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Fang Wu, Emory University
Jialing Wu, Emory University
Andrew D. Nicholson, Emory University
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Marcela Catano, Emory University
Jie An, Emory University
Andrew K. Lee, Emory University
Duc Duong, Emory University
Eric B Dammer, Emory University

Only first 10 authors above; see publication for full author list.

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Tissue-Type Plasminogen Activator Regulates the Neuronal Uptake of Glucose in the Ischemic Brain

Fang Wu¹, Jialing Wu¹,², Andrew D. Nicholson³, Ramiro Echeverry¹, Woldeab B. Haile¹, Marcela Catano¹, Jie An¹,⁴, Andrew K. Lee⁵, Duc Duong⁵, Eric B. Dammer⁵, Nicholas T. Seyfried⁵, Frank C. Tong⁶, John R. Votaw⁷, Robert Medcalf⁸, and Manuel Yepes¹,⁹

¹Department of Neurology & Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, GA, USA

²Department of Neurology, Tianjin Huanhu Hospital, Tianjin, China

³Department of Radiology and Imaging Sciences, Emory University School of Medicine, Atlanta, GA

⁴Department of Pharmacology, Shandong University School of Medicine, Jinan, China

⁵Department of Biochemistry & Center for Neurodegenerative Disease, Emory University, Atlanta, GA

⁶Departments of Radiology and Neurosurgery, Emory University School of Medicine, Atlanta, GA

⁷Departments of Radiology and Physics, Emory University School of Medicine, Atlanta, GA

⁸Australian Center for Blood Diseases, Monash University, Melbourne Victoria, Australia

⁹Department of Neurology, Veterans Affairs Medical Center; Atlanta, GA, USA

Abstract

The ability to sense and adapt to hypoxic conditions plays a pivotal role in neuronal survival. Hypoxia induces the release of tissue-type plasminogen activator (tPA) from cerebral cortical neurons. We found that the release of neuronal tPA or treatment with recombinant tPA (rtPA) promotes cell survival in cerebral cortical neurons previously exposed to hypoxic conditions in vitro or experimental cerebral ischemia in vivo. Our studies using liquid chromatography and tandem mass spectrometry revealed that tPA activates the mammalian target of rapamycin (mTOR) pathway which adapts cellular processes to the availability of energy and metabolic resources. We found that mTOR activation leads to accumulation of the hypoxia-inducible factor-1α (HIF-1α) and induction and recruitment to the cell membrane of the HIF-1α-regulated neuronal transporter of glucose GLUT3. Accordingly, in vivo positron emission tomography studies with 18-fluorodeoxyglucose in mice overexpressing tPA in neurons show that neuronal tPA induces the uptake of glucose in the ischemic brain and that this effect is associated with decrease in the volume of the ischemic lesion and improved neurological outcome following the induction of ischemic stroke. Our data indicate that tPA activates a cell signaling pathway that allows neurons to sense and adapt to oxygen and glucose deprivation.

Address correspondence: Manuel Yepes, Department of Neurology and Center for Neurodegenerative Disease, Whitehead Biomedical Research Building, 615 Michael Street, Suite 505J, Atlanta, Georgia 30322. Telephone: (404) 712 8358. Fax: (404) 727 3728, mypes@emory.edu.

Fang Wu, Jialing Wu and Andrew Nicholson contributed equally to this work

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Introduction

Cerebral cortical neurons require a continuous supply of oxygen and glucose to meet their metabolic demands. Therefore, their survival depends on the activation of mechanisms to detect and adapt to low oxygen and glucose concentrations. The mammalian target of rapamycin (mTOR) is a protein kinase that integrates signals from different pathways to regulate cell survival and growth (Hay and Sonenberg, 2004). Hypoxia and cerebral ischemia cause a rapid inhibition of the mTOR pathway leading to growth arrest and neuronal death (Wouters and Koritzinsky, 2008). Moreover, mTOR activation in neurons has been linked to the development of plasticity (Jaworski and Sheng, 2006) and cell survival and recovery (Shi et al., 2011).

Hypoxia-inducible factor 1 (HIF1) is a transcription factor that plays a central role in hypoxia sensing and adaptation (Sharp and Bernaudin, 2004). HIF1 consists of two subunits, HIF1-α and HIF1-β. HIF1-β is constitutively expressed and does not respond to changes in oxygen tension, whereas HIF1-α is continuously made and rapidly degraded under normoxia (Sharp and Bernaudin, 2004). During hypoxia HIF1-α degradation is inhibited allowing its rapid accumulation and binding to hypoxia-responsive elements thereby activating the expression of hypoxia-responsive genes (Shi, 2009) many of which have a neuroprotective effect in the ischemic brain (Sheldon et al., 2009).

A family of membrane transporter proteins known as GLUTs regulates the passage of glucose across cell membranes. GLUT3 is the main transporter of glucose in neurons (Simpson et al., 2008). GLUT3 is found mostly in synaptic contacts in axons and dendrites and its expression is regulated by HIF1-α (Semenza, 2002) and increased by synaptic activity (Ferreira et al., 2011). The onset of cerebral ischemia is followed by a rapid decrease in the extracellular concentration of glucose in the brain accompanied by an induction in neuronal GLUT3 expression in an attempt to increase glucose transport and utilization. However, this rise in GLUT3 expression is transient and its subsequent decline is followed by neuronal death (Vannucci et al., 1996).

The serine proteinase tissue-type plasminogen activator (tPA) is abundantly expressed in neurons and endothelial cells. Membrane depolarization by hypoxia or cerebral ischemia induces the rapid release of tPA from endothelial cells into the intravascular compartment and from neurons into the synaptic space (Yepes et al., 2000; Yepes et al., 2003; Echeverry et al., 2010). In the intravascular space tPA has a beneficial thrombolytic effect associated with its ability to catalyze the conversion of plasminogen into plasmin. In contrast, the role of tPA released from neurons in response to a hypoxic/ischemic injury is still unclear.

The in vitro and in vivo studies presented here indicate that tPA activates a cell signaling pathway in the synaptic space via a plasminogen-independent mechanism that allows neurons to adapt to low oxygen and glucose concentrations. We found that the release of endogenous tPA or treatment with rtPA activates the mTOR pathway in neurons, leading to HIF-1α accumulation and increase in neuronal uptake of glucose via induction and recruitment to the cell membrane of the HIF-1α-regulated neuronal transporter of glucose GLUT3.

Materials & Methods

Animals and reagents

Murine strains were 8–12 weeks old males, wild-type (Wt) C57BL/6J, tPA deficient (tPA−/−) and plasminogen deficient (Plg−/−) mice, backcrossed at least seven generations into C57BL/6J mice, and their Wt C57BL/6J controls. We also used mice with a 10-fold
increase in tPA expression in neurons (Madani et al., 1999) and their Wt littermate controls (T4 mice, kindly provided by Professor JD Vassalli and Doctor R Mandani, University of Geneva, Switzerland). Experiments were approved by the Institutional Animal Care & Use Committee of Emory University, Atlanta GA. Recombinant murine tPA, inactive tPA (itPA) with an alanine for serine substitution at the active site Ser481 (S481A), human Lys plasmin, and an ELISA kit that detects active tPA were purchased from Molecular Innovations. Other reagents were human recombinant tissue-type plasminogen activator (Genentech Inc.), the phosphoinositide (PI) 3-kinase/Akt inhibitor Wortmannin, methanol, methyl salicylate and triphenyltetrazolium chloride (Sigma Aldrich), the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC), the LDH release assay (Roche), the Receptor-Associated Protein (RAP; kindly provided by Dr. Dudley K. Strickland, University of Maryland), the NMDAR antagonist MK-801 (Tocris Bioscience), rapamycin and the TrkB inhibitor K-252a (Calbiochem), HIF-1α shRNA, scramble shRNA lentiviral particles, anti-GLUT3 antibodies and TRITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), anti-HIF-1α antibodies (Abcam), antibodies against the p70S6 kinase phosphorylated at Thr389 (Cell Signaling), heparin sodium (Abraxis Pharmaceutical Products), blue latex (Connecticut Valley Biological Supply), ApopTag Plus Fluorescein in Situ Apoptosis Detection Kit (Chemicon International), 4'-6-Diamidino-2-phenylindole (DAPI; Invitrogen), triphenyltetrazolium chloride (TTC; Sigma-Aldrich), 2-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG; Molecular Probes), and 18-Fluorodeoxyglucose (PETNET).

Animal model of cerebral ischemia

The dorsal distribution of the middle cerebral artery, the intracerebral circulation and the presence of communications between the anterior (carotid) and posterior (basilar) circulation were studied in T4 mice and their Wt littermate controls (n=4) intravenously injected with blue latex and incubated in methyl salicylate as described elsewhere (Murakami et al., 1998). To induce cerebral ischemia, mice were anesthetized with 4% chloral hydrate (400 mg/kg/IP) and the middle cerebral artery (MCA) was exposed and occluded during 60 minutes with a 6-0 silk suture as described elsewhere (Belayev et al., 1999). Cerebral perfusion (CP) was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc., North Royalton, OH), and only animals with a > 70% decrease in CP after occlusion and complete recovery after suture removal were included in this study. Heart rate, systolic, diastolic and mean arterial blood pressure were controlled with an IITC 229 System (IITC-Lice Science; Woodland Hills, CA). One hour after reperfusion mice were treated with human recombinant tPA 1.0, or 4.5 or 9.0 mg/Kg/IV. Arginin was dialyzed from rtPA with a SnakeSkin Dialysis Tubing (ThermoScientific) prior to its administration. Before tMCAO a sub-group of mice was treated with rapamycin (50 µg IP) or 2 µl of wortmannin 0.1 mM injected at the following coordinates (Paxinos and Franklin, 2001): bregma: − 1 mm, mediolateral: 2 mm, dorsoventral: 2 mm. Twenty-four hours after tMCAO the volume of the ischemic lesion was measured in TTC-stained sections as described elsewhere (Swanson et al., 1990). Each observation was repeated 16 times.

Accelerating rotarod and forelimb grip-strength test

Animals were trained on days -3, -2, and -1 on a rotarod apparatus (LE 8500, Harvard Apparatus) during 30 s with no rotation and thereafter for 2 min with a constant low speed rotation (4 rpm). Before surgery and 6, 12 and 24 hours after tMCAO mice were tested on accelerating conditions from 4 to 40 rpm over 10 min. Each observation was repeated 3 times with an interval of 5 minutes between tests. The time each animal was able to walk on the rod before falling was collected before and at each time point after tMCAO. The forelimb grip-strength was measured using a grip-strength meter (Bioseb) before surgery and 6, 12 and 24 hours after tMCAO. Mice were allowed to grasp a smooth metal triangular
pull bar with their right forelimb and then pulled backward in the horizontal plane. The force applied to the bar at the moment when the grasp was released was recorded as the peak tension (in Newtons). Each test was repeated 5 times and the mean of all trials was recorded as the mean grip strength for that animal. For both tests values are given as a percentage compared to results obtained in the baseline evaluation.

Neuronal cultures, exposure to oxygen-glucose deprivation (OGD) conditions and determination of cell survival

Cerebral cortical neurons were cultured from E19 Wt and tPA−/− mice as described elsewhere (Echeverry et al., 2010). A sub-group of Wt neurons was infected with lentiviral particles with either scramble or HIF-1α shRNA. The efficiency of HIF-1α down-regulation was confirmed by RT-PCR analysis. To study the effect of hypoxia on neuronal survival/death, neurons were exposed in an anerobic chamber (Don Whitley Scientific) to oxygen-glucose deprivation (OGD) conditions (< 0.1% oxygen) for 55 minutes followed 24 hours later by determination of cell survival and death with MTT and LDH release assays, following manufacturer’s instructions. For the MTT assay, results are expressed as a percentage of cell survival in neurons exposed to OGD conditions compared to cells maintained under normoxic conditions. For the LDH release assay results are given as percentage of LDH released into the media compared to LDH released from neurons incubated 24 hours with triton 1%. Wt neurons were exposed to OGD conditions for 55 minutes, and either kept in the incubator for 24 hours or treated 5, 30, 60, 120, 180 or 360 minutes later for the remainder of the experiment with 5 nM of either active or itPA, or 10 nM of plasmin. A sub-group of cells was treated with a combination of 5 nM of tPA and either 10 µM of MK-801, or 60 nM of RAP, or 10 nM of rapamycin, or 20 nM of wortmannin, or 100 nM of the TrkB inhibitor K-252a. A sub-set of neurons infected with scramble or HIF-1α shRNA lentiviral particles was exposed to OGD conditions for 55 minutes, returned to the incubator, and treated 60 minutes later with 5 nM of tPA. To study the protective effect of hypoxic post-conditioning, cell survival was determined with the MTT assay in Wt and tPA−/− neurons exposed to 55 minutes of OGD conditions, returned to the incubator and then exposed 10 minutes later to a second episode of hypoxia (10 minutes OGD, post-conditioning event (PCE)). A sub-group of neurons was incubated with 5 nM of tPA, or 10 µM of MK-801, or 60 nM of RAP, or 20 nM of wortmannin, or 10 nM of rapamycin during the PCE. Each experiment was performed in cultures from three different animals and each observation was repeated 10–20 times. Cell survival/death was determined in each experiment 24 hours later with the MTT and LDH release assays.

TUNEL, HIF-1α and GLUT3 staining

The brains of T4 mice and their Wt littermate controls were harvested 24 hours after tMCAO, cut onto 20 µm sections and stained for TUNEL as described elsewhere (Wu et al., 2010). Observations were made in 3 previously described areas of interest (AOI) (Polavarapu et al., 2007), involving the fronto-parietal (AOI-1) and parieto-temporal (AOI-2) zones, as well as the necrotic core (AOI-3). Results are given as percentage of TUNEL-positive cells in relation to the total number of cells per field. Each observation was repeated 16 times. To study the expression of HIF-1α in the ischemic brain, we performed immunohistochemical analysis with an antibody against HIF-1α in 20 µm brain sections of T4 mice and their Wt littermate controls (n = 4) 1 hour after tMCAO. Observations were made in the 3 AOI described above. To study the expression of GLUT3, Wt cerebral cortical neurons were incubated with 5 nM of tPA during 0 – 3 hours, fixed and stained with a goat anti GLUT3 primary antibody (1:200) and a TRITC-conjugated donkey anti-goat IgG (1:500) for 1 hour at room temperature. Cells were then stained with DAPI and mounted onto a slide for microscopic view. Each observation was repeated 6 times.
**TPA activity assay**

Wt neurons were exposed to 55 minutes of OGD conditions, returned to the incubator, and then exposed 10 minutes later to a post-conditioning event (PCE, 10 minutes OGD). TPA activity was determined in the culture media after 1, 5 and 10 minutes of the onset of the PCE with an ELISA kit following manufacturer’s instructions. As controls, we measured tPA activity at identical time points in sister cultures exposed to OGD conditions without a ensuing PCE. Each experiment was performed with cultures from three different animals and each observation was repeated 8 times.

**Proteomics and Ingenuity Pathways Analysis**

Wt cerebral cortical neurons were incubated 1 hour with 5 nM of tPA or vehicle (control). At the end of tretment neurons were lysed and 50 µg of protein were reduced, resolved on a 10% polyacrylamide SDS gel and each sample was digested in-gel with trypsin. Extracted peptides were loaded onto a C18 column, eluted and sequenced (MS/MS) on an LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA) using data-dependent acquisition. All MS/MS files were searched against mouse database downloaded from the National Center for Biotechnology Information (September, 2009) using the SEQUEST Sorcerer algorithm (version 3.11, SAGE-N). Searching parameters included mass tolerance of precursor ions (±20 ppm) and product ion (±0.5 m/z), semi-tryptic restriction, with a dynamic mass shift for oxidized Met (+15.9949), four maximal modification sites and a maximum of two missed cleavages. To evaluate false discovery rate (FDR), all original protein sequences were reversed to generate a decoy database that was concatenated to the original database (70,368 total entries). To remove false positive matches, assigned peptides were grouped by a combination of trypticity (fully and partial) and precursor ion-charge state. Each group was first filtered by mass accuracy (10 ppm), and by dynamically increasing correlation coefficient (Xcorr) and ΔCn values to reduce protein FDR to less than 1 percent. If peptides were shared by multiple members of a protein family, the matched members were clustered into a single group in which each protein identified by a unique peptide represented a subgroup. Quantitative pair-wise comparison of control and tPA-treated samples was performed using the software DQUAN. The log₂ ratio averaged over all peptides for a particular protein was used to determine the protein expression ratio and a standard deviation. For Ingenuity Pathway analysis, log₂ (1h tPA-treated/control) average protein intensity ratios calculated by DQUAN were centered so that the fit gauss curve midpoint (mean) fell at zero. Log₂ values 1.63 standard deviations from the mean (changed with 95 percent confidence, with absolute value greater than 0.709) were considered as changing and these protein identities and quantifications were considered in the analysis. Resulting ingenuity canonical pathways significantly represented in the list of changing proteins were determined by Ingenuity as a p-value based upon the fraction of total proteins known to be linked to each pathway.

**Western blot analysis**

Wt cerebral cortical neurons were incubated 0 – 180 minutes with 5 nM of either active or inactive tPA, alone or in combination with 20 nM of wortmannin or 60 nM of RAP. Wt mice underwent MCAO and treatment with rtPA 4.5 mg/Kg/IV or a comparable volume of saline solution as described above. A sub-group of mice was treated before tMCAO with 2 µl of a 0.1 nM solution of wortmannin, injected into the third ventricle as the same coordinates described above. Wt and tPA−/− cerebral cortical neurons were exposed to 10 minutes of OGD (post-conditioning event) 10 minutes after exposure to 55 minutes of OGD conditions. As controls, cells were maintained under normoxic conditions or exposed to 55 minutes of OGD conditions without subsequent exposure to a post-conditioning event (10 minutes OGD). In each case extracts from cells and brain lysates were immunoblotted with antibodies against the p70S6 kinase (p70S6K) phosphorylated at Thr389 (1:1000 dilution).
Each observation was repeated 4 – 6 times. To study the expression of HIF-1α, Wt cerebral
cortical neurons were incubated with 5 nM of tPA for 0 – 180 minutes. A sub-set of samples
was co-treated with 10 nM of rapamycin. Wt mice underwent tMCAO and treatment one
hour after reperfusion with rtPA 4.5 mg/Kg/IV or a comparable volume of saline solution. In
each case extracts from cells and brain lysates were immunoblotted with antibodies against
HIF-1α (1:1000 dilution). To study GLUT3 expression Wt neurons were incubated with 5
nM of tPA followed 1, 3 or 6 hours later by membrane extraction with a MEM-PER
mammalian membrane protein extraction kit (Pierce Chemical Co., Rockford, IL). The
brains of Wt and T4 mice were harvested 0 – 3 hours after tMCAO. Lysates from membrane
extracts and brain tissue were immunoblotted with antibodies against GLUT3 (1:500
dilution). Each experiment was repeated 5 times.

Quantification of glucose uptake

To quantify the uptake of glucose in the ischemic brain Wt and T4 mice (n = 4) underwent
tMCAO. Thirty minutes after the onset of reperfusion animals were intravenously treated
with 18.5 MBq $^{18}$FDG, followed 45 minutes later by $^{18}$FDG PET imaging in a Siemens
Inveon micro PET/CT scanner (Siemens AG, Munich, Germany). Images with a spatial
resolution of 1.8 mm FWHM were acquired during 15 minutes and reconstructed in with an
OSEM iterative algorithm. To quantify differences in glucose uptake the differential uptake
ratio images were viewed in coronal sections and regions of interest (ROIs) were placed
over the ischemic and non-ischemic hemispheres. The uptake of $^{18}$FDG in each ROI was
recorded as nCi/cc and the differential uptake ratio (DUR) was calculated three separate
times in 6 slices per mouse using the following formula:

$$\frac{(\text{nCi/cc uptake in ischemic hemisphere}) - (\text{nCi/cc uptake in non–ischemic hemisphere})}{\text{nCi/cc uptake in ischemic hemisphere hemisphere}}$$

To quantify the uptake of glucose in vitro cerebral cortical neurons were cultured in bottom
clear black 96-well plates and incubated with tPA 5nM and the fluorescent deoxyglucose
analog 2-NBDG or with vehicle (control) during 0 – 180 minutes. At the end of each time-
point cells were washed with PBS and the fluorescence in each well was determined at
excitation and emission wavelengths of 485/20 and 528/20 nm, respectively. Values are
given as a mean fold increase in glucose uptake in neurons incubated with tPA in relation
with neurons incubated with vehicle (control) for an identical period of time. Each
experiment was repeated in cultures from three different mice and each experimental group
included 10–12 observations.

Statistical analysis

Values are expressed as percentage or mean ± SD when appropriate. Statistical tests
included the T-test followed by the Wilcoxon signed-ranked test and 2-way ANOVA for
comparisons between groups. P values of less than 0.05 were considered significant.

Results

Characterization of the cerebrovascular anatomy of mice overexpressing neuronal tPA

First we studied the cerebrovascular anatomy of Wt and T4 mice as described in the
Methods section. We found no difference in the dorsal and intracerebral distribution of the
major blood vessels or in the presence of anastomosis between the carotid and
vertebrobasilar systems between both strains of mice (Fig. 1A).
Neuronal tPA has a protective effect in the ischemic brain

Because our previous studies indicate that hypoxia and cerebral ischemia induce the release of tPA from cerebral cortical neurons (Echeverry et al., 2010) we decided to investigate whether this release of tPA has an effect on the ischemic brain. Wt and T4 mice (n = 16) underwent tMCAO followed 6 – 24 hours later by assessment of their performance in the accelerating rotarod test and quantification of the grip strength in the right forelimb (affected by the ischemic lesion). At the end of the last evaluation (24 hours after tMCAO) we measured the volume of the ischemic lesion and the percentage of apoptotic cells/field in each area of interest defined in the Materials and Methods section. We found that compared to their baseline evaluation, the strength in the right forelimb 6, 12 and 24 hours after tMCAO decreased in Wt mice to 61 +/− 11.38 %, 61 +/− 10% and 67 +/− 12 %, respectively, and remained unchanged in T4 animals (p < 0.05; Fig. 1B). Likewise, the performance in the accelerating rotarod at 6, 12 and 24 hours after tMCAO decreased in Wt mice to 55.75 +/− 14 %, 66.89 +/− 12 % and 67.93 +/− 26 % of their baseline, respectively, and remained unchanged in T4 mice (Fig. 1C, p < 0.05). Additionally, we found that compared to Wt littermate controls T4 mice have a 54.91% decrease in the percentage of apoptotic cells/field in the ischemic tissue (27.3 +/− 10.31 % in Wt and 12.3 +/− 8.1 % in T4 mice; p < 0.05; Fig. 1 D & E) and 38 % decrease in the volume of the ischemic lesion (78.90 +/− 9.51 mm$^3$ in Wt mice and 48.92 +/− 11.9 mm$^3$ in T4 mice, p < 0.05; Fig 1F).

Treatment with tPA after exposure to lethal hypoxic conditions or induction of cerebral ischemia promotes neuronal survival

Because our data suggest that tPA has a protective effect in the ischemic brain we decided to use the experimental design depicted in Fig 2A to study cell survival in Wt cerebral cortical neurons treated with 5 nM of tPA 5 – 360 minutes after exposure to 55 minutes of OGD conditions. We found that neuronal survival decreases from 100 +/− 1.1 % in neurons maintained under normoxic conditions to 49.87 +/− 3 % in cells exposed to OGD conditions without subsequent treatment with tPA. Surprisingly, treatment with tPA 5, 30, 60, 120, 180 or 360 minutes after exposure to OGD conditions increased cell survival to 70.8 +/− 2.2 %, 85.3 +/− 3%, 82.4 +/− 1.8 %, 71.6 +/− 1.22 %, 66.4 +/− 2.83 %, and 60.02 +/− 2.96 %, respectively (Fig 2B, n = 20, p < 0.05). To investigate whether this cell survival-promoting effect of tPA requires its proteolytic activity, we performed similar observations in neurons treated with 5 nM of proteolytically inactive tPA (itPA) or 10 nM of plasmin. We found that itPA but not plasmin promotes cell survival in neurons previously exposed to lethal hypoxia (Fig 2C). To confirm our observations with a different assay we used the same experimental design to quantify the release of LDH from Wt cerebral cortical neurons treated with proteolytically active or itPA 30 – 360 minutes after exposure to lethal hypoxia. We found that treatment with either active or itPA decreases the release of LDH into the media from neurons previously exposed to OGD conditions, and that as observed with the MTT assay, this protective effect is maximal when cells are treated 30 – 60 minutes after the hypoxic injury (Fig 2D, n= 20; p < 0.05).

Because in the central nervous system tPA interacts with the NMDAR (Nicole et al., 2001) and the low density lipoprotein receptor-related protein (LRP-1 (Yepes et al., 2003)), we quantified cell survival in Wt neurons exposed to OGD conditions and treated 1 hour later with 5 nM of tPA alone or in combination with either 10 µM of the NMDAR antagonist MK-801 or 60 nM of the receptor-associated protein (RAP, an inhibitor of the binding of members of the LDL receptor family to its ligands). We found that the protective effect of treatment with tPA is abrogated by antagonism of either the NMDAR or LRP1. Because experimental evidence indicate that brain-derived neurotrophic factor (BDNF) promotes neuronal survival (Numakawa et al., 2010) and that tPA cleaves BDNF into its active form (Pang et al., 2004), we performed similar experiments in neurons treated with 5 nM of tPA.
alone or in combination with 100 nM of K-252a (K-252a is an antagonist of the tyrosine kinase receptor B (TrkB), the cognate receptor for BDNF). We found that blockade of the BDNF receptor does not abrogate the protective effect of tPA (Fig 2E; n = 10 ; p < 0.05). To study whether treatment with rtPA also has a neuroprotective effect in vivo, we measured the volume of the ischemic lesion in Wt mice treated one hour after tMCAO with either 1.0 or 4.5 or 9 mg/Kg of rtPA or with a comparable volume of saline solution. We found that compared to animals treated with saline solution, the volume of the ischemic lesion decreases by 13.92+/− 4.4 %, 30.37 +/− 3.92 % and 31.36 +/− 4.6 % in mice treated with 1.0, 4.5 or 9.0 mg/Kg of rtPA, respectively (n = 12; p < 0.05 compared to mice treated with saline solution). To investigate if this protective effect of rtPA in vivo is mediated by tPA’s ability to cleave plasminogen we performed similar observations in Plg−/− mice treated one hour after tMCAO with either rtPA 4.5 mg/kg/IV or saline solution. Our data indicate that treatment with rtPA induces a 22.36 +/− 6% decrease in the volume of the ischemic lesion in Plg−/− mice (Fig 2F; n = 12, p < 0.05). Together, these results indicate that treatment with tPA promotes neuronal survival following exposure to lethal hypoxia or ischemia via a mechanism that does not require the conversion of plasminogen into plasmin but needs the engagement of the NMDAR and a member of the LDL receptor gene family.

The release of neuronal tPA promotes cell survival following exposure to hypoxic conditions

Hypoxic post-conditioning (HP) is a phenomenon whereby exposure to an episode of hypoxia that does not induce cell death (hereinafter referred to as post-conditioning event; PCE) confers neuroprotection to cells previously exposed to an otherwise lethal hypoxic injury (Zhao, 2009). Because the beneficial effect of HP requires the activation of endogenous neuroprotective pathways, we used an in vitro model of HP previously characterized in our laboratory to study whether the release of endogenous tPA also promotes cell survival in neurons previously exposed to lethal hypoxic conditions. In this model, depicted in the upper panel of Fig 3A, exposure to a post-conditioning event (10 minutes of OGD, PCE in Fig 3A) promotes neuronal survival when applied 10 minutes after exposure to an otherwise lethal hypoxic injury (55 minutes of OGD). First, we investigated whether the PCE has an effect on the release of neuronal tPA. Wt neurons were exposed to 55 minutes of OGD conditions and then returned to the incubator and either maintained under normoxic conditions for the remaining of the experiment or exposed 10 minutes later to a PCE. The concentration of tPA in the media was quantified with an ELISA at 1, 5 and 10 minutes after the onset of the PCE, or at similar time points in non post-conditioned neurons. We found that the release of tPA from non post-conditioned neurons is negligible. In contrast, the concentration of tPA in the culture media increased to 10.9 +/- 2.6 pg/ml, 21.49 +/- 2.16 pg/ml and 51 +/- 7.2 pg/ml one, five and ten minutes after the onset of the PCE, respectively (Fig 3A). Then we used this model to investigate whether the release of endogenous tPA during the PCE mediates the neuroprotective effect of HP. Wt and tPA−/− neurons were incubated 55 minutes under OGD conditions and then returned to the incubator and either maintained under normoxic conditions for the remaining of the experiment or exposed 10 minutes later to a PCE. The concentration of tPA in the media was quantified with an ELISA at 1, 5 and 10 minutes after the onset of the PCE, or at similar time points in non post-conditioned neurons. We found that the release of tPA from non post-conditioned neurons is negligible. In contrast, the concentration of tPA in the culture media increased to 10.9 +/- 2.6 pg/ml, 21.49 +/- 2.16 pg/ml and 51 +/- 7.2 pg/ml one, five and ten minutes after the onset of the PCE, respectively (Fig 3A). Then we used this model to investigate whether the release of endogenous tPA during the PCE mediates the neuroprotective effect of HP. Wt and tPA−/− neurons were incubated 55 minutes under OGD conditions and then returned to the incubator for the remaining of the experiment or exposed 10 minutes later to a PCE. A sub-group of neurons was incubated during the PCE with 10 μM of MK-801, or 60 nM of RAP (Wt neurons), or 5 nM of tPA (tPA−/− neurons). Cell survival was determined 24 hours later with the MTT assay. We found that neuronal survival decreased from 100 +/- 1.8% in control cells to 48 +/- 3% in cells exposed to OGD conditions without a subsequent PCE. In contrast, cell survival increased to 72.78 +/- 7.8% in Wt neurons exposed to a PCE 10 minutes after exposure to OGD conditions. However, the protective effect of the PCE was abrogated by genetic deficiency of tPA, or by treatment of Wt neurons with RAP or MK-801. Importantly, incubation with 5 nM of tPA rescued the protective effect of the PCE in tPA−/− neurons (Fig 3B; n = 15). Our data indicate that the release of neuronal tPA promotes survival in cells previously exposed to lethal hypoxia and that as observed
following treatment with rtPA, this effect also requires the engagement of the NMDAR and a member of the LDL receptor gene family.

**TPA activates the mTOR pathway in neurons**

Based on our data, we postulated that tPA activates a neuroprotective cell signaling pathway in cerebral cortical neurons. To test this hypothesis we used liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and quantitative analysis to identify protein changes in Wt cerebral cortical neurons incubated during 60 minutes with 5 nM of tPA or vehicle (control) as described in the Materials and Methods section. We found that compared to vehicle (control)-treated neurons treatment with tPA caused a greater than 50% decrease or increase in abundance in 383 and 206 proteins, respectively (Fig. 4A). Further studies with the Ingenuity Pathway Analysis tool showed that a significant number of these proteins belong to the mammalian target of rapamycin (mTOR) pathway (44 out of 201 proteins in this pathway were down-regulated and 9 proteins were up-regulated by treatment with tPA; p < 0.05). Interestingly, we observed that the greatest effect of tPA on this pathway was on the abundance of the transmembrane protein 127 (TMEM127; \(-4.8 \log_2\)).

Because TMEM127 is a recently described negative regulator of the mTOR pathway (Qin et al., 2010), we decided to investigate the effect of tPA on mTOR pathway activation. Wt cerebral cortical neurons were incubated with 5 nM of either active or inactive tPA followed by a Western blot analysis with antibodies against the p70S6 kinase (p70S6K) phosphorylated at Thr389 (p70S6K phosphorylation is an indicator of mTOR activation). Because Akt mediates the activation of the mTOR pathway and since our earlier studies indicate that tPA induces Akt phosphorylation via LRP1 (An et al., 2008), we performed similar analyses in Wt neurons treated with a combination of tPA and either 20 nM of wortmannin or 60 nM of RAP. We found that incubation with either active or inactive tPA induces mTOR activation in cerebral cortical neurons (Fig. 4B) and that this effect is mediated by activation of the PI3K/Akt pathway via a member of the LDL receptor family (Fig. 4C). Then we used the model of HP described above to study the effect of endogenous tPA on mTOR activation. We found that the application of a PCE induces mTOR activation in Wt but not in tPA−/− neurons (Fig. 4D). To investigate the effect of treatment with rtPA on mTOR activation in Wt mice we studied the expression of (p) p70S6K in the ischemic tissue of Wt mice treated 1 hour after tMCAO with rtPA alone or in combination with wortmannin. We found that treatment with rtPA induces mTOR activation in the ischemic brain and that this effect is abrogated by co-treatment with wortmannin (Fig. 4E). Together, our in vitro and in vivo studies indicate that treatment with rtPA or release of endogenous tPA induces mTOR activation in neurons.

**mTOR activation mediates the neuroprotective effect of tPA**

Because activation of the mTOR signaling pathway plays a pivotal role in the detection, adaptation and survival to hypoxic conditions (Wouters and Koritzinsky, 2008) we used the experimental design described in Fig 5A to investigate whether mTOR mediates the pro-survival effect of tPA in neurons previously exposed to lethal hypoxia. Wt cerebral cortical neurons were exposed to 55 minutes of OGD conditions followed by treatment with 5 nM of tPA, alone or in combination with 10 nM of rapamycin, a selective mTOR inhibitor, and determination of cell survival 24 hours later. Our results indicate that the cell survival promoting effect of treatment with tPA is abrogated by mTOR inhibition (Fig 5A, n = 15, p < 0.05).

To investigate whether the protective effect of endogenous tPA (Fig 3B) is also mediated by mTOR we studied cell survival in Wt neurons exposed to 55 minutes of OGD conditions followed by treatment with 10 nM of rapamycin, a selective mTOR inhibitor, and determination of cell survival 24 hours later. Our results indicate that the cell survival promoting effect of treatment with tPA is abrogated by mTOR inhibition (Fig 5A, n = 15, p < 0.05).
Our results indicate that exposure to OGD conditions decreases neuronal survival from 100 +/- 2.3 % to 52 +/- 3.8 %. In contrast, when neurons were exposed to a PCE cell survival increased to 76 +/- 2.8 %. However, this protective effect was abrogated by mTOR inhibition with rapamycin (54 +/- 4.6 % neuronal survival; Fig. 5B, n = 12, p < 0.05). To determine if the beneficial effect of treatment with rtPA in vivo is also mediated by mTOR activation we measured the volume of the ischemic lesion in Wt mice treated 1 hour after tMCAO with rtPA, alone or in combination with rapamycin 50 µg/IP. Our results indicate that treatment with rtPA induces a 34 +/- 8% decrease in the volume of the ischemic lesion (n = 10, p < 0.05) and that this effect is abrogated by co-treatment with rapamycin (Fig 5C, n = 12, p < 0.05).

**TPA induces mTOR-regulated HIF-1α activation in cerebral cortical neurons**

Because HIF-1α accumulation plays a central role in the adaptation of neurons to hypoxic and ischemic conditions (Semenza, 2002) we decided to investigate whether treatment with tPA has an effect on neuronal HIF-1α. Wt cerebral cortical neurons were incubated with 5 nM of tPA followed 0 – 180 minutes later by detection of HIF-1α expression with Western blot analysis. We found that treatment with tPA induces a progressive increase in HIF-1α accumulation in neurons (Fig 6A). To investigate the association between tPA-induced mTOR activation and tPA-induced HIF-1α accumulation in neurons we performed similar observations in Wt neurons following one hour of incubation with tPA alone or in combination with rapamycin. Our results indicate that the effect of tPA on neuronal HIF-1α is mediated by the mTOR pathway (Fig 6B). Then we studied the effect of genetic overexpression of neuronal tPA on cerebral ischemia-induced HIF-1α accumulation in vivo. Our immunohistochemical studies indicate that HIF-1α expression is virtually undetectable in the ischemic brain of Wt mice following reperfusion. In contrast, we detected in T4 mice a significant increase in HIF-1α accumulation in the areas surrounding the necrotic core in the parietal and frontal lobes (areas of interest 1 and 2) and in the center of the ischemic lesion (area of interest 3; Fig 6C). To investigate the effect of treatment with rtPA on HIF-1α accumulation in the ischemic tissue, we performed a Western blot analysis for HIF-1α expression in brain extracts from Wt mice treated with either rtPA or saline solution 1 hour after tMCAO. We found that treatment with rtPA but not saline solution induces HIF-1α accumulation in the ischemic tissue (Fig 6D).

**HIF-1α mediates the neuroprotective effect of tPA**

To investigate whether HIF-1α mediates the neuroprotective effect of tPA, Wt neurons left untreated or infected with lentiviral particles containing either scramble or HIF-1α shRNA were exposed to 55 minutes of OGD conditions and treated 1 hour later with 5 nM of tPA. Cell survival was quantified 24 hours later with the MTT assay. HIF-1α down-regulation was confirmed by RT-PCR analysis (data not shown). We found that cell survival decreased from 100 +/- 0.9% in controls neurons to 50 +/- 1.2 % in cells exposed to OGD conditions without subsequent treatment. Treatment with tPA 1 hour after exposure to OGD conditions increased neuronal survival in non-infected (76 +/- 2.8%) and scramble-treated neurons (76 +/- 2.8%), but not in cells with HIF-1α down-regulated with shRNA (51 +/- 2.2 % ; Fig 6E; n = 10, p < 0.05). Together, our data indicate that mTOR-regulated HIF-1α accumulation mediates the neuroprotective effect of tPA in cerebral cortical neurons.

**TPA induces the expression of GLUT3 in cerebral cortical neurons and increases the uptake of glucose in the ischemic brain**

Because the expression of the HIF-1α-regulated neuronal transporter of glucose GLUT3 has been linked to neuroprotection following exposure to hypoxic/ischemic conditions (Bergeron et al., 2000; Bernaudin et al., 2002; Sharp and Bernaudin, 2004), we studied the expression GLUT3 in Wt cerebral cortical neurons incubated with 5 nM of tPA. Our results...
indicate that tPA not only increases the expression of GLUT3 in cerebral cortical neurons (Fig 7A) but also induces its recruitment to the cell membrane (Fig 7B). To investigate the functional effect of tPA-induced expression of GLUT3 we measured the uptake of glucose in Wt cerebral cortical neurons treated 0 – 3 hours with 5 nM of tPA or vehicle control. We found that compared to vehicle- treated neurons treatment with tPA during 1 or 3 hours induces a 7.19 +/- 3.14 and 5.20 +/- 3.66 fold-increase in glucose uptake, respectively (n = 12; p < 0.05; Fig 7C).

To determine whether neuronal tPA has an effect on GLUT3 expression in vivo we performed a Western blot analysis for GLUT3 in brain extracts from Wt and T4 mice 0 – 3 hours after tMCAO. Our results show that neuronal overexpression of tPA is associated with an increase in GLUT3 expression in the ischemic brain (Fig 8A). Then we decided to investigate whether neuronal tPA also has an effect on glucose uptake in the ischemic brain in vivo. Wt and T4 mice underwent tMCAO followed 5 minutes later by the intravenous administration of 18FDG and quantification of glucose uptake by PET scanning imaging as described in the Methods section. We found that in Wt mice cerebral ischemia induces a decrease in the differential uptake ratio (DUR) of glucose from 1.01 +/- 0.02 % in non ischemic brains to – 18.4 +/- 5 % indicating a sharp decrease in the uptake of glucose in the ischemic hemisphere (p < 0.05). In contrast, the DUR in T4 mice was + 8.0 +/- 4.2 % demonstrating not only a lack of decrease but instead an increase in the uptake of glucose in the ischemic hemisphere associated with neuronal overexpression of tPA (Fig 8B; p < 0.05).

Discussion

A decline in the supply of oxygen and glucose induces the rapid release of tPA from cerebral cortical neurons in vitro (Echeverry et al., 2010) and in vivo (Wang et al., 1998; Yepes et al., 2000). However, a relation between tPA and neuronal survival during hypoxic and ischemic conditions remains controversial. Indeed, whereas some studies have proposed that tPA mediates the harmful effects of hypoxia and cerebral ischemia on neuronal death (Wang et al., 1998; Nagai et al., 1999), more recent evidence using different experimental designs indicates that tPA may instead have a neuroprotective effect (Flavin and Zhao, 2001; Lee et al., 2007; Echeverry et al., 2010; Haile et al., 2012).

Our results show that treatment with rtPA or the induction of the release of endogenous tPA promotes cell survival in neurons previously exposed to lethal hypoxia in vitro or cerebral ischemia in vivo via a mechanism independent of tPA’s ability to catalyze the conversion of plasminogen into plasmin. This observation is of significant clinical importance. Indeed, our data suggest that a large number of acute ischemic stroke patients that currently remain untreated due to the existence of contraindications to receive proteolytically active tPA as a thrombolytic may still benefit from the administration of proteolytically inactive tPA as a neuroprotector. Also important, our in vivo data indicate a protective effect with doses of rtPA significantly lower than those used so far in experimental studies with animal models of cerebral ischemia and instead very similar to the doses administered to treat acute ischemic stroke patients (Hacke et al., 2008).

Treatment with rtPA is associated with neurological recovery in a significant number of acute ischemic stroke patients. Interestingly, although it is believed that the recovery observed in rtPA-treated patients is entirely due to plasmin-mediated lysis of the clot, their improvement in neurological function is observed only 90 days later (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Hacke et al., 2008) suggesting that in addition to thrombolysis, other mechanisms may be responsible for rtPA’s beneficial effect. These observations are in agreement with our data indicating that tPA has a neuroprotective effect in the ischemic brain independently of its thrombolytic properties.
Our work is in apparent discrepancy with reports by others indicating that tPA has a neurotoxic effect in the ischemic brain (Wang et al., 1998; Nagai et al., 1999). We believe that this disagreement may be explained at least in part by differences between experimental designs. However, our previous work indicates that the concentrations of tPA used in some of these studies are highly unlikely to be found in vitro in neuronal cultures exposed to OGD conditions or in vivo in the ischemic brain following tMCAO, even after treatment with rtPA (Echeverry et al., 2010; Haile et al., 2012). Our finding that mice overexpressing tPA in neurons have a better neurological outcome following tMCAO than their Wt littermate controls not only supports our hypothesis that tPA has a neuroprotective effect in the ischemic brain but also disagrees with previous studies indicating that mice genetically deficient in tPA in all cell types also have a decrease in the volume of the ischemic lesion following the induction of experimental cerebral ischemia (Wang et al., 1998). We believe that this discrepancy may be explained by a pleiotropic cell type-specific role of tPA in the ischemic brain. Indeed, experimental work in animal models of cerebral ischemia (Yepes et al., 2003) and neuroradiological studies in acute ischemic stroke patients (Kidwell et al., 2008) have demonstrated that tPA also increases the permeability of the BBB with the development of cerebral edema and hemorrhagic transformation. Therefore, it is possible that the decrease in the volume of the ischemic lesion observed in tPA−/− mice is due to preservation of the barrier function of the BBB instead of a direct cell survival promoting effect associated with tPA deficiency. Our data is also in apparent conflict with earlier studies indicating that neuroserpin has a neuroprotective effect in the ischemic brain (Yepes et al., 2000; Cinelli et al., 2001). Despite the fact that neuroserpin has been considered the inhibitor of tPA in the CNS, it has also been shown that tPA activity remains unchanged in the brain of mice overexpressing neuroserpin (Madani et al., 2003) and that neuroserpin also protects neurons genetically deficient in tPA from the deleterious effects of a hypoxic injury (Wu et al., 2010). Together, these observations suggest that neuroserpin has a tPA-independent neuroprotective effect in the CNS.

The mTOR pathway plays a central role adapting cellular processes to the availability of energy and metabolic resources. Our proteomic studies show that tPA causes a significant down-regulation of the transmembrane protein-127 which was recently identified as a negative regulator of the mTOR pathway (Qin et al., 2010). In agreement with these observations we found that the release of endogenous tPA and treatment with rtPA are very efficient activators of the mTOR pathway in neurons via a plasminogen-independent mechanism. The clinical relevance of this finding is underscored by the observation that mTOR inhibition in the ischemic brain has been associated with neuronal death and mTOR activation in neurons has been linked to recovery after injury (Abe et al., 2010). Our previous studies showed that tPA is an efficient activator of the PI3K/Akt pathway (An et al., 2008). The work presented here shows that Akt activation mediates the effect of tPA on the mTOR pathway.

HIF-1α regulates the expression of genes that facilitate the adaptation to low oxygen concentrations. Despite the fact that HIF-1α is continuously made, it only accumulates in hypoxic cells (Sharp and Bernaudin, 2004). Indeed, in normoxic cells HIF-1α is continuously hydroxylated by the activity of prolyl-4-hydroxylases in the cytoplasm and nucleus, abrogating its intracellular accumulation. In contrast, hypoxia inhibits these hydroxylases leading to the rapid accumulation of HIF-1α in the cytoplasm. We found that tPA induces mTOR-regulated HIF-1α accumulation in neurons and that this effect does not require of hypoxic conditions. This hypoxia-independent effect on HIF-1α accumulation has also been observed with some growth factors and cytokines (Semenza, 2002).

GLUT3 mediates the transport of glucose in neurons and its expression is regulated by HIF-1α and induced by synaptic activity (Simpson et al., 2008) via NMDAR activation.
Our results indicate that tPA not only induces the expression of neuronal GLUT3 but also its recruitment to the cell membrane suggesting a direct effect of tPA on the uptake of glucose. This hypothesis is supported by our observation that either treatment with tPA in vitro or overexpression of neuronal tPA in vivo increases the uptake of glucose in cerebral cortical neurons.

In summary, our results indicate that tPA plays a pivotal role in the detection, adaptation and survival to hypoxic and ischemic conditions. Based on our data, here we propose a model where either endogenous tPA released in response to a hypoxic injury or rtPA intravenously administered following the onset of acute ischemic stroke promotes neuronal survival via mTOR-mediated HIF-1α-regulated increase in the uptake of glucose in neurons. This effect is independent of tPA’s ability to cleave plasminogen into plasmin and observed with doses of tPA not associated with hemorrhagic transformation in the ischemic brain.

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References


Figure 1. Neuronal tPA has a protective effect in the ischemic brain
A. Circle of Willis (upper row), dorsal middle cerebral artery (middle row) and intracerebral vasculature (lower row) in Wt and T4 mice. B & C. Mean change (percentage) compared to baseline evaluation in right forelimb strength (B) and performance in accelerating rotarod test (C) in Wt (white circles) and T4 mice (black triangles) 6, 12 and 24 hours after tMCAO. n = 16; * in B: p < 0.05 compared to Wt mice 6 hours after tMCAO. ** in B: p < 0.05 compared to Wt mice 12 hours after tMCAO. *** in B: p < 0.05 compared to Wt mice 24 hours after tMCAO. * in C: p < 0.05 compared to Wt mice 6 hours after tMCAO. ** in C: p < 0.05 compared to Wt mice 12 hours after tMCAO. *** in C: p < 0.05 compared to Wt mice 24 hours after tMCAO.
mice 24 hours after tMCAO. Lines denote SD. D & E. Micrographs of TUNEL staining in area of interest-2 (AOI-2) (D) and mean percentage of TUNEL-positive cells per field in the three AOI (E) in Wt and T4 mice 24 hours after tMCAO. Blue is DAPI; green is TUNEL. n = 16; * in E: p < 0.05 compared to Wt controls. F. Mean volume of the ischemic lesion and representative TTC-stained sections in T4 and Wt mice 24 hours after tMCAO. n = 16. Lines denote SD. * p < 0.05 compared to Wt mice.
Figure 2. Treatment with tPA promotes cell survival in cerebral cortical neurons exposed to hypoxic conditions and in the ischemic brain following tMCAO

A. Experimental design used to study the effect of tPA on neuronal survival. Letters denote time of treatment with tPA after exposure to oxygen-glucose deprivation (OGD) conditions. B–D. Mean cell survival (panels B & C) and release of LDH into the culture media (panel D) in Wt cerebral cortical neurons treated with 5 nM of proteolytically active (panels B & D) or inactive tPA (itPA; panels C & D), or 10 nM of plasmin (panel C), 5, or 30, or 60, or 120, or 180, or 360 minutes after exposure to 55 minutes of OGD conditions. n = 20 in B and 15 in C and D. * in B: p < 0.05 compared to neurons exposed to OGD conditions without subsequent treatment with tPA. * in C: p < 0.05 compared to neurons left untreated.
after exposure to OGD conditions. Ns: non-significant. * in D: p < 0.05 compared to cells exposed to OGD conditions without subsequent treatment. Lines denote SD. E. Mean cell survival in Wt cerebral cortical neurons exposed to 55 minutes of OGD conditions and treated 1 hour later with 5 nM of tPA, alone or in combination with either 10 µM of MK-801 or 60 nM of the receptor associated protein (RAP), or 100 nM of the tyrosine kinase receptor B (TrkB) inhibitor K-252a. n = 10. * p < 0.05 compared to neurons treated with tPA alone, or with a combination of tPA and K-252a. Lines denote SD. F. Mean volume of the ischemic lesion in Wt and Plg−/− mice treated one hour after tMCAO with saline solution (white bars) or rtPA 1 – 9 mg/Kg/IV (gray bars). n = 10 per group. * p < 0.05 compared to Wt mice treated with saline solution. ** p < 0.05 compared to mice treated with 1 mg/Kg/IV of rtPA. *** p < 0.05 compared to Plg−/− mice treated with saline solution. Bars depict mean volume of the ischemic lesion in mm³. Lines denote SD.
Figure 3. Endogenous tPA promotes cell survival in cerebral cortical neurons exposed to hypoxic conditions

A. Upper panel: model of hypoxic post-conditioning (HP). Lethal hypoxia indicates the time interval during which neurons are exposed to 55 minutes of OGD conditions. R1: recovery. Indicates the moment when neurons are returned to the incubator for 10 minutes. PCE: post-conditioning event. Denotes the time interval during which neurons are exposed to a second episode of OGD conditions (10 minutes). R2: recovery. Indicates the moment when cells are returned to the incubator before quantification of cell survival. Lower panel: mean concentration of active tPA in the culture media of Wt cerebral cortical neurons during the PCE. n = 8 per time point. * p < 0.05 and ** p < 0.05 compared to tPA concentration in the culture media of sister cultures not exposed to a PCE after lethal OGD. Lines denote SD.

B. Wt and tPA−/− cerebral cortical neurons were exposed to 55 minutes of OGD conditions and exposed 10 minutes later to a PCE in the presence of either 10 mM of MK-801, or 60 nM of RAP, or 5 nM of tPA, or vehicle (control) followed by determination of cell survival 24 hours later. Lines denote SD. n = 15 per experimental group. * p < 0.05 compared to non post-conditioned Wt neurons, or with post-conditioned in the presence of RAP or MK-801. ** p < 0.05 compared to tPA−/− neurons post-conditioned in absence of recombinant tPA.
**Figure 4. tPA induces activation of the mTOR pathway in cerebral cortical neurons**

A. Histogram analysis of the population distribution of quantified proteins in cerebral cortical neurons treated with 5 nM of tPA or vehicle (control). Thin traces represent theoretical Gaussian distributions (Gauss1) that fit the raw data representing 92% of proteins within the population. The dashed Gaussian 2 (Gauss2) traces represent a unique subset of proteins that decrease in the tPA treated sample compared to control (shaded area). The sum of the two Gaussian distributions is represented by the thick traces (2 Gauss Fit).

B. & C. Representative Western blot analysis with an antibody that detects p70S6K phosphorylated at Thr389 ((p) p70S6K) in cell extracts from Wt cerebral cortical neurons left untreated (C, control) or incubated 5 – 180 minutes with 5 nM of either active tPA or proteolytically inactive tPA (itPA) (B) or during 1 hour with a combination of 5 nM of tPA and either 20 nM of wortmannin (Wort) or 60 nM of RAP (C). D. Representative Western blot for (p) p70S6K expression in cell extracts from Wt cerebral cortical neurons exposed to 55 minutes of OGD conditions and either returned to the incubator for the remaining of the experiment or exposed 10 minutes later to hypoxic post-conditioning (HP, 10 minutes OGD). E. Representative Western blot analysis for (p) p70S6K expression in brain extracts from Wt mice 1 hour after tMCAO and the intravenous administration of rtPA alone or in combination with wortmannin (Wort).
Figure 5. mTOR activation mediates the neuroprotective effect of tPA
A. The upper panel describes the experimental design used to investigate whether mTOR pathway activation mediates the neuroprotective effect of tPA. The lower panel indicates mean cell survival in Wt cerebral cortical neurons incubated with 5 nM of tPA alone or in combination with 10 nM of rapamycin 30 (A), or 60 (B), or 180 (C), or 360 (D) minutes after exposure to 55 minutes of OGD conditions. n = 15 per experimental condition. * p = < 0.05 compared to cultures treated with a combination of tPA and rapamycin at similar time points after OGD, respectively. Lines denote SD.

B. Mean cell survival in Wt cerebral cortical neurons subjected to HP alone or in the presence of 10 nM of rapamycin, 10 minutes
after exposure to 55 minutes of OGD conditions. n = 12. * p < 0.05 compared to cells preconditioned in absence of rapamycin. Lines denote SD. C. Wt mice underwent tMCAO followed 1 hour later by the intravenous administration of either saline solution (black bar), or rtPA (white bar), or a combination of rtPA and rapamycin (RAPA; gray bar). The volume of the ischemic lesion was determined 24 hours later as described in Methods. n = 12. * p < 0.05 compared to mice not treated with rtPA after tMCAO. ** p = 0.03 compared to mice treated with rtPA alone.
Figure 6. TPA promotes neuronal survival via mTOR-regulated HIF-1a activation

A. Representative Western blot analysis for HIF-1a expression in extracts from Wt cerebral cortical neurons incubated 5 – 180 minutes with 5 nM of tPA. B. Representative Western blot analysis for HIF-1a expression in extracts from Wt cerebral cortical neurons incubated 60 minutes with 5 nM of tPA alone or in combination with 10 nM of rapamycin. C. Representative micrographs of HIF-1α expression in the fronto-parietal area of the ischemic tissue of T4 mice and their Wt littermate controls 1 hour after tMCAO and complete reperfusion. Magnification 40X. Blue is DAPI, red is HIF-1α. D. Representative Western blot analysis for HIF-1α expression in brain extracts from the ischemic tissue of Wt mice treated with wither rtPA or a comparable volume of saline solution 1 hour after tMCAO and complete reperfusion. E. Mean cell survival in Wt cerebral cortical neurons left uninfected, or infected with lentiviral particles containing either HIF-1α shRNA or scramble shRNA and treated with 5 nM of tPA one hour after exposure to 55 minutes of OGD conditions. n = 8 per condition. Lines denote SD. * p < 0.05 compared to cell survival in Wt neurons either uninfected (white bar) or treated with scramble shRNA (gray bar).
Figure 7. TPA induces the expression of GLUT3 and the uptake of glucose in cerebral cortical neurons

A. Representative micrographs of GLUT3 staining in Wt cerebral cortical neurons incubated during 3 hours with 5 nM of tPA (tPA +) or vehicle (control; tPA −). Blue is DAPI and red is GLUT3. Magnification is 20X in a – e and 100 X in e – h.

B. Representative Western blot analysis for GLUT3 expression in membrane extracts from cerebral cortical neurons incubated 0 – 6 hours with 5 nM of tPA.

C. Mean fold increase in the uptake of a fluorescent deoxyglucose analog (2-NBDG) in cerebral cortical neurons following 0 – 3 hours of treatment with 5 nM of tPA. Data is presented as mean fold increase in glucose uptake in neurons treated with tPA in relation with glucose uptake in neurons treated with vehicle.
(control) for similar periods of time. n = 12 per experimental condition. * p < 0.05 compared to neurons treated with vehicle control during 1 hour. ** p < 0.05 compared to neurons treated with vehicle control for 3 hours.
Figure 8. TPA induces the expression of GLUT3 and the uptake of glucose in cerebral cortical neurons

A. Representative Western blot analysis for GLUT3 expression in the ischemic tissue of Wt and T4 mice 1 hour after tMCAO. B & C. Representative pictures of ¹⁸FDG-PET scan imaging (B) and quantification of the Differential Uptake Ratio (DUR) of glucose (C) in Wt and T4 mice 1 hour after tMCAO and the intravenous injection of 18-Fluorodeoxyglucose. Arrows in B denote the area of the brain affected by the ischemic injury. n = 12 observations per animal. * p < 0.05 compared to non ischemic brains; ** p < 0.05 compared to nonischemic brains and with brains from Wt mice.