenic role.

INTRODUCTION

In the central nervous system (CNS), glial cells, mainly composed by astrocytes and microglia, are the major support for neurons and play critical roles during physiological and pathological situations. Astrocytes secrete factors needed for the proliferation of microglial cells and for neuronal survival, and microglia are considered as the primary immune cells in the brain (Kreutzberg, 1996). After injury, microglia become activated and acquire a phagocytic phenotype in order to present antigen and phagocyte cell debris, resulting in neuroprotection (Ladeby et al., 2005; Streit et al., 1999). However, activated microglia can also be detrimental by promoting and/or mediating neurodegeneration through the release of cytotoxic molecules such as chemokines, cytokines, reactive nitrogen/oxygen species, and proteases (Colton and Gilbert, 1987; Chao and Hu, 1994; Chao et al., 1995; Espey et al., 1997). Indeed, local inflammatory reaction of microglia has been implicated in the pathogenesis of a variety of neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, stroke, multiple sclerosis, Creutzfeld-Jacob disease and HIV-associated dementia (Eikelenboom et al., 2002; Gonzalez-Scarano and Baltuch, 1999; Perry et al., 2010; Tansey and Goldberg, 2010; Wood, 1995). Therefore, identification of the molecular mechanisms responsible for microglial activation is an important area of research in order to find new treatments for neurodegenerative disorders.

Several studies support that tissue plasminogen activator (tPA) is involved in the initiation of microglial activation (Rogove et al., 1999; Siao and Tsirka, 2002; Siao et al., 2003; Zhang et al., 2009). tPA is an extracellular serine protease whose main physiological source are endothelial cells, but it is also present in the brain parenchyma, where it is expressed by both neurons and glial cells. The best known function of tPA is fibrinolysis by plasminogen conversion into plasmin (Collen and Lijnen, 2004) and

these clot clearance properties have determined its use as the first election drug for the treatment of acute myocardial infarct and thrombotic stroke (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). For this reason, most of the studies in the tPA field have been focused in its catalytic activity and thrombolysis. However, in the last decade, emerging data have shown that tPA is a pleiotropic protein that can exert many other functions, both in a catalytic-dependent and independent manner. Thus, in the CNS, tPA mediates excitotoxic neurodegeneration by a mechanism involving plasmin generation and subsequent laminin degradation (Chen and Strickland, 1997; Tsirka et al., 1995; 1996; 1997). In contrast, we have previously demonstrated that tPA can also exert neurotoxic effects in a catalytic-independent way by activating the ERK1/2 signalling pathway and that this molecular mechanism could be responsible for the neuronal death induced in mouse hippocampal neurons after A β treatment (Medina et al., 2005). tPA-mediated microglial activation is also catalytic-independent (Rogove et al., 1999). This tPA cytokine-like activity is mediated by its finger domain and the interaction with Annexin A2 receptor (Siao and Tsirka, 2002), although the underlying molecular mechanism has not been characterized.

In this report, we show that amyloid- β (A β) treatment induces tPA expression in glial cells and, using microglial cells from tPA $-/-$ mice, we demonstrate that tPA is required for glial activation and TNF α release after A β treatment, indicating an important role for tPA during the inflammatory response associated to Alzheimer's disease. We also identify the molecular signalling pathways responsible for tPA-mediated microglial activation. Using mouse primary mixed cultures (astrocytes and microglia) or purified microglial cells, we show that tPA, in a catalytic-independent manner, activates ERK1/2, JNK, PKB/AKT and p38 signalling pathways leading to

microglial activation and TNF α production. Furthermore, using blocking strategies against different tPA receptors, we demonstrate that the activation of these signalling pathways and tPA-stimulated glial inflammation is triggered by AnnexinA2 (AnxA2) and Galectin-1 (Gal1). These findings emphasize the role of tPA as a cytokine in glial cells and suggest that inhibition of tPA signalling would offer new approaches to avoid neurotoxic effects of microglial inflammation found in some neurological diseases.

MATERIALS AND METHODS

Cell Culture

Glial cells were isolated from cerebral cortices from 2-day-old OF1 (Charles River, France), C57BL/6, tPA^{-/-} or Gal1^{-/-} (in 100% C57BL/6 genetic background, provided by Dr. P. Carmeliet (tPA^{-/-}), Katholieke Universiteit Leuven, Belgium, and F. Poirier (Gal1^{-/-}), Institut Jacques Monod, Paris, France) mice. Two types of primary glial cultures were prepared: Mixed glial (containing around 70% astrocytes and 30% microglia) and purified microglial cultures (> 98% microglial cells). Mixed glial cultures were made as previously described (Giulian and Baker, 1986). Briefly, cerebral cortices were dissected, carefully stripped of their meninges, digested with 0.25% Trypsin, dissociated by trituration and plated onto 24 well plates at a density of 250.000 cells/ ml and cultured at 37°C for 15 days. Mouse purified microglial cultures were prepared by mild trypsinization as described (Saura et al., 2003). Briefly, confluent mixed glial cultures were treated for 30 min with a low trypsin concentration (0.06%). This treatment results in the detachment of an intact layer of cells containing virtually all the astrocytes and leaves a population of attached cells identified as > 98% microglia. The microglia was used 24 h after this treatment.

Cells were grown in Dulbecco's modified Eagle medium (DMEM)-F12 nutrient mixture (Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), penicillin 100 U/ml and streptomycin 100 µg/ml (Invitrogen).

All procedures were approved by the Ethic Committee of the Parc de Recerca Biomedica de Barcelona (PRBB).

A β treatment

Amyloid peptide A β -42 (Bachem) was initially dissolved at a concentration of 2 mM in DMSO and diluted to 100 mM in sterile PBS. A β -42 was incubated at 37 °C for 5 days to induce its aggregation (Lorenzo and Yankner, 1994). Mixed glia cultures from C57Bl/6 (wt) or tPA *KO* (tPA^{-/-}) mice were incubated with A β -42 for 30 min to 24 h and TNF α mRNA expression was analyzed. For rescue experiments, tPA^{-/-} mixed glial cultures were treated with 500 ng/ml of recombinant tPA (Actilyse®, Boehringer Ingelheim) 24 h prior to addition of A β -42 peptide.

tPA Zymography assay

Supernatants from mixed cultures were electrophoresed on a 10% nonreducing sodium dodecyl sulfate (SDS) polyacrylamide gel, which was washed for 30 min in 2.5% Triton X-100–PBS and for 30 min in distilled water. Gels were subsequently placed in contact with a casein gel containing 2% nonfat dry milk in 0.25 mM Tris-HCl, pH 7.6, 2% agarose and 15 μ g/ mL plasminogen (Chromogenix), and incubated in a humid chamber at 37°C until caseinolytic bands were visualized and photographed.

Morphological analysis of activated microglia

Confluent mixed glial or purified microglial cell cultures were incubated for 24 h or 48 h with 20 μ g/ml of recombinant tPA (Actilyse®, Boehringer Ingelheim), 100 ng/mL of LPS (*E. coli* LPS serotype 055:B5, Sigma) and combinations of tPA and LPS, in a 24-well plate. Glial activation was visualized by morphological changes using phase contrast microscopy.

TNF α mRNA and protein detection

Reverse transcription and quantitative real time PCR (RT-qPCR) was used to analyze TNF α mRNA expression in glial cells. Cells were incubated with tPA or LPS from 30 min to 24 h. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) and 1 μ g of total RNA was reverse transcribed into cDNA using the revert aid First Strand cDNA Synthesis kit (Fermentas). TNF α mRNA expression in mouse primary mixed and purified microglia cells was determined by Reverse transcription and Quantitative real time PCR. The following TNF α primers were used: 5'-GGG GCC ACC ACG CTC TTC TGT C -3' (forward) and 5'-TGG GCT ACG GGC TTG TCA CTC G-3' (reverse). PCR solutions were prepared with RNA-se free water containing primers and SYBR $^{\circledR}$ Green Master mix (Applied Biosystems). Assays were run in triplicate on the ABI Prism 7900HT system. All quantifications were normalized to β -actin values (endogenous control).

TNF α concentrations of the cell culture supernatants (100 μ l of conditioned medium from each sample) were determined using an enzyme linked-immuno-sorbent assay (ELISA) kit specific for mouse TNF α (eBioscience) using the standard and instructions supplied by the manufacturer.

Measurement of Nitric Oxide (NO) release

NO production by mixed glial and purified microglial cells was evaluated by a nitrite assay employing the Griess reaction (Green et al., 1990). 100 μ l conditioned medium were incubated with 100 μ l of Griess reaction and optical density at 540 nm was measured. Sodium nitrite (0-50 μ M) was used as a standard.

Western Blot (WB) Analysis of Signalling Pathways

In both cultures, cells were starved by replacement of their normal growth medium to DMEM-F12 containing 0.1% FBS during 72 h before treatments. Recombinant tPA (Actilyse®, Boehringer Ingelheim) or recombinant catalytically inactive mutant tPA (S481A tPA) (Loxo GMBH) were added at 20 µg/ml for different times and activation of signaling pathways was determined by WB. Cells treated with 10% FBS or untreated were used as positive and negative controls, respectively.

Cells were lysed in 67 mmol/L Tris, pH 6.8, 2% sodium dodecyl sulfate at 4°C, centrifuged, and proteins in the supernatant were quantified by Bradford assay (Bio-Rad Laboratories). Cell extracts were resolved on 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following primary antibodies were used; total ERK1/2 (Upstate Biotechnology International), phospho-ERK1/2 (Thr202,Tyr204), total PKB/AKT, phospho-PKB/AKT (Ser473, Thr308), total SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185) and phospho-p38 (Thr180 Tyr182) (Cell Signalling). A secondary rabbit antibody conjugated to horseradish peroxidase (HRP) (Dako) followed by enhanced chemiluminescence method (Amersham) and autoradiography were used for the detection of proteins. Quantification was done on the scanned autoradiographies using ImageJ software.

Immunofluorescence Analysis of Signalling Pathways

Mixed glial and purified microglial cells obtained as mentioned before were plated onto glass coverslips (200.000 cells/ ml). Treatments with tPA at 20 µg/ml were carried out from 5 min to 4 h. Treatment with 10% FBS for 5 min or untreated cells were used as positive and negative controls respectively. After treatment, coverslips were washed with PBS and fixed with 4% PFA for 10 min at RT. Free formaldehyde groups were quenched by treatment with NH₄Cl (30 min, RT), and 0.1% Triton X-100 in PBS (10

min, RT) was used to permeabilize. Cells were blocked with 1% BSA in PBS (1 h, RT) and incubated overnight at 4°C with the following primary antibodies: rabbit anti-ERK1/2, rabbit anti-PKB/AKT and rabbit anti-JNK (Cell Signalling), rabbit anti-Iba1 (Wako) and mouse anti-GFAP (Sigma). The secondary antibodies used were Alexa-488-goat anti-rabbit and Alexa-555-goat anti-mouse (Invitrogen) (10 µg/ mL, 1 h, RT). Non-immune mouse and rabbit IgG purified immunoglobulins (Sigma) were used as negative controls. Finally, coverslips were washed three times in PBS, rinsed in water, air-dried, mounted with Fluoromount-G (Southern Biotechnology Associates) and analyzed by fluorescence microscopy. Images were acquired with a Leica DMRB microscope adapted to a DC300F camera (Leica Lasertechnik GmbH).

Treatment with Signalling Pathways Inhibitors

Mixed glial and purified microglia cells were incubated for 6 h with 20 µg/ml of recombinant mutant S481A tPA (Loxo GMBH) in the presence or absence of the following signalling pathway inhibitors: U0126 (inhibitor of mitogen-activated protein kinase kinase (MEK) 1/2), (10 µmol/L), JNKI1 (inhibitor of SAPK/JNK kinase pathway) (100 µmol/L) and wortmannin (inhibitor of PKB/AKT) (1 µmol/L). U0126 and JNKI1 were added 1 h before tPA treatment, whereas wortmannin was added 6 h before tPA treatment and these inhibitors were maintained in the medium during all the experiment. Cells were also treated with the signalling inhibitors alone to analyze whether they have any direct effect on TNFα expression. After treatments, total RNA was extracted and processed by RT-qPCR as describe above in order to analyze TNFα expression levels.

AnxA2 and Gal1 analysis and Pull-Down Assays

Mixed glial or purified microglial cultures cells were lysed in buffer A (50 mmol/L Tris, pH 7.4, 0,1% Triton X-100 plus protease inhibitors (200 mmol/L sodium vanadate, 1 mmol/L sodium fluoride and 10 mmol/L sodium pyrophosphate) at 4°C and expression of AnxA2 and Gal1 proteins was determined by SDS/PAGE and WB as described above. Rabbit polyclonal anti-AnxA2 (raised in our laboratory, Aguilar S et al., 2004) or rabbit anti-Gal1 (kindly provided by Dr. H. J. Gabius, Ludwig-Maximilians-Universität, München, Germany) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig (Dako) was used as secondary antibody. tPA interaction with AnxA2 or Gal1 proteins in mixed glial or purified microglial cultures was determined using pull-down assays as described (Roda et al., 2009). Briefly, cell lysates were incubated with tPA-Sepharose or BSA-Sepharose and centrifuged. Unbound proteins were collected in the supernatant and sepharose beads were washed and bound proteins were eluted with Laemmli buffer. Proteins were resolved by 10% SDS/PAGE and transferred to nitrocellulose filters for WB analysis as described above. 30% of the bound fraction (B) and 3% of the unbound lysate (U) of both tPA and BSA pull-downs were loaded.

Knockdown of AnnexinA2 expression by siRNA

Mixed glia cells were transfected with siRNA SMARTpool® against AnnexinA2 (siAnxA2), or with non-targeting siRNA pool® (siIrrel) (Dharmacon) using the Oligofectamine system (Invitrogen). The siRNA concentration used was of 25 nM in all conditions. Downregulation of AnnexinA2 expression was confirmed by WB. 24 h after transfection, medium was replaced with medium containing 0.1% FBS and, 24 h later, cells were untreated (negative control), treated with 20 µg/ml S481A tPA for 1 h or with 10% FBS for 5 min (positive control). Cell extraction and detection of phosphorylated

kinases ERK1/2 (P-ERK1/2), JNK (P-JNK), AKT (P-AKT) and p38 (P-p38) levels by WB were performed as described above. Total ERK1/2 (T-ERK1/2) was used as loading control.

Statistical analysis

Results are expressed as mean \pm s.d. of triplicates and Student's t-test was used to compare the effects of the treatments with the control experimental conditions, except for A β 42 and LPS treatments of tPA $-/-$ mixed glial cells (Fig. 1B and 1C) where statistic analysis was performed comparing tPA $-/-$ values versus C57Bl/6 (wt) values or tPA $-/-$ values versus tPA $-/-$ + rtPA (rescue) values. Unless indicated, 3 independent experiments were always performed. p values < 0.05 were considered statistically significant.

RESULTS

1.- tPA is involved in A β -mediated glial activation

A β peptides, the major component of senile plaques in AD, are known to activate microglia and to induce the expression of proinflammatory molecules (Combs et al., 2001; Floden and Combs, 2006; Li et al., 1996; Lindberg et al., 2005; Lue et al., 2001; Yates et al., 2000), highlighting the important role of microglial activation in AD pathogenesis. Although tPA has been proven to be involved in the initiation of microglial activation after in vivo kainate-induced excitotoxicity (Rogove and Tsirka, 1998) and after culture treatment with LPS (Rogove et al., 1999), there is no data regarding the involvement of tPA in glial activation in response to A β . In order to analyze whether tPA was playing any role in A β -induced glial activation, primary cultures of mixed glial cells were treated with A β (20 μ M) and the levels of tPA were analyzed by zymography. We show that A β induces a strong increase in tPA activity in glial cells (Fig. 1A). These data are in agreement with previous results showing that A β can induce tPA expression in neurons (Medina et al., 2005; Tucker et al., 2000a). In order to get further insights in the involvement of tPA in A β -mediated inflammatory response in microglial cells, we analyzed the effects of A β in mixed glial primary cultures from wild type (wt) and tPA deficient (tPA $-/-$) mice. A β treatment of tPA deficient glial cells results in a dramatic reduction of inflammation, analyzed by TNF α expression, (Fig. 1B, circles) in comparison to wt cells (Fig. 1B, triangles). The involvement of tPA in these effects was further demonstrated by the addition of exogenous tPA (rtPA) to tPA $-/-$ glial cells (Fig. 1B, squares), which restored the values of TNF α to similar levels as in wt cells. LPS treatment, which has been previously described to require tPA for microglial activation (Rogove et al., 1999), was used as positive control. As expected, tPA $-/-$ glial cells treated with LPS showed a marked

decrease of TNF α levels in comparison to cells with normal tPA levels (wt) and addition of recombinant tPA to tPA $-/-$ cells rescued the TNF α levels to those observed in wt cells (Fig. 1C). These results demonstrate that tPA is involved in A β -mediated inflammatory response of glial cells.

2.- tPA induces glial activation and triggers ERK, JNK and AKT intracellular signalling cascades.

Taken into account the important role that tPA seems to play in glial activation in response to different stimuli including A β (see above), excitotoxicity and LPS (Rogove et al., 1999), we next aimed to further characterize the tPA molecular mechanisms responsible for these effects. Two types of primary cell cultures were used for this study: i) mixed cultures, that contained around 70% astrocytes and 25% microglia (Supplementary Fig. 1); and ii) purified microglial cultures (\geq 98% microglial cells, Saura et al., 2003). The rationale for using these two types of cultures was that whereas mixed cultures will mimic the *in vivo* situation, maintaining the crosstalk between astrocytes and microglial cells and therefore representing a more physiological scenario, the purified microglial cultures would help to discriminate the specific contribution of this population to the effects analyzed.

First, we compared tPA effects in glial activation by analyzing changes in cell morphology – an indication of microglial activation (Davis et al., 1994)– after tPA treatment. In mixed glial cell cultures, addition of recombinant tPA induced a moderate effect in cell morphology (Fig. 2A, b) that was much more evident in purified microglia, where tPA increased the round-shaped cells and induced the acquisition of an ameboid/phagocytic phenotype (Fig. 2A, b'). Treatment of both cell cultures with LPS was used as positive control and led to clear morphological changes related to glial

activation as previously reported (Kreutzberg, 1996) (Fig. 2A, c and c'). Addition of tPA together with LPS resulted in a synergistic effect and strongest glial activation both in mixed (Fig. 2A, d) and purified microglial cultures (Fig. 2A, d'). Altogether these data demonstrate that tPA induces morphological changes associated to cell activation in mixed and purified microglial cultures, although microglial cells seem to be more susceptible to tPA effects.

We next analyzed the molecular pathways that can account for tPA-mediated microglial activation. Mitogen-activated protein kinase (MAPK) superfamily - comprised of three signalling pathways: extracellular signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs) and p38 - have been implicated in mechanisms of glial activation in both primary glial cultures (Bhat et al., 1998; Schumann et al., 1998) and microglial cell lines (McDonald et al., 1998; Pyo et al., 1998). Microglial activation can be induced by other signalling pathways as PKB/AKT. For instance, the activation of AKT has been linked to the promotion of microglial cell proliferation (Ito et al., 2005; Suh et al., 2005) and the expression of AKT in the CNS increases in response to cellular stress or injury, suggesting a role for AKT in cell survival (Chong et al., 2005). Furthermore it has been recently reported that tPA induces AKT phosphorylation in astrocytes during ischemia (An et al., 2008). In order to define the signalling pathways that are downstream of tPA stimulation in glial activation, we examined the activation of the mentioned major signalling pathways in mixed and purified microglial cell cultures. Western blot analysis of phosphorylated ERK1/2, JNK and AKT showed that tPA treatment induced a time-dependent activation of all three kinases in both mixed and purified microglial cultures (Fig. 2B). We found that tPA induced a rapid (5 min) and sustained (up to 4 h) activation of ERK1/2 and JNK pathways both in mixed glia (Fig. 2B, left) and purified microglia (Fig. 2B, right). In contrast, PKB/AKT signalling

pathway required longer time of tPA treatment to show a peak of activation (4 h) (Fig. 2B). Quantification of time-dependent activation of all three kinases in response to tPA in mixed and purified microglial cultures is shown in Supplementary Fig.S2. Under the same conditions, we could not observe activation of p38MAPK neither in mixed glia nor in purified microglia cultures when cells were treated with tPA (data not shown). The activation of ERK1/2, JNK and AKT was further confirmed using immunofluorescence, as it has been reported that activation of these signalling pathways results in their translocation from the cytosol to the nucleus (Borgatti et al., 2000; Brunet et al., 1999; Mielke and Herdegen, 2000). We then analyzed the subcellular localization of activated ERK1/2, JNK and AKT in mixed and purified microglia primary cultures after tPA treatment. Immunofluorescence studies showed a diffuse and slight cytoplasmic staining for phosphorylated ERK, JNK and AKT in untreated cells in mixed (Fig. 2C, b, e, h, respectively) and purified microglia (Fig. 2C, b', e', h', respectively) whereas a nuclear staining was clearly observed after tPA treatment in both cell populations (Fig. 2C, c, c'; f, f'; i, i'). Positive controls treated with FBS also showed nuclear translocation of P-ERK, P-JNK and P-AKT as expected (Fig. 2C, a, a'; d, d'; g, g'). Nuclear localization was also detected by double immunofluorescence using DAPI staining (Supplementary Fig. S3). Altogether these data indicate that tPA induces activation of ERK, JNK and AKT signalling cascades in mixed glial and purified microglial cell cultures.

3.- tPA-induced activation of glial inflammatory response and activation of ERK, JNK and AKT signalling pathways is independent of its catalytic activity.

Activation of microglia by tPA has been reported to be independent of its catalytic activity (Kim et al., 1999; Rogove et al., 1999; Tsirka et al., 1997). In order to

test in our experimental system whether glial activation induced by tPA requires its catalytic activity, we treated mixed and purified microglial cell cultures with the catalytically inactive S481A mouse recombinant tPA for different times (30 min to 24 h) and TNF α expression was analyzed. Treatment with S481A tPA induced a strong increase in TNF α mRNA expression, detected by RT-qPCR, that reached its maximal level at 2 h (mixed glia) or 6 h (purified microglia) (Fig. 3A). The induction of TNF α expression was also detected at the protein level by ELISA, being the maximum level of activation at 12 h both in mixed and purified microglia (Fig. 3B). LPS treatment was used as a positive control and induced an increase of TNF α mRNA and protein levels with similar kinetics, although in general, higher values than S481A tPA treatment (Fig. 3A and 3B). Finally, nitric oxide (NO) release, another hallmark of microglial activation, was also analyzed in mixed and purified microglial cell cultures treated with S481A tPA. In this case, catalytically inactive tPA induced an increase in NO release in mixed glial cultures and purified microglial cells although the release only reached statistic significance in mixed cultures (Fig. 3C). LPS treatment was used as positive control and showed similar results, although higher levels of NO release in comparison to tPA (Fig. 3C). These data support our previous results showing that although microglia are the main source of NO, the presence of astrocytes strongly potentiate NO production by microglial cells (Sola et al., 2002).

Activation of signalling pathways by tPA has been shown to be catalytic-independent in neurons (Medina et al., 2005) and other cell types (De Petro et al., 1994; Maupas-Schwalm et al., 2004; Welling et al., 1996). Therefore, we wondered whether the above reported tPA-mediated induction of the ERK1/2, JNK and AKT intracellular signalling pathways could be mediated through a mechanism independent of plasmin generation. To validate this hypothesis, we investigated the effects of mutant S481A

tPA in primary mixed glia and purified microglia cultures. We found that ERK1/2, JNK and AKT pathways were all activated after treatment with S481A non-catalytic tPA in both cell cultures (Fig. 3D). Surprisingly, although the kinetics of activation were similar to those induced by wild-type tPA, catalytically-inactive S481A tPA induced higher levels of activation for ERK1/2, JNK and AKT, that became more evident in the purified microglia cells. In addition, although we did not observe any effect on p38 in mixed glia and purified microglia cultures treated with recombinant wt tPA, mutant S481A tPA induced a strong p38 activation in both cell cultures (Fig. 3D). Quantification of time-dependent activation of ERK1/2, JNK, AKT and p38 in response to tPA in mixed and purified microglial cultures is shown in Supplementary Fig. S4. All these differences between wt and S481A tPA effects on signalling pathways activation might be explained by a higher stability of the catalytically inactive protein, rendering it more active for signalling. These results strongly support for a tPA catalytically independent cytokine function by activation of ERK1/2, JNK, AKT and p38 intracellular signalling pathways in mixed and purified microglial cells.

Altogether our data demonstrate that tPA induces activation of microglia in a catalytic-independent way, resulting in TNF α increase, NO release and activation of MAPK and AKT intracellular signalling pathways.

4.- Microglial activation induced by tPA is mediated by ERK1/2 and JNK signalling pathways.

We next aimed to validate whether the intracellular signalling cascades triggered by tPA, in a catalytic independent way, are responsible for its effects in microglial inflammatory response. To address this point we measured the expression of TNF α by RT-qPCR after the treatment with S481A tPA alone or in the presence of inhibitors for

the different signalling pathways. Treatment of mixed or purified microglia cells with S481A tPA in the presence of MEK/ERK1/2 (U0126) or JNK (JNKI1) pathways inhibitors, resulted in a dramatic decrease in the induction of TNF α (Fig. 4), indicating that these two pathways are involved in this effect. In contrast, when tPA was added in the presence of wortmannin, an inhibitor of AKT pathway, we did not observe any inhibitory effect on the induction of TNF α expression but a strong increase (Fig. 4). Cells treated with the signalling inhibitors alone were also analyzed and no differences were observed in comparison to untreated cells (data not shown).

5.- AnxA2 and Gal1 receptors are required for tPA-induced signalling activation and inflammatory response in glial cells.

Activation of intracellular signalling pathways by tPA requires its interaction with cell surface receptors that would mediate intracellular signal transduction. Several receptors have been found to be involved in tPA-mediated triggering of intracellular cascades in different cell types (An et al., 2008; Bacskai et al., 2000; Herz, 2001; Hu et al., 2006; Lin et al., 2010; Medina et al., 2005; Nicole et al., 2001; Ortiz-Zapater et al., 2007; Pawlak et al., 2005; Roda et al., 2009), although in microglial cells this aspect has never been characterized. We therefore decided to study the involvement of cell membrane receptors in tPA signalling in our experimental system. Our group has previously identified that ERK1/2 activation by tPA in pancreatic cancer cells requires AnnexinA2 (AnxA2) and Galectin-1 (Gal1) receptors (Ortiz-Zapater et al., 2007; Roda et al., 2009). We analyzed the expression of these two receptors in our cultures. We found that both AnxA2 and Gal1 are highly expressed in mixed and purified microglial cells (Fig. 5A). To further investigate the putative role of these two proteins as tPA receptors in glial cells, we performed pull down experiments using tPA-coupled to

sepharose incubated with total extracts from primary mixed and purified microglial cultures. We found that AnxA2 and Gal1 are able to interact with tPA as they could be detected in fractions bound to tPA-sepharose but not in those bound to BSA-sepharose, indicating binding specificity (Fig. 5B). To examine the functional role of these receptors in tPA-induced signalling, we downregulated AnxA2 or Gal1 expression in mixed and purified microglial cells. Downregulation of AnxA2 using siRNA technology resulted in around 70% (mixed) or 48% (purified microglia) reduction of total protein levels when used AnxA2 specific sequences (siAnxA2) in comparison to irrelevant random sequences (siIrrel) (Fig 6A). We found that tPA treatment of mixed or purified microglia in the presence of siRNA for AnxA2 abolished ERK1/2, JNK, AKT and p38 signalling pathways activation, in comparison to the activation levels observed in the presence of irrelevant siRNA (Fig. 6B), indicating that AnxA2 is required to mediate tPA-induced signalling activation in mixed and purified microglia. Considering our previous data regarding the involvement of MAPK signalling pathways in tPA-induced glial inflammation (Fig. 4), we next aim to analyze whether AnxA2 is required for this effect. Downregulation of AnxA2 in mixed glia cultures using siRNA led to a significant decrease in TNF α production (Fig. 6C) and NO release (Fig. 6D). These data demonstrate that AnxA2 is involved in tPA-stimulated glial inflammatory response.

We next addressed the involvement of Gal1 in tPA signalling using purified microglia cells from Gal1 $-/-$ mice. Our results showed that absence of Gal1 expression induces a clear delay in the activation of intracellular signalling pathways. Whereas tPA treatment of microglial cells from wild type C57Bl/6 mice (wt) resulted in a rapid and sustained activation of ERK1/2, AKT, JNK and p38, microglia from Gal1 $-/-$ mice showed later activation of all these pathways (Fig. 6E). In order to investigate the effects of Gal1 depletion in tPA-induced microglial inflammation, we analyze TNF α and NO levels in

response to tPA using purified microglia cells from wt or Gal1 -/- mice. We found that abolishment of Gal1 results in a significant decrease of tPA-mediated inflammatory effects, detected as a reduction in TNF α (Fig. 6F) and NO (Fig. 6G) levels. Taken together these data demonstrate that both AnxA2 and Gal1 are involved in tPA-mediated signalling and pro-inflammatory responses in glial cells.

DISCUSSION

Microglial activation is a key contributor to neuronal death in a variety of neurodegenerative diseases. Importantly, tPA is directly involved in microglial activation after various stimuli such as excitotoxicity or LPS (Rogove et al., 1999) and brain ischemia (Wang et al., 1998; Zhang et al., 2009). In AD, tPA has been reported to play a dual role: it can play a beneficial function through plasmin production and subsequent A β degradation (Exley and Korchazhkina, 2001; Lee et al., 2007; Melchor et al., 2003; Tucker et al., 2000a; 2000b), but it can also mediate neuronal death by a NMDA receptor-mediated catalytic independent mechanism (Fernandez-Monreal et al., 2004; Medina et al., 2005; Nicole et al., 2001). Although the contribution of inflammation to AD pathogenesis is well documented (Akiyama et al., 2000) and A β deposits or senile plaques are frequently associated with reactive microglia and astrocytes (Itagaki et al., 1989; Miyazono et al., 1991), the contribution of tPA to A β -associated glial inflammation has been poorly analyzed. Here, we show that A β treatment increases the release of tPA by glial cells and, using glial cultures from tPA-deficient mice, we demonstrate that tPA is required for glial activation after A β stimulation. Induction of tPA by A β (Medina et al., 2005; Tucker et al., 2000a) and its involvement in neuronal apoptosis has been previously reported in neurons (Medina et al., 2005) but this is the first evidence for a direct link between tPA and the glial inflammatory reaction in response to A β . Taken into account that A β is the major component of senile plaques and one of the major causes of synaptic dysfunction and neurodegeneration in AD, we propose the following scenario in this pathology: A β aggregates will stimulate neurons and glial cells to produce tPA which, in turn, will generate plasmin in order to degrade A β peptides (Ledesma et al., 2000; Tucker et al., 2000a; 2000b). However, in AD patients, catalytic activity of tPA is highly impaired

(Aoyagi et al., 1992; Ledesma et al., 2000; Melchor et al., 2003), leading to an inefficient A β clearance and therefore A β deposition and senile plaque formation. Accumulation of A β aggregates would lead to a local increase of tPA in the extracellular space that will trigger, in a catalytic-independent way, intracellular signalling pathways responsible for neuronal death (Medina et al., 2005) and microglial activation. This situation would be similar to that reported for glutamate-mediated excitotoxicity, where neurons can release tPA which, in a paracrine way, activates microglia in order to scavenge damaging molecules and cell debris (Siao et al., 2003; Tsirka et al., 1995). On the other hand, activated microglia can secrete additional tPA leading to an autocrine loop of inflammation (Rogove et al., 1999; Siao and Tsirka, 2002) and to neurodegeneration via plasmin generation (Chen and Strickland, 1997; Flavin et al., 2000; Nagai et al., 1999; Siao et al., 2003). Therefore, low and high levels of tPA can lead to different effects in both neurons and glia, suggesting that tPA release by these cells should be tightly regulated in order to ensure a right balance between beneficial and detrimental effects.

Previous data have reported that tPA is required but not sufficient for the initiation of microglial activation and that it acts in synergy with other stimuli, as LPS or other factors, to activate microglia (Siao and Tsirka, 2002). However, we found here that tPA alone is able to induce morphological changes in microglial cells (round-shaped cells with phagocytic phenotype) that reflect an activated state (Fig. 2A, b'). This apparently contradictory data can be explained by differences in tPA doses, mouse strains or methods of purified microglial cultures generation used in previous reports. tPA-mediated microglial activation has been also reported to be catalytic independent (Rogove et al., 1999; Zhang et al., 2009), but the underlying molecular mechanisms are poorly understood. We show for the first time that exposure of microglial cell cultures

to tPA results in the activation of the ERK1/2, PKB/AKT, JNK and p38 signal transduction pathways. Furthermore, we demonstrate that tPA catalytic activity is not required for this activation, as the same effects were observed when we add catalytically inactive mutant S481A tPA. Activation of intracellular signalling pathways by tPA in a non-catalytic way has been reported in several systems, including epithelial cells (Ortiz-Zapater et al., 2007; Roda et al., 2009), fibroblasts (Hu et al., 2006) and neurons (Medina et al., 2005), leading to the classification of tPA as a novel cytokine. The results presented here are consistent with this cytokine-like role of tPA in microglial activation and inflammatory response by activating ERK1/2, PKB/AKT, JNK and p38 intracellular signalling pathways independently of its catalytic activity. Furthermore, using two different cellular systems, mixed glial (astrocytes and microglia) or purified microglial cell cultures, we have analyzed which cell population is involved in these events. Our data suggest that microglial cells are the main responsible for tPA-induced signalling, as a stronger activation of ERK1/2, PKB/AKT and JNK signalling pathways was detected in microglial-enriched cultures in comparison to mixed cultures (Figs. 3, 4). These results could be explained considering two possibilities. First, that microglia, and not astrocytes, would be the only population responding to tPA and therefore, mixed cultures will show lower signal due to dilution of the “responsive” population in the total sample. Second, both astrocytes and microglia might respond to tPA-induced signalling activation but, as a consequence of the cross-talk between both cell types and the modulating effect of astrocytes (Saura, 2007; Sola et al., 2002), the effects could be lower than those observed in microglia alone. Taking into account the already reported AKT activation in astrocytes by tPA (An et al., 2008), and our immunofluorescence studies showing activation of signalling pathways in most of the cells (Fig. 2C) in mixed glial cultures (where around 70% cells are astrocytes, Supplementary Figure S1).

the second possibility seems to be the most plausible.

Importantly, our data using chemical inhibitors demonstrates that glial inflammation in response to tPA is mediated by the activation of ERK1/2, JNK or AKT signalling pathways. We found that tPA treatment of mixed glial and purified microglial cells results in a marked increase of TNF α expression, a cytokine with a well-known role in glial inflammatory response (Clark et al., 2010). Interestingly, addition of MEK or JNK inhibitors abolishes tPA-induced TNF α increase, whereas wortmannin, an inhibitor of PKB/AKT signalling pathway, results in an increase of the production of TNF α . We can then hypothesize that, in response to tPA, glial cells activate ERK1/2 and JNK signalling pathways that will be responsible of TNF α production and inflammation but, in parallel, they will also activate AKT pathway, that would inhibit glial activation in order to avoid excessive inflammation and cellular damage. Indeed, we found that activation of AKT occurs later than ERK1/2 and JNK (Figs. 2B and 3D, and Suppl. Figs. S2 and S4) suggesting an AKT-mediated compensatory mechanism to prevent an exacerbated inflammatory response. In this regard, a similar AKT protective role has been described during LPS-induced TNF α expression in monocytes (Guha and Mackman, 2002) and in neurons after cellular stress (Datta et al., 1999; Dudek et al., 1997) or ischemia (An et al., 2008; Echeverry et al., 2010).

tPA is a secreted protease which lacks an intracellular or transmembrane domain, therefore activation of intracellular signalling cascades by tPA requires its interaction with cell membrane receptors. Several receptors, or receptor complexes, have been reported to mediate tPA signalling in different cellular contexts (An et al., 2008; Bacskai et al., 2000; Fernandez-Monreal et al., 2004; Herz, 2001; Hu et al., 2006; Lin et al., 2010; Medina et al., 2005; Nicole et al., 2001; Ortiz-Zapater et al., 2007; Pawlak et al., 2005). In microglia, AnxA2 participates in the catalytically independent

tPA-mediated activation although the underlying molecular mechanism has not been characterized (Siao and Tsirka, 2002). Here, using blocking strategies, we show that tPA-stimulated activation of ERK1/2, JNK, AKT and p38 and glial inflammatory reaction are mediated by AnxA2 and Gal1. Downregulation of AnxA2 expression using siRNA technology (Fig. 5C) blocks tPA-induced ERK1/2, JNK, AKT and p38 phosphorylation indicating that this receptor is required for tPA signalling in glial cells. In agreement with previous data reporting that an antibody against AnxA2 did block microglial activation induced by tPA/LPS (Siao and Tsirka, 2002) we found that AnxA2 downregulation by siRNA results in a significant reduction of TNF α and NO levels, indicating that this receptor is involved in tPA-mediated glial inflammation. Importantly, we show for the first time that Gal1, a protein that is highly expressed in glial cells and that we have recently identified as a new receptor for tPA in pancreas (Roda et al., 2009), is also involved in tPA-induced signalling and activation of microglia. Gal1 is a carbohydrate-binding protein with high affinity for β -galactosides (Ahmad et al., 2004) that exerts a wide range of biological activity, including regulation of cell growth, migration, adhesion and invasion (Camby et al., 2006; Gabius et al., 2002; Scott and Weinberg, 2004). In addition, Gal1 has been described as a master regulator of immune responses and inflammation and its expression in neuronal and glial cells correlates with the regenerative capacity after injury (McGraw et al., 2005; Wada et al., 2003). Therefore, our finding that Gal1 is involved in tPA-mediated microglial activation and inflammation agrees with previous data available for this receptor. Interestingly, our data using glial cells from Gal1^{-/-} mice show that absence of Gal1 does not abolish tPA-mediated signalling activation but delays the process of activation (Fig. 5E). These results indicate that Gal1, although not essential, is required for a proper time activation of ERK1/2, JNK, AKT and p38 pathways after tPA

stimulation in glial cells, resulting in impaired inflammatory response. One possible mechanism to explain the delay in signalling activation observed in Gal1^{-/-} microglial cells could be related to the already reported interaction between Gal1 and Ras leading to Ras-GTP stabilization in the membrane (Elad-Sfadia et al., 2000; Paz et al., 2001). In this regard, inactivation of Gal1 in microglial cells might result in less efficient Ras mobilization to the membrane and delayed signalling activation. Furthermore, it is also possible that integrins (Fischer et al., 2005) or other known (Camby et al., 2006) or even not identified Gal1 partners may be responsible for the effects found in Gal1^{-/-} microglial cells. Moreover, taking into account that both AnxA2 and Gal1 lack canonical signalling domains, it is tempting to speculate that additional receptors can be involved in a multiprotein complex that would mediate tPA signalling in glial cells, as previously reported in pancreatic cancer (Diaz et al., 2004; Ortiz-Zapater et al., 2007; Roda et al., 2009). A suggestive candidate would be the low-density lipoprotein receptor-related protein 1 (LRP1), a receptor that has been previously reported to be a tPA signal transducer in neurons (Bacskai et al., 2000; Herz, 2001) and astrocytes (An et al., 2008). In microglial cells, however, the role of LRP1 in tPA-mediated activation is controversial as it has been reported to participate in microglial activation and inflammation after brain ischemia (Zhang et al., 2009) but not after LPS treatment (Siao and Tsirka, 2002). Additional work will be required to clarify whether LRP1 or other molecules participate, together with AnxA2 and Gal1, in tPA signal transduction during microglial activation.

The role of glial inflammatory response in brain pathophysiology is a matter of debate; it has been proposed to act in a neuroprotective manner by phagocytosing cell debris after injury (Banati and Graeber, 1994; Kreutzberg, 1996) but on the other hand a sustained and exacerbated microglial activation can potentiate neurodegeneration by

secreting neurotoxic substances as glutamate and reactive oxygen species (Colton and Gilbert, 1987; Chao et al., 1995; Piani et al., 1991; 1992). Our results demonstrate that addition of exogenous tPA to glial cells results in the activation of signalling cascades that in turn are responsible of the induction of an inflammatory response (measured as TNF α release). Our *in vitro* data can mimic pathological situations such as AD (Medina et al., 2005) or ischemia (Zhang et al., 2009), where it has been reported that neurons release high amounts of tPA which activates microglia cells in a paracrine manner. Therefore, we propose that inhibition of tPA signalling could be considered as a therapeutic strategy to avoid undesirable effects of this protease in CNS diseases related to exacerbated inflammatory response of microglia. Furthermore, this study alerts on the possible undesirable effects that the use of recombinant tPA in stroke patients (Ingall et al., 2004; The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995) may have, inducing neurotoxicity by a direct effect on neurons (Medina et al., 2005) and by microglial activation and induction of an inflammatory process that might exacerbate neuronal damage.

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FOOTNOTES

Abbreviations list

¹The abbreviations used are: A β , Amyloid- β ; AD, Alzheimer's disease; AnxA2, Annexin A2; CNS, Central nervous system; ERK, Extracellular signal-regulated kinase; Gal1, Galectin-1; JNK, Jun N-terminal kinase; LRP1, low-density lipoprotein receptor-related protein 1; NO, nitric oxide; TNF α , Tumor Necrosis Factor- α ; tPA, tissue plasminogen activator; WB, Western blot.

FIGURE LEGENDS

Figure 1.- A β -42 aggregates induce tPA release and tPA-mediated glial activation

of mixed glial cultures. (A) Mixed glial cultures were treated for 30, 60, 90 and 120 min with 20 μ M fibrillar A β -42 and tPA secreted in the conditioned medium was determined by zymography. Figure shows results of one representative experiment out of three performed. (B) Mixed glia cultures obtained from C57BL/6 (wt), tPA knock out (tPA $-/-$) mice, or from tPA $-/-$ mice preincubated with 1 μ g/ml of rtPA for 24h (+rtPA), were treated with 20 μ M fibrillar A β -42 at indicated times and relative TNF α mRNA levels, referred to β -actin mRNA, were analyzed by RT-qPCR. Data shown correspond to results of one of three independent experiments performed and values correspond to mean \pm s.d. of triplicates, $p < 0.05$ (*) or $p < 0.01$ (**). (C) Mixed glia cultures obtained from C57BL/6 (wt), tPA knock out mice (tPA $-/-$) or from tPA $-/-$ mice preincubated with 1 μ g/ml of rtPA for 24h (+rtPA) were treated with 100 ng/ml LPS at indicated times and relative TNF α mRNA levels, referred to β -actin mRNA, were analyzed by RT-qPCR. Data shown correspond to results of one of three independent experiments performed and values correspond to mean \pm s.d. of triplicates, $p < 0.05$ (*) or $p < 0.01$ (**).

Figure 2.- tPA treatment induces glial activation and phosphorylation of ERK,

JNK and AKT. (A) Effects in cell morphology in mixed and purified microglial cultures after 48h treatment with 20 μ g/ml of tPA (tPA), 100 ng/ml of LPS (LPS) or both (tPA+LPS) compared to controls (control). Images were taken in bright field microscope. Bars: 100 μ m (a-d) and 50 μ m (a'-d'). (B) WB analysis of total lysates (10 μ g/lane) from mixed (left) and purified microglia (right) cultures treated with 20 μ g/ml

of tPA for 5 min, 30 min, 1 h, 2 h and 4 h. Figure shows results of one representative experiment out of three performed. Detection of activated ERK1/2, JNK and AKT was performed using antibodies against phosphorylated (P-ERK1/2, P-JNK and P-AKT) or total proteins (T-ERK1/2, T-JNK, T-AKT). 5 min 10% FBS treatment was used as a positive control (+), and non treated cells were used as negative control (-). *Mr* markers, expressed in kDa, are shown on the right. (C) Immunofluorescence analysis of P-ERK, P-JNK and P-AKT of mixed (a-i) and microglia (a'-i') cell cultures untreated (-), (b, e, h; b', e', h'), treated with 20 µg/ml of tPA for 1h (tPA) (c, f, i; c', f', i') or treated for 5 min with 10% FBS, as positive control (+) (a, d, g; a',d',g'). Bar, 50 µm. Figure shows results of one representative experiment out of three performed.

Figure 3.- tPA-mediated glial activation and phosphorylation of ERK, JNK, AKT and p38 kinases does not require its catalytic activity. (A) Mixed glial and purified microglial cultures were treated for 30 min, 1 h, 2 h, 6 h, 12 h and 24 h with 20 µg/ml of S481A rtPA (tPA), 100 ng/ml of LPS (LPS) or non-treated (-). RNA was collected and relative TNFα mRNA levels, referred to β-actin mRNA, were analyzed by RT-qPCR. Data shown correspond to results of one of three independent experiments performed and values correspond to mean ± s.d. of triplicates, $p < 0.05$ (*) or $p < 0.01$ (**). (B) Mixed and purified microglial cultures were treated for 30 min, 1 h, 2 h, 6 h, 12 h and 24 h with 20 µg/ml of S481A rtPA (tPA), 100 ng/ml of LPS (LPS) or non-treated (-). TNFα protein expression was analyzed by ELISA. Data shown correspond to the results of one of the three independent experiments performed and values correspond to mean ± s.d. of triplicates, $p < 0.05$ (*) or $p < 0.01$ (**). (C) Measurement of NO release after 12, 24 and 48 h of treatment with 20 µg/ml of S481A rtPA (tPA), 100 ng/ml of LPS (LPS) or non-treated (-). Data shown represent the mean ± s.d. of three different

experiments, $p < 0.05$ (*) or $p < 0.01$ (**). **(D)** WB analysis of whole lysates (10 $\mu\text{g}/\text{lane}$) from mixed (left) and purified microglial (right) cultured cells treated with 20 $\mu\text{g}/\text{ml}$ of catalytically inactivated mouse mutant tPA (S481A tPA) for 30 min, 1 h and 4 h. Detection of activated ERK1/2, JNK, AKT and p38 was performed using antibodies against phosphorylated (P-ERK1/2, P-JNK, P-AKT and P-p38) or total proteins (T-ERK1/2, T-JNK, T-AKT). 5 min treatment with 10% FBS was used as positive control (+) and non treated cells as negative control (-). Mr markers, expressed in kDa, are shown on the right.

Figure 4.- Signalling pathways activated by tPA are responsible for glial TNF α release. Mixed and purified microglial cultures were treated for 6 h with 20 $\mu\text{g}/\text{ml}$ of S481A tPA alone (tPA) or S481A tPA in the presence of 10 μM U0126 (tPA+U0), 5 μM of JNK inhibitor 1 (JNKI1) (tPA+JNKI1) or 1 μM of Wortmannin (tPA+Wor), or left untreated (-). Relative TNF α mRNA levels, referred to β -actin mRNA, were analyzed by RT-qPCR. tPA treatment values were adjusted to 100% and the rest of data were referred as percentage of tPA treatment. Data shown represent the mean \pm s.d. of three different experiments, $p < 0.05$ (*) or $p < 0.01$ (**).

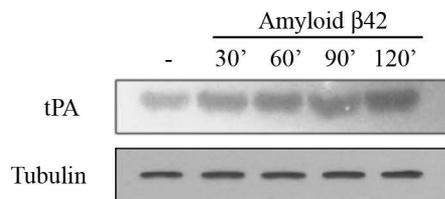
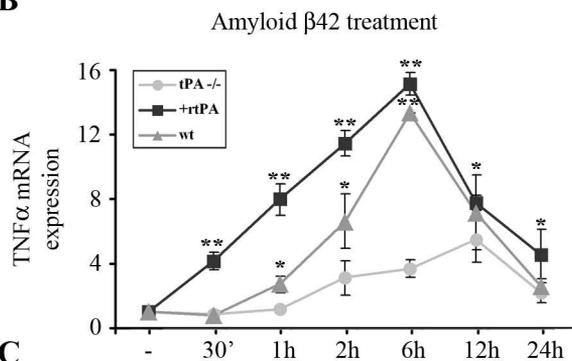
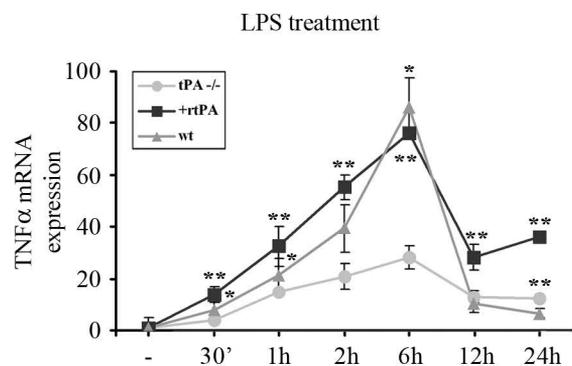
Figure 5.- AnxA2 and Gal1 receptors interact with tPA in glial cells. **(A)** WB analysis of AnxA2 and Gal1 protein levels in mixed and purified microglia cultures. Tubulin levels were determined as loading control **(B)** AnxA2 and Gal1 pulldown assays using tPA-coupled Sepharose. Mixed and purified microglia total cell lysates were incubated with tPA-Sepharose (tPA) or BSA-Sepharose (BSA), and Gal1 and AnxA2 in the bound (B) and unbound (U) fraction were determined by WB. Gal1 and

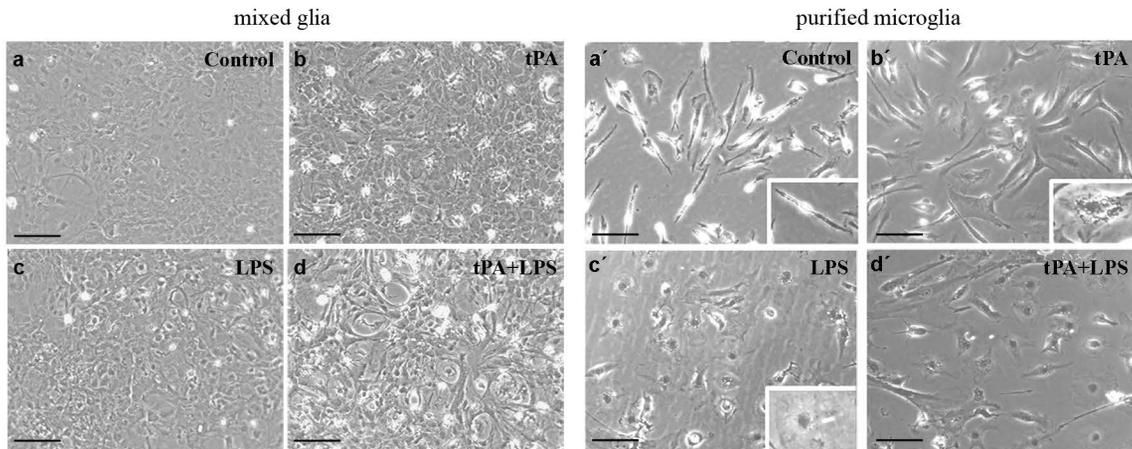
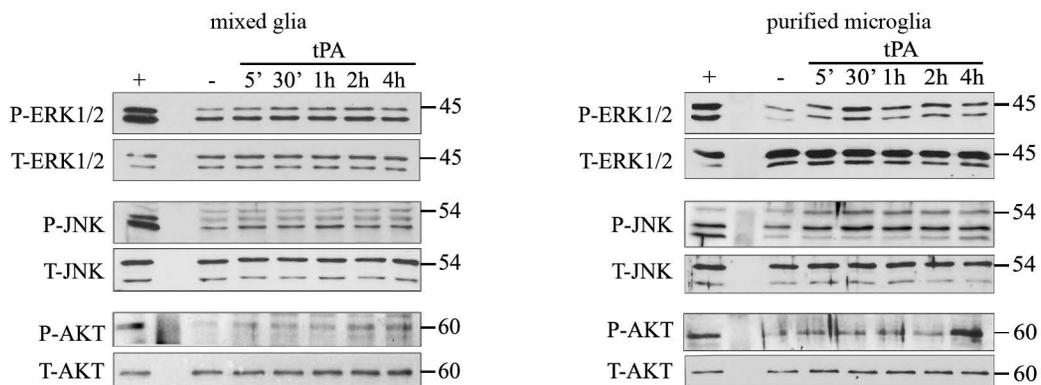
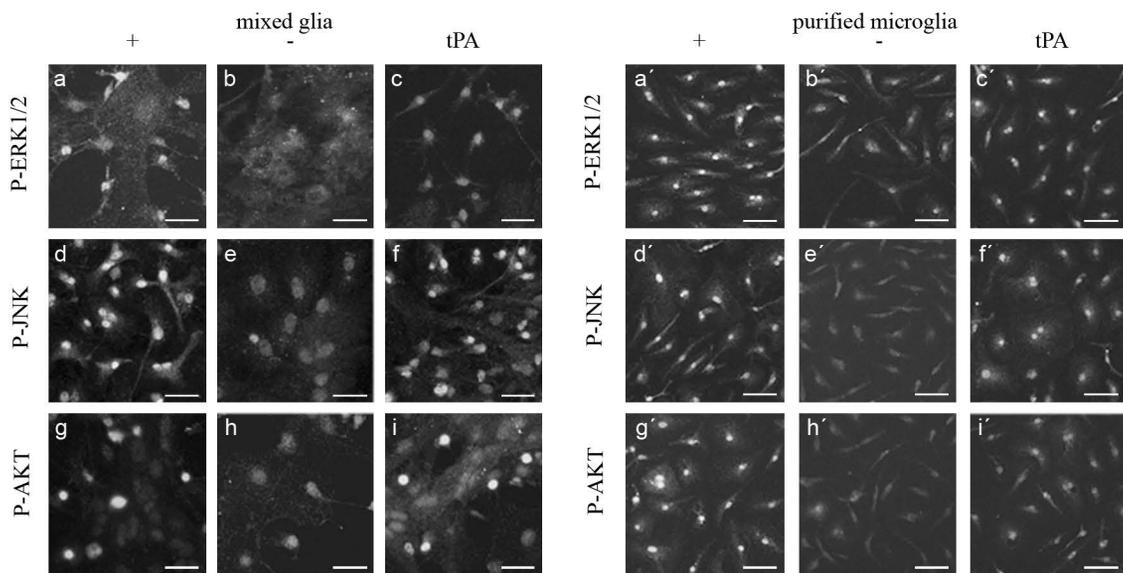
AnxA2 were specifically detected in the bound fraction of tPA-Sepharose. Mr markers, expressed in kDa, are shown on the right.

Figure 6.- Role of AnxA2 and Gal1 receptors in tPA-induced signalling activation and inflammatory response in glial cells.

(A) Down regulation of AnxA2 expression using siRNA. Mixed and purified microglia cells were transfected with siRNA targeting AnxA2 (siAnxA2) or an irrelevant target (siIrrel); after 24 h, medium was replaced with serum-free medium, and 24 h later, cells were left untreated (-), treated with 20 µg/ml S481A rtPA for 1 h (tPA) or treated with 10% FBS for 5 min (+). Decrease of AnxA2 after siRNA transfection was analysed by WB. Tubulin levels were assayed as loading control. Mr markers, expressed in kDa, are shown on the right. **(B)** Phosphorylated-ERK1/2 (P-ERK1/2), -JNK (P-JNK), -AKT (P-AKT) and -p38 (P-p38) and total ERK1/2 (T-ERK1/2) levels were determined by WB in mixed and purified microglia cells transfected with siRNA targeting AnxA2 (siAnxA2) or an irrelevant target (siIrrel) shown in panel A. Mr markers, expressed in kDa, are shown on the right. **(C)** Mixed glia cultures obtained from OF1 mice were transfected with siIrrel or siAnxA2, treated for 30 min, 1 h, 2 h, 6 h, 12 h and 24 h with 20 µg/ml of S481A rtPA and relative TNFα mRNA levels, referred to β-actin mRNA, were analyzed by RT-qPCR. Data shown correspond to the results of one of the three independent experiments performed and values correspond to mean ± s.d. of triplicates, p < 0.05 (*) or p < 0.01 (**). **(D)** Mixed glia cultures obtained from OF1 mice were transfected with siIrrel or siAnxA2, treated for 12 h, 24 h and 48 h with 20 µg/ml of S481A rtPA and NO release was measured. Data shown represent the mean ± s.d. of three different experiments, p < 0.05 (*) or p < 0.01 (**). **(E)** Gal1 modulates activation of ERK1/2, JNK, AKT and p38 induced by tPA in purified microglia cells. Blot analysis of whole lysates (10 µg/lane) of purified microglial cultures, obtained from C57BL/6 (wt) and Gal1 knock out mice (Gal1 -/-)

treated with 20 $\mu\text{g/ml}$ of S481A rtPA for 5 min, 30 min, 1 h, and 4 h. Detection of P-ERK, P-JNK, P-AKT, P-p38 and total ERK1/2 was performed using antibodies against phosphorylated proteins, and against total ERK (T-ERK1/2). 5 min 10% FBS treatment was used as a positive control (+), and non tPA treated cell were used as negative control (-). Figure shows results of one representative experiment out of three performed. Mr markers, expressed in kDa, are shown on the right. **(F)** Microglial purified cultures obtained from C57BL/6 (wt) and Gal1 knock out mice (Gal1 -/-) were treated with 20 $\mu\text{g/ml}$ of S481A rtPA (tPA) at indicated times and relative TNF α mRNA levels, referred to β -actin mRNA, were analyzed by RT-qPCR. Data shown correspond to the results of one of the three independent experiments performed and values correspond to mean \pm s.d. of triplicates, $p < 0.05$ (*) or $p < 0.01$ (**). **(G)** Purified microglial cultures from C57BL/6 (wt) or Gal1 knock out mice (Gal1 -/-) were treated with 20 $\mu\text{g/ml}$ of S481A rtPA (tPA) during 12, 24 and 48 h and NO release was measured. Data shown represent the mean \pm s.d. of three different experiments, $p < 0.05$ (*) or $p < 0.01$ (**).

A**B****C****Figure 1**

A**B****C****Figure 2**

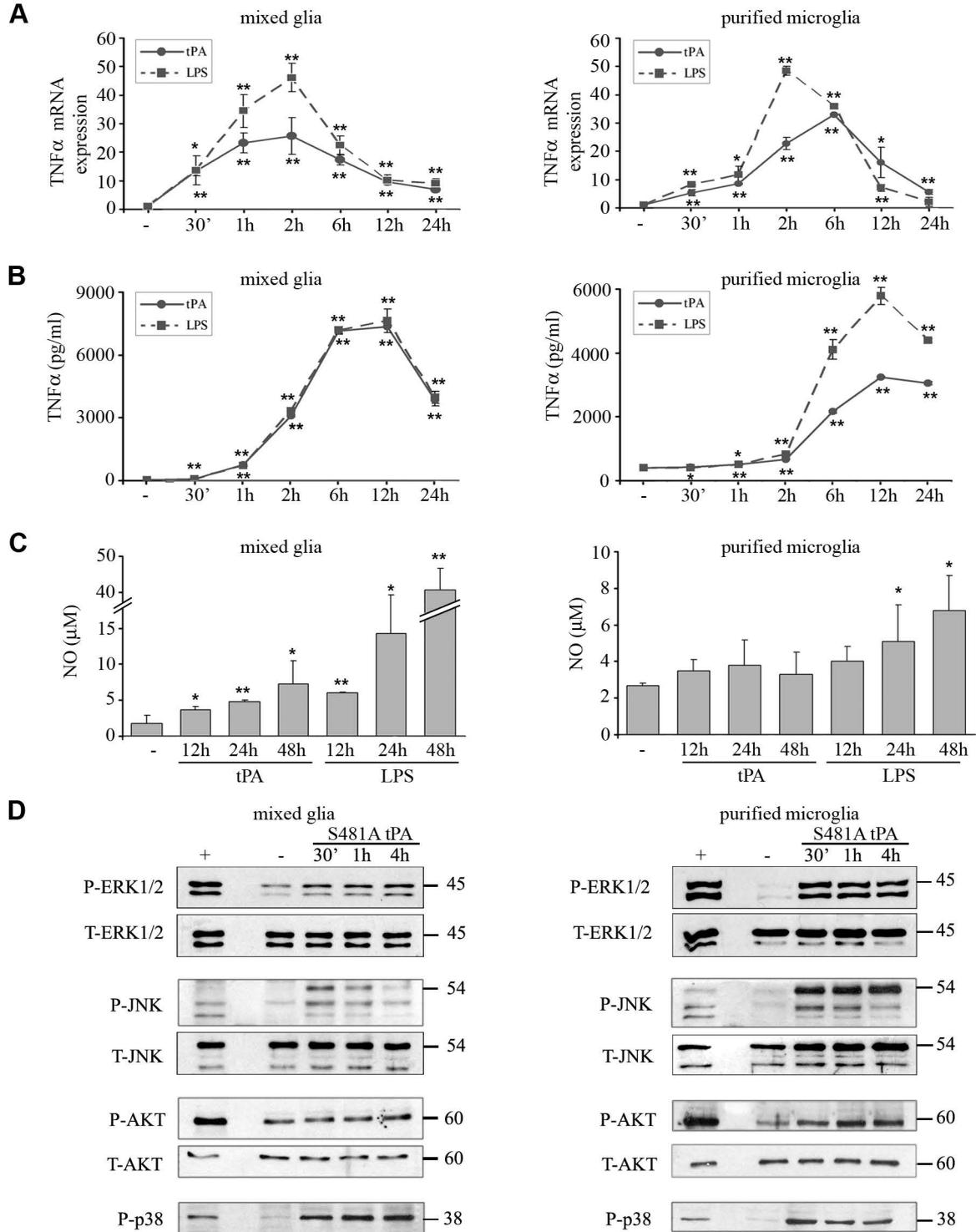
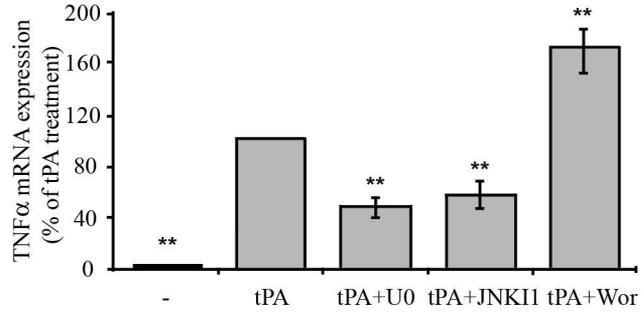


Figure 3

mixed glia



purified microglia

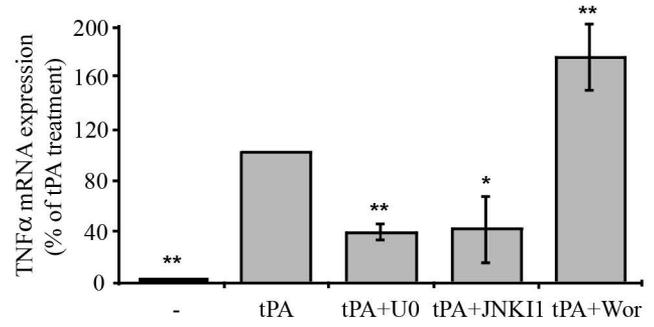
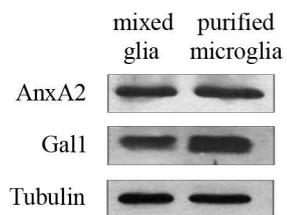
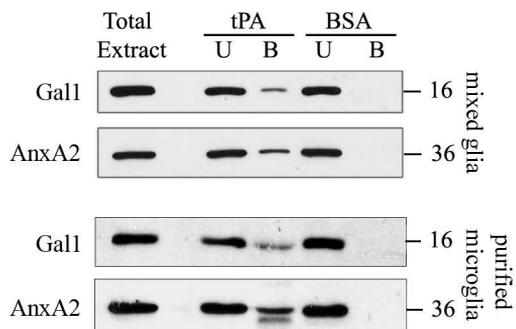
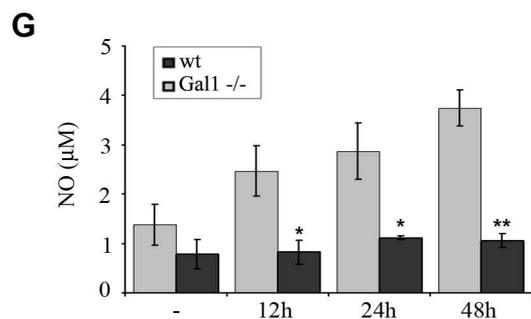
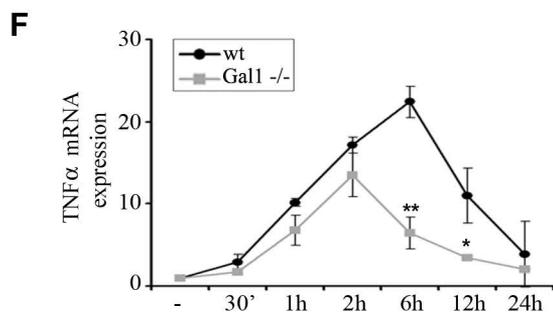
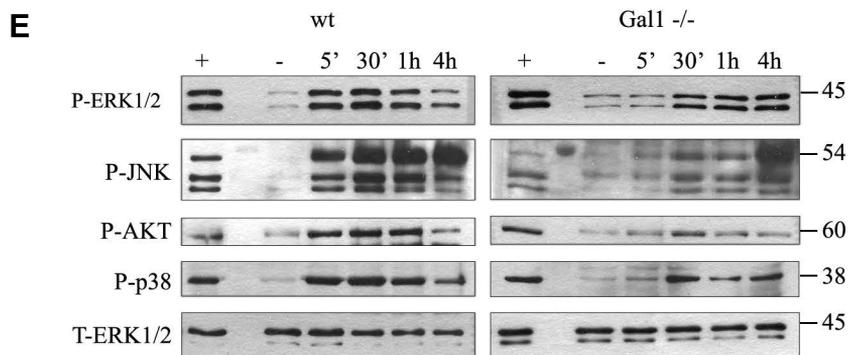
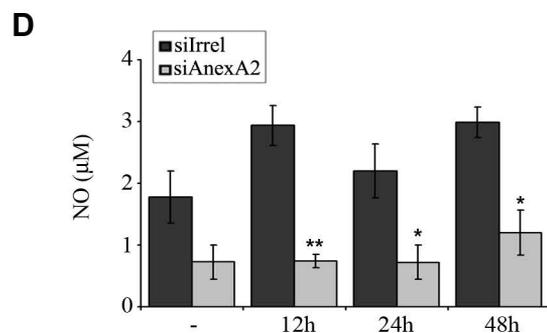
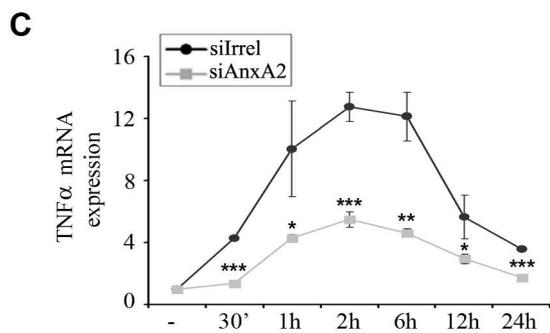
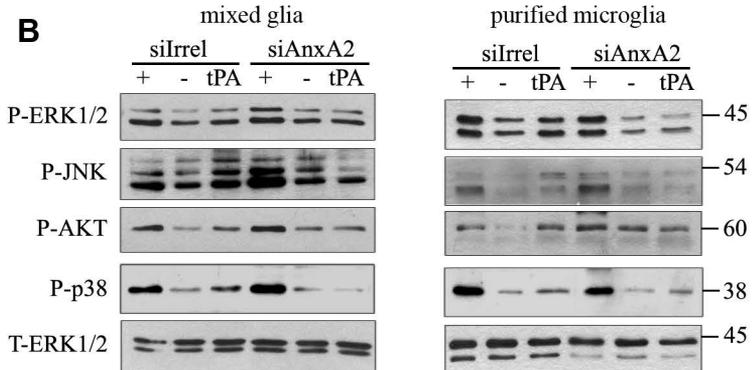
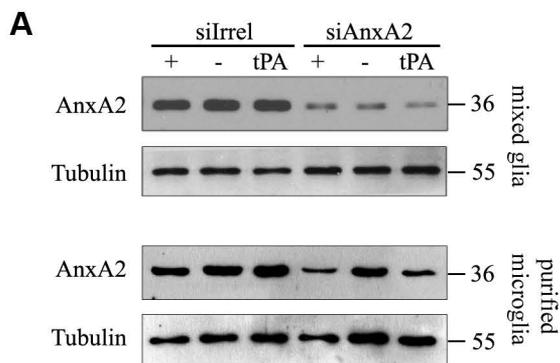


Figure 4

A**B****Figure 5**



Tissue plasminogen activator (tPA) induces microglial inflammation via a non-catalytic molecular mechanism involving activation of MAPKs and AKT signalling pathways and AnnexinA2 and Galectin-1 receptors.

David Pineda^{1, 2}, Coral Ampurdanés¹, Manel G. Medina¹, Joan Serratosa³, Josep Maria Tusell³, Josep Saura⁴, Anna M. Planas³ and Pilar Navarro^{1#}.

SUPPLEMENTARY INFORMATION

Figure S1.- Immunofluorescence analysis of mixed glia cultures. Microglia and astrocytes were detected by double immunofluorescence using Iba1 and GFAP as specific markers, respectively. Merge images show the enrichment in astrocytes (around 70%) found in our experimental conditions. Bar, 50 μ m.

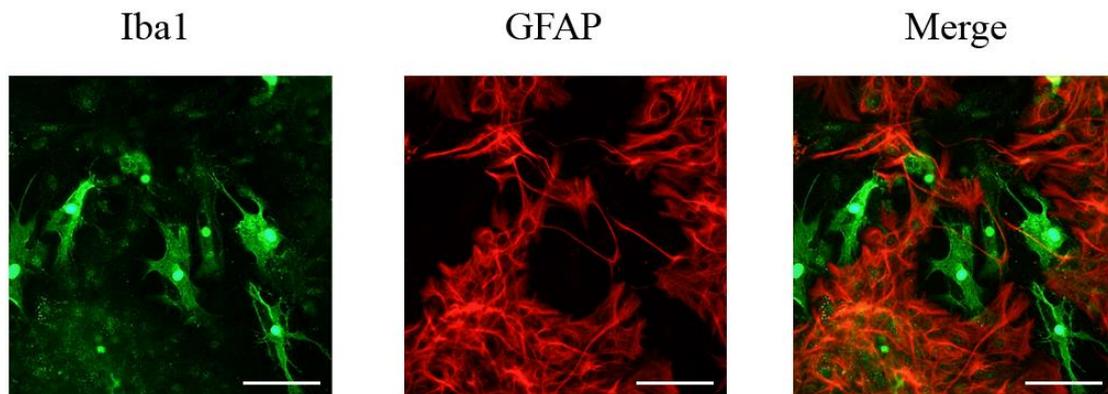


Figure S2.- Quantification of signalling pathways induced by tPA treatment of mixed glial and purified microglial cultures. Graphs show the densitometric analysis of activated P-ERK1/2, P-JNK and P-AKT versus the levels of total kinases (T-ERK, T-JNK and T-AKT, respectively) after treatment with 20 $\mu\text{g/ml}$ rtPA at the indicated times. Values are from three independent experiments (mean \pm s.d). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

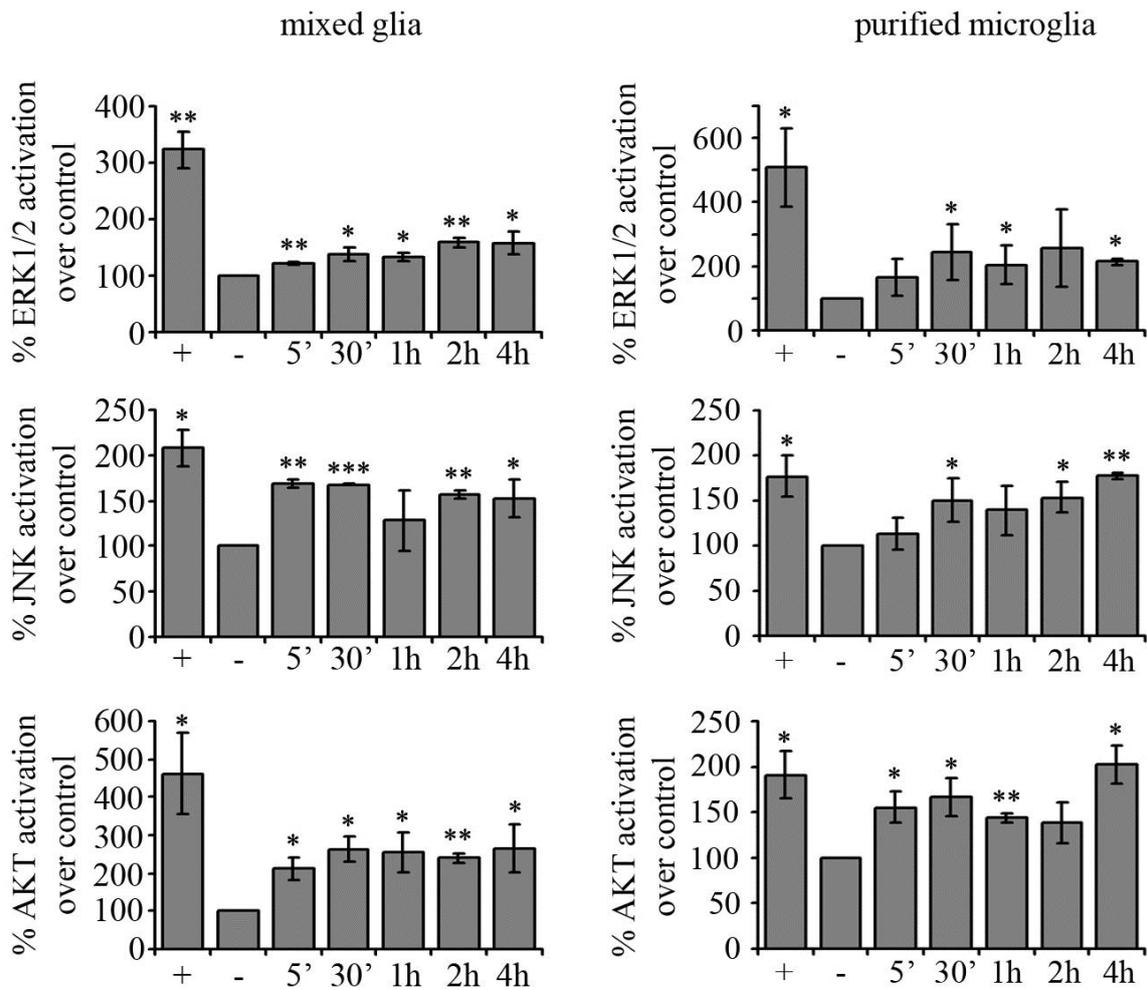


Figure S3.- tPA treatment induces nuclear translocation of ERK, JNK and AKT in mixed glia. (A) Double immunofluorescence of P-ERK, P-JNK or P-AKT and DAPI staining in mixed glial cell cultures treated with 20 $\mu\text{g/ml}$ of rtPA for 1h. Merge images show kinase internalization into the nuclei (B) Negative controls using rabbit IgG and mouse IgG as primary antibody and rabbit Alexa 488 and mouse Alexa 555 as secondary antibodies. DAPI staining is shown in the right for nuclei detection. Bar, 50 μm .

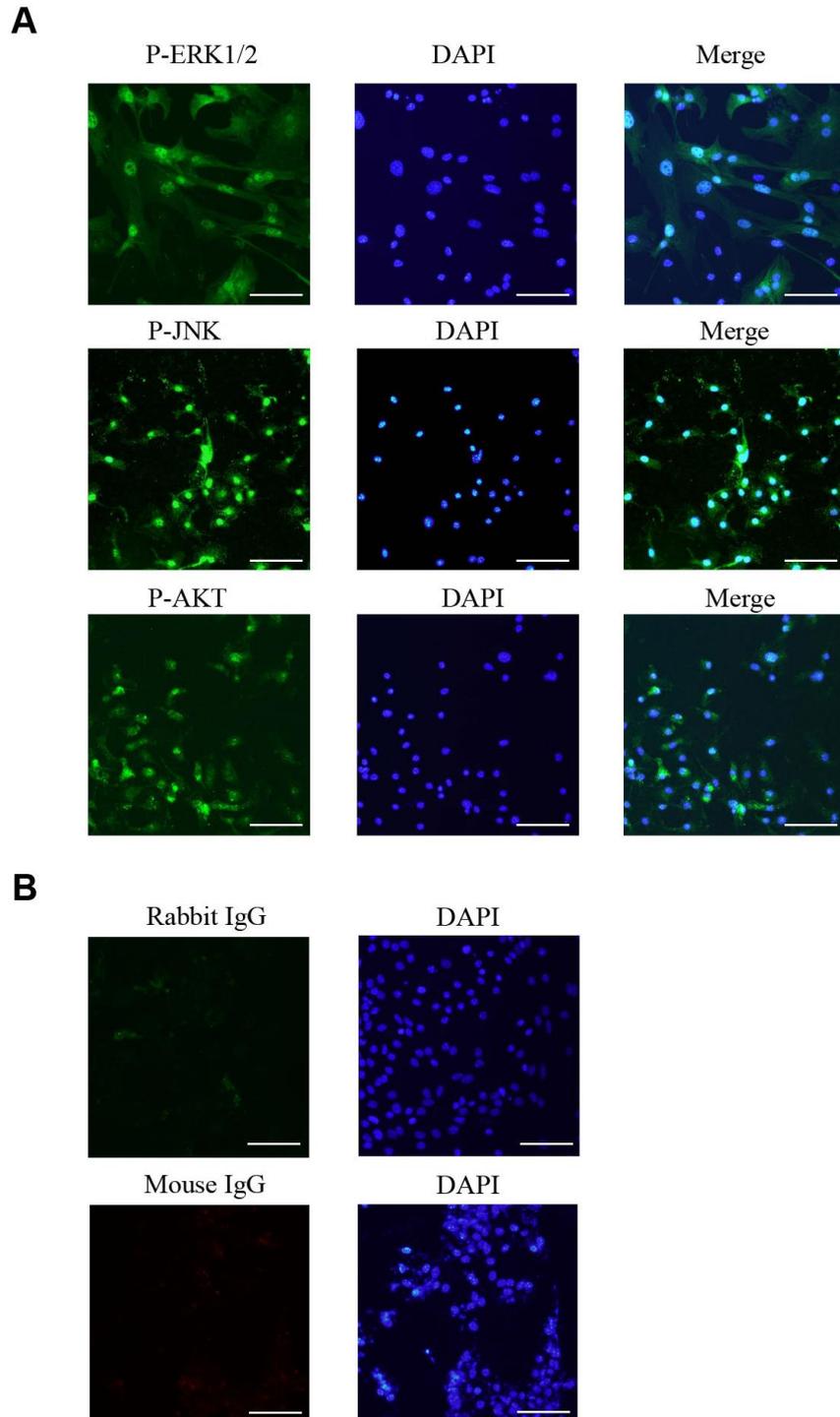


Figure S4.- Quantification of signalling pathways induced by catalytically inactivated mouse mutant tPA (S481A tPA) treatment of mixed glial and purified microglial cultures. Graphs show the densitometric analysis of activated P-ERK1/2, P-JNK, P-AKT and P-p38 versus the levels of its respective total kinases after treatment with 20 $\mu\text{g/ml}$ S481A tPA at the indicated times. Values are from three independent experiments (mean \pm s.d). * $p < 0.05$; ** $p < 0.01$.

