THE ROLE OF THE PLASMINOGEN ACTIVATING SYSTEM IN NEUROBIOLOGY

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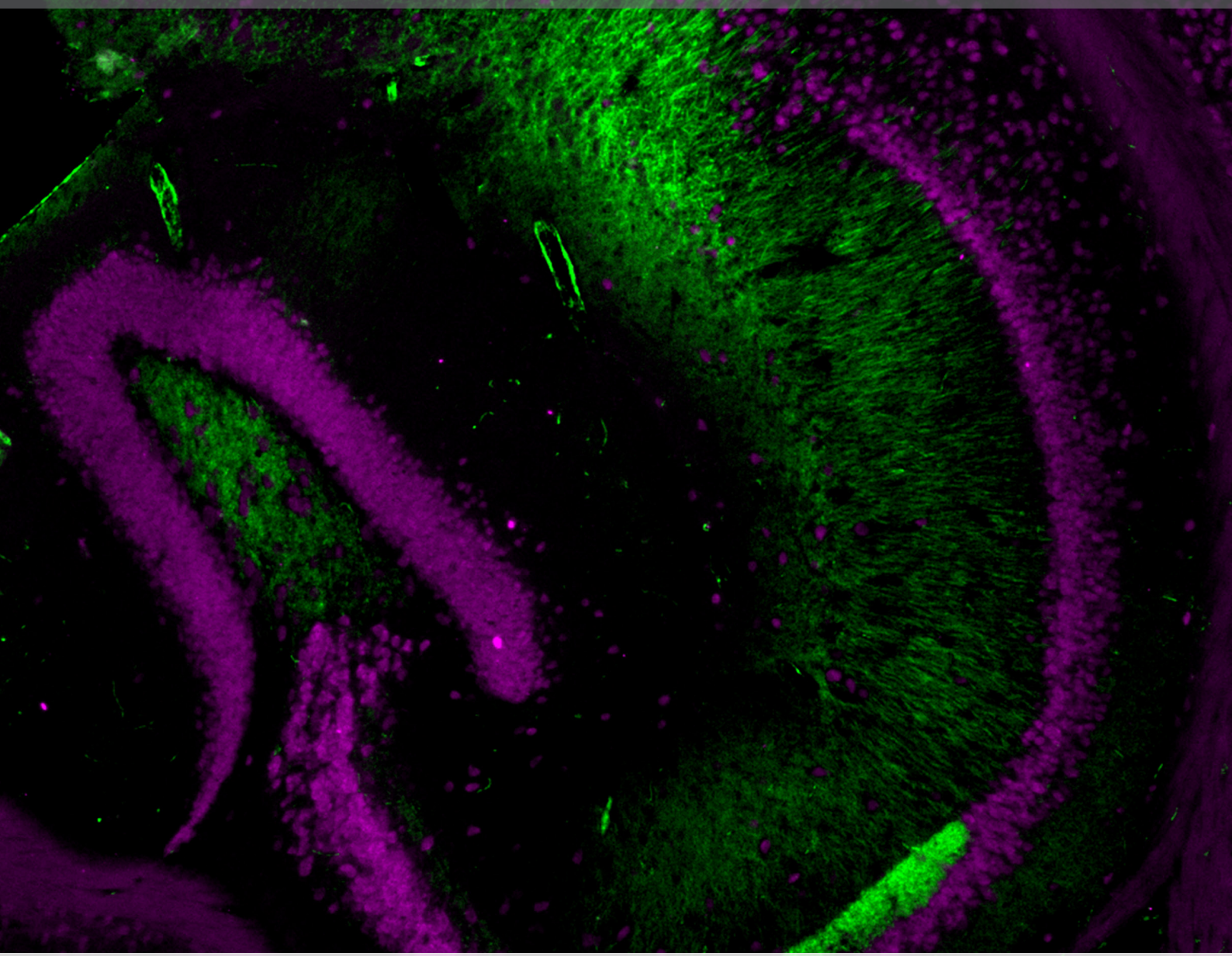

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THE ROLE OF THE PLASMINOGEN ACTIVATING SYSTEM IN NEUROBIOLOGY

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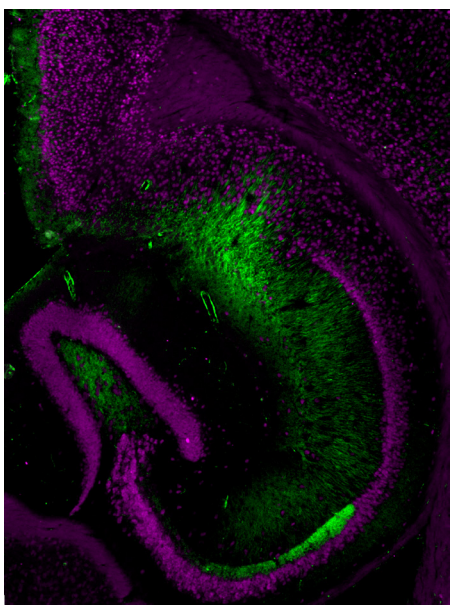

Horizontal cross-section of the murine hippocampus and entorhinal cortex. Section was stained with antibodies to the neuronal marker NeuN (magenta) and tissue-plasminogen activator (tPA, green). tPA expression is present in the hilus, the mossy fiber projections that terminate in stratum lucidum, and stratum radiatum. Prominent tPA expression is also noticeable in vessels, such as those intensely stained for tPA in the hippocampal fissure.
Image by Tamara K Stevenson.

This ebook contains a series of original publications, reviews and mini-reviews by leaders in the field that address the growing importance of the plasminogen activating system in neurobiology. The articles included cover the role of the plasminogen activating system as a key modulator of blood brain barrier permeability, and the implications of this in traumatic brain injury and in ischemic stroke. State-of-the-Art manuscripts are also included that address the regulatory mechanisms that control this important process.

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Table of Contents

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EDITORIAL
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Editorial: The Role of the Plasminogen Activating System in Neurobiology

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Keywords: plasminogen, plasminogen activators, blood brain barrier, ischaemic stroke, traumatic brain injury (TBI), neurotoxicity, neuroprotection

The Editorial on the Research Topic

The Role of the Plasminogen Activating System in Neurobiology

The plasminogen activating system has been well-appreciated for its roles in fibrinolysis and metastatic cancer for over 30 years. These observations lead to the clinical development of the key plasminogen activators, namely urokinase (u-PA), and tissue-type plasminogen activator (t-PA) as thrombolytic agents, initially for myocardial infarction in the mid-1980's, and a decade later for use in patients with ischaemic stroke following the approval of tPA (Ninds, 1995). Similarly various attempts were made to modulate cell surface plasminogen activation in an effort to reduce metastatic spread with varying success, although various components of this system have become biomarkers for some malignancies (McMahon and Kwaan, 2015).

While many laboratories continue to work in these classical areas, and with due reason, a growing list of publications dating from the early 1980's revealed that the main components of the plasminogen activating system were expressed in almost all cell types and were regulated by agonists linked to almost all signal transduction pathways identified (Medcalf, 2007). While these reports were consistent with a broadening role of the plasminogen activating system in physiology, other findings also from the early 1980's reported strong expression of components of the plasminogen activating system in the central nervous system (Krystosek and Seeds, 1981; Soreq and Miskin, 1981). While these were largely descriptive studies, and without any clear connection to conventional fibrinolysis or metastatic cancer, speculation arose as to the role of the plasminogen activating system in the CNS (Yepes and Lawrence, 2004), particularly given the fact that the normal brain is devoid of fibrin. A decade or so later, CNS focused reports of activity dependent expression of t-PA in the brain added substantial fuel to notion of a critical role for t-PA in normal brain function, with increases in t-PA gene expression in the CNS correlated with long term potentiation (Qian et al., 1993; Huang et al., 1996); and motor learning (Seeds et al., 1995). Soon after, reports using t-PA deficient mice provided evidence for surprising neurotoxic effects of t-PA where t-PA, via plasmin was shown to be necessary to facilitate glutamate-mediated toxicity *in vivo* (Chen and Strickland, 1997). These reports were published at about the same time that t-PA was approved for therapeutic use in patients with ischemic stroke and raised concerns with the clinical use of t-PA given the fact t-PA administration in ischemic stroke was not risk-free.

It soon became apparent that t-PA was influencing numerous other aspects of brain function including modulation of memory (Huang et al., 1996) and learning (Seeds et al., 2003) and the response to drugs of addiction (Pawlak et al., 2005; Bahi and Dreyer, 2008; Maiya et al., 2009). Another landmark discovery made in the early 2000's reported a potent effect of t-PA at promoting

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BBB disruption in rodent models of cerebral ischemia (Yepes et al., 2003), an effect that has since been documented in a subset of human stroke patients who receive thrombolysis (Kidwell et al., 2008). This further added to the debate of t-PA as a safe thrombolytic in patients with ischaemic stroke. The enhancing effect of t-PA on BBB permeability not only directed many laboratories to uncover the mechanism behind this (Su et al., 2008; Niego et al., 2012), but also raised interest in other areas of brain pathology where BBB integrity was compromised, namely in traumatic brain injury (TBI, Mori et al., 2001). Initial research into the role of t-PA at influencing outcome following TBI resulted in a number of publications supporting the notion that brain-derived t-PA, as opposed to exogenous t-PA (as in ischemic stroke), was also promoting BBB permeability and subsequent deleterious outcome following TBI (Sashindranath et al., 2012; Su et al.). It soon became apparent that t-PA was indeed a major modulator of BBB permeability (Niego and Medcalf, 2014), even under non-ischemic or traumatic conditions (Fredriksson et al., 2016).

With the realization of these various roles of t-PA in the CNS, questions arose as to how t-PA was implementing these effects and how it was being regulated. t-PA modulating agents i.e., neuroserpin (Lebeurrier et al., 2005), critical signaling systems i.e., tyrosine kinase (Su et al., 2008), and Rho kinase pathways (Niego et al., 2012), and receptors i.e., LRP-1 (Yepes et al., 2003; Samson et al., 2008), and PDGFRα (Fredriksson et al., 2004) in the CNS were later identified by various groups to participate in this new frontier of plasminogen activation biology. Although these findings pushed the field further, controversy also arose. Conflicting reports on how t-PA promoted neurotoxicity (Nicole et al., 2001; Matys and Strickland, 2003; Samson et al., 2008), or its opposite effect (i.e., neuroprotection) via non-proteolytic means (Kim et al., 1999), or proteolytically at low concentrations (Echeverry et al., 2010; Wu et al., 2012) continued to pepper the literature, particularly in recent years (Yepes). The diverse reach of the plasminogen activators in the brain also posed the question as to whether there was a common mechanistic element behind these various, seemingly unrelated events (Fredriksson et al., 2016).

This themed issue of *Frontiers in Cellular Neuroscience* entitled "The role of the plasminogen activating system in Neurobiology" contains 12 contributions from key scientists in this field that includes topics ranging from basic neurobiology, ischaemic stroke, and TBI. Data is presented to implicate t-PA in neurovascular development, how parallel protease systems (i.e., the MMPs) may participate in some aspects of t-PA's effects in the CNS, novel approaches to attenuate t-PA mediated BBB permeability in TBI, and new insights into the biology of the major brain t-PA inhibitor, neuroserpin. We have endeavored to cover areas of controversy, particularly in relation to the purported roles of t-PA at promoting both neurotoxicity and neuroprotection while at the same time include state-of-the-art reviews, including the insights as to how the coagulation and the fibrinolytic systems can modulate the neurovascular unit and how this can in turn have an impact on the immune response.

This themed issue also includes clinical and basic science perspectives which are likely to seed further innovation to future research in this field. At the time of writing this Editorial, these 12 articles have amassed over 15,000 article views and nearly 3000 downloads within ∼14 months since publication, providing clear evidence that this particular topic continues to be vibrant, appealing, and important. The relatively recent entry of the plasminogen activator into the field of neurobiology has certainly been an eye-opener. As technology ultimately advances, it is almost certain that the subsequent years will not only uncover novel mechanistic insights into the how the plasminogen activating system functions in the CNS, but it will also uncover important roles for this enzyme system in other key areas of neurobiology.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Imatinib treatment reduces brain injury in a murine model of traumatic brain injury

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Current therapies for Traumatic brain injury (TBI) focus on stabilizing individuals and on preventing further damage from the secondary consequences of TBI. A major complication of TBI is cerebral edema, which can be caused by the loss of blood brain barrier (BBB) integrity. Recent studies in several CNS pathologies have shown that activation of latent platelet derived growth factor-CC (PDGF-CC) within the brain can promote BBB permeability through PDGF receptor α (PDGFRα) signaling, and that blocking this pathway improves outcomes. In this study we examine the efficacy for the treatment of TBI of an FDA approved antagonist of the PDGFRα, Imatinib. Using a murine model we show that Imatinib treatment, begun 45 min after TBI and given twice daily for 5 days, significantly reduces BBB dysfunction. This is associated with significantly reduced lesion size 24 h, 7 days, and 21 days after TBI, reduced cerebral edema, determined from apparent diffusion co-efficient (ADC) measurements, and with the preservation of cognitive function. Finally, analysis of cerebrospinal fluid (CSF) from human TBI patients suggests a possible correlation between high PDGF-CC levels and increased injury severity. Thus, our data suggests a novel strategy for the treatment of TBI with an existing FDA approved antagonist of the PDGFRα.

Keywords: traumatic brain injury, TBI outcome, blood brain barrier, platelet derived growth factor-CC, platelet derived growth factor receptor α, Imatinib, cerebral edema, Morris water maze

Introduction

The Centers for Disease Control estimate that every year in the United States approximately 2.5 million people sustain a Traumatic brain injury (TBI). There are approximately 53,000 TBI related deaths and 283,000 hospitalizations annually, with many patients suffering permanent disability (Frieden et al., 2014). Additionally, TBI is a contributing factor in nearly a third of all injury-related deaths in the United States and is a leading cause of death in North America for individuals between the ages of 1–45 (Rutland-Brown et al., 2006; Hemphill and Phan, 2013b; Byrnes et al., 2014). TBI also accounts for more lost work time than cancer and

cardiovascular diseases combined (Thurman et al., 1999; Ma et al., 2014). Over the past two decades our understanding of the complex pathobiology of TBI has improved significantly. However, despite numerous studies in animal models of TBI and clinical trials of various therapeutic strategies, no effective therapy for TBI patients has emerged (Grumme et al., 1995; Marmarou et al., 1999; Bramlett and Dietrich, 2004; Yurkewicz et al., 2005; Maas et al., 2006, 2010). The pathophysiology of TBI is complex and involves both primary and secondary insults (Hemphill and Phan, 2013b; Finnie, 2014). Primary injury to the brain can be induced by numerous mechanisms, such as brain contusion, hematoma, and shearing or stretching of the brain tissue caused by motion of the brain structures relative to the skull. Secondary injury development includes multifaceted biochemical and physiological processes that are initiated by the primary insult and manifest over a period of hours to days and even months (Cernak, 2005; Finnie, 2014; Logsdon et al., 2015).

The lack of effective pharmacological treatments for TBI patients despite the many clinical trials in the past two decades suggests that the development of improved therapies for the treatment of TBI will depend upon a better understanding of the underlying mechanisms that drive secondary neuronal injury during the acute phase of TBI. One of the most serious and difficult to control secondary effects of TBI is the development of cerebral edema. Cerebral edema leads to brain swelling and increased intracranial pressure (ICP), which in severe cases can result in cistern compression, brain herniation, and even death. The causes of edema in TBI patients are complex but it is well appreciated that the loss of the blood brain barrier (BBB) is a significant factor in the development of vasogenic edema (Chodobski et al., 2011). Our recent studies, and those of others, have shown that signaling through the PDGF receptor α (PDGFRα) in the neurovascular unit (NVU) can promote BBB permeability and neuronal injury in several different neuropathological settings, including ischemic and hemorrhagic stroke, spinal cord injury, MS, and seizures (Su et al., 2008; Ma et al., 2011; Abrams et al., 2012; Adzemovic et al., 2013; Fredriksson et al., 2015). In our previous studies of ischemic stroke we have found that the protease tissue-type plasminogen activator (tPA) induces opening of the BBB through proteolysis of latent platelet derived growth factor-CC (PDGF-CC), generating an active form of PDGF-CC that binds to the PDGFRα and induces cell signaling (Su et al., 2008). The PDGFRα is localized to astrocytes in the NVU (Su et al., 2008; Fredriksson et al., 2015), and blocking this pathway with either the PDGFRα antagonist Imatinib or neutralizing antibodies to PDGF-CC reduces BBB dysfunction and improves outcome after ischemic stroke (Su et al., 2008). Similar results have been obtained by blocking PDGFRα signaling in animal models of hemorrhagic stroke, spinal cord injury, MS, and seizures (Ma et al., 2011; Abrams et al., 2012; Adzemovic et al., 2013; Fredriksson et al., 2015). These latter studies suggest that blocking PDGFRα signaling may provide benefit in diverse CNS pathologies through protection of the BBB. Consistent with this suggestion our recent work indicates that tPA can promote post-traumatic cerebrovascular damage including increased BBB leakage (Sashindranath et al., 2012). However, it is not known whether the PDGFRα pathway also plays a role in TBI-related injuries.

In the study presented here we used two versions of a well-established mouse model of TBI, controlled cortical impact (CCI; Sinz et al., 1999; Gilmer et al., 2008; Loane et al., 2009) to demonstrate for the first time that Imatinib treatment after TBI reduces BBB opening and significantly improves outcomes. Our data suggest that PDGF signaling contributes to the development of vasogenic edema by increasing BBB opening after TBI and that both vasogenic edema and cognitive impairment can be reduced by Imatinib treatment. These findings identify novel targets for TBI treatment and contribute to our understanding of the relationship between BBB leakage and the downstream secondary injuries associated TBI. In addition, we demonstrate the potential effectiveness of Imatinib, an existing FDA approved inhibitor of the PDGFRα pathway, for the treatment of acute TBI, suggesting the possibility of rapid translation of these results.

Material and Methods

TBI Models

Ten-week-old male C57BL/6J mice were anesthetized with 2% isoflurane and placed in a stereotatic frame (Kopf, Tujunga, CA, USA). Core body temperatures were maintained at 37.0° C for the entire procedure. For the unilateral TBI experiments, a 3.5 mm craniotomy was made over the right parietotemporal cortex with an electric drill (Harvard Apparatus) and the bone flap was removed. Vertically directed CCI was performed using a pneumatic impactor (Precision Systems and Instrumentation, VA) with a 3 mm flat-tip. The impact speed, tissue displacement and impact duration were set at 3.65m/s, 1 mm, and 400 ms respectively. A cap made from Dental Acrylic was glued to cover the craniotomy. To generate a larger bilateral injury, a previously described TBI model was used where the CCI is delivered to the midline (Liu et al., 2013). For this model a 5 mm circular craniotomy was made with center near bregma −2.5 and the impact speed, tissue displacement and impact duration were set at 3.00m/s, 1.1 mm, and 50 ms respectively. After the impact, the circular bone fragment from the craniotomy was glued back to the cranial window. For the unilateral TBI experiments, animal group sizes were n = 10 for the Evans blue (EB) Assays, n = 6 in the T2 and apparent diffusion co-efficient (ADC) analysis, and n = 5 for volumetric tissue loss after 21 days. In the bilateral TBI experiments, animal group sizes were n = 5 in the T2 and ADC analysis, n = 5 for volumetric tissue loss after 21 days, and n = 7–9 in the Morris water maze (MWM) studies. Separate groups of animals were used in each experiment and were not overlapped with the exception that the T2 and ADC analysis at 24 h and 7 days were performed on the same mice. For sham surgeries, all animals underwent the same surgical procedures except the craniotomy and CCI. All animals received the analgesic carprofen (5 mg/kg by subcutaneous injection) immediately prior to surgery, and post-surgery care consistent with the "*Guide for the Care and Use of Laboratory Animals*". Briefly, mice were kept on a 37°C warming pad overnight during recovery and were monitored daily for any distress

behavior until the end of the study, receiving analgesics after surgery as needed. All animal experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan, and conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. In general, the mice tolerated the procedures well. They were lethargic in the first few hours after surgery, and mice with the bilateral injury took longer to recover from anesthesia than mice in the unilateral model and remained lethargic for a longer period of time during the first day. There were no deaths or other complications with the unilateral model, but there was 1 death out of 52 mice subjected to the bilateral injury.

Evans Blue Analysis

For analysis of cerebrovascular permeability after TBI, mice were injected with 100 microliters of 4% EB (intravenous, Sigma-Aldrich) in lactated Ringer's solution 1 h before the animals were sacrificed by transcardial perfusion with phosphate buffered saline (PBS) for 8 min. The brains were removed and separated into hemispheres ipsilateral and contralateral to the TBI. Each hemisphere was then homogenized in N, N-dimethylformamide (Sigma-Aldrich) and centrifuged for 45 min at 25,000 rcf. The supernatants were collected and quantitation of EB extravasation performed as described (Yepes et al., 2003). Briefly, EB levels in each hemisphere were determined from the formula:

$$(A_{620nm} - ((A_{500nm} + A_{740nm})/2))/\text{mg wet weight}.$$

MRI Scan

After CCI, animals were anesthetized with 2% isoflurane/air mixture for T2 scans (7.0T Varian MR, 183 mm horizontal bore, Varian, Palo Alto, CA, USA). A double-tuned volume radiofrequency coil was used to scan the head region of the mice. Axial T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time (TR)/effective echo time (TE), 4000/60 ms; echo spacing, 15 ms; number of echoes, 8; field of view (FOV), 20 × 20 mm; matrix, 256 × 128; slice thickness, 0.5 mm; number of slices, 25; and number of scans, 1. The protocol for diffusion weighted imaging (DWI) utilized the following parameters: TR/TE, 4000/47 ms; FOV, 20 × 20 mm; matrix, 128 × 64 and the same slice package as the above spin-echo sequence.

For the data analysis, Image J software (NIH) was used to calculate the lesion volume from T2 scan, and Matlab software (MathWorks, Natick, MA, USA) was used to calculate the apparent diffusion coefficient from DWI scan. To calculate volumetric tissue loss 21 days after TBI, ROIs from MRI slices corresponding to the hippocampal region were calculated by Image J. The volume scales used (mm^3) for all T2 scans were the same in each model.

Imatinib Treatment

To block PDGFRα activation, mice were treated twice daily by oral gavage with the tyrosine kinase inhibitor Imatinib (200 mg/kg) starting 45 min after TBI and repeated for 5 days. Lactated Ringer's solution was used as vehicle control.

Histology

Deeply anaesthetized mice were perfused transcardially 21 days after TBI with PBS for 2 min and followed by 4% paraformaldehyde for 5 min. After perfusion, the brains were quickly removed from the skull and post-fixed for 1 h in buffered 4% paraformaldehyde (+4 °C), and embedded in OCT and stored at −70 °C until cut. Brains were cut in 14 μm-thick coronal sections with a sliding microtome and stained with hematoxylin and eosin. Images were visualized using Nikon Eclipse TE-2000e and captured with a digital camera (Q-Imaging Micropubliser RTV version 5.0). These captured images from each section were then stitched together by metamorph software (version 7.7.4.0).

Morris Water Maze

The MWM was performed as previously described (McKinney et al., 2008; White et al., 2008; Perkowski and Murphy, 2011). The pool was 1.2 meters in diameter and filled with water made opaque with white non-toxic paint. The escape platform consisted of a 10 cm platform that was submerged 0.5 cm below the surface of the water in the center of one of the quadrants. Water was maintained at 25 ± 2° C. The walls surrounding the pool were adorned with high-contrast posters for use as distal cues. The room was lit by indirect white light (200 lux in center of pool).

For 10 days prior to training, mice were handled for 2–3 min once daily. Every training trial began with the mouse on the platform for 15 s. The mouse was then placed into the water facing the wall of the pool and allowed to search for the platform. The trial ended either when the mouse climbed onto the platform or when 60 s had elapsed. At the end of each trial the mouse was allowed to rest on the platform for 15 s. Mice were given 6 trials per day (in blocks of two trials, 1 min inter-trial intervals and 1 h inter-block intervals) for 4 days, with the starting position chosen pseudo-randomly among 6 start positions. A probe trial was conducted 24 h after the end of training (on day 5). During the probe trial, the escape platform was removed and mice were placed in the pool at the start location directly opposite of where the platform was previously located and allowed to swim for 60 s. To control for motivation and sensory deficits mice were also examined in the visible-platform version of the MWM 24 h after the probe trial. The visible-platform version consisted of a single day of training with 6 trials during which the platform was moved to a new location and marked with a distinct local cue. All MWM data was acquired with a digital video camera 1.5 meters from the water surface. Images from the digital camera were processed and stored on a desktop PC using Actimetrics WaterMaze Software (Actimetrics, Wilmette, IL). Water maze performance was quantified using proximity. Proximity measurements were calculated by the tracking software at a rate of 1 Hz as the instantaneous distance (in centimeters) from the designated platform location. We prefer to use proximity measures because they are independent of swim speed (Gallagher et al., 1993) and moreover, the average proximity measure is the most sensitive probe trial measurement (Maei et al., 2009). Performance during training was assessed using a cumulative proximity measure.

Cumulative proximity is the sum of all of the instantaneous distance measurements minus the distance calculated as a perfect swim path which is represented as a straight line between the start location and the platform location. Performance during the probe trial was assessed using an average proximity measure. Average proximity is the average of all of the instantaneous distance measurements recorded during the probe trial with lower values reflecting a more selective search strategy.

Human CSF Samples

This study was conducted in accordance with the National Health and Medical Research Council of Australia National Statement on Ethical Conduct in Research Involving Humans and approved by Human Ethics Committees of the Alfred Hospital and Austin Hospital, Melbourne, Australia. Cerebrospinal fluid (CSF) was obtained from 17 TBI patients (16 male; 1 female) aged between 15 and 58 years (mean 31.1 years) recruited via the Trauma Service of the Alfred Hospital with delayed informed consent. Patients who required an external ventricular drain (EVD) for monitoring ICP were included. The CSF was collected within the first 36 h of admission in bags over 24 h in cooled containers then centrifuged at 2000 g for 15 min at 4°C and stored at −80°C. Non-TBI control CSF samples obtained from 4 patients recruited to the Alfred hospital for elective neurosurgery, or via lumbar puncture from 5 patients recruited to the Austin Hospital (Melbourne) suspected of having multiple sclerosis. CSF samples were centrifuged as above and the supernatants stored at −80°C.

Western blot analysis was performed to detect PDGF-CC levels in human CSF samples. CSF (30 μl of undiluted CSF) were subjected to SDS-PAGE electrophoresis under denaturing conditions and transferred to nylon membranes using standard conditions. To detect PDGF-CC, membranes were hybridized with a polyclonal goat anti-human PDGF-C antibody (RnD Systems, AF1560) diluted 1:200. After washing, membranes were hybridized with HRP tagged secondary rabbit anti-goat IgG antibody (1:5000 dilution). PDGF-CC signals were revealed using enhanced chemiluminescence (SuperSignal West Pico; Thermo Scientific, Rockford, IL, USA). The relative amount of PDGF-CC in each sample was estimated from the intensity of the PDGF-CC band in each lane and assigned a value from 0 to 10, with 0 being undetected and 10 being the most intense. The average for all the samples at each extended glasgow outcome scale (GOSE) score was plotted against the GOSE score.

Statistical Analysis

The data are presented as mean ± SEM. All statistical analysis was performed using GraphPad Prism statistical software. Significant outliers were identified and excluded based on Grubb's test with significance level alpha set to 0.05. Analysis for significance was performed as indicated in the figure legends. All neuropathology experiments were repeated at least two independent times and *n* indicates the number of individual mice used in the study. *P* values less than 0.05 were considered statistically significant and are indicated in the figures by an asterisk.

Results

Imatinib Inhibits the BBB Leakage after TBI

Since both TBI and ischemic stroke share the common pathophysiologic mechanisms of loss of BBB control and the development of cerebral edema (Bouma et al., 1991; Marion et al., 1991; Zauner et al., 1996; Bramlett and Dietrich, 2004; Lescot et al., 2010), we hypothesized that, as is the case with ischemic stroke, PDGFRα signaling might also play a role in TBI. To test this hypothesis we investigated if blocking PDGFRα signaling after TBI could reduce BBB leakage. For these studies we examined the efficacy of treatment with the PDGFRα antagonist Imatinib in mice subjected to CCI in the right parietotemporal cortex. The mice were treated with either vehicle or 200 mg/kg of Imatinib by gavage 45 min after CCI and again 22 h after injury. The mice were then analyzed for BBB integrity by EB extravasation 24 h after TBI. We predicted that if PDGFRα signaling contributes to loss of BBB integrity after TBI then Imatinib treatment should result in less BBB leakage 24 h after TBI. Consistent with our hypothesis, we found that compared to vehicle-treated controls, Imatinib treatment significantly reduced EB leakage in the ipsilateral hemisphere 24 h after TBI (**Figures 1A,B** >70%). We also found that the injury resulted in BBB leakage in the contralateral hemisphere, but at levels that were nearly 8-fold lower than in the ipsilateral hemisphere (3.4 ± 0.6 ipsi vs. 0.43 ± 0.08 contra). Similar to the effects observed in the ipsilateral hemisphere Imatinib treatment was also effective at reducing BBB leakage in the contralateral hemisphere (**Figure 1C** >75%). These results demonstrate that like its effects in stroke, spinal cord injury and MS (Su et al., 2008; Ma et al., 2011; Abrams et al., 2012; Adzemovic et al., 2013), Imatinib treatment preserves BBB integrity following TBI.

Imatinib Reduces Lesion Size and Vasogenic Edema after TBI

Since brain edema is a common and serious consequence of TBI that contributes to lesion expansion and secondary brain damage (Dietrich et al., 1994; del Zoppo and Mabuchi, 2003) and since the rise in edema after TBI is thought to be due, at least in part, to the loss of the BBB control, we examined if Imatinib treatment also reduced lesion size and edema 24 h and 7 days after TBI. For these studies mice were subjected to CCI in the right parietotemporal cortex and then treated with either vehicle or 200 mg/kg of Imatinib by gavage 45 min after TBI and then twice a day for 5 days following injury. T2 and DWI MRI scans were obtained at 24 h and 7 days post-injury. The T2 scans were used to calculate the lesion volume and showed that by 24 h after CCI there was conspicuous tissue damage within the region surrounding the impact site (**Figure 2A**). The T2 scans also revealed that by 7 days post-injury there was extensive tissue loss and cavitation in the cortex of untreated mice that was significantly reduced in the Imatinib treated group (**Figure 2A**). Quantification of these data demonstrated that at 24 h after injury the mean T2 lesion volume was 22.7 ± 2.9 mm^3 in the vehicle-treated group and 11.1 ± 1.2 mm^3 in the Imatinib-treated group. By 7 days after injury, the T2 signal was reduced in both groups.

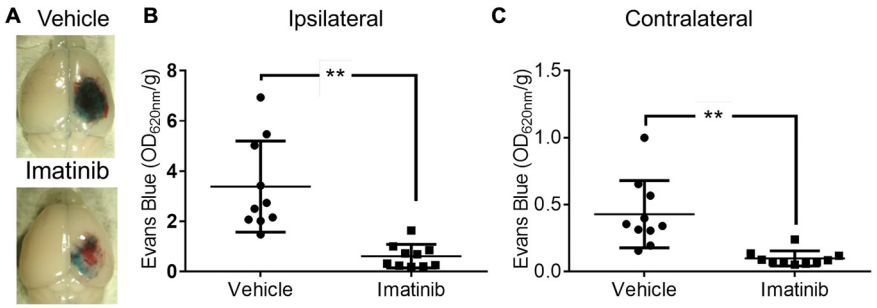

FIGURE 1 | Imatinib reduces blood brain barrier (BBB) leakage after Traumatic brain injury (TBI). Mice were treated with vehicle or Imatinib (200 mg/kg, daily p.o. for 1 day) starting 45 min after unilateral controlled cortical impact (CCI). Twenty three hours after unilateral CCI, Evans blue (EB) dye was injected intravenously as a bolus and animals were perfused with phosphate buffered saline (PBS) 1 h later. **(A)** Representative brains from vehicle and Imatinib treated animals are shown. **(B,C)** Quantitative analysis of EB leakage in the ipsilateral **(B)** and contralateral **(C)** hemispheres. Data are expressed as mean ± SEM (n = 10) and double asterisks indicate $p < 0.01$.

However, the Imatinib-treated mice still showed a significant reduction in lesion size of approximately 50% compared to the vehicle-treated group (12.5 ± 2.4 mm^3 vs. 6.0 ± 0.8 mm^3, **Figure 2B**).

To examine the effect of Imatinib treatment on edema in the injured hemisphere, the DWI scans of these mice were used to calculate hemispheric ADC values, which measure the impedance of water molecule diffusion within tissue. These results are shown in **Figure 2C**. In both groups the ADC values were reduced by 24 h after TBI compared to uninjured mice (Rau et al., 2006) (the normal ADC value is shown by the dashed line in **Figure 2C**). This indicates the presence of cytotoxic edema in both vehicle- and Imatinib-treated mice that was not significantly different between the groups. By day 7 there was a pseudo-correction of the ADC above the normal value indicating the development of vasogenic edema in the vehicle-treated mice. However, in the Imatinib-treated group the ADC value was significantly lower, indicating that there was much less vasogenic edema 7 days after injury in Imatinib treated mice (0.944 ± 0.065 mm^2/s vs. 0.799 ± 0.046 mm^2/s, **Figure 2C**).

Imatinib Reduces Cavitation and Tissue Loss after TBI

Since neuronal loss in the hippocampus has been linked to pathological outcomes of TBI in both humans (Tate and Bigler, 2000; Swartz et al., 2006) and in animal models of TBI (McIntosh et al., 1989; Dixon et al., 1991; Lowenstein et al., 1992; Hall et al., 2008), we next investigated whether intervention in the acute phase of TBI with 5 days of Imatinib treatment translated into long term tissue preservation. For these studies mice were subjected to CCI in the right parietotemporal cortex and then treated with either vehicle or 200 mg/kg of Imatinib by gavage 45 min after CCI and then twice a day for 5 days following injury. Previous studies have demonstrated that hippocampal neurons undergo regional alterations and remodeling after TBI (Witgen et al., 2005; Cohen et al., 2007). Consistent with this, hematoxylin and eosin staining of brain sections showed that there was dramatic tissue loss and remodeling in the cortex and hippocampus 21 days after TBI, including large cavitations (**Figure 3A**). As with BBB permeability, lesion size, and vasogenic edema, Imatinib substantially reduced cavitations (**Figure 3B**). To quantify the extent of the cavitations, MRI T2 scans were performed 21 days after TBI. These scans confirmed the loss of tissue noted in the H&E stains, showing large areas of hyper-intense signals indicative of cavitation (**Figures 3C,D**). Quantification of the hyper-intense signals showed that Imatinib treatment significantly reduced tissue loss (vehicle 3.6 ± 0.3 mm^3 vs, Imatinib 2.3 ± 0.4 mm^3 **Figure 3E**). This suggests that early intervention with Imatinib may impact long term TBI progression.

Imatinib Protects in a Bilateral TBI Model

Since neuronal loss and remodeling can affect long term memory and cognitive function, mice that were subjected to an identical injury and treatment were subsequently examined in the MWM test. However, neither the vehicle- nor the Imatinib-treated mice showed a consistent deficit, suggesting that the injury to one hemisphere may not be extensive enough to result in measurable behavioral changes (not shown). Consistent with this interpretation several studies have suggested that when an undamaged unilateral hippocampus is present, behavioral changes in mice are minimized in memory and cognition tests (Shipton et al., 2014). Based on these recent studies, we hypothesize that the lack of significance in our MWM test is due to the presence of an intact hippocampus on the contralateral side resulting in the lack of a measureable cognitive deficit in the control cohort. We therefore extended our study with a second, more severe, TBI model where CCI is delivered to the midline producing a larger bilateral TBI (Liu et al., 2013).

To characterize the extent of injury in the midline CCI model and to examine the potential efficacy of Imatinib in a more severe model of TBI we performed midline CCI followed by Imatinib treatment and analyses as above. Briefly, mice were gavaged 45 min after TBI and then twice a day for 5 days following injury with either vehicle or 200 mg/kg of Imatinib. T2 and DWI

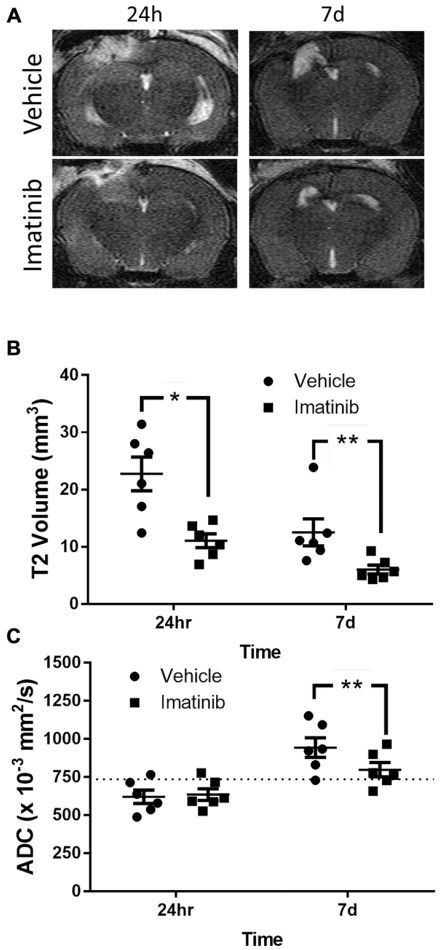

FIGURE 2 | Imatinib reduces lesion volume and edema after TBI. Mice were treated with vehicle or Imatinib (200 mg/kg, twice daily p.o. for 5 days) starting 45 min after unilateral CCI and examined for lesion volume and cerebral edema by MRI 24 h and 7 days after unilateral CCI. **(A)** Representative T2-weighted MRI images. **(B)** Quantitative analysis of the lesion volume determined from theT2-weighted hyper-intense signal. **(C)** The relative extent of cerebral edema in the ipsilateral hemisphere was determined by calculating the apparent diffusion co-efficient (ADC) values from diffusion weighted imaging (DWI) scans. Data are expressed as mean ± SEM ($n = 6$); single asterisk indicates $p < 0.05$, double asterisks indicate $p < 0.01$, and the dotted line indicates the normal ADC value of 736 × 10^{-3} mm^2/s reported for age matched mice (Rau et al., 2006).

MRI scans were obtained at 24 h and 7 days post-injury. Lesion volumes were calculated from the T2 scans (**Figures 4A,B**). As expected, the midline CCI resulted in a bilateral injury that was nearly twice as large as the unilateral injury at 24 h after CCI (39.8 ± 5.6 mm^3 vs. 22.7 ± 2.9 mm^3) and remained larger 7 days after injury (18.9 ± 3.2 mm^3 vs. 12.5 ± 2.4 mm^3) (compare **Figure 2B** with **Figure 4B**). Imatinib treatment was also effective at reducing lesion size in the midline CCI model, producing a non-significant trend toward a smaller lesion at 24 h but a significant decrease in lesion volume by 7 days post injury (18.9 ± 3.2 mm^3 vs. 8.0 ± 3.2 mm^3).

ADC analysis was also performed with this model; however unlike the unilateral model where the ADC values were calculated only in the injured hemisphere, in the bilateral TBI model the ADC values were calculated for the entire brain. Similar to the lesion size, both cytotoxic and vasogenic edema were increased in the midline CCI model compared to unilateral CCI model (compare **Figures 2C–4C**). These data also demonstrated that even with the more severe injury, Imatinib treatment was effective at reducing both cytotoxic edema at 24 h after injury (0.559 ± 0.009 mm^2/s vs. 0.635 ± 0.018 mm^2/s) and vasogenic edema at day 7 (1.577 ± 0.040 mm^2/s vs. 1.391 ± 0.028 mm^2/s) (the normal ADC value is shown by the dashed line in **Figure 4C**).

Mice subjected to midline CCI were also analyzed for long term loss of neuronal tissue due to cavitation 21 days after injury. These data are shown in **Figure 5** and indicate that unlike the differences in initial lesion size, that were nearly 2-fold at 24 h after injury (39.8 ± 5.6 mm^3 vs. 22.7 ± 2.9 mm^3), the final difference in tissue loss due to cavitation was only ∼9% between the two models (3.9 ± 0.3 mm^3 vs. 3.6 ± 0.3 mm^3, compare **Figures 3E**, **5E**). Nonetheless, H&E staining of brain sections showed that there was much more hippocampal loss and remodeling in the bilateral TBI model compared to the unilateral model, where the left hippocampus remained largely intact (compare **Figures 3A–5A**). Similar to the results in lesion size, and edema, Imatinib treatment significantly reduced cavitations in the bilateral TBI model (**Figures 5D,E**) and appeared to preserve hippocampal structure (**Figure 5B**). This suggests that even in a severe model of TBI early intervention with Imatinib may have long term benefit in TBI.

Imatinib Preserves Cognitive Function after Bilateral TBI

Cognitive function in mice exposed to midline CCI was assessed by examining spatial learning and memory in the MWM. Data comparing mice with bilateral CCI to Sham treated mice are presented in **Figures 6A,C,E,G**. Data comparing injured mice treated with Imatinib or vehicle are presented in **Figures 6B,D,F,H**. All data are presented in terms of proximity which is a measure of "search error" and therefore lower scores indicate a more selective search strategy. Mice in the Sham group exhibited typical performance across the four days of training (**Figure 6A**) during which time their cumulative proximity scores significantly decreased. Conversely, the performance of mice in the TBI group did not improve over time and was significantly worse compared to the Sham group ($p < 0.001$). To assess long-term memory, a probe trial was conducted 24 h after the last training trial was completed on day 4. Similar to the cumulative proximity, the average proximity measure is a search error measure which is independent of swim speed. The duration of the probe trial is fixed at 60 s; therefore an average measure is made (c.f. cumulative for the variable length training trials). Again, a lower number indicated a more selective search strategy. Injured mice in the TBI group were significantly impaired when compared to the mice in the Sham treated group. In addition, the injured mice in the TBI group performed significantly worse even when the platform was clearly marked (**Figure 6E**) and exhibited slower swim speeds during the

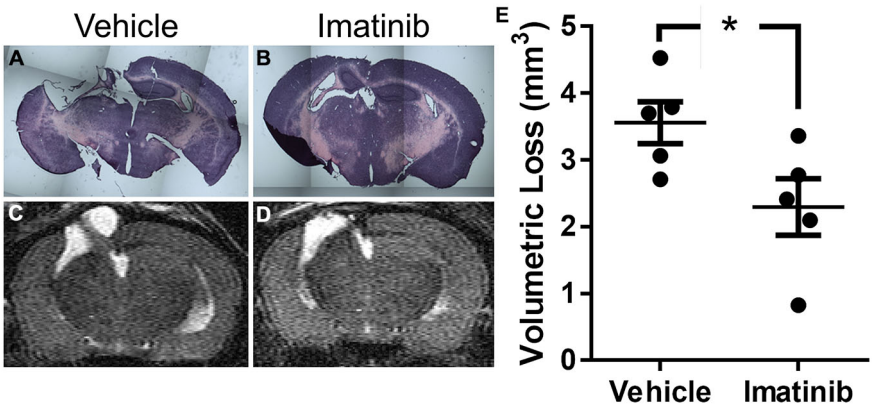

FIGURE 3 | Imatinib reduces tissue loss after TBI. Mice were treated with vehicle or Imatinib (200 mg/kg, twice daily p.o. for 5 days) starting 45 min after unilateral CCI. Twenty one days later, animals underwent MRI T2 scan and then were perfused and fixed in paraformaldehyde and stained with H&E. **(A,B)** Representative photomicrographs of coronal sections near bregma −1.5 (10× objective) from vehicle-treated **(A)** and Imatinib-treated **(B)** mice. **(C,D)** Representative T2-weighted MRI images from vehicle-treated **(C)** and Imatinib-treated mice **(D). (E)** Quantification of volumetric loss of tissue determined from theT2-weighted hyper-intense signal. Data are expressed as mean ± SEM ($n = 5$) and the single asterisk indicates $p < 0.05$.

probe trial (**Figure 6G**). In a separate experiment, mice were exposed to bilateral CCI and then treated with either vehicle or 200 mg/kg of Imatinib by gavage 45 min after CCI and then twice a day for 5 days prior to the start of the water maze. Across the 4 days of training (**Figure 6B**) injured mice that were treated with Imatinib exhibited significantly better performance when compared to the vehicle treated mice ($p < 0.01$). Similarly, mice treated with Imatinib outperformed vehicle treated mice during the probe trial (**Figure 6D**). Treatment with Imatinib did not alter performance on the visible platform version of the water maze (**Figure 6F**) or swim speed (**Figure 6H**).

PDGF-CC is Increased in Human CSF after TBI

Although our data suggest that the BBB leakage, controlled by PDGF signaling in the NVU, may contribute to TBI progression in animal models, it is not known if PDGF-CC protein levels are altered in human TBI. Therefore, we examined PDGF-CC levels in the CSF of human TBI patients and in control non-TBI patients by immunoblotting. For these studies we obtained CSF from 17 TBI patients and 9 non-TBI patients. The TBI patients had GOSE ranging from 1 to 8, where 1 is the most severe, indicating that the patient died of their injuries, and 8 is the mildest injury and the patients have a good recovery (Lu et al., 2010). Immunoblot analysis of these samples is shown in **Figure 7** along with a platelet releasate sample as a positive control for PDGF-CC. Note that the lanes with the strongest PDGF-CC signal are all from TBI patients with the lowest (most severe) GOSE scores. The relative intensity of the PDGF-CC staining in each lane was ranked from 0 to 10 for both the TBI patients and non-TBI patients with 0 being undetectable and 10 representing the most intense PDGF-CC staining, and these data were then plotted against the GOSE score (**Figure 7B**). This semi-quantitative analysis suggests that there may be a correlation between TBI severity and the presence of PDGF-CC in the CSF. These data are also similar to a positive correlation reported between PDGF-CC levels in plasma and worse outcomes in ischemic stroke patients (Rodríguez-González et al., 2013), and with our previous study showing elevated tPA-inhibitor complexes in the CSF of the most severe TBI patients (Sashindranath et al., 2012).

Discussion

The loss of BBB integrity is a common feature of severe TBI with nearly 50% of the patients examined in a recent study showing biochemical evidence of BBB disruption (Ho et al., 2014). Damage to the BBB can be immediate due to trauma-induced hemorrhage (Logsdon et al., 2015), or can occur rapidly following TBI in the absence of overt hemorrhage, with significant increases in permeability observed within 3 h of injury (Sashindranath et al., 2012). This early rise in BBB permeability can affect the molecular injury pathways associated with secondary brain injury in TBI, increasing cerebral edema by disrupting parenchymal fluid homeostasis and by increasing neuroinflammation (Shlosberg et al., 2010; Thal and Neuhaus, 2014; Logsdon et al., 2015). A principal focus in the management of TBI is limiting the occurrence of cerebral edema and the attendant increase in ICP (Hemphill and Phan, 2013a), therefore targeting pathways that promote BBB disruption in TBI should be beneficial. Our earlier studies demonstrating that the pathway regulated by the protease tPA, acting through PDGF-CC and the PDGFRα in the NVU, can promote pathologic increases in BBB permeability (Yepes et al., 2000, 2003; Fredriksson et al., 2004, 2005, 2015; Su et al., 2008; Abrams et al., 2012; Adzemovic et al., 2013) led us to test the hypothesis that treatment with Imatinib, an antagonist of the PDGFRα, could reduce BBB permeability after TBI and improve outcomes. Consistent with this hypothesis an earlier study has shown that mice deficient in tPA have reduced BBB disruption 6 h after injury, reduced edema 24 h after injury and smaller lesions 7 days

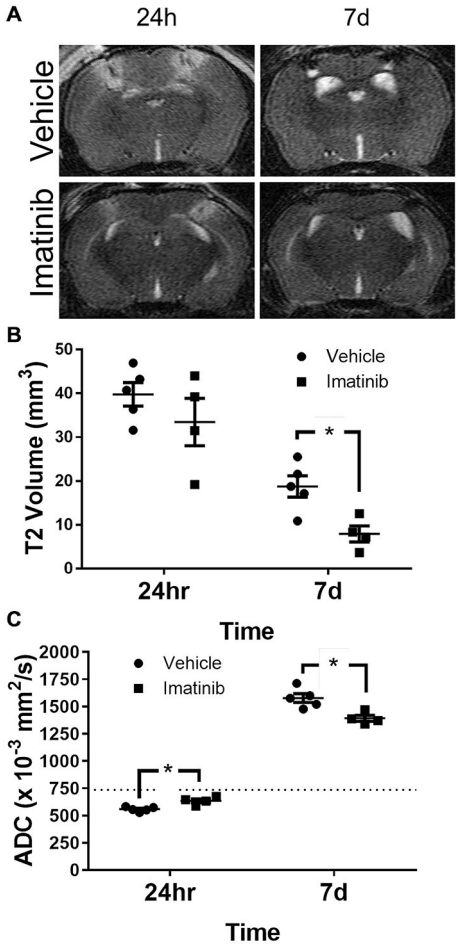

FIGURE 4 | Imatinib reduces lesion volume and edema after Bi-lateral TBI. Mice were treated with vehicle or Imatinib (200 mg/kg, twice daily p.o. for 5 days) starting 45 min after bilateral CCI and examined for lesion volume and cerebral edema by MRI 24 h and 7 days after bilateral CCI. **(A)** Representative T2-weighted MRI images. **(B)** Quantitative analysis of the lesion volume determined from theT2-weighted hyper-intense signal. **(C)** The relative extent of cerebral edema in both hemispheres was determined by calculating the ADC values from DWI scans. Data are expressed as mean ± SEM (n = 4–5), the single asterisk indicates $p < 0.05$, and the dotted line indicates the normal ADC value of 736 × 10^{-3} mm^2/s reported for age matched mice (Rau et al., 2006).

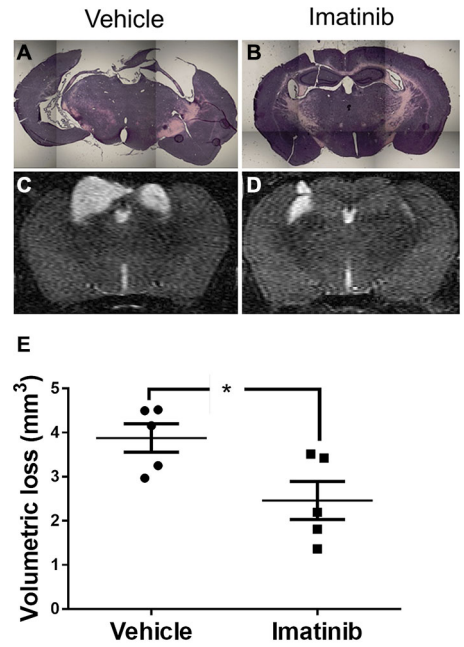

FIGURE 5 | Imatinib reduces tissue loss after Bi-lateral TBI. Mice were treated with vehicle or Imatinib (200 mg/kg, twice daily p.o. for 5 days) starting 45 min after bilateral CCI. Twenty one days later, animals underwent MRI T2 scan and then were perfused and fixed in paraformaldehyde and stained with H&E. **(A,B)** Representative photomicrographs of coronal sections near bregma −1.5 (10× objective) from vehicle-treated **(A)** and Imatinib-treated **(B)** mice. **(C,D)** Representative T2-weighted MRI images from vehicle-treated **(C)** and Imatinib-treated mice **(D). (E)** Quantification of volumetric loss of tissue determined from the T2-weighted hyper-intense signal. Data are expressed as mean ± SEM (n = 5) and the single asterisk indicates $p < 0.05$.

following TBI (Mori et al., 2001). Whereas we have shown that overexpression of tPA results in a significant increase in BBB leakage and lesion volume 3 h after TBI (Sashindranath et al., 2012).

Our data presented here demonstrate for the first time that Imatinib dramatically reduces BBB permeability 24 h after TBI by approximately 80% (**Figure 1**). This is similar to the extent of BBB protection from Imatinib in a model of spinal cord injury (Abrams et al., 2012) and even better than the approximately 30% reduction in BBB permeability we observed in stroke (Su et al., 2008). Consistent with the view that BBB permeability contributes to increased cerebral edema, Imatinib treatment for 5 days also significantly reduced vasogenic edema in both unilateral and bilateral injury models (**Figures 2C**, **4C**). Imatinib also significantly reduced cytotoxic edema 24 h after injury in the bilateral TBI model but was not significantly different from vehicle in the unilateral model. This was likely because the milder injury in the unilateral model produced less cytotoxic edema at 24 h than did the bilateral injury (unilateral 0.620 ± 0.043 mm^2/s vs. bilateral 0.559 ± 0.009 mm^2/s).

The reductions in BBB permeability and cerebral edema induced with Imatinib treatment were also correlated with significant reductions in lesion size during the first week following injury and with tissue preservation at 21 days after CCI (**Figures 2**–**5**). Examination of the hematoxylin and eosin stained sections demonstrated that Imatinib treatment was remarkably effective at preserving brain tissue compared to the vehicle treated mice. In fact in the vehicle treated mice the loss of tissue due to vacuole formation or cavitation was so extensive that it was not possible to quantify the magnitude of tissue loss in either the unilateral or midline CCI model (**Figures 3**, **5**). Therefore, T2 MRI scans were utilized to quantify the extent of cavitation after CCI and the effects of 5 days of Imatinib treatment on tissue preservation. The preservation of brain tissue at 21 days following injury is significant for the long term recovery from TBI. Previous studies have shown that neuronal tissue loss several weeks after injury, especially in the hippocampus, is a common pathology in TBI that is

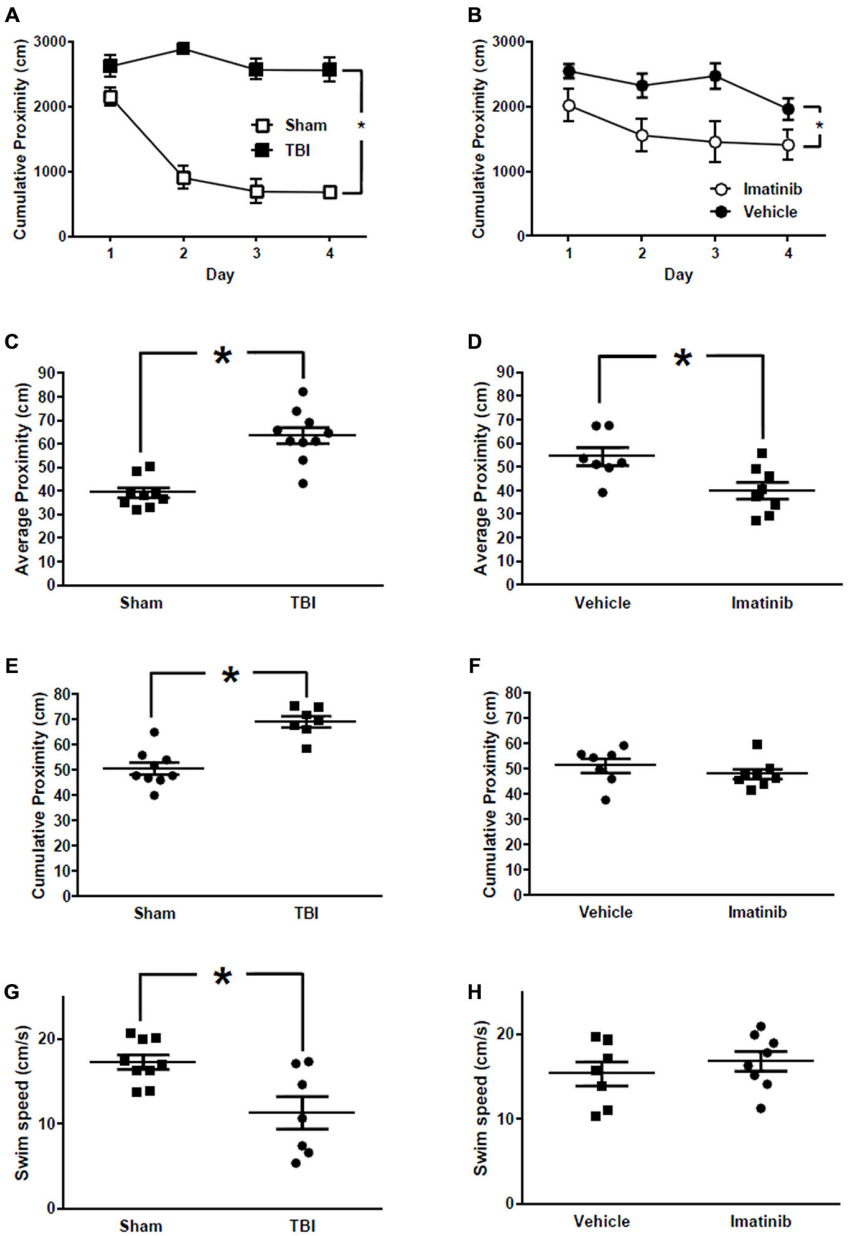

FIGURE 6 | Imatinib improves neurological outcome in Morris Water Maze (MWM) after TBI. Imatinib ameliorates TBI associated impairments in spatial learning and memory. **(A)** Cumulative proximity scores are plotted across 4 days of training for TBI (n = 7) and Sham (n = 9) treated mice. **(B)** Cumulative proximity scores are plotted across 4 days of training for TBI mice treated with vehicle or Imatinib. **(C)** Average proximity during the probe trial which was completed 24 h after the last training trial plotted for Sham and TBI treated mice. **(D)** Average proximity during the probe trial which was completed 24 h after the last training trial is plotted for mice subjected to TBI and then treated with Imatinib (n = 8) or Vehicle (n = 7). **(E)** Cumulative proximity measured during the visible platform version of the water maze is plotted for Sham and TBI treated mice. Data represent the average of 6 trials. **(F)** Cumulative proximity measured during the visible platform version of the water maze is plotted for mice subjected to TBI and then treated with Imatinib or vehicle. **(G)** Swim speed measured during the probe trial in Sham and TBI treated mice. **(H)** Probe trial swim speed of mice subjected to TBI and then treated with Imatinib or vehicle. Data are presented as mean ± SEM. Single asterisk in **(A,B)** indicates $p < 0.05$ for main effect of treatment evaluated using a two-way repeated measures ANOVA with treatment and training days as factors. Single asterisk in **(C–H)** indicates $p < 0.05$, two tailed unpaired t-test.

associated with memory deficit and cognitive decline (Witgen et al., 2005). Our results demonstrate that Imatinib treatment preserves significantly more tissue compared to vehicle including tissue in the hippocampus. This is particularly apparent when comparing **Figures 5A,B**. The exact mechanism by which 5 days of Imatinib treatment is able reduce cavitation 21 days after injury is uncertain. However, it is likely that early reduction in BBB leakage together with the reductions in cerebral edema achieved with Imatinib treatment contributed to reductions in tissue loss.

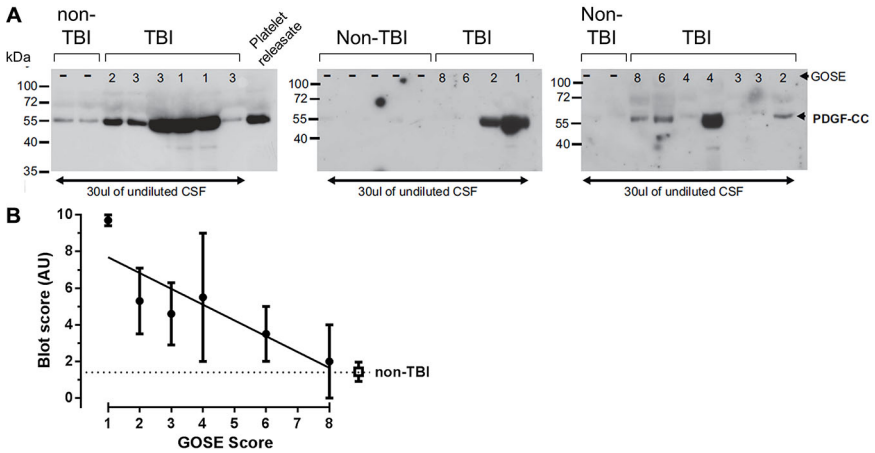

FIGURE 7 | Analysis of platelet derived growth factor-CC (PDGF-CC) in human TBI cerebrospinal fluid (CSF) samples. Human CSF Samples obtained from patients within the first 24 h after TBI were immunoblotted for PDGF-CC. **(A)** Immunoblots from 17 TBI patients and nine controls are shown. As a control for PDGF-CC a sample of releasate from human platelets is shown in the last lane of the first blot. The extended glasgow outcome scale (GOSE) score of each patient is shown at the top of each lane (GOSE 1–8) and the position of molecular mass size markers are indicated at the left of each blot. **(B)** The relative amount of PDGF-CC in each sample was estimated from the intensity of the PDGF-CC, assigned a value from 0 to 10 and the average for each GOSE score was plotted against the GOSE score. The filled circles are the TBI samples and the open square represents the non-TBI samples. Each point is the mean ± SEM and the line is a linear regression fit of the TBI samples, a Spearman's correlation analysis of these data yields a one tailed $p = 0.0292$.

Behavioral impairment in the unilateral CCI model was not consistently observed even in the vehicle treated mice. However, midline cortical impact clearly disrupted performance in the water maze. Compared to Sham treated mice, mice in the TBI group exhibited significantly degraded search strategies as measured by cumulative proximity during training and average proximity during the probe trial. Treatment with Imatinib significantly ameliorated these deficits. Injured mice also exhibited, on average, slower swim speeds during the probe trial and were impaired in the visible platform version of the water maze. Interestingly, injured mice treated with vehicle or Imatinib performed to the same level in the visible platform task and did not differ with regard to their swim speeds. Furthermore, performance in both groups appears to be similar to that observed in the Sham treated mice. This likely reflects the reduction in stress and anxiety produced by the additional handling associated with the daily dosing of the mice in the Imatinib experiment (Fridgeirsdottir et al., 2014). Interpreted this way, these data would suggest that at least some of the performance deficits observed in the TBI mice may be more related to affective state and not due to deficits in motor performance. This interpretation would be consistent with previous work in the field demonstrating that TBI in mice produces deficits in behavioral tests of anxiety-like behaviors (Washington et al., 2012). In light of these observations we conclude that Imatinib improves performance in the water maze by ameliorating TBI associated cognitive impairments and not by altering affective state or motor output.

Together, these data support the view that a dysfunctional BBB and ensuing edema may contribute directly to TBI progression including tissue loss and cognitive impairment, and that treatment with Imatinib reduces these pathologic effects in injured mice. Analysis of human CSF samples from TBI patients suggests that PDGF-CC levels may correlate with the extent of injury. Importantly though, it should be noted that these data are semi-quantitative, and while they suggest that this pathway could be active in human TBI, additional studies will be needed to directly test this hypothesis. Nonetheless, it is interesting to note that in ischemic stroke patients treated with tPA there is a significant positive association between increased plasma PDGF-CC and hemorrhagic transformation (Rodríguez-González et al., 2013), suggesting that in humans PDGF-CC may be linked to cerebrovascular damage.

In conclusion, the data presented here support the hypothesis that the PDGFRα pathway may play a role in the loss of BBB integrity following TBI, and that inhibiting this pathway may improve TBI outcomes. In addition, we demonstrate the potential effectiveness of Imatinib, an existing FDA approved antagonist of the PDGFRα pathway, for the treatment of acute TBI, suggesting the possibility of the relatively rapid translation of Imatinib into a clinical trial in TBI.

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Conflict of Interest Statement: Drs. U. Eriksson, D.A. Lawrence, E.J Su, and L. Fredriksson hold a patent on modulating blood-neural barrier using PDGFR-alpha antagonist. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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tPA Deficiency in Mice Leads to Rearrangement in the Cerebrovascular Tree and Cerebroventricular Malformations

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The serine protease tissue-type plasminogen activator (tPA) is used as a thrombolytic agent in the management of ischemic stroke, but concerns for hemorrhagic conversion greatly limits the number of patients that receive this treatment. It has been suggested that the bleeding complications associated with thrombolytic tPA may be due to unanticipated roles of tPA in the brain. Recent work has suggested tPA regulation of neurovascular barrier integrity, mediated via platelet derived growth factor (PDGF)-C/PDGF receptor-α (PDGFRα) signaling, as a possible molecular mechanism affecting the outcome of stroke. To better understand the role of tPA in neurovascular regulation we conducted a detailed analysis of the cerebrovasculature in brains from adult tPA deficient (*tPA*$^{-/-}$) mice. Our analysis demonstrates that life-long deficiency of tPA is associated with rearrangements in the cerebrovascular tree, including a reduction in the number of vascular smooth-muscle cell covered, large diameter, vessels and a decrease in vessel-associated PDGFRα expression as compared to wild-type (WT) littermate controls. In addition, we found that ablation of tPA results in an increased number of ERG-positive endothelial cells and increased junctional localization of the tight junction protein ZO1. This is intriguing since ERG is an endothelial transcription factor implicated in regulation of vascular integrity. Based on these results, we propose that the protection of barrier properties seen utilizing these *tPA*$^{-/-}$ mice might be due, at least in part, to these cerebrovascular rearrangements. In addition, we found that *tPA*$^{-/-}$ mice displayed mild cerebral ventricular malformations, a feature previously associated with ablation of PDGF-C, thereby providing an *in vivo* link between tPA and PDGF signaling in central nervous system (CNS) development. Taken together, the data presented here will advance our understanding of the role of tPA within the CNS and in regulation of cerebrovascular permeability.

Keywords: tPA, PDGF, blood-brain barrier, neurovascular unit, neurovascular coupling, lateral ventricles, ERG, vascular permeability

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INTRODUCTION

The serine protease tissue-type plasminogen activator (tPA) is primarily known for its role in fibrinolysis via proteolytic activation of plasminogen into plasmin (Collen, 2001). The observation that tPA directly binds to fibrin (van Zonneveld et al., 1986; Verheijen et al., 1986) and thereby facilitates localized generation of plasmin has led to the use of tPA as a thrombolytic agent for treatment of acute myocardial infarction and ischemic stroke (Collen, 2001). In fact, tPA administration is the only FDA-approved thrombolytic therapy for acute ischemic stroke (The national institute of neurological disorders and stroke rt-PA stroke study group, 1995; Su et al., 2009). The use of thrombolytic tPA in ischemic stroke is however markedly limited due to concerns for hemorrhagic complications and the requirement that it is administered within a few hours of onset of symptoms (The national institute of neurological disorders and stroke rt-PA stroke study group, 1995; Ahmed et al., 2010). The mechanism by which thrombolytic tPA might lead to hemorrhagic transformation of ischemic stroke is not completely understood, but it appears to be due in part to unique activities that tPA has in the brain beyond its well established role in fibrinolysis (Nicole et al., 2001; Wang et al., 2003; Yepes et al., 2003; Su et al., 2009).

The role of tPA within the central nervous system (CNS) is controversial (Su et al., 2009; Yepes et al., 2009; Lemarchant et al., 2012; Schielke and Lawrence, 2012). It has been proposed that tPA directly affects multiple processes, including neuronal development/plasticity/excitotoxicity (Tsirka et al., 1996; Seeds et al., 2003; Li et al., 2013), microglial activation (Tsirka et al., 1997; Rogove and Tsirka, 1998), as well as regulation of cerebrovascular permeability (Yepes et al., 2003; Su et al., 2008). In a recent paper, we proposed that the neurovascular events regulated by tPA might provide a unifying pathway for many of these pleotropic effects of tPA in the CNS (Fredriksson et al., 2015). We argued that tPA-induced changes in cerebrovascular permeability might lead to a loss of precise control of the extracellular environment, which in turn, might promote dysregulation of neuronal signaling pathways (Fredriksson et al., 2015).

To better understand the role of tPA in cerebrovascular regulation we have conducted a detailed analysis of the cerebrovasculature and the neurovascular unit in brains from adult tPA deficient ($tPA^{-/-}$) mice. We demonstrate that tPA deficiency is associated with abnormal cerebral vascularization, including a reduction in the amount of large diameter vessels. In addition, we found a significant reduction of platelet derived growth factor receptor-α (PDGFRα) expression around cerebral vessels of $tPA^{-/-}$ mice which is particularly interesting given our previous findings showing that the effect of tPA in regulation of cerebrovascular permeability is mediated via activation of platelet derived growth factor (PDGF)-C/PDGFRα signaling on perivascular astrocytes (Fredriksson et al., 2004, 2005; Su et al., 2008). Intriguingly, we also found that ablation of tPA results in increased number of ERG-positive endothelial cells. ERG (ETS related gene) is a member of the ETS family of transcription factors implicated in vascular development (reviewed in Randi et al., 2009) and overexpression of ERG in vivo was recently shown to reduce vascular permeability (Birdsey et al., 2015). In addition, we found that $tPA^{-/-}$ mice displayed mild cerebral ventricular malformations, a feature previously associated with ablation of PDGF-C (Fredriksson et al., 2012), thereby providing an *in vivo* link between tPA and PDGF signaling in CNS development. These findings will aid in our understanding of the role of tPA in the CNS.

MATERIALS AND METHODS

Animal Strains

Age- and gender-matched tPA deficient mice ($tPA^{-/-}$; Carmeliet et al., 1994), back-crossed at least 10 generations into C57BL/6J background, and their wild-type (WT) littermate controls obtained from heterozygous breedings were used. All animal experiments were approved by the local committee for animal experiments at the University of Michigan, USA, and the studies were conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Immunostaining and Confocal Microscopy

Tissue preparation for sectioning and immunostaining were conducted using standard protocols. Mice were anesthetized with isoflurane and following transcardial perfusion fixation with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) the brains were removed and postfixed in 4% PFA 1 h at room temperature (RT) and then kept in 30% sucrose, 4°C overnight.

For immunofluorescence, vibratome sections (50 μm) were cut and stained free floating in 24-well plates. The sections were permeabilized and blocked with 1% bovine serum albumin (BSA) in 0.5% TritonX-100/PBS overnight at 4°C, followed by incubation with primary antibodies in blocking solution overnight at 4°C (1:200 dilution). After thorough washes the sections were incubated with fluorescent-conjugated secondary antibodies overnight at 4°C. The specific primary antibodies used were: aquaporin 4 (AB2218; Millipore, Billerica, MA, USA), α-smooth muscle actin (ASMA)-Cy3 (C6198; Sigma-Aldrich, St. Louis, MO, USA), CD13 (MCA2183; AbD Serotec, Oxford, UK), CD31 (553370; BD Biosciences, Franklin Lakes, NJ, USA), ERG1 (ab92513; Abcam Plc, Cambridge, UK), collagen IV (21501470; AbD Serotec), glial fibrillary acidic protein (GFAP; Z0334; Dako, Glostrup, Denmark), glucose transporter 1 (GLUT1; sc-1605; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PDGFRα (AF1062; R&D Systems, Minneapolis, MN, USA), podocalyxin (AF1556; R&D Systems), and ZO1 (339100; Invitrogen, Molecular Probes). For S100B (Z0311; Dako) antigen retrieval was performed by boiling 10 min in target retrieval solution (S1700; Dako). Appropriate fluorophore-conjugated secondary antibodies were used for multi color detection (Alexa Fluor 488, 568 and 647; Life Technologies, Molecular Probes, Grand Island, NY, USA) and DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride, 0.2 μg/ml) was included in the last PBS wash to visualize the nuclei. The sections were then

mounted on Superfrost Plus slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) with ProLong Gold Antifade reagent (P36930; Life Technologies). All stainings were repeated two-four independent times.

All images were acquired at RT with a Zeiss LSM700 confocal microscope or a Zeiss Axio Observer Z1 inverted microscope and the ZEN 2009 software (Carl Zeiss Microimaging GmbH, Jena, Germany). Stained brain sections from $tPA^{-/-}$ mice ($n = 5$) and WT littermates ($n = 5$) were analyzed by two independent investigators blinded to the study group. In addition, as a control of the quantification at least one set of images from each respective staining was reanalyzed by a second blinded investigator. *n* indicates the number of individual mice used in the study. The individual observations are based on analysis of four-eleven fields of view (same number of images per animal and settings for each respective staining within an individual experiment). The fields of view were taken in comparable anatomic positions in each animal and the anatomic position to be imaged was identified using the DAPI channel. The anatomic positions analyzed were from brain regions where high levels of tPA expression has been reported, including cortex, hippocampus and amygdala (Yu et al., 2001). We did not find any evidence of sub-regional effects or differences during our analysis. The images were processed and analyzed using Volocity 3D image analysis software (PerkinElmer, Waltham, MA, USA), Photoshop CS5 (Adobe, San Jose, CA, USA) or ImageJ64 (National Institutes of Health, Bethesda, MD, USA). For quantification of antibody immunoreactivity using intensity, all images were acquired using the same settings (within the respective staining experiment) and the number of pixels above a set threshold was determined. Each field of view analyzed was from a maximum intensity *Z*-stack image (15–22 μm stack) or from an epifluorescent image. The result from all the fields of view in a given animal was averaged to obtain the value for that individual. Individual values and group mean ± SEM are shown. The images shown are representative of the respective staining. Detailed information regarding all the stainings and analysis performed is summarized in the supplementary table 1 (Table S1). Brightness and contrast settings were changed to generate the final image and were applied equally to the entire image and within the same set of images.

Magnetic Resonance Imaging Scans

All magnetic resonance imaging scans (MRI) were conducted at the Center for Molecular Imaging, Department of Radiology, University of Michigan. During the imaging procedure the mice were anesthetized with isoflurane and the body temperature was maintained using forced heated air. The MRI examinations were performed in a 7.0T Varian MR scanner (183-mm horizontal bore, Varian, Palo Alto, CA, USA). A double-tuned volume radiofrequency coil was used to scan the head region of the mice. Coronal T2-weighted images were acquired using a fast spin-echo sequence with the following parameters: repetition time (TR)/effective echo time (TE), 4000/60 ms; echo spacing, 15 ms; number of echoes, 8; field of view (FOV), 20×20 mm; matrix, 256×128; slice thickness, 0.5 mm; number of slices, 25; and number of scans, 1 (total scan time ∼2 min.). Photoshop and Image J were used to assess differences in ventricular area and the analysis was conducted blinded.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 statistical software (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by student's unpaired *t*-test and *P* values less than 0.05 were considered statistically significant and are indicated in the figures by asterisks.

RESULTS

tPA Deficiency Affects Cerebral Vessel Size

A growing body of evidence is showing that tPA is both necessary and sufficient to regulate cerebrovascular permeability (Yepes et al., 2003; Su et al., 2008; Fredriksson et al., 2015). In order to gain a better understanding of the role of tPA in controlling cerebrovascular events we performed a thorough analysis of the vascular bed in brain sections from tPA deficient ($tPA^{-/-}$) mice. Immunofluorescent stainings using CD31 antibodies, a marker for vascular endothelial cells, revealed an abnormal vascularization in the brains of adult $tPA^{-/-}$ mice ($n = 5$) compared to littermate WT controls ($n = 5$; **Figure 1A**). In $tPA^{-/-}$ brains the vascular bed showed an apparent decrease in large diameter vessels (arrowheads) and increase in small diameter vessels (arrows) relative to WT mice (**Figure 1A**). This was confirmed by quantification of the CD31 stainings, showing a significant decrease ($P < 0.01$) in vessel diameter in $tPA^{-/-}$ mice (4.75 ± 0.2 μm) as compared to WT littermate controls (6.03 ± 0.3 μm; **Figure 1B**). This decrease in average vessel size in $tPA^{-/-}$ mice was due to a significant reorganization of the vascular bed in $tPA^{-/-}$ brains relative to WT littermate controls, with increased number of small diameter vessels (<5 μm; WT = 54 ± 6% vs. $tPA^{-/-}$ = 67 ± 4%, $P < 0.05$) and fewer large diameter vessels (>10 μm; WT = 10 ± 1% vs. $tPA^{-/-}$ = 1 ± 0.8%, $P < 0.01$; **Figure 1C**). This was accompanied with an overall, but non-significant ($P = 0.31$), reduction in the total amount of CD31 staining in $tPA^{-/-}$ brains (80 ± 12% of WT) compared to WT littermate controls (**Figure 1D**). Staining with podocalyxin antibodies, another marker of vascular endothelial cells, confirmed the loss of large diameter vessels (arrowheads) and increase in small diameter vessels (arrows) in $tPA^{-/-}$ brains relative to WT littermate controls (**Figures 1E,F**).

tPA Deficiency is Associated with Increased Number of ERG-Positive Endothelial Cells and Enhanced Junctional Localization of ZO1 in the Murine Brain

In order to characterize whether the cerebrovascular changes seen in $tPA^{-/-}$ mice were associated with altered number of endothelial cells we conducted immunofluorescent stainings using antibodies against the endothelial transcription factor ERG. Unexpectedly, we found an apparent increase in the number of ERG-positive cells in the brains of $tPA^{-/-}$ mice ($n = 5$) relative to WT littermate controls ($n = 5$; **Figure 2A**). This was confirmed

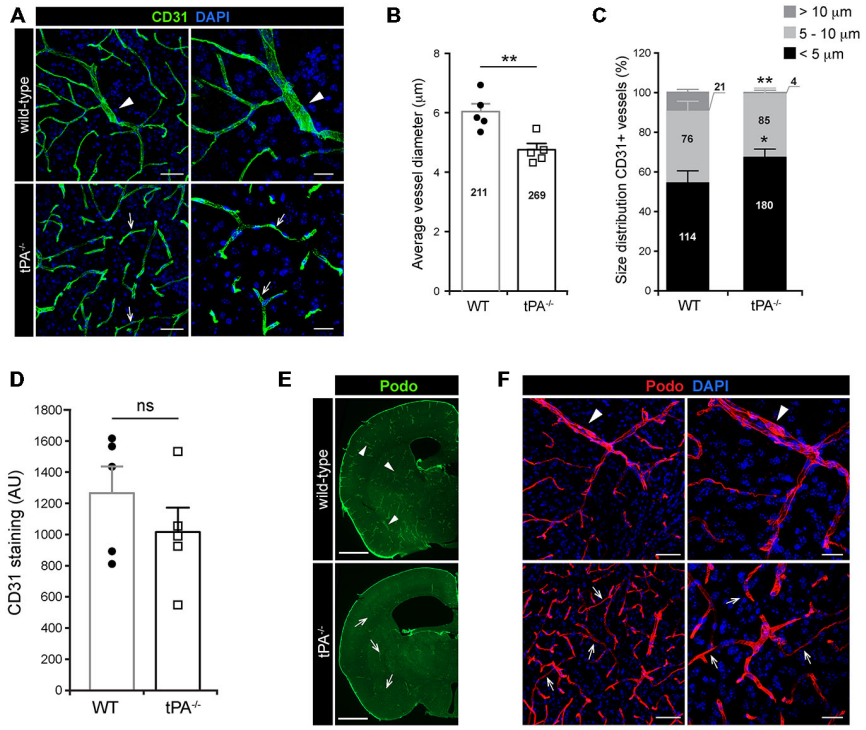

FIGURE 1 | Decreased number of large diameter vessels in tPA$^{-/-}$ brains. Immunofluorescent staining of murine brain sections from *tPA*$^{-/-}$ deficient mice ($n = 5$) and wild-type (WT) littermate controls (WT, $n = 5$) with **(A–D)** the endothelial cell marker CD31 and **(E–F)** podocalyxin (Podo) showed fewer large diameter vessels (arrowheads) and increased number of small diameter vessels (arrows) in *tPA*$^{-/-}$ mice compared to WT littermate controls. Quantification of the CD31 staining from four confocal *Z*-stacks per animal revealed that the average vessel diameter was significantly lower in *tPA*$^{-/-}$ brains as compared to WT littermate controls **(B)** and that there was a significant redistribution toward smaller diameter vessels in the cerebrovasculature of *tPA*$^{-/-}$ mice **(C)**. The number of vessels analyzed is displayed on the respective bars. There was no significant difference in the overall amount of CD31 positive staining as quantified by pixel intensity **(D)**. Staining with podocalyxin confirmed the results seen with staining for CD31 **(E–F)**. The data shown are representative quantifications of four to nine maximum intensity confocal *Z*-stacks per animal from four independent staining experiments with CD31 and Podocalyxin, respectively. The images display **(A,F)** maximum intensity projections generated from confocal *Z*-stacks (22 µm) and **(E)** stitched tiling of epifluorescent images (taken with 10× objective). Cell nuclei were visualized with DAPI. Data presented as mean ± SEM. Statistical significance was determined by student's unpaired *t*-test. $^{*}P < 0.05$; $^{**}P < 0.01$; *ns* = non significant relative to control. Scale bars **(A,F)** left panels, 50 µm; right panels, 20 µm, **(E)** 1 mm. Arbitrary units, A.U.

by quantification of the number of ERG-positive cells, showing a significant ($P < 0.01$) overall increase in the *tPA*$^{-/-}$ mice (20.2 ± 1.2 ERG+ cells/field of view) compared to WT littermate controls (15.2 ± 0.6 ERG+ cells/field of view; **Figure 2B**). To ensure that the increase of ERG-positive cells was not due to an increase in the number of small vessels in *tPA*$^{-/-}$ brains, we normalized the number of ERG+ cells to the amount of CD31 staining. This verified that there were significantly more ($P < 0.01$) ERG+ cells in the cerebral vessels of *tPA*$^{-/-}$ mice (149 ± 11% of WT; **Figure 2C**). This was particularly interesting since ERG has been implicated in vascular development, control of vascular permeability and maintenance of the integrity of the endothelial junctions (Randi et al., 2009; Birdsey et al., 2015). We therefore investigated the appearance of endothelial tight junctions in *tPA*$^{-/-}$ mice by immunofluorescent stainings using antibodies against the tight junction protein ZO1. We found that ZO1 appeared to show an increased junctional localization in vessels in *tPA*$^{-/-}$ brains compared to WT littermate controls (**Figure 2D**), suggesting that *tPA*$^{-/-}$ mice might have tighter endothelial cell-cell junctions.

tPA Deficiency Leads to a Redistribution in Vascular Mural Cell Coverage

Mural cells on the cerebral vascular tree include vascular smooth muscle cells (vSMCs) and capillary pericytes. These cells are known to play important roles in cerebrovascular events, including maintenance of the blood-brain barrier (BBB; Armulik et al., 2010; Daneman et al., 2010) and in regulation of vessel diameter and blood flow (Kornfield and Newman, 2014; Hill et al., 2015). To assess the distribution of vascular mural cell coverage on cerebral vessels in *tPA*$^{-/-}$ mice we performed immunofluorescent stainings using antibodies against ASMA, a marker for vSMCs, and CD13, a marker for pericytes. The stainings showed an apparent shift in the size of ASMA+ vessels, with more ASMA+ small diameter vessels (arrows) and fewer ASMA+ large diameter vessels (arrowheads) in *tPA*$^{-/-}$ brains ($n = 5$) compared to WT littermate controls ($n = 5$; **Figure 3A**). There was no significant difference in the overall amount of ASMA+ staining ($P = 0.46$; **Figure 3B**) but the average diameter of ASMA+ vessels was significantly smaller ($P < 0.01$) in *tPA*$^{-/-}$ mice (15.4 ± 0.7 µm) as compared to WT littermate controls

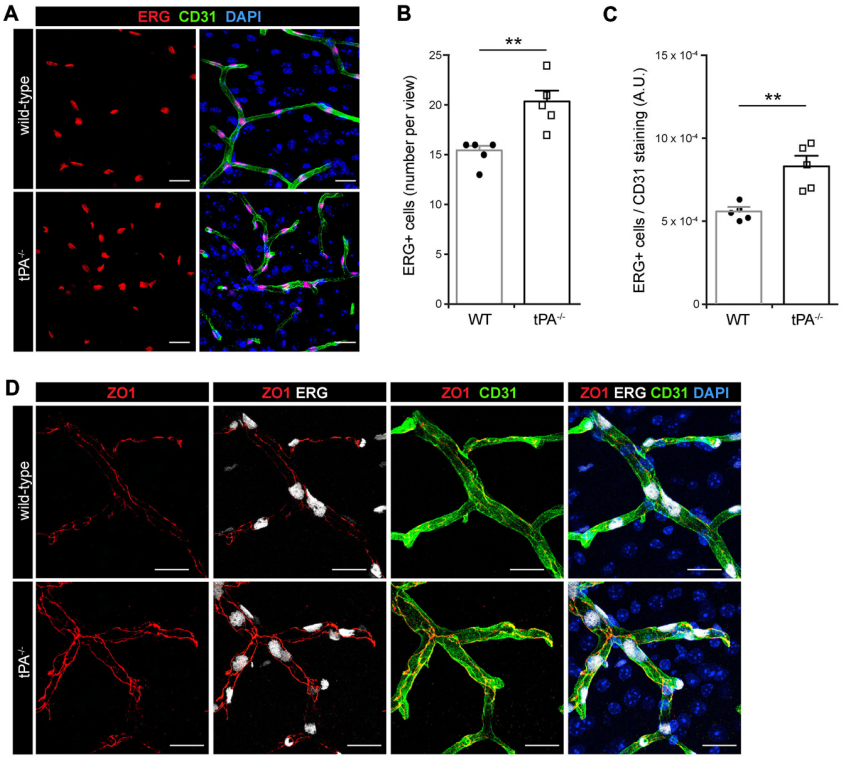

FIGURE 2 | Increased number of ERG+ endothelial cells and enhanced tight junction staining in tPA$^{-/-}$ mice. (A) Immunofluorescent stainings of murine brain sections from *tPA*$^{-/-}$ mice ($n = 5$) and WT littermate controls (WT, $n = 5$) with antibodies for the endothelial transcription factor ERG showed what appeared to be increased number of endothelial cells in *tPA*$^{-/-}$ mice as compared to WT littermate controls. **(B–C)** This was confirmed through quantification of the number of ERG+ nuclei from four maximum intensity confocal *Z*-stacks per animal. This revealed that there was significantly more ERG-positive cells in *tPA*$^{-/-}$ brains compared to WT littermate controls **(B)**, also when normalized to CD31 staining as quantified by pixel intensity (arbitrary units, A.U.) **(C)**. The data shown are representative quantifications of four to nine maximum intensity confocal *Z*-stacks per animal from two independent staining experiments. **(D)** Staining with antibodies against the tight junction protein ZO1 illustrated increased junctional expression in cerebral vessels in *tPA*$^{-/-}$ mice as compared to WT littermate controls. The images display maximum intensity projections generated from confocal *Z*-stacks (22 μm). Data presented as mean ± SEM. Statistical significance was determined by student's unpaired *t*-test and $^{**}P < 0.01$ relative to control. Scale bars **(A,D)** 20 μm.

(22.9 ± 1.3 μm; **Figure 3C**). *tPA*$^{-/-}$ mice displayed significantly increased numbers of ASMA+ small diameter vessels (<15 μm; WT = 17 ± 8% vs. *tPA*$^{-/-}$ = 61 ± 6%, $P < 0.01$) and fewer large diameter vessels (>30 μm; WT = 19 ± 3% vs. *tPA*$^{-/-}$ = 5 ± 1%, $P < 0.01$; **Figure 3D**). The microvascular capillaries in the adult murine brain of *tPA*$^{-/-}$ mice appeared to have normal coverage of pericytes as visualized by immunostainings with CD13 antibodies (arrows, **Figure 3E**). However, CD13 antibodies also stain vSMC in larger vessels (arrowheads, **Figure 3E**) and these vessels are essentially lost in *tPA*$^{-/-}$ mice, resulting in an overall reduction in CD13 staining relative to WT littermate controls ($P < 0.05$; **Figure 3F**).

The Basement Membrane and Astrocyte Distribution is not Affected by tPA Deficiency

To determine whether tPA deficiency affects the basement membrane and/or perivascular astrocyte distribution we analyzed sections from *tPA*$^{-/-}$ and WT littermate control brains stained with antibodies directed against collagen IV, a marker of the basement membrane; GFAP, a marker for astrocytes; and aquaporin 4 (AQP4), a marker of perivascular astrocytic endfeet. These analyses revealed what appeared as normal distribution of basement membrane around similar sized vessels in *tPA*$^{-/-}$ mice (**Figure 4A**). Further, we detected similar distribution of GFAP+ astrocytes in the parenchyma (arrowheads) as well as GFAP+ perivascular astrocytes associated with comparable sized vessels (arrows) in the brains of *tPA*$^{-/-}$ mice relative to WT littermate controls (**Figure 4B**). This was supported by stainings of astrocytic endfeet with AQP4 showing apparently normal perivascular astrocyte coverage in vessels of comparable size (**Figure 4C**).

tPA Deficiency is Associated with Reduced Levels of Vessel-Associated PDGFRα Expression

Due to our previous findings showing that the effect of tPA in regulation of cerebrovascular permeability is mediated via activation of PDGFRα signaling on perivascular astrocytes in the neurovascular unit (Fredriksson et al., 2004, 2005, 2015;

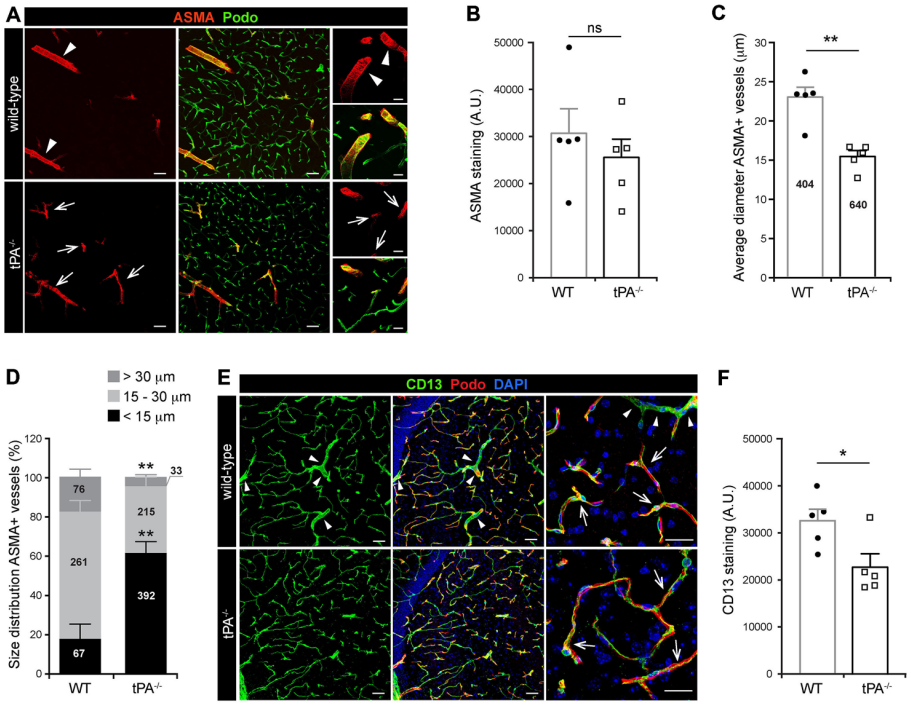

FIGURE 3 | Decreased number of large diameter ASMA+ vessels in tPA$^{-/-}$ brains. (A) Immunofluorescent staining of murine brain sections from mice *tPA*$^{-/-}$ (n = 5) and WT littermates (n = 5) with the vascular smooth muscle cell (vSMC) marker for alpha-smooth muscle actin (ASMA) displayed a redistribution of ASMA-positive (ASMA+) vessels in *tPA*$^{-/-}$ mice compared to WT littermate controls. There appeared to be more ASMA+ vessels with smaller diameter (arrows) in *tPA*$^{-/-}$ brains and fewer large ASMA+ diameter vessels (arrowheads) commonly seen in WT brains. **(B–D)** Quantification of the ASMA staining from eleven confocal images per animal revealed that there was no significant difference in the overall amount of ASMA staining as quantified by pixel intensity **(B)**, although the average vessel diameter was significantly lower in *tPA*$^{-/-}$ brains as compared to WT littermate controls **(C)**. In addition, there was a significant redistribution toward smaller diameter ASMA+ vessels in the cerebrovasculature of *tPA*$^{-/-}$ mice compared to WT littermate controls **(D)**. The number of vessels analyzed is displayed on the respective bars. The data shown are representative quantifications of four to eleven confocal images per animal from three independent staining experiments. **(E)** Staining with the vascular mural cell marker CD13, which stains pericytes, revealed that large diameter vessels positive for CD13, normally present in WT control brains (arrowheads), was essentially lost in *tPA*$^{-/-}$ brains. However, the pericyte coverage of capillaries appeared normal (arrows). **(F)** The loss of large diameter CD13+ vessels resulted in a significant reduction in CD13 staining in *tPA*$^{-/-}$ brains (n = 5) compared to WT littermate controls (n = 5). Quantification of CD13 pixel intensity was made from nine confocal Z-stacks per animal. The data shown are representative quantifications of four to nine maximum intensity confocal Z-stacks per animal from three independent staining experiments. The images display **(A,E)** maximum intensity projections generated from confocal Z-stacks (22 µm). Cell nuclei were visualized with DAPI and vessels with podocalyxin (Podo). Data presented as mean ± SEM. Statistical significance was determined by student's unpaired t-test. *$P < 0.05$; **$P < 0.01$; *ns* = non significant relative to control. Scale bars **(A,E)** left and middle panels, 50 µm; right panels, 20 µm. Arbitrary units, A.U.

Su et al., 2008) we investigated the expression of PDGFRα in *tPA*$^{-/-}$ brains. Immunofluorescent stainings for PDGFRα displayed a reduction of PDGFRα expression around vessels (arrows) in *tPA*$^{-/-}$ mice compared to WT littermate controls (**Figure 5A**). Quantification of the total amount and vessel-associated PDGFRα expression revealed that there was no significant difference ($P = 0.38$) in the total amount of PDGFRα+ staining between *tPA*$^{-/-}$ mice and WT littermate controls (**Figure 5B**). Perivascular PDGFRα expression was on the other hand significantly reduced in *tPA*$^{-/-}$ mice (21 ± 8% of WT) as compared to WT littermate controls ($P < 0.05$; **Figure 5C**).

Asymmetry of the Cerebral Lateral Ventricles and Distorted Ependymal Lining in tPA Deficient Mice

During the characterization of the *tPA*$^{-/-}$ mice we noted that these mice presented with asymmetric LV (**Figure 6**) similar to what we recently reported for *Pdgfc*$^{-/-}$ mice on C57BL/6 background (Fredriksson et al., 2012). Analysis of brain sections from *tPA*$^{-/-}$ mice (n = 5) by DAPI staining revealed that the abnormal LV (arrowheads) coincided with a hypoplastic septum, a defect that was not noted in any WT animals (n = 5; arrow, **Figure 6A**). To ensure that the ventricular abnormalities were not artifacts of tissue preparation, *in vivo* MRI in live mice was employed. **Figure 6B** shows the montage from MRI scans of one adult WT mouse (upper panel) and two *tPA*$^{-/-}$ mice (middle and lower panels). These MR scans illustrate displacement of the septum towards the side of the smaller ventricle in one of the *tPA*$^{-/-}$ brains (arrowhead) and a hypoplastic septum in the other *tPA*$^{-/-}$ mouse (arrow, **Figure 6B**). This displacement of the septum in the *tPA*$^{-/-}$ mice made the smaller ventricle appear compressed. Comparing the total area of the smallest to the largest ventricle from the MR scans revealed significant asymmetry of the LV ($P < 0.05$) in the *tPA*$^{-/-}$ mice

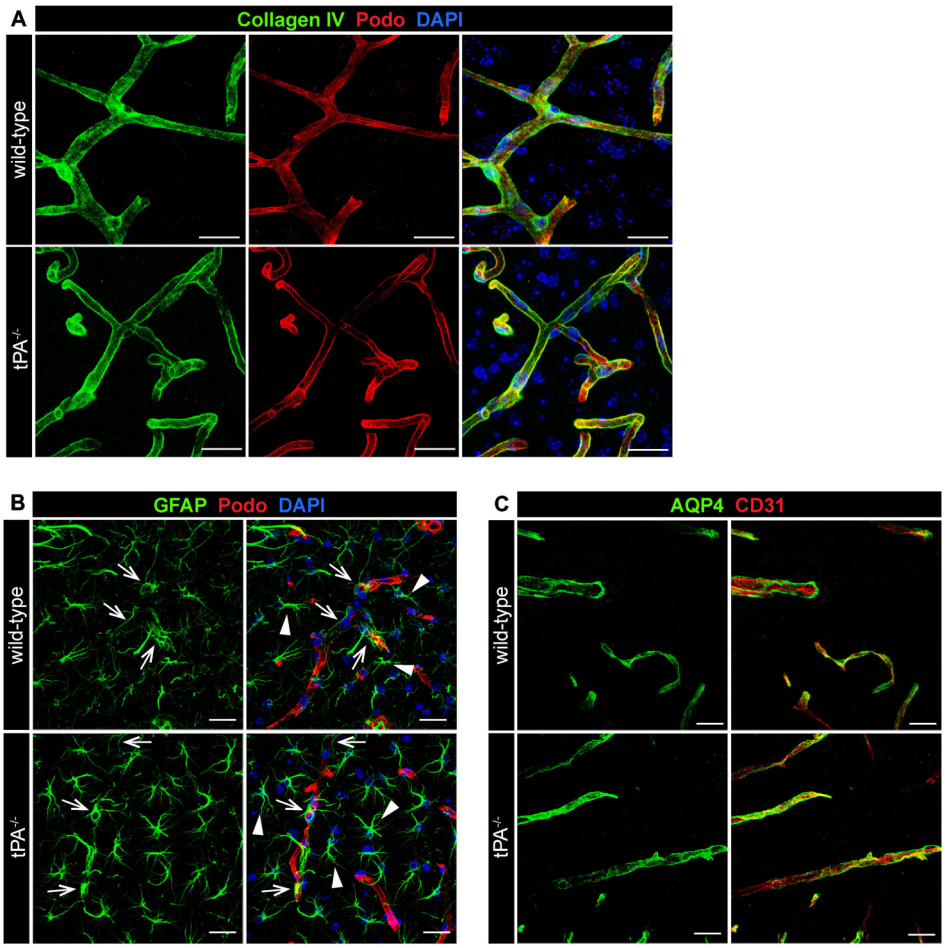

FIGURE 4 | Normal basement membrane and astrocyte distribution in tPA$^{-/-}$ mice. Immunofluorescent staining of murine brain sections from *tPA*$^{-/-}$ mice (n = 5) and WT littermate controls (n = 5) was conducted with antibodies directed against **(A)** collagen IV, a marker of the basement membrane, **(B)** glial fibrillary acidic protein (GFAP), a marker for astrocytes, and **(C)** aquaporin 4 (AQP4), a marker of perivascular astrocytic endfeet. These analyses revealed normal distribution of basement membrane **(A)** and perivascular astrocytes (arrows, **B,C)** around similar sized vessels in *tPA*$^{-/-}$ mice and WT littermates. In addition, normal distribution of parenchymal astrocyte staining (arrowheads, **B**) was detected. The pictures are representative maximum intensity projections images generated from confocal *Z*-stacks from stainings on brain sections from WT and *tPA*$^{-/-}$ mice and the stainings have been repeated at least two independent times. Cell nuclei were visualized with DAPI and vessels with podocalyxin (Podo) or CD31. Scale bars 20 μm.

(70 ± 5%, n = 13) as compared to WT littermate controls (86 ± 4%, n = 10; **Figure 6C**), thus confirming the observation from the DAPI analysis. The ventricular abnormalities seen in the *tPA*$^{-/-}$ mice appeared to be milder when compared to the asymmetry of the LV reported in *Pdgfc*$^{-/-}$ mice (56 ± 6%; Fredriksson et al., 2012).

In adult mammals, the cerebral ventricles are normally lined by a single layer of uninterrupted ciliated squamous to columnar ependymal cells, which form the interface between the brain parenchyma and the ventricular cavities (Bruni, 1998). Based on the above findings of lateral ventricle abnormalities in the *tPA*$^{-/-}$ mice we then examined the integrity of the ependymal cell lining in adult tPA mutant mice. In the *tPA*$^{-/-}$ mice (n = 5) we noted that the ependymal lining, here visualized by the ependymal marker S100B (Bruni, 1998), was distorted and that the normal cuboidal shape of ependymal cells was often lost in the *tPA*$^{-/-}$ animals (arrows, **Figure 6D**). The ependymal lining appeared pleomorphic in *tPA*$^{-/-}$ mice and did not form a uniform single layer as seen in WT littermate controls (n = 5; arrowheads, **Figure 6D**). This was further confirmed by immunostaining with podocalyxin (Podo) showing that the strong apical border staining seen in WT ependyma was abnormal and distorted in *tPA*$^{-/-}$ mice (**Figure 6D**, middle panels). Unlike what we saw in the *Pdgfc*$^{-/-}$ mice, we did not find any signs of ependymal denudation or decreased expression in glucose transporter 1, GLUT1, (a marker for mature ependyma; Silva-Alvarez et al., 2005) in the ventricular wall of *tPA*$^{-/-}$ mice. On the contrary, our analysis showed an increase in GLUT1 staining in the ventricular wall (indicated by the dashed lines) in *tPA*$^{-/-}$ mice (n = 5) compared to WT littermate controls (n = 5; **Figure 6E**). This was

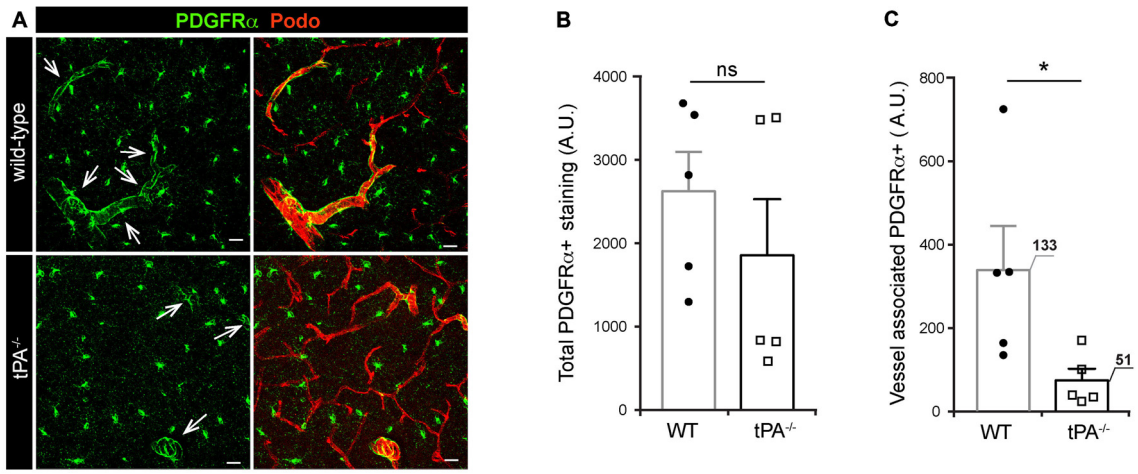

FIGURE 5 | Decreased expression of perivascular PDGFRα in brains of tPA$^{-/-}$ mice. (A) Immunofluorescent staining of murine brain sections from *tPA*$^{-/-}$ deficient mice (n = 5) and WT littermate controls (WT, n = 5) with antibodies directed against PDGFRα displayed fewer PDGFRα-positive (PDGFRα+) vessels (arrows) in *tPA*$^{-/-}$ mice compared to WT littermate controls. **(B–C)** Quantification of the PDGFRα staining by pixel intensity from nine maximum intensity confocal *Z*-stacks per animal revealed that there was no significant difference in the overall amount of PDGFRα staining **(B)**, but that perivascular PDGFRα expression was significantly reduced in *tPA*$^{-/-}$ mice as compared to WT littermate controls **(C)**. The number of PDGFRα+ vessels analyzed is displayed on the respective bars. The data shown are representative quantifications of nine maximum intensity confocal *Z*-stacks per animal and images from epifluorescent tiles of the entire brain from three independent staining experiments. The images display maximum intensity projections generated from confocal *Z*-stacks (22 μm). Cell nuclei were visualized with DAPI and vessels with podocalyxin (Podo). Data presented as mean ± SEM. Statistical significance was determined by student's unpaired *t*-test and $^*P < 0.05$; *ns* = non significant relative to control. Scale bars 20 μm. Arbitrary units, A.U.

confirmed by quantification of the ventricular GLUT1 staining ($P < 0.05$; *tPA*$^{-/-}$ = 580 ± 144% of WT; **Figure 6F**). Further, the ependymal cells in the ventricular walls normally display a punctate pattern of ZO1 distribution, indicative of discontinuous tight junctions (Petrov et al., 1994). This punctate pattern was seen in the WT littermate controls (arrowheads, **Figure 6G**), but in *tPA*$^{-/-}$ mice, the ZO1 staining indicated continuous tight junctions between the ependymal cells (arrows, **Figure 6G**). Quantification of the ZO1 staining showed significantly increased levels of ZO1 ($P < 0.01$) in the ventricular wall of *tPA*$^{-/-}$ mice (n = 5) compared to WT littermate controls (n = 5; *tPA*$^{-/-}$ = 363 ± 62% of WT; **Figure 6H**).

Taken together these results demonstrate that ablation of tPA affects the cerebrovascular bed as well as ventricular development and ependymal integrity.

DISCUSSION

Thrombolytic treatment with tPA only benefits a limited number of patients with ischemic stroke, and the limitations appear to be due in part to unique activities of tPA in the brain beyond its well established role in fibrinolysis (Nicole et al., 2001; Wang et al., 2003; Yepes et al., 2003; Su et al., 2009). Development of improved therapies for stroke will therefore benefit from understanding the unique characteristics of the role that endogenous tPA plays in the CNS. Previous work has demonstrated an effect of tPA as a necessary and sufficient regulator of cerebrovascular permeability and that targeting tPA-induced opening of the BBB improves outcome of disease (Yepes et al., 2003; Su et al., 2008; Fredriksson et al., 2015). To advance our understanding of the role of tPA in regulation of cerebrovascular integrity we performed a detailed characterization of the cerebrovasculature in tPA deficient mice. We demonstrate that tPA ablation leads to reorganization of the vascular bed in the adult murine brain, with a significant decrease in average vessel diameter, mainly due to a reduction in the number of large and medium sized vessels. These vessels are normally enveloped by PDGFRα+ perivascular astrocytes (Fredriksson et al., 2015), and we believe that the loss of these larger vessels in *tPA*$^{-/-}$ brains accounts for the observed reduction in vessel-associated PDGFRα expression. This is particularly interesting since our previous work has shown that the effect of tPA in regulation of cerebrovascular permeability is mediated via tPA-catalyzed activation of PDGF-C, and subsequently PDGFRα signaling on perivascular astrocytes in the neurovascular unit (Fredriksson et al., 2004, 2005; Su et al., 2008). The data presented here suggests that the protection of barrier properties seen utilizing these *tPA*$^{-/-}$ mice might be due, at least in part, to the cerebrovascular rearrangements, further supporting our idea that tPA-induced changes in cerebrovascular permeability proceeds dysregulation of neuronal signaling pathways (Fredriksson et al., 2015). In support of the notion, that congenital cerebrovascular rearrangements contribute to increased cerebrovascular integrity in *tPA*$^{-/-}$ mice, is our intriguing observation that ablation of tPA results in increased number of ERG-positive endothelial cells. Overexpression of this endothelial transcription factor *in vivo* has recently been

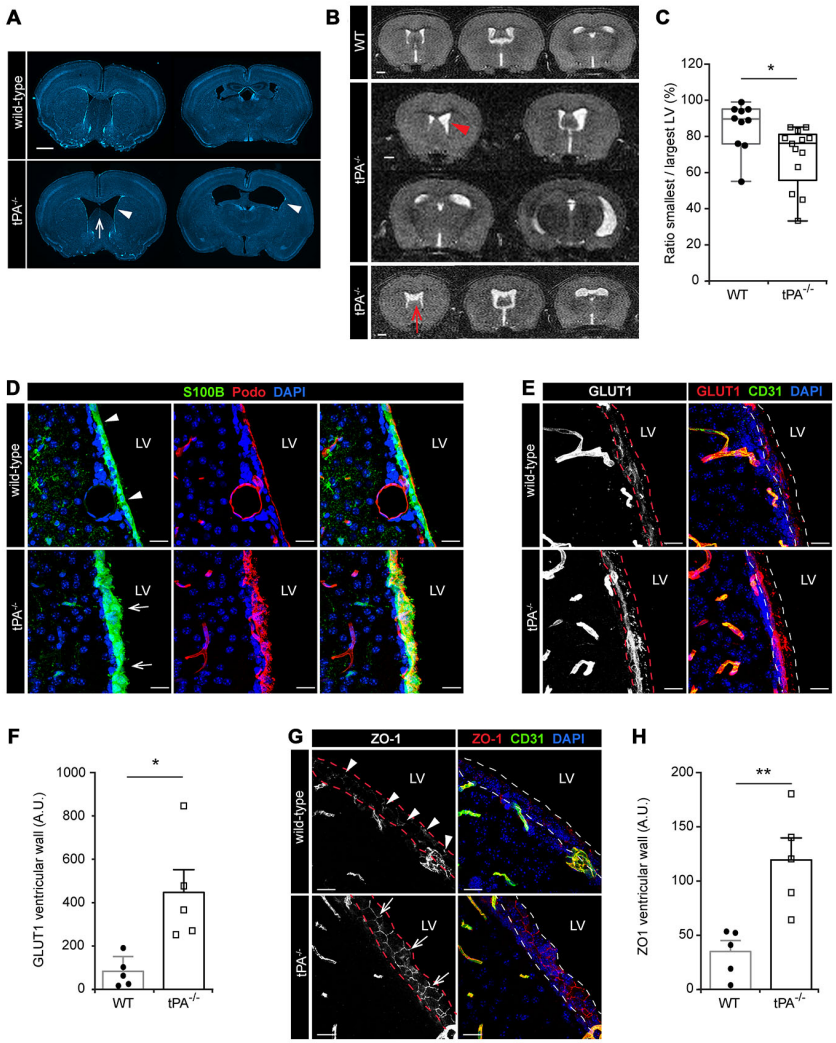

FIGURE 6 | Lateral ventricular defects and abnormal ependymal lining in the brains of tPA$^{-/-}$ mice. Lateral ventricular abnormalities (arrowheads) and hypoplastic septum separating the lateral ventricles (LV; arrows) were seen in *tPA*$^{-/-}$ mice in **(A)** *ex vivo* sections visualized with DAPI and with **(B)** *in vivo* magnetic resonance imaging (MRI) of WT and *tPA*$^{-/-}$ adult mice. **(B)** Montage from MR scans of an adult WT female mouse (upper panel) and two *tPA*$^{-/-}$ mice (middle panels, images from a male adult *tPA*$^{-/-}$ mouse and lower panel, images from a female adult *tPA*$^{-/-}$ mouse). **(C)** Quantification of the ratio between the smallest to the largest ventricular size from the MRI scans confirmed that *tPA*$^{-/-}$ mice (n = 13) displayed a significant asymmetry in lateral ventricular size as compared to WT littermate controls (n = 10). The quantifications in **(C)** were made using Image J to trace the entire area of the right and left ventricle respectively. **(D–H)** Abnormal ependymal lining of the LV in *tPA*$^{-/-}$ mice were revealed through immunofluorescent stainings and confocal microscopy on brain sections from WT (n = 5) and *tPA*$^{-/-}$ (n = 5) mice. Analysis of the ependymal cell lining of the lateral wall of the LV was conducted using antibodies against S100B and Podocalyxin (Podo) **(D)**, GLUT1 **(E–F)** and ZO1 **(G–H)**. Note the thicker ependymal lining in *tPA*$^{-/-}$ mice (arrows, **D**) compared to WT littermate controls (arrowheads, **D**) and the punctate pattern of ZO1 staining in WT (arrowheads, **G**) compared to the continuous tight junctions between ependymal cells in *tPA*$^{-/-}$ mice (arrows, **G**). Quantification of ependymal GLUT1 and ZO1 staining (between the dashed lines in **E,G**) by pixel intensity from six (GLUT1) and four (ZO1) maximum intensity confocal Z-stacks per animal revealed an significant increase of both GLUT1 **(F)** and ZO1 **(H)** staining in the ventricular wall of *tPA*$^{-/-}$ mice compared to WT littermate controls. The data shown are representative quantifications of two to six maximum intensity confocal Z-stacks per animal from two independent staining experiments. The images display **(A)** stitched tiling of epifluorescent images (taken with 10× objective) and **(D,E,G)** maximum intensity projections generated from confocal Z-stacks (22 μm). Each picture is a representative from different individuals. Cell nuclei were visualized with DAPI and vessels with podocalyxin (Podo) or CD31. Data presented as **(C)** box-and-whiskers plot for distribution with min to max variance and **F,H**) mean ± SEM. Statistical significance was determined by student's unpaired t-test and $^{*}P < 0.05$; $^{**}P < 0.01$ relative to control. Scale bars **(A–B)** 1 mm and **(D,E,G)** 20 μm. LV, Lateral ventricles. Arbitrary units; A.U.

shown to reduce vascular permeability during VEGF-induced angiogenesis (Birdsey et al., 2015). It should be noted however, that tPA is sufficient to directly control cerebrovascular permeability, not only due to congenital rearrangements in the cerebrovascular bed, since intraventricular injection of tPA induces BBB opening in WT mice (Yepes et al., 2003). Further, cerebrovascular permeability induced by experimental ischemic stroke can be blocked by neuroserpin (Yepes et al., 2003), the specific inhibitor of tPA in the brain (Fredriksson et al., 2015).

In addition to its role in regulation of cerebrovascular permeability (Fredriksson et al., 2015), tPA has been implicated in other cerebrovascular responses, including neurovascular coupling (Park et al., 2008; Su et al., 2009). Neurovascular coupling refers to increases in cerebral blood flow in response to neuronal activity and ensures coupling between energy demand and supply (Iadecola, 2004; Hamel, 2006). In the study by Park et al. (2008) they utilized $tPA^{-/-}$ mice to demonstrate that cerebral blood flow in the corresponding barrel cortex of these mice showed a sustained attenuation after whisker stimulation compared with WT mice. Another study has recently demonstrated that neurovascular coupling of local neural activity to local blood flow takes place exclusively at ASMA+ SMC-covered arterioles, but not in pericyte-covered capillaries (Hill et al., 2015). Interestingly, tPA has been shown to be expressed around arterioles in the CNS (Levin and del Zoppo, 1994; Su et al., 2008; Fredriksson et al., 2015). Given the decreased number of large diameter ASMA+ vessels observed in $tPA^{-/-}$ mice it therefore seems plausible to suggest that the cerebrovascular rearrangements associated with tPA ablation may explain the attenuated neurovascular coupling response in $tPA^{-/-}$ mice. Arguing against this are the findings that ectopic administration of tPA to $tPA^{-/-}$ mice restored the neurovascular coupling response (Park et al., 2008) and that *in vivo* vasomotion in awake mice occurs in vessels covered by SMCs, regardless of their diameter (Hill et al., 2015). In addition, the add-back experiment (Park et al., 2008) implicates there may be another downstream target of tPA than PDGFRα signaling in the neurovascular coupling response since vessel-associated expression of the receptor is reduced in $tPA^{-/-}$ mice.

Our previous findings established a novel, previously unknown, role for PDGF-C/PDGFRα signaling in cerebroventricular development (Fredriksson et al., 2012). This was later confirmed by a study showing that aberrant PDGFRα signaling in primary cilia, and a subsequent loss of NG2(+)PDGFRα(+) neural progenitor cells in the subventricular zone, is associated with ventricular malformations (Carter et al., 2012). The findings that $tPA^{-/-}$ mice displayed mild cerebroventricular malformations is especially intriguing since this provides an *in vivo* link between tPA and PDGF-C signaling during CNS development. As reported for the PDGF-C deficient ($Pdgfc^{-/-}$) mice (Fredriksson et al., 2012), where the smaller ventricle appeared to arise from displacement of the septum, i.e., the structure separating the LV, this also seemed to be the case in $tPA^{-/-}$ mice. The overall effect on cerebroventricular assymmetry seemed less affected by ablation of tPA compared to PDGF-C ablation. Our analysis did however identify a few cases of severe assymetry and incomplete development of the septum, a feature reminiscent of cavum septum pellucidum in humans. A cavum of the septum is often present at birth but eliminated as the septal leaves fuse postnatally (Raine et al., 2010) and preservation of the cavum in adults has been suggested to be due to underdevelopment of limbic structures such as the hippocampus, amygdala and septal nuclei (Nopoulos et al., 2000; Kim et al., 2007), places where high expression of tPA is seen. Contrary to our earlier study, showing that PDGF-C ablation causes defects in the ependymal lining of the LV, including ependymal denudation and reduced expression of GLUT1 in the ventricular wall (Fredriksson et al., 2012), we did not find any signs of ependymal denudation in $tPA^{-/-}$ mice and instead noted an increase in ventricular expression of GLUT1 and ZO1. This is indicative of a more mature ventricular lining with higher integrity in $tPA^{-/-}$ mice compared to WT littermate controls and $Pdgfc^{-/-}$ mice. The opposing effects of tPA and PDGF-C ablation on ependymal development, and in cerebrovascular development where $Pdgfc^{-/-}$ mice, contrary to $tPA^{-/-}$ mice, display a significant overall increase in vessel diameter and ASMA+ expression, suggests additional, independent roles, of the two in cerebral development. Whether the congenital defects observed in $tPA^{-/-}$ mice are directly controlled by tPA-mediated processes, or an indirect consequence of tPA ablation, remains to be established. It will therefore be interesting to utilize the $tPA^{-/-}$ mice, $Pdgfc^{-/-}$ mice (Fredriksson et al., 2012) and conditional PDGFRα knockout mice (Carter et al., 2012) to further unravel aspects and underlying molecular mechanisms of cerebral vascular, ventricular and ependymal development.

In conclusion, our data demonstrate that tPA plays an important role in normal cerebral vascularization and normal cerebral ventricular formation. Thus, this study enhances our understanding of the role of tPA in the CNS, in particular the role in regulation of cerebrovascular integrity, and might help explain how vascular barrier defects contribute to stroke evolution. In addition, since increased cerebrovascular permeability constitutes a significant pathologic factor in the development of other neurologic diseases, including seizures (Friedman et al., 2009) and traumatic brain injury (Shlosberg et al., 2010), and since tPA-mediated PDGFRα signaling has been implicated in regulation of vascular integrity in these diseases (Fredriksson et al., 2015; Su et al., 2015), our findings might be of importance not only for stroke but also for unrelated CNS disorders where vascular integrity is compromised.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00456/abstract

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frontiers in Cellular Neuroscience

MINI REVIEW
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Breaking boundaries—coagulation and fibrinolysis at the neurovascular interface

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Blood proteins at the neurovascular unit (NVU) are emerging as important molecular determinants of communication between the brain and the immune system. Over the past two decades, roles for the plasminogen activation (PA)/plasmin system in fibrinolysis have been extended from peripheral dissolution of blood clots to the regulation of central nervous system (CNS) functions in physiology and disease. In this review, we discuss how fibrin and its proteolytic degradation affect neuroinflammatory, degenerative and repair processes. In particular, we focus on novel functions of fibrin—the final product of the coagulation cascade and the main substrate of plasmin—in the activation of immune responses and trafficking of immune cells into the brain. We also comment on the suitability of the coagulation and fibrinolytic systems as potential biomarkers and drug targets in diseases, such as multiple sclerosis (MS), Alzheimer's disease (AD) and stroke. Studying coagulation and fibrinolysis as major molecular pathways that regulate cellular functions at the NVU has the potential to lead to the development of novel strategies for the detection and treatment of neurologic diseases.

Keywords: fibrinogen, blood-brain barrier, microglia, autoimmunity, neuroinflammation, neurodegeneration, multiple sclerosis, Alzheimer's disease

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Fibrin Formation and Degradation in the CNS

The plasminogen activation (PA) system is an enzymatic cascade with key regulatory functions in fibrinolysis and degradation of extracellular matrix proteins (Syrovets and Simmet, 2004; Castellino and Ploplis, 2005; Kwaan, 2014). Plasminogen circulates in the blood as an inactive zymogen that is converted into active plasmin by tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). The serine protease tPA is an immediate-early response gene expressed in the brain (Bignami et al., 1982; Qian et al., 1993; Sappino et al., 1993; Carroll et al., 1994; Tsirka et al., 1995). The activity of tPA is controlled by plasminogen activator inhibitor 1 (PAI-1). Upon activation, plasmin binds

Abbreviations: Akt, Protein kinase B; APC, Antigen-presenting cells; CCL2, Chemokine (C-C motif) ligand 2; CSPG, Chondroitin sulfate proteoglycan; CXCL10, C-X-C motif chemokine 10; EAE, Experimental autoimmune encephalomyelitis; EGFR, Epidermal growth factor receptor; ERK1/2, Extracellular signal-regulated kinase 1/2; FIE, Fibrinogen-induced encephalomyelitis; ICAM-1, Intercellular adhesion molecule 1; MCP-1, Monocyte chemoattractant protein 1; MEK, Mitogen-activated protein kinase kinase 1; NF-κB, Nuclear factor "kappa-light-chain-enhancer" of activated B-cells; PI3K, Phosphoinositide 3-kinase; ROS, Reactive oxygen species; Smad2, SMAD family member 2; TCR, T-cell receptor; TGFβ, Transforming growth factor beta; TJ, Tight junction; TLR4, Toll-like receptor 4; VCAM-1, Vascular cell adhesion molecule-1; VE, Vascular endothelial.

its main substrate fibrin(ogen) and degrades insoluble fibrin deposits that form intravascularly during blood clotting, as well as in the central nervous system (CNS) parenchyma after vascular rupture (Cesarman-Maus and Hajjar, 2005; Davalos et al., 2012). Fibrin controls plasmin activity through its capacity to bind plasminogen (Plg) as well as tPA or tPA/PAI-1 complexes to facilitate their proximate interaction (Wagner et al., 1989; Kaczmarek et al., 1993; Kim et al., 2012).

The pivotal fibrinolytic functions of the PA system were discovered in Plg-deficient mice, which show impaired wound healing, severe thrombosis, early lethality and delayed nerve regeneration (Bugge et al., 1995; Akassoglou et al., 2000). Interestingly, this phenotype is rescued by fibrinogen deficiency, suggesting that fibrin(ogen) is the main physiologic substrate for plasmin *in vivo* (Bugge et al., 1996; Akassoglou et al., 2000). Besides binding plasmin, fibrin(ogen) interacts with cell surface receptors expressed by different cell types in the CNS, including microglia (Adams et al., 2007; Davalos et al., 2012; Ryu et al., 2015), neurons (Schachtrup et al., 2007), astrocytes (Schachtrup et al., 2010) and Schwann cells (Akassoglou et al., 2002; reviewed in Davalos and Akassoglou, 2012; Ryu et al., 2009). Thus, fibrinogen acts as a molecular switch linking the PA system to activation of cell intrinsic signaling pathways involved in immune response and CNS homeostasis/neuronal functions (**Figure 1**).

The multifaceted and central functions of fibrin(ogen) in the PA system are highlighted by studies showing that fibrin acts: (1) as a main substrate of plasmin during fibrinolysis; (2) as a feed-back regulator of PA by binding tPA/PAI-1 or Plg directly; and (3) as a signaling molecule for cell activation in the CNS. By highlighting the PA system as a molecular link between coagulation, fibrinolysis and inflammation, this review will focus on cellular mechanisms and molecular signaling pathways driven by fibrin deposition and fibrinolysis in the CNS, specifically at the neurovascular unit (NVU).

The Plasminogen System in Blood-Brain Barrier Dynamics

Under healthy conditions, plasma proteins like fibrinogen and Plg are not found in the brain parenchyma—a relatively immune-priviledged environment sealed by the selectively permeable blood-brain-barrier (BBB). Activation of the Plg system in the CNS parenchyma occurs in response to BBB disruption in which components from the blood enter the brain milieu (**Figure 1**). The BBB is an emergent property of the brain vasculature controlled by endothelial cells ensheathed by pericytes and astrocytic endfeet. The brain vasculature with an intact BBB plays essential roles in maintaining flow of nutrients into the brain, as well as protecting the brain from invasinto the brain, as well as protecting the brain from invasion by toxins, pathogens and inflammatory cells (Zlokovic, 2008; Daneman and Prat, 2015).

BBB opening can result from tight junction (TJ) complex disassembly or downregulation, increased transcellular transport, or physical damage to the blood vessel (Stamatovic et al., 2008). Disruption of the BBB is observed in a variety of neurological conditions in humans and in their animal models, such as stroke (Elster and Moody, 1990; Belayev et al., 1996), traumatic brain injury (Tanno et al., 1992; Conti et al., 2004; Shlosberg et al., 2010), epilepsy (Sokrab et al., 1990; Liu et al., 2012) and chronic neuroinflammation and neurodegeneration, including multiple sclerosis (MS; Paterson, 1976; Grossman et al., 1988; Miller et al., 1988; Adams et al., 2004; Gaitán et al., 2011) and Alzheimer's disease (AD; van Oijen et al., 2005; Ahn et al., 2010; Cortes-Canteli et al., 2010; Oh et al., 2014b). BBB opening is also a hallmark of normal aging (Tucsek et al., 2014; Montagne et al., 2015). Indeed, contrast-enhanced MRI showed an age-dependent BBB breakdown in the hippocampus, a region critical for learning and memory that is affected in neurodegenerative diseases, such as AD (Montagne et al., 2015).

Multiple components of the PA system and in particular tPA function in BBB homeostasis (Vivien et al., 2011). tPA opens the BBB via mechanisms that include activation of platelet-derived growth factor-CC (PDGF-CC) signaling (Su et al., 2008), astrocyte remodeling through plasmin (Niego et al., 2012) and phosphorylation of BBB proteins claudin-5 and occludin (Kaur et al., 2011), as well as through a mechanism independent of its catalytic activity toward Plg (Abu Fanne et al., 2010). tPA may also open the BBB via low density lipoprotein receptor–related protein 1 (LRP-1) signaling (Yepes et al., 2003), which may be mediated by matrix metalloproteinase (MMPs; Wang et al., 2003; Lakhan et al., 2013). In contrast, PAI-1, the primary inhibitor of tPA, enhances barrier tightness in *in vitro* BBB models (Dohgu et al., 2011). tPA may also regulate the BBB through annexin-2 (Cristante et al., 2013). These studies show that tPA regulates several potentially overlapping pathways involved in BBB dysfunction. Evidence for tPA in maintaining vascular integrity can also be found in the clinic, as tPA treatment for thrombotic stroke increased hemorrhagic risk (Fugate and Rabinstein, 2014). Similarly, anticoagulants, such as clopidogrel, which inhibit platelet functions, increase the risk of brain hemorrhage after a stroke (Morrow et al., 2012).

In addition to the fibrinolytic system, molecular players promoting clot formation also regulate the BBB. Thrombin, the catalyst of fibrin formation, may disrupt the BBB (Lee et al., 1997; Liu et al., 2010) and in a human brain endothelial cell line can induce upregulation of intercellular adhesion molecule 1 (ICAM-1), Vascular cell adhesion molecule-1 (VCAM-1) and cytokines chemokine (C-C motif) ligand 2 (CCL2) and CX3CL1 (Alabanza and Bynoe, 2012). Fibrinogen increases endothelial cell permeability *in vitro*, in part by reducing expression of TJ proteins (Tyagi et al., 2008; Patibandla et al., 2009). The likelihood of BBB opening in response to fibrinogen may be increased under pathological conditions in which fibrinogen/fibrin accumulates on the blood vessel wall and in the parenchyma. A positive feedback loop whereby a precipitating event transiently opens the BBB, leading to the activation of the Plg and coagulation systems in the CNS, the components of which then further act to exacerbates BBB dysfunction can be envisaged. In sum, many pathologies are associated with BBB breakdown, indicated by persistent fibrin deposition inside the CNS. Therefore, fibrin has emerged as a potential target for development of diagnostic tools and therapeutic strategies

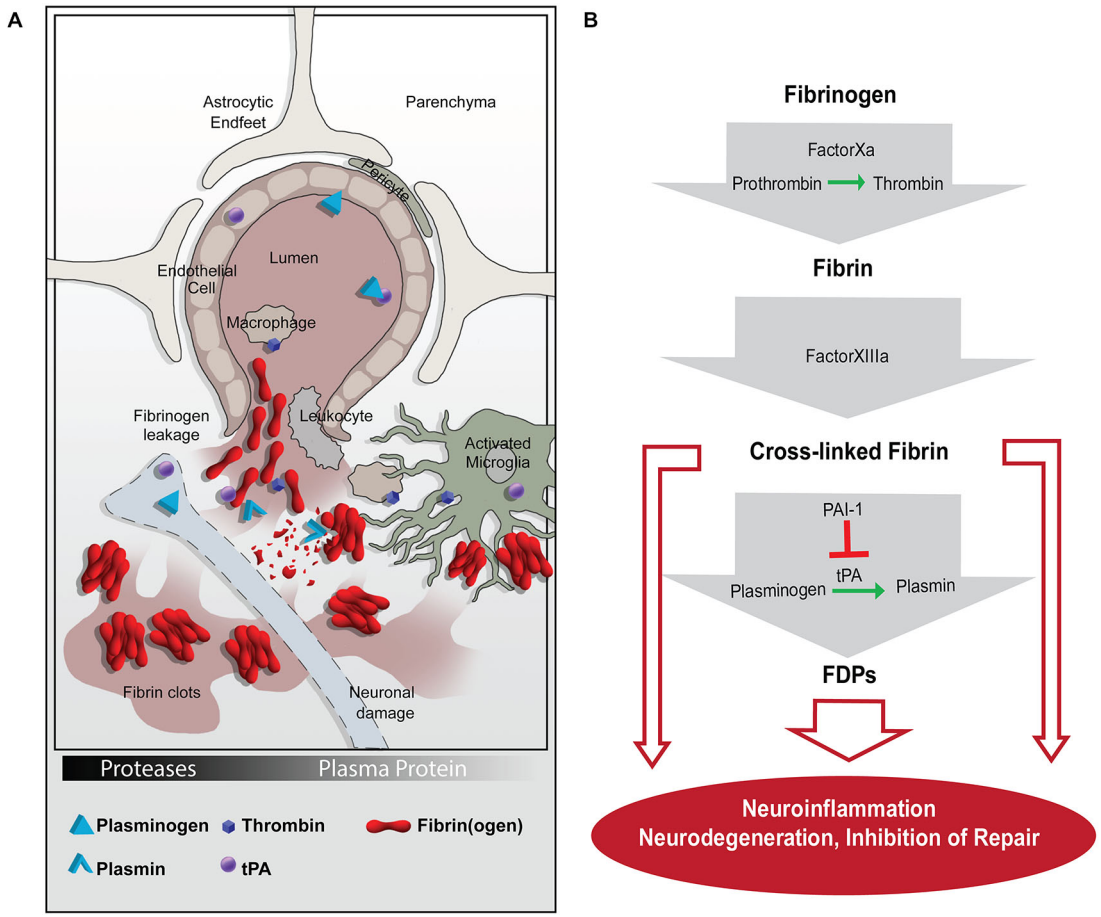

FIGURE 1 | The coagulation and proteolytic cascades at the neurovascular interface. (A) Fibrinogen leakage in the central nervous system (CNS) and activation of the plasminogen activation (PA) system occur following blood-brain-barrier (BBB) disruption. The molecular network of fibrin and the PA system enable inflammation and neurodegeneration via activation of microglia, macrophages, and leukocytes. **(B)** A series of proteolytic events converts extravasated fibrinogen into insoluble fibrin, which can be cleaved into FDPs. Fibrin and FDPs interact with cellular receptors to induce inflammation, degeneration, and repair inhibition in the nervous system. tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor-1; FDPs, fibrin degradation products.

(Conti et al., 2004; Adams et al., 2007; Craig-Schapiro et al., 2011; Ahn et al., 2014; Davalos et al., 2014).

Plasminogen Activation and Fibrin Degradation in CNS Inflammation

Insofar as fibrin is necessary to stop hemorrhage, and plasmin can remove fibrin clots that block vital blood flow, the PA system has a beneficial role in the brain. However, dysregulation of the PA and coagulation systems are linked to inflammation, which is a common hallmark of many CNS pathologies, including the autoimmune disease MS (East et al., 2005; Marik et al., 2007; Han et al., 2008), as well as other chronic neuroimmune and neurodegenerative disorders (van Oijen et al., 2005; Paul et al., 2007).

MS is an autoimmune disease in which the myelin-producing oligodendrocytes are targeted for destruction by the immune system. Histopathology of human brain tissue shows focal fibrin deposition in MS plaques, indicative of perivascular inflammation and BBB disruption (Gay and Esiri, 1991; Kirk et al., 2003; Vos et al., 2005; Marik et al., 2007) that is also observed in MS mouse models (Paterson et al., 1987; Adams et al., 2004, 2007). Proteomic analysis of chronic active plaques from MS patients revealed a set of coagulation proteins uniquely present in active plaques, suggesting a role for the coagulation cascade in the development of MS pathology (Han et al., 2008). Indeed, MS lesions have increased levels of PAI-1 and less fibrin degradation and, thus, more sustained fibrin deposition than normal control tissue (Gveric et al., 2003). Fibrin depletion provides protection in a wide range of MS mouse models (Paterson, 1976; Akassoglou et al., 2004; Adams et al., 2007; Yang et al., 2011; Davalos et al., 2012). Studies of other Plg cascade components also support the hypothesis that fibrin deposition is a major instigator of experimental autoimmune encephalomyelitis (EAE). $tPA^{-/-}$ mice have increased disease

severity in EAE, which may be due to accumulated fibrin deposits and/or loss of fibrin-independent tPA functions in the CNS (Lu et al., 2002; East et al., 2005). Exacerbation of demyelination in $tPA^{-/-}$ or $plg^{-/-}$ mice after peripheral nerve injury is fibrin-dependent, since fibrin depletion rescues the damaging effects of tPA or Plg deficiency (Akassoglou et al., 2000). Furthermore, $PAI\text{-}1^{-/-}$ mice have reduced EAE severity associated with increased fibrinolysis (East et al., 2008). It is important to underscore that fibrin and the tPA/plasmin system act in concert to exert the full effect of vascular-driven neuroinflammation. For example, inflammation and fibrin-induced neurodegeneration are reduced in $plg^{-/-}$ mice, suggesting that multiple molecular players from the coagulation and fibrinolytic systems are needed for a full inflammatory and degenerative response (Hultman et al., 2014).

Emerging evidence suggests a pivotal role of fibrin in the regulation of CNS innate and adaptive immune responses (Davalos et al., 2012; Ryu et al., 2015; **Table 1**). Fibrin(ogen) interactions with microglia, macrophages, and neutrophils via integrin receptor CD11b/CD18 (also known as Mac-1, Complement Receptor 3 or integrin $\alpha_M\beta_2$) were identified as direct activation pathways of innate immune response (Davalos and Akassoglou, 2012). Extravascular fibrin deposition stimulates recruitment and perivascular clustering of microglia in EAE lesions (Davalos et al., 2012), while deletion of fibrin or blockade of fibrin signaling protects from microglial activation and axonal damage in EAE (Akassoglou et al., 2004; Adams et al., 2007). A recombinant mutant thrombin analog similarly ameliorated EAE progression, corroborating the regulatory functions of thrombin-mediated fibrinogen/fibrin conversion during neuroinflammation (Verbout et al., 2015). Fibrin-induced activation of microglia via CD11b/CD18 induced secretion of cytokines and chemokines that stimulate recruitment of peripheral monocytes/macrophages (Ryu et al., 2015). Importantly, fibrin in the CNS white matter was sufficient to induce the infiltration and activation of myelin-specific T cells, suggesting a fibrin-induced innate immune-mediated pathway that triggers CNS autoimmunity (Ryu et al., 2015). Potential direct effects of fibrin on T cells might also play a role in autoimmune responses (Takada et al., 2010). Moreover, PA-mediated opening of the BBB and extracellular

TABLE 1 | Fibrin(ogen) cellular targets at the NVU in neurologic diseases.

Target	Functions	Receptors/Signaling pathways	Model	Reference
Resident cells				
Microglia	Activation – Phagocytosis – Perivascular clustering – Chemokine and proinflammatory gene expression – ROS release	CD11b/CD18 RhoA, Akt, PI3K	*In vitro*: microglia cultures *In vivo*: EAE; FIE, AD animal models	Adams et al. (2007) Davalos et al. (2012) Ryu et al. (2015) Paul et al. (2007)
Astrocytes	Gliosis – Scar formation	TGFβ, Smad2, CSPGs CSPGs	*In vivo*: stab wound injury; cortical fibrinogen injection	Schachtrup et al. (2010)
Neurons	– Axonal damage – Inhibition of neurite outgrowth	β3-integrin, EGFR	*In vitro*: neuronal cultures *In vivo*: EAE, spinal cord injury, ischemic stroke	Schachtrup et al. (2007) Davalos et al. (2012) Ill-Raga et al. (2015)
Endothelial cells	– Increased permeability – Infiltration of leukocytes	ICAM-1, α5β1 F-actin, TJ proteins, MEK, ERK, VE-cadherin, fibrin fragment E and Bβ15–42, RhoGTPase	*In vitro*: endothelial cell cultures	Tyagi et al. (2008) Patibandla et al. (2009) Jennewein et al. (2011) Muradashvili et al. (2011)
CNS infiltrating cells				
T cells	– Recruitment – Activation – Proliferation – Th1 differentiation	APC CD11b/CD18 CXCL10, IL12, IFN-γ	*In vitro*: T cell/APC co-cultures *In vivo*: FIE; 2D2 TCR MOG transgenic mice	Ryu et al. (2015)
Macrophages	– Recruitment – Chemokine expression – Infiltration	CD11b/CD18 TLR4 CXCL10, CCL2, MCP-1	*In vitro*: macrophage cultures *In vivo*: FIE	Ryu et al. (2015) Smiley et al. (2001)

proteolysis facilitates T-cell extravasation and migration (Cuzner and Opdenakker, 1999; Yepes et al., 2003). Genetic and pharmacologic evidence point to CD11b/CD18 as the major receptor mediating the *in vivo* proinflammatory effects of fibrin in the CNS (Adams et al., 2007; Davalos et al., 2012; Ryu et al., 2015). In addition to CD11b/CD18, *in vitro* evidence indicates a role for toll-like receptor 4 (TLR4) in fibrin-induced macrophage activation (Smiley et al., 2001). Moreover, *in vitro* evidence suggests a role for fibrinogen in neutrophil activation (Skogen et al., 1988; Rubel et al., 2001). The relative contributions of these proinflammatory pathways in the CNS *in vivo* remain to be determined. Overall, fibrin(ogen) and tPA/plasmin can be potent modulators of neuroinflammation.

Plasminogen Activation and Fibrin Degradation in Neurodegeneration and Repair

The PA system plays a critical role in normal cognitive function (e.g., regulation of synaptic plasticity) and neural dysfunction (Melchor and Strickland, 2005). For example, tPA can modulate neurotoxicity as $tPA^{-/-}$ mice exhibit less neuronal death after hippocampal kainate injection or after ethanol withdrawal, both of which induce neurodegeneration (Tsirka et al., 1995; Skrzypiec et al., 2009). Unlike tPA and plasmin, fibrinogen is not present in the healthy brain. However, fibrinogen is detected in the brains of patients with MS (Gay and Esiri, 1991; Kirk et al., 2003; Vos et al., 2005; Marik et al., 2007), schizophrenia (Körschenhausen et al., 1996), HIV-encephalopathy (Dallasta et al., 1999), ischemia (Massberg et al., 1999), AD (Paul et al., 2007; Ryu and McLarnon, 2009) and normal aging (Viggars et al., 2011), all conditions which have transient or long-lasting BBB opening.

AD is a common aging-related neurodegenerative disease of dementia and is characterized by extracellular aggregates of beta-amyloid (Aβ) plaques and intracellular neurofibrillary tangles of tau protein (Huang and Mucke, 2012). Co-localization of microhemorrhages and amyloid plaques in human AD brains suggests that bleeding can precipitate or promote plaque deposition (Cullen et al., 2006). Fibrin deposits colocalize with areas of neurite dystrophy in human AD tissue and AD mouse models (Cortes-Canteli et al., 2015). Individuals with high levels of plasma fibrinogen have an increased risk for developing AD and dementia (van Oijen et al., 2005; Xu et al., 2008). Furthermore, AD patients with two alleles of *apoE ε4*, which is the strongest genetic risk factor for AD (Mahley and Huang, 2012), have significantly more fibrin deposition than AD patients with ε2 or ε3 apoE alleles (Hultman et al., 2013). Fibrin depletion in AD model mice via genetic and pharmacological methods ameliorates the disease pathology and cognitive impairment (Paul et al., 2007; Cortes-Canteli et al., 2010, 2015). AD model mice lacking one allele for *tPA* develop more severe Aβ plaque deposition and cognitive impairment (Oh et al., 2014a). This effect may be due to reduced fibrinolysis, but there is also evidence that tPA is neuroprotective via a fibrin-independent mechanism by promoting Aβ degradation (Melchor et al., 2003), perhaps by activating microglia to phagocytose Aβ plaques. The physical association of fibrin and Aβ impairs fibrin degradation, which has the potential to induce chronic inflammation (Ahn et al., 2010; Cortes-Canteli et al., 2010; Zamolodchikov and Strickland, 2012). This interaction seems to be instrumental in the disease process as administration of a peptide that inhibits fibrin-Aβ interaction rescues cognitive decline in AD mice (Ahn et al., 2014). An important question to address is whether Aβ plaques associated with fibrin exacerbate neurodegeneration.

Studies indicate that fibrinogen and the PA system also impacts nervous system repair through regulation of neuron-glia interactions. Regeneration in the CNS may be limited by the development of astrogliosis via fibrin-induced transforming growth factor beta (TGF-β) signaling in astrocytes (Schachtrup et al., 2010) or by fibrinogen-mediated inhibition of neurite outgrowth (Schachtrup et al., 2007; **Table 1**). In the peripheral nervous system, fibrin impedes remyelination by inhibiting Schwann cell migration and differentiation into myelinating cells (Akassoglou et al., 2002, 2003). The increased severity of nerve injury in $tPA^{-/-}$ or $plg^{-/-}$ knock-out mice in the sciatic nerve crush model is rescued by genetic or pharmacological fibrinogen depletion (Akassoglou et al., 2000; Siconolfi and Seeds, 2001), supporting the concept that fibrin accumulation is an important trigger for inhibition of remyelination. While these findings are highly suggestive of new pathways for fibrin and tPA/plasmin in regeneration, more work will be needed to determine their contribution as inhibitors of nervous system repair.

Future Directions

Emerging evidence from the fields of neuroscience, immunology, and vascular biology have aimed the spotlight on fibrin and the fibrinolytic system for their pleiotropic functions in neurological diseases. Although current evidence points to fibrin as a major contributor to neuroinflammation and neurodegeneration, it is possible that other components of the coagulation cascade are activated upon neurologic disease and play a role in CNS diseases via fibrin-dependent and potentially fibrin-independent mechanisms (Akassoglou, 2015). For example, a novel molecular probe for thrombin identified increased thrombin activity in animal models of stroke (Chen et al., 2012) and MS (Davalos et al., 2014). In accordance, depletion of thrombin by anti-coagulants inhibits fibrin formation and is protective in MS animal models (Adams et al., 2007; Han et al., 2008; Davalos et al., 2012). It is now timely for the fields of neuroscience and neurology to explore the contribution of the coagulation cascade in inflammatory, degenerative, and repair processes in the CNS.

Fibrin degradation products (FDPs) are commonly used as biomarkers to assess the severity of trauma after injury, in sepsis, or myocardial infract. Components of the coagulation cascade and FDPs have been detected in MS patients (Aksungar et al., 2008; Han et al., 2008; Liguori et al., 2014), in patients with mild cognitive impairment (Xu et al., 2008), and in human AD (Cortes-Canteli et al., 2015; Zamolodchikov et al., 2015). However, most of these studies have been performed in small population cohorts without availability of imaging data, response to treatments, and disease duration. Studies in large patient cohorts would

be required to assess whether components of coagulation or the fibrinolytic cascade correlate with disease progression in neurologic diseases. Although coagulation and fibrinolysis could trigger and perpetuate neurologic disease, animal models of vascular-driven inflammation and neurodegeneration are currently lacking. Inducing neuroinflammation in the CNS in Fibrinogen-induced encephalomyelitis (FIE) by introducing fibrinogen in the brain (Ryu et al., 2015), or perhaps by manipulating PA, or by transgenic or pharmacological models that increase BBB permeability could lead to vascular-driven experimental settings to study disease pathogenesis in the CNS.

Several FDA-approved drugs target different aspects of the coagulation cascade leading to reduced fibrin formation. Although new generation anticoagulants have reduced hemorrhagic effects, target-based drug design would be preferable to selectively inhibit the pathogenic effects of coagulation in the CNS. Indeed, pharmacologic inhibition of fibrin interactions with CD11b/CD18 using a fibrin peptide suppressed EAE pathology without adverse effects in blood clotting (Adams et al., 2007; Davalos and Akassoglou, 2012). Future studies will determine whether pharmacologic reagents can be developed to selectively target the pathogenic effects of fibrin and perhaps other components of the coagulation cascade in the CNS without affecting their beneficial effects in blood clotting.

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REVIEW
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A Review of the Mechanisms of Blood-Brain Barrier Permeability by Tissue-Type Plasminogen Activator Treatment for Cerebral Ischemia

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Cerebrovascular homeostasis is maintained by the blood-brain barrier (BBB), which forms a mechanical and functional barrier between systemic circulation and the central nervous system (CNS). In patients with ischemic stroke, the recombinant tissue-type plasminogen activator (rt-PA) is used to accelerate recanalization of the occluded vessels. However, rt-PA is associated with a risk of increasing intracranial bleeding (ICB). This effect is thought to be caused by the increase in cerebrovascular permeability though various factors such as ischemic reperfusion injury and the activation of matrix metalloproteinases (MMPs), but the detailed mechanisms are unknown. It was recently found that rt-PA treatment enhances BBB permeability not by disrupting the BBB, but by activating the vascular endothelial growth factor (VEGF) system. The VEGF regulates both the dissociation of endothelial cell (EC) junctions and endothelial endocytosis, and causes a subsequent increase in vessel permeability through the VEGF receptor-2 (VEGFR-2) activation in ECs. Here, we review the possibility that rt-PA increases the penetration of toxic molecules derived from the bloodstream including rt-PA itself, without disrupting the BBB, and contributes to these detrimental processes in the cerebral parenchyma.

Keywords: brain ischemia, blood-brain barrier permeability, endothelial endocytosis, intracranial bleeding, tissue-type plasminogen activator, vascular endothelial growth factor

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INTRODUCTION

The recombinant tissue-type plasminogen activator (rt-PA), a serine proteinase, is a thrombolytic agent that degrades fibrin clots through the activation of plasminogen to plasmin (Lijnen and Collen, 1987). Although rt-PA given within 3 h from the onset of ischemic stroke improves patients' clinical outcome, it induces a 10-fold increase of symptomatic intracranial

Abbreviations: BBB, blood-brain barrier; BSA, bovine serum albumin; CNS, central nervous system; EC, endothelial cell; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HIF, hypoxia-inducible factor; ICB, intracranial bleeding; LRP, low-density lipoprotein receptor-related protein; MCA, middle cerebral artery; MMP, metalloproteinase; ROS, reactive radical oxide species; rt-PA, recombinant tissue-type plasminogen activator; VEGF, vascular endothelial growth factor; TJ, tight junction; ZO, zonula occludens.

hemorrhage (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Furthermore, rt-PA treatment delayed beyond 3 h is associated with an increased risk of hemorrhagic transformation with enhanced brain injury (Clark et al., 1999). Subsequently, the European Cooperative Acute Stroke Study showed that rt-PA administered between 3 and 4.5 h after the onset of symptoms significantly improved the clinical outcomes of patients with acute ischemic stroke, but it increased the risk of symptomatic intracranial hemorrhage. It was confirmed that delayed treatment beyond 4.5 h was not associated with a statistically significant benefit (Hacke et al., 2008). A number of clinical studies using magnetic resonance imaging provided evidence that rt-PA treatment is associated with blood-brain barrier (BBB) breakdown (Kastrup et al., 2008; Kassner et al., 2009), which also correlates with an increased risk of hemorrhagic transformation during thrombolysis in ischemic stroke (Kassner et al., 2009). These findings strongly suggest a causal relationship between rt-PA and BBB breakdown in the ischemic human brain.

Although the deleterious effect of rt-PA after ischemic stroke has been widely accepted, it remains unclear whether many blood-derived rt-PAs penetrate the brain and contribute to these detrimental processes in the cerebral parenchyma. Intravenously administered rt-PA has been shown to cross brain endothelial cells (ECs) via two ways: (1) by binding to the surface; and (2) transcytosis without compromising the BBB integrity (Benchenane et al., 2005; López-Atalaya et al., 2007). rt-PA was also found to enter the parenchyma under pathological conditions where it further affects BBB breakdown (Su et al., 2008). Some hypotheses have been proposed to explain how rt-PA within the parenchyma exacerbates intracranial bleeding (ICB) after ischemic stroke. Extracellular rt-PA can mediate some of its actions either through plasmin; the degradation of laminin, one of the extracellular matrix (ECM) proteins (Chen and Strickland, 1997); the activation of microglia (Rogove and Tsirka, 1998); or the excessive induction of vascular remodeling and angiogenesis via overactivation of metalloproteinases (MMPs; Suzuki et al., 2007; Yamashita et al., 2009; Won et al., 2014) and the vascular endothelial growth factor (VEGF; Kanazawa et al., 2011; Suzuki et al., 2015). There is also evidence that rt-PA may have a direct toxic effect on the ischemic brain (Wang et al., 1998; Nagai et al., 1999), possibly through activation of the N-methyl-D-aspartate receptor (NMDAR; Nicole et al., 2001). This may be of particular importance given that rt-PA diffused within the cerebral parenchyma after ischemia can have detrimental effects including enhancing the neurotoxic processes (Kaur et al., 2004).

In the present review, we firstly described BBB breakdown by rt-PA, and then discussed the role of the enhancement of BBB permeability without compromising BBB integrity on BBB breakdown. Especially, we focus on the involvement of VEGF in ECs as the first step in BBB breakdown by the deleterious effect of rt-PA after ischemic stroke. In addition, it is described the possibility that an inhibition the enhancement of BBB permeability without compromising BBB integrity may extend the therapeutic time widow by rt-PA for ischemic stroke.

BBB AND ENDOTHELIAL TIGHT JUNCTIONS

The BBB is formed by endothelial tight junctions (TJs) together with pericytes, perivascular astrocytes, and basement membrane in the vasculature. Furthermore, as cerebrovascular function is regulated by the neuronal environment, the BBB and neurons form a functional unit called the neurovascular unit. TJs are constituted by multiple protein components that involve transmembrane proteins (e.g., occludin, claudins, and junction-associated molecules) linked to the actin cytoskeleton via cytoplasmic zonula occludens (ZO) proteins. Transmembrane proteins, occludin and claudins are critical for paracellular function at the BBB (Hawkins and Davis, 2005). Claudins are small transmembrane proteins (20–24 kDa) that span the membrane four times; claudin-1, -3, and -5 are expressed in ECs of the BBB. Occludin is a 60–65 kDa phosphoprotein highly expressed in cerebral endothelia, but it is sparsely distributed in peripheral endothelia (Hirase et al., 1997). The overexpression of claudins can induce the formation of TJs, but the expression of occludin does not lead to the formation of TJs. Thus, it is likely that claudins form the primary seal of TJs, and occludin acts as an additional support structure. Claudin and occludin are anchored to the actin cytoskeleton via ZO-1 (Hawkins and Davis, 2005).

rt-PA and Reperfusion Injury

In patients with ischemic stroke, rt-PA increases the risk of ICB via BBB breakdown through a number of mechanisms. One of these mechanisms is thought to be by reperfusion after the degradation of occlusive blood clots by rt-PA. Brain parenchymal damage occurs because of a complex series of events in the setting of ischemia followed by reperfusion injury. These events start due to an interruption in blood flow to the affected tissue followed by the depletion of cellular energy resources and glycolysis at an anerobic substrate level, with subsequent lactic acidosis, failure of the sodium potassium pump, the release of glutamate, cytotoxic edema, and free radical formation (Nour et al., 2013). The activation of both innate and adaptive immune responses also creates free radicals. This excessive generation of free radicals overwhelms the system, which then becomes inefficient in scavenging these molecules, leading to BBB breakdown. Furthermore, ICB associated with ischemic infarction is recognized due to ischemia followed by reperfusion, and both the rate of ICB and stroke outcome can be increased by the duration of reperfusion from the onset of vessel occlusion due to ischemic stroke (Jickling et al., 2014). Additional injury is extensively shown in the brain and in other tissues, which is mediated by reactive radical oxide species (ROS). ROS contribute to BBB disruption by several mechanisms: oxidative damage to cellular molecules (i.e., proteins, lipids, and DNA); the activation of MMPs and subsequent degradation of basement membrane; cytoskeletal reorganization of ECs; the modulation of TJ proteins and upregulation of inflammatory mediators; and subsequent additional and extensive reperfusion injury (Kahles and Brandes, 2012).

rt-PA Promotes ICB Through Mechanisms Beyond its Role in Thrombolysis and Reperfusion

rt-PA increases BBB permeability via degradations of basement membrane and TJ proteins. These degradations are associated with plasmin activation, low density lipoprotein receptor associated protein-1 (LRP-1) stimulation, and MMPs induction (Yamashita et al., 2009; Won et al., 2014). As a result, rt-PA exacerbates ischemic brain damage and ICB by increasing BBB permeability.

Plasmin, which is activated by rt-PA, can directly degrade fibrin clots and basement membrane components such as collagen IV (Mackay et al., 1990; Lukic-Panin et al., 2010), laminin (Chen and Strickland, 1997) and fibronectin (Marchina and Barlati, 1996), or via the activation of MMPs (Lijnen, 2001), which possess similar basement membrane dismantling capabilities and damages the TJs (Jin et al., 2010).

LRP is one of the major binding sites of rt-PA (Bu et al., 1992) on the cell surface. LRP, a member of the lipoprotein receptor family, is a scavenger receptor that binds a variety of biological ligands associated with the ECM and is thought to be primarily involved in lipoprotein metabolism (Herz et al., 1988), and in the clearance of protease-inhibitor complexes in the adult brain (Bu et al., 1992). Furthermore, the increase in BBB permeability by rt-PA occurs via the activation of LRP (Yepes et al., 2003; Benchenane et al., 2005; Su et al., 2008; Suzuki et al., 2009; Niego and Medcalf, 2014). LRP is selectively upregulated in ECs under ischemic stress, and LRP activation by binding to rt-PA stimulates signal pathways such as the nuclear factor κB pathway (Suzuki et al., 2009).

MMPs, a family of zinc endopeptidases, contribute to tissue remodeling through the degradation of ECM proteins. Although clarifying the precise timing and release of cells to MMP after ischemia requires further study, MMPs, including MMP-9 (gelatinase B), MMP-2 (gelatinase A), and MMP-3 (stromelysin-1), are thought to be key molecules involved in BBB opening and ICB after ischemic stroke (Rosenberg et al., 1995; Castellanos et al., 2003; Wang et al., 2003; Suzuki et al., 2007; Mishiro et al., 2012; Jickling et al., 2014) together with other brain proteases (i.e., plasmin, endogenous t-PA, and urokinase; Wang and Shuaib, 2007). Claudin-5 and occludin, components of the TJs, contain extracellular MMP cleavage sites and are a direct substrate of MMPs (Wachtel et al., 1999; Yang et al., 2007), suggesting that MMPs can degrade TJs directly. As the structural disruption of the interaction between occludins and actin filaments can lead to the perturbation of paracellular permeability (Madara et al., 1986), the degradation of occludin by MMPs is likely to trigger BBB opening. MMPs also degrade components of basement membrane and contribute to BBB impairment, vasogenic edema and hemorrhagic transformation (Rosell et al., 2008). MMP-9 especially is believed to play a major role in BBB disruption during ischemic stroke because ischemic stress induces MMP-9 and the plasma MMP-9 concentration, which strongly correlate with patients' stroke severity (Horstmann et al., 2003; Jin et al., 2010; Reuter et al., 2013).

MMP-3 is also thought to be involved in ICB due to delayed rt-PA treatment. MMP-3 is produced by pericytes (Yang et al., 2013) and ECs (Suzuki et al., 2007) after ischemic stroke. The increase in ICB caused by the delayed rt-PA treatment was impaired in mice with a gene deficiency of MMP-3, and a broad spectrum MMP-inhibitor suppressed ICB in wild-type mice but not in MMP-3 deficient mice (Suzuki et al., 2007). MMP-3 can be activated by plasmin (Lijnen, 2001), and it has a broad-spectrum substrate specificity, including pro-MMP-9, which is activated by limited cleavage (Nagase and Woessner, 1999). Furthermore, rt-PA treatment induced MMP-3 selectively in ECs at the ischemic damaged area in a mouse stroke model (Suzuki et al., 2007), and MMP-3 was increased in a postmortem human stroke brain (Jickling et al., 2014). These findings indicate that MMP-3 is also involved in degrading the barrier of blood vessels and contributing to ICB.

Increase in BBB Permeability by Paracellular Transport and Transcytosis

rt-PA treatment is thought to enhance ICB via the acceleration of BBB disruption after ischemic stroke. However, there is still a possibility that rt-PA treatment enhances BBB permeability without BBB disruption after ischemic stroke.

The BBB is composed of blood vessels whose ECs display extremely low rates of transcellular vesicular transport (transcytosis) due to pinocytic activity (Reese and Karnovsky, 1967; Pun et al., 2009; Saunders et al., 2012; Siegenthaler et al., 2013). In concert with pericytes and astrocytes, this unique brain endothelial barrier seals the central nervous system (CNS) and controls substance influx and efflux (Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010). BBB permeability is regulated in response to various stimulators or stressors, which can exert beneficial or deleterious effects on the brain depending on the context, timing, and functional cellular outcomes of signaling (Roux and Couraud, 2005). BBB permeability can be increased via two processes. The first is paracellular transport, which is associated with loosening the TJs between ECs. The expression of occludin, a component of TJs, is highly suppressed by a number of pathological stresses, including oxidative stress, and a decrease in the occludin expression results in the increase in BBB permeability (Ramirez et al., 2009; Lochhead et al., 2010). To enhance paracellular transport, rt-PA seems to decrease occludin through ROS generation associated with reperfusion and/or through the activation of MMPs.

The second process is transcytosis, which is consistent with endocytosis and involves vesicle transport to the opposite side of the cell and exocytosis. Peripheral ECs display active vesicle trafficking to deliver nutrients to peripheral tissues, whereas CNS ECs express transporters to selectively traffic nutrients across the BBB (Saunders et al., 2012; Siegenthaler et al., 2013). However, it is still unclear when and how ECs are transported by transcytosis.

VEGF

VEGF stimulates endocytosis and transcytosis (Horowitz and Seerapu, 2012; Nakayama and Berger, 2013). VEGF binds to two receptor-coupled protein tyrosine kinases (Tyr), VEGF receptor 1 (VEGFR-1, Flt-1) and VEGFR-2 (i.e., fetal liver kinase 1 [Flk-1] or the kinase insert domain receptor). VEGF regulates the dissociation of TJs under ischemic condition (Fischer et al., 2002; **Figure 1**) and endocytosis (Horowitz and Seerapu, 2012), and the subsequent increase in vessel permeability through VEGFR-2 activation, which contributes to cerebral swelling at the early stage after ischemic stroke (Abumiya et al., 2005). VEGF also has a fundamental role in vascular remodeling and angiogenesis by increasing EC proliferation, migration, and microvascular hyperpermeability (Brown et al., 1992). The downstream elements of VEGFR-2 signaling include the Ras/Raf/MEK pathway, which leads to EC proliferation; PI3K-AKT/PKB pathway, which supports EC survival; and p38/MAPK-HSP27 pathway, which promotes EC migration (Obermeier et al., 2013). Vascular remodeling is an important component of recovery after stroke, although it makes vessels to be leakier and prone to intracranial hemorrhage (Durukan et al., 2009). Thus, they may also promote intracranial hemorrhage.

VEGF is expressed in the normal adult brain, mainly in epithelial cells of the choroid plexus, as well as in astrocytes and neurons, such as granule cells of the cerebellum (Monacci et al., 1993; Marti and Risau, 1998). VEGF expression is regulated by extensive signaling pathways. Among them, hypoxia is a strong inducer of VEGF messenger (m)-RNA expression in many cells *in vitro* and *in vivo* (Banai et al., 1994; Ikeda et al., 1995; Kovács et al., 1996; Hayashi et al., 1997). The two transcription factors, hypoxia-inducible factor-1 (HIF-1) and HIF-2 are involved in the regulation of VEGF expression. HIF-1 is composed of a hypoxia-regulated α-subunit and a β-subunit, and is a basic helix-loop-helix heterodimeric transcription factor activated by reduced oxygen tension (Wenger and Gassmann, 1997). HIF-1α is continuously produced and rapidly degraded under normoxia (Sharp and Bernaudin, 2004), whereas it is degraded slowly under hypoxia, and allowed rapid accumulation and binding to hypoxia-responsive elements (Shi, 2009). HIF-2, a homolog of HIF-1, is also involved in the regulation of VEGF gene expression (Ema et al., 1997). HIF-2 has an additional role in the regulation of VEGFR-2 (Kappel et al., 1999). Although VEGF is induced in ECs through HIF-1α (Tang et al., 2004), and the expression of HIF-1 and HIF-2 are increased in the border area of ischemic stroke (Marti et al., 2000), the mechanisms by which VEGF gene expression is regulated during cerebral ischemia remain unclear.

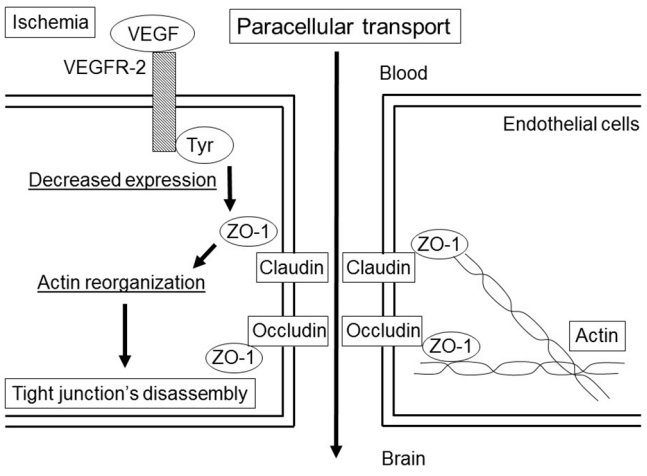

FIGURE 1 | Paracellular transport in the blood-brain barrier (BBB) by the vascular endothelial growth factor (VEGF) under ischemic condition. The binding of VEGF to the VEGF receptor-2 (VEGFR-2) induces the activation of tyrosine kinase (Tyr), which leads to a decrease in the expression of zonula occludens (ZO)-1. The changes in ZO-1 correlate with small changes in action distribution, which induce disassembly of the tight junction (TJ).

One mechanism for the increase in VEGF secretion by cells exposed to ischemia is an increase in its transcription rate mediated by the binding of HIF-1 to a hypoxia-responsive element in the 5′-flanking region of the VEGF gene, as is observed in PC12 cells (Levy et al., 1995), bovine pulmonary artery ECs (Liu et al., 1995), and Hep3B cells (Forsythe et al., 1996). Other mechanisms include the increase in VEGF mRNA stability (Ikeda et al., 1995) and efficient translation of VEGF mRNA through an internal ribosome entry site (Stein et al., 1998).

rt-PA, HIF-1, and VEGF

rt-PA increases VEGF expression in bone marrow-derived myeloid cells, cultured cerebral cortical neurons, and ECs (Ohki et al., 2010; Wu et al., 2012; Duan and Ni, 2014; Suzuki et al., 2015). In addition, rt-PA affects HIF-1α regulation. rt-PA induces HIF-1α accumulation in the ischemic brain and accelerates HIF-1α accumulation mediated by the mammalian target of rapamycin in cultured neurons (Wu et al., 2012). rt-PA treatment after ischemia does not enhance the expression of VEGF through the nuclear accumulation of HIF-1α in a transformed mouse brain EC line, bEnd.3 (Suzuki et al., 2015); instead, the overexpression of t-PA stimulates VEGF expression in ECV304, a human immortalized EC due to the stimulation of ERK/p38 signaling pathways (Duan and Ni, 2014). These findings suggest a possibility that rt-PA accelerates the expression of VEGF via HIF-1α or the signal pathway for the upregulation of HIF-1α in parallel without VEGF induction.

The inhibition of VEGF signaling reduces rt-PA-related ICB (Kanazawa et al., 2011; Suzuki et al., 2015), suggesting that VEGF has a deleterious role in ICB. Similarly, VEGF administered within 1–24 h from stroke onset increases in the rate of BBB breakdown and hemorrhagic transformation, and the size of infarction in rodents (Zhang et al., 2000; Abumiya et al., 2005). Taken together that VEGF increases microvascular permeability to blood plasma proteins within minutes after its administration (Dvorak et al., 1995), the induction of VEGF after ischemic stroke may enhance the detrimental effects. In contrast, VEGF administered 48 h from

stroke onset enhances angiogenesis and improves neurologic recovery, and improves cerebral blood flow 28 days after stroke (Zhang et al., 2000). The effect of VEGF on angiogenesis is longer than on permeability. Newly formed vessels in ischemic mouse brains are first visible within 4 days (Dellian et al., 1996) and there is an increase in newly formed vessels in ischemic mouse brains 10 days after VEGF treatment but not in contralateral non-ischemic brains (Zechariah et al., 2013). Thus, VEGF seems to have biphasic roles in stroke, it promotes BBB breakdown and hemorrhagic transformation in the early stage, within 24 h, but promotes BBB integrity and vascular function in the late stage, over 48 h, after ischemic stroke.

Paracellular Permeability by rt-PA

Increased paracellular permeability is correlated with the disruption of TJs (Kevil et al., 2000; Mark and Davis, 2002; Lee et al., 2004). Until now, however, the role of TJs in vascular permeability of either plasma components or circulating cells was supported by the results of only a few studies (Martìn-Padura et al., 1998; Pedram et al., 2002). During ischemic stroke, temporal hypoxemia for 10 min increased BBB permeability associated with alterations in TJ protein expression (Witt et al., 2003). Accordingly, immunoreactivity of ZO-2 or claudin-5 was significantly reduced in infarct regions compared with non-infarct regions 24 h after ischemia (Fischer et al., 2007). However, according to ultrastructural analyses at 5 and 25 h after ischemia, fluorescein isothiocyanate (FITC)-albumin was extravased around vessels with intact TJs, whereas the endothelium exhibited an enhanced transcellular vesicle trafficking (Krueger et al., 2013). Additionally, the morphology of TJ components identified by antibodies against occludin and claudin-5 appears to be regularly maintained in regions where FITC-albumin massively leaked into the neuropil 25 h after ischemia (Krueger et al., 2013). A conclusive time frame for TJ reassembly following the disruption of ischemia is currently lacking. In Madin-Darby canine kidney cells, permeability is returned to the same levels of the initial condition 5 h after ATP repletion. After ATP depletion for 1 h and repletion for 3 h, occludin was once again found almost exclusively at the level of the TJs. This reversible shift is inhibited by the chelation of intracellular calcium. In contrast, ZO-1 is not significantly altered during ATP depletion or repletion (Ye et al., 1999). However, VEGF specifically down-regulates claudin-5, occludin protein, and mRNA. In the mouse cerebral cortex, the microinjection of VEGF disrupted claudin-5 and occludin, and induced loss of barrier function (Argaw et al., 2009). The continuity of the ZO-1 expression was significantly disrupted during 1.5 h of hypoxia in primary cultures isolated from porcine brain ECs. Furthermore, VEGF alone or α-lipoic acid alone did not change ZO-1 localization in the primary culture, however, VEGF in combination with α-lipoic acid decreased the ZO-1 expression to nearly the same extent as 3 h of hypoxia (Fischer et al., 2002). These results suggest that ischemia increases the paracellular flux via the release of VEGF, which in turn leads to the dislocalization, decreased expression, and enhanced phosphorylation of TJs. As TJ proteins are responsible for the paracellular permeability across the BBB, VEGF induced by ischemia may increase paracellular permeability of BBB via suppression of the expression of TJ proteins in the early stage of ischemic stroke (Fischer et al., 2002; Argaw et al., 2009, 2012). However, it is possible that BBB permeability is increased by TJs loosening in the early stage of ischemic stroke because a study found intact TJs at 24 h after stroke (Krueger et al., 2013).

Delayed rt-PA treatment enhances the fragmentation of occludin and claudin-5 24 h after middle cerebral artery (MCA) occlusion in rats (Won et al., 2014), and reduces claudin-5 at 24 h (Ishiguro et al., 2010) or occludin and ZO-1 at 48 h after ischemia in mice (Mishiro et al., 2012). Furthermore, it was found that blood levels of TJ proteins were higher in patients with hemorrhagic transformation than in those without hemorrhagic transformation (Kazmierski et al., 2012). These findings indicate that delayed rt-PA treatment results in the enhancement of ICB due to stroke by the loss of TJ proteins at a relatively later stage, over 24 h, on ischemic stroke. However, the role of TJ proteins in early ICB is still unclear. Delayed rt-PA treatment may enhance the BBB permeability through the paracellular pathway by the degradation of TJ proteins without BBB breakdown as well as ischemia without rt-PA treatment.

rt-PA Treatment and Endocytosis

The intravenous treatment of rt-PA does not increase either albumin extravasation or ICB in naive mice (Cheng et al., 2006; Su et al., 2008; Suzuki et al., 2015). Additionally, the intraventricular injection of rt-PA does not increase Evans blue extravasation in sham-operated mice (Yepes et al., 2003). In contrast, an intravenous injection of rt-PA slightly increases the Evans blue extravasation in native mice (Turner and Vink, 2012). Furthermore, an intravenous injection of biotinylated rt-PA with fluorescent dextran (77 kDa) is detected the extravasation of rt-PA in the brain parenchyma of nonlesioned animals (Benchenane et al., 2005), suggesting that rt-PA itself and other plasma molecules can cross the intact BBB via transcytosis. Similarly, we observed that delayed rt-PA treatment dramatically increased endocytosis of cerebral ECs at the ischemic border region together with an increase in the existence of gold-labeled bovine serum albumin (BSA) administered intravenously at the vascular lumen, inside ECs, in the basement layer of the ECM and extravascular space without obvious TJ defects in mice (Suzuki et al., 2015). This indicates that delayed rt-PA treatment increased extravasation of BSA at the ischemic border region in the early period after MCA occlusion by the additional acceleration of transcytosis rather than by the degradation of vascular structures. It is likely that the administration of rt-PA accelerates extravasation of phagocytic vesicles, including rt-PA, and interacting plasma plasminogen in the parenchyma by the upregulation of transcytosis increases the likelihood of a plasmin-dependent BBB alteration at the perivascular space. Because this study does not provide direct evidence for the involvement of exocytosis, another component of the transcytosis process, in the ischemic border region of the extravasation of BSA, there is still

the possibility that endothelial endocytosis is independent of BBB opening. Additionally, transcytosis may possibly be involved in the increase in BBB permeability by the delayed treatment of rt-PA after ischemic stroke (**Figure 2**).

Endocytosis and the Multifunction of LRP

As previously described, LRP is a scavenger receptor that binds a variety of biological ligands associated with the ECM, and it is a major binding protein of rt-PA (Bu et al., 1992). LRP acts as a membrane receptor of rt-PA, and its activation induces the expressions of MMPs and VEGF (Wang et al., 2003; Suzuki et al., 2009, 2015). The invasion of carcinoma cells is also decreased by LRP silencing with RNA interference despite a strong stimulation of pericellular MMP-2 and urokinase-type plasminogen activator proteolytic activities (Dedieu et al., 2008). However, the precise role of LRP in the regulation of ECM remodeling is still unclear.

During endocytosis, LRP does not act alone, as it has membrane partners that vary according to numerous parameters, including the cell origin, ECM composition, and pathological conditions (Etique et al., 2013). LRP-mediated endocytosis of soluble ligands is usually followed by intracellular lysosomal routing and catabolism. LRP emerges as an endocytic receptor regulating cellular matrix attachment sites and coordinating the balance of adhesion/deadhesion. It has been known that a small number of transmembrane proteins are associated with LRP. As this association is thought to be involved in the endocytosis and subsequent turnover of the membrane proteins, the mechanisms are insufficient to be understood. As LRP is a major binding protein of t-PA, the administration of rt-PA may stimulate ECs and accelerate the endocytosis of plasma proteins via LRP and subsequent extravasation of proteins into the parenchyma.

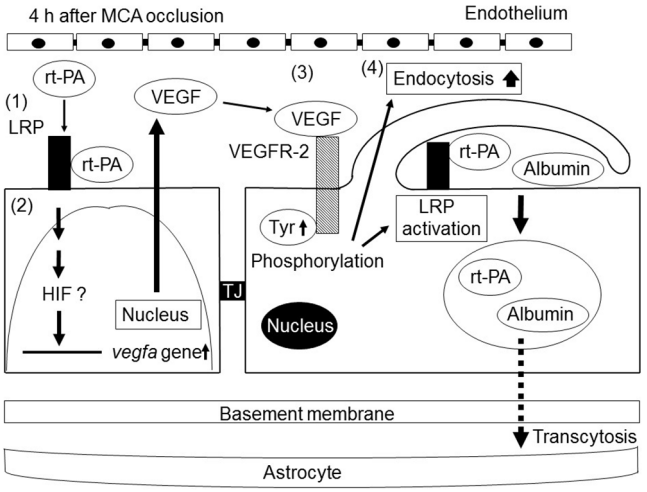

FIGURE 2 | Schematic of the mechanisms of the increase in BBB permeability by recombinant tissue-type plasminogen activator (rt-PA) treatment after ischemic stroke. rt-PA activates the low-density lipoprotein receptor-related protein (LRP), which is upregulated in endothelial cells (ECs) by ischemic stress (1). The activation of LRP induces the transcriptional upregulation of VEGF. Secreted VEGF binds to VEGFR-2 on the surface of ECs through an autocrine mechanism and induces its phosphorylation (3). The activation of VEGFR-2 leads to an increase in endocytosis and to the activation of LRP, resulting in enhanced BBB permeability by endocytosis and subsequent transcellular transport of proteins into cerebroparenchyma (4). Tyr, TJ.

rt-PA, Plasmin, and the Substrate for Plasmin

It is unclear whether the involvements of rt-PAs during ischemia, except for clot lysis, are associated with plasmin. Plasminogen is essentially present in both blood and the brain under most pathologic brain scenarios, especially together with rt-PA during its utilization in ischemic stroke. Plasminogen is exclusively localized in neurons of the cerebral cortex, hippocampus, hypothalamus, and the cerebellum in rodents (Tsirka et al., 1997; Basham and Seeds, 2001; Taniguchi et al., 2011). Hence, it is not likely to be obviously assumed that brain-derived plasminogen is activated at the BBB during stroke by endogenous t-PA. Blood-derived plasminogen may be more readily available by passing the BBB, and it may be activated at the BBB under an ischemic condition. Therefore, brain-derived plasminogen in the neuronal pathology may not be completely associated with BBB permeability.

As previously described, plasmin can directly degrade basement membrane components or cause degradation of basement membrane components via the activation of MMPs (Lijnen, 2001), which possess similar basement membrane dismantling capabilities and damages the TJs (Jin et al., 2010). rt-PA treatment did not alter ICB associated with stroke in mice deficient in plasminogen and MMP-3, suggesting that plasmin may be required to activate MMP-3 by rt-PA in ECs during stroke (Suzuki et al., 2007).

Plasmin (and plasminogen) binds a wide array of cell-surface receptors or binding proteins and cleaves a variety of biologic substrates (Kwon et al., 2005; Miles and Parmer, 2013). As a result of this capacity, plasmin has been understood to play a role in many cellular responses, including cell migration, wound healing, tissue remodeling, apoptosis, cancer invasion, cancer metastasis, and inflammation and immunity, as extensively reviewed elsewhere (Kwon et al., 2005; Syrovets et al., 2012; Miles and Parmer, 2013). Together, these characters attribute a position to plasmin as a sound candidate that participates in the remodeling of cerebral blood vessels, especially during stroke, when the BBB weakens and blood components gain access to the BBB. The direct intracortical injection of concentrated plasmin resulted in substantial lesion formation 6 h later, accompanied by the oxidation of proteins and DNA, degradation of occludin and collagen IV (basement membrane), and elevation of MMP-9, without the loss of ECs (Lukic-Panin et al., 2010). These findings indicate that plasmin can evidently influence TJ proteins and basement membrane. Therefore, it is likely that the administration of rt-PA accelerates the likelihood of a plasmin-dependent BBB alteration.

CONCLUSIONS

Delayed rt-PA treatment increases BBB permeability through a number of mechanisms. Although the mechanisms depend on

the degree of cell damage after ischemia, rt-PA has the possibility of increasing BBB permeability without compromising BBB integrity and causing subsequent BBB breakdown. rt-PA increases BBB permeability via the induction of VEGF, which at least partially mediates the subsequent increase in endothelial endocytosis. Furthermore, an increase in BBB permeability by endocytosis is likely the first step in BBB breakdown by delayed rt-PA treatment combined with ischemic stroke because various plasma proteins in the bloodstream are taken up by the parenchyma across the compromised BBB.

AUTHOR CONTRIBUTIONS

YS and NN: planning and writing the review. KU: supervisor.

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frontiers in Cellular Neuroscience

REVIEW
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Implications of MMP9 for Blood Brain Barrier Disruption and Hemorrhagic Transformation Following Ischemic Stroke

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Numerous studies have documented increases in matrix metalloproteinases (MMPs), specifically MMP-9 levels following stroke, with such perturbations associated with disruption of the blood brain barrier (BBB), increased risk of hemorrhagic complications, and worsened outcome. Despite this, controversy remains as to which cells release MMP-9 at the normal and pathological BBB, with even less clarity in the context of stroke. This may be further complicated by the influence of tissue plasminogen activator (tPA) treatment. The aim of the present review is to examine the relationship between neutrophils, MMP-9 and tPA following ischemic stroke to elucidate which cells are responsible for the increases in MMP-9 and resultant barrier changes and hemorrhage observed following stroke.

Keywords: neutrophils, MMP-9, tPA, blood-brain barrier, ischemic stroke, hemorrhagic transformation, cerebral edema

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INTRODUCTION

Over the last decade the matrix metalloproteinases (MMPs) have been widely investigated for their role in disruption of the blood-brain barrier (BBB), particularly the extracellular matrix (ECM), following stroke (Romanic et al., 1998; Rosenberg et al., 1998; Fujimura et al., 1999; Gasche et al., 1999; Gidday et al., 2005) and other cerebral pathologies such as traumatic brain injury (Planas et al., 2001) and neoplasm (Lukes et al., 1999; Turba et al., 2007). MMPs are a family of zinc and calcium-dependent endopeptidases that are capable of degrading all components of the ECM including laminin, collagen and fibronectin, amongst many other targets (Van den Steen et al., 2002). At least 23 MMPs have been identified to date (Sternlicht and Werb, 2001), with MMP-2 and MMP-9 the most widely studied in stroke. In particular, MMP-9 has been implicated, not only in the pathogenesis of BBB breakdown and subsequent vasogenic edema formation following stroke (Fujimura et al., 1999; Gasche et al., 1999; Rosenberg and Yang, 2007), but also in hemorrhagic transformation (HT) in the setting of tissue plasminogen activator (tPA) therapy (Lapchak et al., 2000; Wang et al., 2009). Cerebral edema and HT of the infarct are significant problems in clinical stroke, which are associated with poor outcome and contribute to the morbidity and mortality of this condition (Hacke et al., 1996; Fiorelli et al., 1999). Elucidating the mechanisms of such deleterious events is the key to developing targeted, more effective clinical therapies.

Numerous clinical and experimental studies have confirmed an increase in serum MMP-9 following stroke (Clark et al., 1997; Romanic et al., 1998; Yushchenko et al., 2000; Montaner et al., 2003a; Ning et al., 2006). However, the cellular source of this MMP-9 remains controversial. Although it is generally accepted that MMP-9 is increased following stroke, there is debate as to which cells are responsible, whether it be resident brain cells, cells of the vasculature or circulating immune cells, such as neutrophils. However, the aim of the present review was to explore the potential relationship between neutrophil-derived MMP-9 and complications such as BBB disruption and HT following stroke to elucidate the cellular source of MMP-9 in ischemic stroke.

Matrix Metalloproteinases

MMPs regulate many aspects of cellular activity with functions ranging from ECM degradation, cell proliferation, adhesion, and migration to release of ECM-sequestered molecules by proteolysis, shedding of cell-surface proteins that transduce signals from the ECM (Cunningham et al., 2005) and activation of pro-inflammatory cytokines (Candelario-Jalil et al., 2009). As such, recognized targets of MMP-9 include components of the ECM, tight junction components, growth factors and their precursors, cell surface receptors and cell adhesion molecules (Bajor and Kaczmarek, 2013; Vandooren et al., 2013; Conant et al., 2015). The MMPs may have pleiotropic actions on target tissues, with MMPs integrally involved in the normal remodeling of tissue during development and homeostasis but dysregulation of MMPs is implicated in disease states and has repercussions for BBB integrity, tissue injury and cell death (Agrawal et al., 2008). However, action of the MMPs, including MMP-9, have been well documented to play critical roles in tissue repair and remodeling following stroke (Lenglet et al., 2015), particularly in angiogenesis and re-establishment of cerebral blood flow with long-term MMP inhibition shown to markedly reduce neuronal plasticity and impair vascular remodeling (Zhao et al., 2006, 2007).

Given that uncontrolled expression of MMPs can result in tissue injury and destruction, the catalytic activity of MMPs is regulated at four points, which are: gene expression level, compartmentalization of the MMPs, pro-enzyme activation, and enzyme inactivation (Ra and Parks, 2007). Cleavage of the prop-peptide renders the MMP proteolytically active. However, given that one cysteine residue in the pro-peptide domain coordinates the catalytic site, disruption of this site via S-nitrosylation can also activate MMP-9 (Gu et al., 2002; Manabe et al., 2005; McCarthy et al., 2008). MMP is activity is further controlled by the availability and affinity of substrates. Indeed, MMPs are normally expressed at very low levels under normal conditions with localized expression induced when remodeling of the ECM is required. An in depth discussion of the transcription and regulation of MMPs is beyond the scope of the present review; we refer readers to some excellent reviews on the control of MMP activity (Ra and Parks, 2007; Clark et al., 2008; Fanjul-Fernández et al., 2010). Furthermore, MMPs are tightly regulated at both the transcriptional and post-transcriptional level by transcription factors and inhibitor proteins (Clark et al., 2008). In particular, endogenous tissue inhibitors of metalloproteinases (TIMPs), through high affinity non-covalent binding to the MMP catalytic domain, inhibit the activity of MMPs. To date, four TIMPs have been identified, with TIMP-1 having a specific affinity for MMP-9 (Clark et al., 2008; Fujimoto et al., 2008). In addition, MMPs, such as MMP-9, are secreted as inactive zymogens (proforms) that require activation through cleavage of the pro-peptide. This cleavage produces a conformational change, enabling a water molecule to associate with MMP-9, rendering it proteolytically active (Clark et al., 2008), and thereby providing another level of control for MMP function. In particular, pro-MMP-9 may be activated by a number of molecules including MMP-2, MMP-3, plasmin, urokinase-type plasminogen activator (uPA), and tPA (Rosenberg et al., 2001; Cunningham et al., 2005). Indeed, another level of control is conferred by the influence of growth factors, cytokines, and chemokines on both MMP and TIMP transcription (Yan and Boyd, 2007), with a response at the transcriptional level typically occurring within a few hours of stimulation.

Certainly MMP-9 has been the most widely studied MMP family member in both the experimental and clinical stroke literature, which may in part be due to the fact that it can easily be assessed using techniques such as gelatin zymography. Nevertheless, the advancement of other techniques such as multiplex ELISAs and proteome arrays has allowed the identification and quantification of the role of other MMP family members following stroke and their contribution to tissue injury.

MMP-9 IN EXPERIMENTAL ISCHEMIC STROKE

Increased expression of pro/active MMP-9 has been detected within hours to days following stroke in non-human primates (Heo et al., 1999), rats (Romanic et al., 1998; Rosenberg et al., 1998; Justicia et al., 2003), and mice (Fujimura et al., 1999; Gasche et al., 1999; Asahi et al., 2001a). Following stroke, increased levels of MMP-9 have been detected in both peripheral and central cells including neurons, glia, endothelial cells and neutrophils, with each of these cell types having a unique MMP secretion/expression profile (Rosenberg, 2002; Van den Steen et al., 2002; Gasche et al., 2006). However, the expression of MMPs is highly dependent upon the type, duration and severity of the ischemic insult, in addition to the animal species and strain used, with the temporal profile of MMP expression varying widely amongst studies. Another level of complexity in comparison of studies is that both pro-MMP and active MMP levels are not always reported in concert. Altogether, these issues highlight the complexity in targeting this protease with treatment following ischemic stroke.

Indeed, alterations in other MMPs beyond MMP-9 have been observed following stroke. For example, increased levels of pro/active MMPs including MMP-2 (Amantea et al., 2008; Lenglet et al., 2014), MMP-3 (Kim et al., 2005; Si-Tayeb et al., 2006; Yang et al., 2011; Lenglet et al., 2014), MMP-4 (Lenglet et al., 2014), MMP-10 (Lenglet et al., 2014), and MMP-13 (Cuadrado et al., 2009). Such alterations in MMPs (and their endogenous inhibitors) has been linked to activation of astrocytes

and microglia (Kim et al., 2005; Yang et al., 2011), increased levels of circulating inflammatory cytokines (Amantea et al., 2014) and enhanced thrombolysis (Orbe et al., 2011).

Blood-Brain Barrier Breakdown

Disruption of the BBB is a key event in the secondary injury cascade following stroke, one that exacerbates injury through a number of mechanisms including permitting the entry of peripheral immune cells into the brain to enhance the neuroinflammatory response, and hasten the development of vasogenic edema (Kuroiwa et al., 1985). Given their ability to degrade the ECM and tight junction components, MMPs have been implicated in BBB permeability alterations post-stroke.

Following stroke, a biphasic opening of the BBB is well established (Kuroiwa et al., 1985). The first alteration in BBB permeability occurs within hours of stroke onset, with the second occurring some 24–48 h later (Rosenberg et al., 1998). Such early and late alterations in barrier permeability are consistent with the increased expression of MMP-2 and MMP-9, which are the main MMPs that have been shown to be altered following both stroke and traumatic brain injury (Mun-Bryce and Rosenberg, 1998; Romanic et al., 1998; Fujimura et al., 1999; Gasche et al., 1999; Asahi et al., 2000; Planas et al., 2001; Rosenberg et al., 2001). Specifically, in one study increased levels of MMP-2 have been observed, in concert with an early and reversible disruption to the BBB (Chang et al., 2003), with late BBB disruption at 24–48 h following stroke observed in conjunction with increased MMP-9 levels (Sandoval and Witt, 2008). The early BBB disruption attributable to increased MMP-2 levels is deemed reversible, as although the tight junction components loosen, they remain within the endothelial cleft, and thus can be reassembled to reverse such permeability changes (Yang et al., 2007). In contrast, the delayed breach in BBB integrity in the setting of elevated MMP-9 expression is associated with complete degradation of the basal lamina (Mun-Bryce and Rosenberg, 1998) and tight junction components (Asahi et al., 2001b) resulting in and gross barrier disruption. This late barrier disruption persists for several days and is associated with complete breakdown of the BBB and HT (Romanic et al., 1998; Rosenberg et al., 1998; Asahi et al., 2001b). Due to the integral function of the BBB in the development of cerebral oedema, changes in MMP-9 levels have also been shown to correlate with the severity of cerebral edema, in a rodent model of stroke (Li et al., 2013). This scenario of early MMP2 and late MMP9, however, has been challenged by a number of other studies described below that implicate neutrophil MMP9 in early BBB opening (Montaner et al., 2008).

In addition, despite the involvement of MMP-2 in early barrier disruption, MMP-2 inhibition does not confer protection against BBB disruption (Asahi et al., 2001b; Gidday et al., 2005). Indeed, administration of the specific MMP-2/9 inhibitor SB-3CT failed to confer protection against a hypoxic-ischemic insult in neonatal rats (Ranasinghe et al., 2012). In contrast, MMP-9 inhibition provides robust protection against changes in BBB permeability (Svedin et al., 2007), suggesting that MMP-9 is the dominant protease acting at the BBB following ischemic stroke (Dejonckheere et al., 2011). MMP-3 immunoreactivity has been observed in pericytes following stroke and given that MMP-3 activates MMP-9 *in vivo*, MMP-3 knockout mice have been investigated to explore the effects of MMP-9 in ischemic stroke (Gurney et al., 2006). MMP-3 knockout reduced the levels of active MMP-9 and subsequent BBB disruption and was associated with a significantly reduced number of neutrophils infiltrating the stroke lesion.

Oxidative stress is known to play a significant role in the evolution of injury following stroke. Through studies in superoxide dismutase (SOD) 1-knockout mice, a role for oxidative stress in the mediation of BBB disruption has been revealed (Gasche et al., 2001). Specifically, higher levels of pro-MMP-9 and active MMP-9, in conjunction with profound BBB disruption were observed in SOD1 knockouts, an effect reversed with MMP inhibition, with comparable findings also reported in SOD2 knockouts (Maier et al., 2004). Subsequent studies have revealed nitric oxide and ROS to be the specific components of the oxidative stress response that lead to MMP-9 activation (Gu et al., 2002), validated by the significant decrease in infarct volume, vascular damage and MMP-9 activation following treatment with a non-selective nitric oxide inhibitor (Gürsoy-Ozdemir et al., 2000).

Hemorrhagic Complications

As many as 88% of strokes are ischemic in type and therefore may benefit from thrombolysis with tPA to recanalize the occluded cerebral artery (Adibhatla and Hatcher, 2008). In addition to its actions as a thrombolytic agent, tPA, via activation of MMP-9, may also damage the basal lamina and tight junctions of the cerebral blood vessels, resulting in increased permeability of the BBB, cerebral edema, and hemorrhagic complications (Lapchak et al., 2000; Sumii and Lo, 2002). MMP-induced degradation of the ECM is problematic as it weakens vessels, making them more prone to rupture and increases risk of cerebral hemorrhage (Heo et al., 1999; Lapchak et al., 2000; Montaner et al., 2001a; Sumii and Lo, 2002; Rosenberg and Yang, 2007; Rosell et al., 2008). Though a number of proteases may activate MMP-9, plasmin and tPA are two of the most important in the setting of stroke as they have implications in hemorrhagic complications. Indeed, such secondary disturbances to the BBB and microvascular damage precede such HT. Thrombolysis-induced hemorrhage is classified into two main types: PH and HT. In PH there is a discrete loss of microvascular integrity and extravasation of red blood cells into the brain parenchyma, whereas HT involves gross disruption to the cerebral vasculature and hematoma development. However, it must be noted that tPA-induced activation of MMP-9 may be beneficial in the late reparative phase of stroke to assist in the vascular remodeling, angiogenesis, neurogenesis and axonal regeneration response.

In addition to driving BBB disruption, MMP-9 has also been implicated in HT following ischemic stroke. Treatment with tissue-type tPA increases MMP-9 levels after embolic stroke in rodents, thereby implicating MMPs in tPA-induced hemorrhage (Sumii and Lo, 2002). Furthermore, MMP inhibitors have been shown to reduce the incidence and severity of tPA-induced hemorrhagic complications (Lapchak et al., 2000; Sumii and Lo, 2002). Minocycline (either low dose IV or high dose IP) inhibited MMP-9 upregulation induced by tPA treatment (Machado et al.,

2009) and was shown to extend the 3 h time window for tPA administration to 6 h in a embolic model of ischemic stroke in rats (Murata et al., 2008).

MMP Inhibition and Knockout Studies

Reduced breakdown of the BBB has been reported with broad inhibition of MMPs (Ferry et al., 1997; Fernandez-Patron et al., 2001) or targeted MMP-9 gene deletion (Asahi et al., 2000; Gasche et al., 2001; Rosenberg, 2002). Indeed, elevated active MMP-9 was observed as early as 3 h post-stroke and peaking at 18 h post-stroke onset, and thus preceded marked BBB disruption at 6 h and cerebral edema at 24 h. Increased MMP-9 levels were involved in such events as MMP inhibition with GM6001 ameliorated BBB permeability changes and cerebral edema (Shigemori et al., 2006). Specifically, MMP-9 knockout animals have reduced infarct volumes, incidence of HT, volume of cerebral edema, and functional deficits compared to wild type animals (Romanic et al., 1998; Asahi et al., 2000, 2001b; Lee et al., 2003; Hu et al., 2009; Wang et al., 2009). Similarly, MMP inhibitor treatment reduced MMP-9 activation and attenuated disorganization of tight junction proteins, including occludin and zona-occludens-1, leading to a reduction in vascular leakage and preservation of BBB integrity (Bauer et al., 2010). Furthermore, MMP inhibition with GM6001 ameliorated BBB disruption at 6 h and cerebral edema at 24 h post-stroke (Shigemori et al., 2006). Studies administering MMP inhibitors prior to stroke have also shown benefit (Asahi et al., 2000). However, as these molecules are unable to cross the intact BBB it is likely that they are acting on inflammatory cells or endothelial cells, providing it can gain access to them. The fact that such agents are beneficial suggests that an effect on circulating leukocytes with a subsequent reduction in MMP-9 is plausible.

MMP-9 IN CLINICAL ISCHEMIC STROKE

In keeping with the experimental literature, studies of clinical stroke patients have revealed increased levels of MMP-9 following ischemic stroke in humans (Anthony et al., 1997; Montaner et al., 2003b; Ning et al., 2006; Rosell et al., 2008) with elevated MMP-9 levels observed compared to healthy controls (Lucivero et al., 2007). Gene expression studies of peripheral blood from stroke patients reveled that MMP-9 was one of the key genes upregulated in response to stroke (Tang et al., 2006). Furthermore, MMP-9 has been shown to be elevated in the serum of stroke patients and is correlated with a worsened outcome (Montaner et al., 2001b; Copin et al., 2005; Ning et al., 2006). This elevation in MMP-9 was determined to be a marker of stroke for patients arriving within 12 h of stroke onset (Reynolds et al., 2003), with MMP-9 levels even proposed as a marker that could predict the probability of stroke (Lynch et al., 2004). Indeed, elevations in other MMPs, such as MMP-10, have been observed in clinical patients following stroke. Increased serum pro-MMP-10 levels were observed in both tPA-treated and non-tPA-treated patients compared to age-matched controls. Such alterations in pro-MMP-10 were associated with large infarct volumes, development of severe brain edema, neurological deterioration, and poor outcome at 3 months but interestingly, elevations in pro-MMP10 were not associated with hemorrhagic transformation (Rodríguez et al., 2013). Such studies highlight the breadth of MMP alterations following stroke and the implications for infarct evolution and both the development and treatment of complications.

Outcome Following Stroke

In ischemic stroke patients, a correlation between plasma MMP-9 levels and final National Institute of Health Stroke Scale (NIHSS) score, which is used to objectively quantify the impairment cause be stroke, has been observed (Montaner et al., 2001b). MMP-9 expression correlated with stroke severity and poor outcome, as assessed by the NIHSS (Inzitari et al., 2013) at 48 h post-stroke (Montaner et al., 2001b) but was shown to correlate with NIHSS score early as 24 h post-stroke onset (Rosell et al., 2005). Furthermore, there was a positive correlation between MMP-9 levels and NIHSS score and a negative correlation with the Barthel Index, a measure of activities of daily living (Vukasovic et al., 2006). Although a subsequent study by Montaner showed that high MMP-9 levels correlated with NIHSS score at admission this did not identify a specific stroke etiology (Montaner et al., 2008). Potential explanations for the differences in the outcomes of these studies may include the composition of the patient cohorts and variations in stroke size and type. In regards to long-term outcomes, MMP-9 was associated with a poor neurological outcome at 3 months post-stroke (Rodríguez-Yáñez et al., 2006) and hyperacute levels of MMP-9 correlated with worse Rankin outcome at 3 months post-stroke (Ning et al., 2006). This poor outcome and increased levels of MMP-9 were subsequently correlated with both infarct volume and stroke severity (Ning et al., 2006). A single study has reported that both MMP-2 and MMP-9 levels correlated with clinical severity and the extent of the infarct (Sotgiu et al., 2006).

Infarct Volume

In keeping with the role of MMP-9 in hastening BBB disruption and resultant injury, a non-significant increase in MMP-9 levels was observed in those tPA-treated patients that developed severe brain edema (Moldes et al., 2008). Furthermore, MMP-9 levels were shown to directly relate to stroke infarct volume (Horstmann et al., 2003; Rosell et al., 2005; Sotgiu et al., 2006; Vukasovic et al., 2006), a correlation that was observed at 24 h post-stroke (Rosell et al., 2005), but was apparent as early as 6 h post-stroke (Montaner et al., 2003b), with MMP-9 identified as the only marker that accurately predicted final infarct volume (Montaner et al., 2003b). As has been observed in the experimental stroke literature, MMP-9 levels increase over time following clinical stroke. MMP-9 levels in ischemic stroke patients were significantly elevated at 7d compared to 1d post-stroke (Kurzepa et al., 2006), with a temporal profile study out to 12d post-stroke revealing that levels of MMP-9 increased steadily over time following the onset of cerebral ischemia (Horstmann et al., 2003).

Hemorrhagic Complications

A relationship between baseline MMP-9 levels and late HT has been established, where high baseline levels were predictive

of late hemorrhagic events (Montaner et al., 2001c), further supported by the observation that MMP-9 levels were higher in patients who developed HT of their infarct (Heo et al., 2003). Furthermore, MMP-9 levels were significantly higher in those patients with HT, compared to those without (Castellanos et al., 2003). Levels of MMP-9 even differed between those patients who developed symptomatic HT compared to non-symptomatic HT (Castellanos et al., 2003). MMP-9 levels were also a good predictor of petechial hemorrhage (PH) in tPA-treated ischemic stroke patients (Castellanos et al., 2007). Indeed, elevated MMP-9 levels were associated with increased symptomatic intracerebral hemorrhage (ICH) or death (Inzitari et al., 2013), leading to the suggestion that MMP inhibitors may of clinical use when administered in conjunction with thrombolysis to reduce or prevent such hemorrhagic complications.

Activation of MMP-9 by tPA

tPA has been shown to directly activate MMP-9 (Wang et al., 2003; Benarroch, 2007), with rt-PA treatment increasing MMP-9 activity in the serum of ischemic stroke patients (Golab et al., 2014). Such tPA-activation of MMP-9 may further amplify the MMP-9 response to stroke and involvement in injury pathways (Tsuji et al., 2005). Indeed, MMP-9 levels were shown to correlate with the levels of free radicals, measured as a marker of oxidative stress, this was observed in both tPA-treated and non-tPA treated patients (Kelly et al., 2006). However, tPA administration increases plasma MMP-9 levels, with co-administration of the free radical scavenger Edaravone having no effect on MMP-9 levels (Tsuruoka et al., 2014), although this may have been attributable to the low-dose Alteplase (0.6 mg/kg) used. An increased rate of HT on day 1 following acute ischemic stroke was observed in patients who underwent thrombolysis, compared to untreated controls (Carbone et al., 2015), with thromboylsis with tPA shown to increase venous blood levels of MMP-9 (Ning et al., 2006). Indeed, significantly higher levels of active MMP-9 have been observed in areas of HT, compared to both non-hemorrhagic, and non-ischemic tissue (Rosell et al., 2008). Indeed, MMP-9 levels were higher in patients that developed HT (Castellanos et al., 2003; Heo et al., 2003), with differences in MMP-9 levels even observed between symptomatic and asymptomatic HT (Castellanos et al., 2003). However, it has also been reported that neither baseline MMP-9 levels nor the rate of MMP-9 increase had any association with the risk of HT in the setting of ischemic stroke (Tsuruoka et al., 2014). Clearly this relationship requires further exploration. Nevertheless, it has been suggested that early HT is attributable to ROS, blood-derived MMP-9, and brain-derived MMP-2, whereas delayed HT is likely attributable to brain-derived MMP-9 and MMP-3, amongst other proteases, vascular remodeling and neuroinflammation (Jickling et al., 2014). Accordingly, early inhibition of MMP-9 may reduce hemorrhagic complications. MMP inhibitors may maintain the integrity of the BBB following stroke by limiting the access of tPA to brain parenchyma, thereby reducing hemorrhagic complications (Rosenberg and Yang, 2007).

Post-Mortem Studies

Valuable insights into the changes in MMPs following human stroke was gained from post mortem studies of brain tissue from stroke patients. Fresh brain tissue from ischemic and hemorrhagic stroke patients examined within 6 h of death revealed higher levels of MMP-9, compared to control brains (Rosell et al., 2006). A further study by Rosell et al. (2008) of ischemic stroke patients with hemorrhagic complications revealed elevated MMP-9 levels within the stroke lesion. Within the infarct core, MMP-9 was localized to perivascular tissue and was associated with neutrophil infiltration whereas in the peri-infarct tissue microglial cells were found to highly express MMP-9 (Rosell et al., 2006). In hemorrhagic stroke, tissue surrounding the hematoma was shown to have increased levels of MMP-9. In both studies, no changes in MMP-2 levels were observed (Rosell et al., 2006, 2008). Neutrophils were identified as the main source of MMP-9 in areas of hemorrhage. Neutrophils were observed surrounding microvessels in conjunction with severe degradation of basal lamina type IV collagen and extravasation of blood into the surrounding brain parenchyma. As such, microvessel inflammation and MMP-9 appear to be key events associated with the development of hemorrhagic complications following ischemic stroke.

Overall, the clinical studies reveal that higher levels of MMP-9 are present in patients with acute ischemic stroke, compared with controls, and that this is a predictor for the development of hemorrhagic complications (both PH and HT), with MMP-9 levels correlating with larger infarct volume, increasing severity of stroke and poor functional outcome (Ramos-Fernandez et al., 2011). In this way, MMP-9 is proposed as a marker for ongoing brain ischemia and evolution of the stroke lesion, making it a potential candidate for inclusion in a stroke biomarker panel. As such, it is universally accepted that profound changes in MMP-9 expression and activity occur following ischemic stroke in both humans and animals. However, controversy still remains as to which cells are responsible for the bulk of the MMP-9 load following ischemic stroke.

TIMP-1 FOLLOWING STROKE

TIMPs are the endogenous inhibitors of MMPs present under normal conditions in tissues to regulate the activity of MMPs (Cunningham et al., 2005). However, just as MMP-9 levels are elevated following stroke, the expression of TIMP-1, the endogenous inhibitor of MMP-9, has also been shown to be dysregulated following stroke (Rivera et al., 2002). Furthermore, the MMP-9/TIMP-1 ratio has been proposed as a marker of stroke. Indeed, the correlation of MMP-9/TIMP-1 ratio with cerebral edema was even stronger than that of MMP-9 alone following ischemic stroke in rats (Li et al., 2013). Similar findings were observed in a clinical study with the relative increase in MMP-9/TIMP-1 ratio independently associated with symptomatic ICH in ischemic stroke patients (Piccardi et al., 2015). In keeping with the role of MMP-9 and TIMP-1 in the pathogenesis of stroke, mild hypothermia provided protection from ischemia/reperfusion injury via decreasing the expression

of MMP-9 (Burk et al., 2008; Zhao et al., 2013) and TIMP-1 (Zhao et al., 2013).

Transient global cerebral ischemia led to TIMP-1 expression within 4 h of onset within the dentate gyrus of the hippocampus and progressed to involve other regions of the hippocampus including CA1 by 24 h post-stroke, a region especially vulnerable to ischemic injury. Such elevated TIMP-1 expression was observed in conjunction with elevated MMP-9 levels at 24 h following stroke onset (Fujimoto et al., 2008). Such increased expression of TIMP-1 was apparent in neurons and glial cells following global cerebral ischemia. This elevation in TIMP-1 may be a protective response to the ischemic insult to combat the increased levels of MMP-9 in an attempt to not only preserve endothelial barrier function but also participate in the long-term reorganization of the tissue following injury. Although the protective effects of TIMP-1 inhibition are thought to extend beyond simply its inhibition of MMP-9, as neuroprotection through TIMP-1 inhibition could not be reproduced with MMP inhibition alone (Chesler et al., 1995).

Rendering TIMP-1 inactive abolished the neuroprotective effects of TIMP-1 application to hippocampal cultures exposed to an excitotoxic insult (Tan et al., 2003). TIMP-1 knockout mice demonstrated significantly elevated Evan's Blue extravasation, indicative of profound BBB disruption and vasogenic edema, along with larger infarct volumes, in keeping with increased MMP-9 expression and activity in the absence of inhibition by TIMP-1 (Fujimoto et al., 2008). Presumably, most of the exogenously administered TIMPs act on endothelial cells and circulating immune cells, such as neutrophils, to inhibit MMP-9 activity, rather than the brain tissue itself as these molecules are unable to cross the intact BBB. Such a hypothesis certainly fits with the argument that neutrophils are the cellular source of MMP-9.

CELLULAR SOURCE OF MMP-9 FOLLOWING STROKE

Increased MMP-9 levels have been well documented following both experimental and clinical stroke. However, the cellular source of this MMP-9, which is driving barrier permeability changes, cerebral edema, and hemorrhagic complications remains to be elucidated. A number of studies have sought to investigate what cells are releasing MMP-9 in stroke, with both peripheral and central candidates examined. A wide range of cell types have been shown to express MMP-9 following stroke, including neurons, microglia, and endothelial cells (Rosell et al., 2008). However, the main or initiating source of the MMP-9 remains unclear.

Neutrophils and MMP-9

Following stroke, the chemokines and cytokines released in response to the ischemic insult promote the chemotaxis of inflammatory cells, such as neutrophils, to the ischemic site (Rodrigues and Granger, 2015). These cells transmigrate into the ischemic tissue where they contribute to tissue injury. In order to gain access to the brain tissue, the well-orchestrated expression of cellular adhesion molecules, integrins, and chemokines are required to enable neutrophils to roll along the endothelium, adhere to the endothelium and transmigrate through the endothelial cell barrier into brain tissue (Wang and Doerschuk, 2002; Schnoor and Parkos, 2008; Alcaide et al., 2009; Choi et al., 2009). Neutrophils are the main inflammatory cell type that responds to the inflammatory stimulus following stroke (Tang et al., 2006). Transmigration of neutrophils is thought to be reliant on three proteinases released from neutrophilic granules: neutrophil elastase (NE), collagenase, and gelatinase granules. These proteinases act in concert to degrade the ECM and allow passage of neutrophils into the tissue. Indeed, neutrophils are equipped with MMP-9 in secretory granules, which enables them to dissolving the ECM and basal lamina and "burrow" their way into the tissue (Choi et al., 2009). However, it has been suggested that neutrophils only require MMP-9 for stage 5 of transmigration only, where the neutrophils are migrating between the endothelial cells (Oda et al., 1995). Nonetheless, exocytosis of MMP-9 from gelatinase granules is likely to be important for the transmigration of neutrophils into tissues (Keck et al., 2002; Lee et al., 2003; Khandoga et al., 2006) with cell culture studies demonstrating that gelatinase activity is required for the migration of neutrophils through Matrigel and amnion membranes (Bakowski and Tschesche, 1992; Steadman et al., 1997). Once neutrophils gain access to the tissue they are then able to stimulate the release/production of MMP-9 from other cells types including resident brain cells, further perpetuating the effects of MMP-9 activity. Indeed, neutrophil neurotoxicity is dependent upon MMPs, reactive oxygen species (ROS) and cytokines such as tumor necrosis factor-α (TNF-α) (Nguyen et al., 2007).

The expression of MMP-9 by neutrophils occurs late in their development and thus following transcription, pro-MMP-9 is stored in cytoplasmic granules, along with neutrophil gelatinase-associated lipocalin, which protects the pro-MMP-9 molecules from degradation (Van den Steen et al., 2002). The presence of pre-made MMP-9 within these neutrophil granules allows the rapid release (within minutes) of MMP-9 into the extracellular milieu, as opposed to the mechanism of increased expression in other cell types that requires transcription and takes in excess of several hours (Opdenakker et al., 2001; Van den Steen et al., 2002). This suggests that neutrophils may be responsible for the early increases in MMP-9 observed in plasma following stroke. Following degranulation, pro-MMP-9 is released into the extracellular space where it is activated through removal of its pro-peptide by proteolysis (Opdenakker et al., 2001; Sternlicht and Werb, 2001). Although MMP-2 is generally constitutively expressed in most cell types, neutrophils are unique in that they do not express MMP-2, nor do they express TIMPs.

Neutrophils as the Cellular Source of MMP-9 Following Stroke

Inflammatory cells have been implicated as important sources of MMP-9 in a variety of other pathological conditions including tumor progression, asthma, and hepatic injury (Yushchenko et al., 2000; Turba et al., 2007; Fujimura et al., 2009). Previous

studies have suggested that neutrophils are the major source of MMP-9 acting on the BBB (Tang et al., 2006). Indeed, MMP-9 is expressed almost exclusively in neutrophils in peripheral blood (Tang et al., 2006) and increased BBB permeability induced by leukocyte-derived MMP-9 has been shown in ischemia-reperfusion injury to correlate with peak neutrophil infiltration (Sandoval and Witt, 2008), suggesting that neutrophils are a good candidate to be the main source of MMP-9 released following ischemic stroke.

Experimental Studies on MMP-9 Source

Investigation of the role of MMP-9 and neutrophils in cerebral ischemia has taken two approaches, either knockout of MMP-9 or depletion of neutrophils prior to induction of stroke. MMP-9 knockout significantly attenuates leukocyte recruitment into brain tissue following stroke, implicating a pro-inflammatory role for MMP-9 in the recruitment of leukocytes to reperfused brain tissue (Gidday et al., 2005). Gidday et al. (2005) was the first group to report on the cellular source of MMP-9 following stroke but since this paper there have been a number of other studies that have investigated whether neutrophils are the main contributors to the increased MMP-9 load observed following stroke. In their paper, Gidday et al. (2005) reported that depletion of neutrophils prior to induction of stroke markedly reduced vasogenic edema, collagen IV degradation and the extent of cerebral infarction, suggesting the MMP-9 promotes neutrophil transmigration into the brain and resultant injury. Such results provide compelling evidence for neutrophils as the source of MMP-9 following stroke.

One such study in support of the neutrophil-derived MMP-9 theory was carried out in rats with neutropenia treatment prior to induction of stroke to deplete neutrophils (Justicia et al., 2003). They reported an increase in 95 kDa MMP-9 in neutrophils and of 88 kDa MMP-9 in brain tissue following 1 h MCAO. Neutropenia treatment prior to stroke markedly reduced MMP-9 expression and prevented infiltration of neutrophils into the ischemia tissue. Treatment with an anti-intercellular adhesion molecule-1 (expressed on endothelial and immune cells) antibody, in addition to neutropenia, still produced low levels of MMP-9 expression, which was attributed to expression of MMP-9 by other cell types within the ischemic tissue. Accordingly, this group concluded that neutrophil infiltration was essential for the rise in MMP-9 expression and activity observed, and that neutrophils significantly contribute to the increase in MMP-9 in cerebral tissue through the release of pro-MMP-9.

In contrast, a few studies have reported that neutrophils are not the cellular source of MMP-9 following stroke (Maier et al., 2004; Harris et al., 2005; Zozulya et al., 2008). Specifically, 3 h MCAO was shown to produce a significant increase in MMP-9 and MMP-2 within the ischemic hemisphere, however prior neutrophil depletion did not affect MMP-9 protein levels, nor was there any benefit in terms of infarct volume, HT, cerebral edema, or functional outcome as measured by the neuroscore (Harris et al., 2005). Accordingly, this group proposed that neutrophils are not an important contributor to MMP-9 expression in the setting of cerebral ischemia and do not have a significant effect on neurovascular damage or neurological function and therefore is unlikely to be a key player in early microvascular damage and hemorrhagic complications following stroke. They suggest that neutrophils may be more important in ischemia models where there is a lower degree of injury, certainly the 3 h MCAO used in this study is at the severe end of the ischemia spectrum. Maier et al. (2004) found MMP-9 expression to be variable within the ischemic hemisphere and proposed that various cell types contribute differently in a region dependent manner. All myeloperoxidase (MPO, major component of neutrophil azurophilic granules)-positive cells were found to be MMP-9-postive and these cells were present in high numbers. Micgroglia were also shown to express MMP-9, but not all microglia were MMP-9 positive. MMP-9 immunoreactivity was clearly visible in vessels demonstrating Evan's blue extravasation. However, they did not observe any difference in the number of MPO positive cells over time, from the 24–72 h time-points, nor between the wild type of SOD2 animals examined. Furthermore, the MMP-9 response was shown to be bi-phasic, which did not coincide with neutrophil infiltration into the stroke lesion. Although not discounting the contribution of neutrophil-derived MMP-9 in barrier disruption and evolution of injury following stroke, this group suggest that neutrophils are not the primary source of MMP-9 following stroke and that other cell types such as microglia, astrocytes, and endothelial cells may be more appropriate targets for MMP inhibition. Such findings suggest that neutrophils may be the cells that initiate the release of MMP-9 in/directly from these other cell types, thereby potentiating MMP-9 release within ischemic tissue. As such, neutrophils may release MMP-9 locally at the BBB and do not need to migrate into the brain parenchyma to initiate the release of MMP-9 from resident brain cells to perpetuate the injury cascade. Indeed, studies of cultured endothelial cells showed that under normal conditions neutrophils weakly express MMP-9 but when co-cultured with pericytes an increase in MMP-9 secretion is observed, indicating that pericytes may be responsible for the increase in MMP-9 (Zozulya et al., 2008).

Neutrophils themselves are a significant source of ROS (Rodrigues and Granger, 2015). In a cell culture model, using neutrophil conditioned medium, neutrophils were shown to have high MMP-9 expression and release high levels of superoxide, hydrogen peroxide, and TNF-α (Nguyen et al., 2007). Treatment of the cells with the MMP-9 inhibitor GM6001 produced an 80% reduction in MMP-9 activity and an accompanying decrease in hydrogen peroxide and increase in TNF-α. Such findings indicate the neutrophil-derived MMP-9 can promote cell death by increasing the levels of ROS and also through the regulation of NE that increases endothelial cell death.

Clinical Studies on MMP-9 Source

Gene expression analysis of peripheral blood taken from ischemic stroke patients revealed that MMP-9 was significantly upregulated in neutrophils early after stroke (Tang et al., 2006). A human study measuring MMP-9 levels in the CSF and serum of controls and patients with various neurological disorders showed that MMP-9 was not expressed in the CSF of controls but was markedly increased in the CSF of patients. This increase in

MMP-9 was attributable to an increased in neutrophils within the CSF (Yushchenko et al., 2000), whereas monocytes/macrophages and lymphocytes were only shown to be weak producers of MMP-9.

Not only have neutrophils been implicated as a source of MMP-9 following stroke which contributes to injury and barrier dysfunction, they have also been suggested to be the source of MMP-9 following tPA treatment. This was supported by the observation that tPA treatment can induce the release of pro-MMP-9 from ischemic brain (Justicia et al., 2003) and more recent observations that tPA can directly initiate the release of MMP-9 (Cuadrado et al., 2008), along with other MMPs and TIMPs. Given that tPA promotes neutrophil degranulation and the release of MMP-9. These inflammatory cells are good candidates to be the main source of MMP-9 post-stroke in the setting of tPA treatment, and may be responsible, at least in part, for tPA-induced hemorrhage (Cuadrado et al., 2008). In thrombolysed ischemic stroke patients a peak of neutrophil degranulation was observed 30 min following tPA administration (Carbone et al., 2015). The tPA-induced degranulation of neutrophils induced a combined release of the contents from primary (MPO and NE), secondary (collagenases such as MMP-8) and tertiary granules (MMP-9) (Carbone et al., 2015). Such increases in MMP-9 were seen in microvessels and neutrophils associated with the hemorrhagic tissue. Neutrophils were found to be an important source of MMP-9, as indicated by MPO staining, with total brain MMP-9 levels correlating with the number of MMP-9 positive neutrophils. In terms of HT, it appears that it is not the neutrophils adhered to the endothelium but rather those that transmigrate into the cerebral tissue that are responsible for such hemorrhagic complications (Gautier et al., 2009). As such, the location of the neutrophils, for example neutrophils within the cerebral vasculature, those localized to the BBB or neutrophils that have extravasated into the brain parenchyma, may determine the effect of MMP-9 release on the local tissue. However, others have suggested that neutrophils are not necessary for the acute development of HT (Harris et al., 2005). Human brain endothelial cells were shown to participate in MMP-mediated BBB breakdown during an ischemic insult, although their data did not support a role of MMP-9 in this process. Instead, MMP-2 was identified as the MMP that was elevated in ischemia.

COMPARISON BETWEEN EXPERIMENTAL AND CLINICAL DATA

The experimental and clinical literature is in agreeance that profound elevations in MMP-9 occur early following stroke that can hasten and exacerbate injury. Specifically, in the experimental studies, elevations in MMP-9 are observed as early as 3 h following stroke (Shigemori et al., 2006) and persist for days following stroke onset. Such alterations in MMP-9 are associated with increased BBB permeability (Romanic et al., 1998; Rosenberg et al., 1998; Asahi et al., 2001b; Sandoval and Witt, 2008), the development of cerebral edema (Li et al., 2013), hemorrhagic transformation (Romanic et al., 1998; Rosenberg et al., 1998; Asahi et al., 2001b; Sandoval and Witt, 2008), increased infarct volume (Romanic et al., 1998; Asahi et al., 2000, 2001b; Lee et al., 2003; Hu et al., 2009; Wang et al., 2009; Li et al., 2013), and poor outcomes (Romanic et al., 1998; Asahi et al., 2000, 2001b; Lee et al., 2003; Hu et al., 2009; Wang et al., 2009; Li et al., 2013) in animal models. Indeed, the clinical studies report a comparable picture with MMP-9 levels elevated as early as 6 h post-stroke in post-mortem tissue (Rosell et al., 2006) and within 12 h in patients (Reynolds et al., 2003) with such alterations and persisting for many days thereafter with these changes associated with large infarct volumes (Horstmann et al., 2003; Rosell et al., 2005; Sotgiu et al., 2006; Vukasovic et al., 2006), hemorrhagic transformation of the infarct (both with and without tPA treatment), and poor outcomes (Rosell et al., 2005; Ning et al., 2006; Rodríguez-Yáñez et al., 2006; Vukasovic et al., 2006) in clinical stroke patients. Furthermore, the experimental and clinical literature also largely agree that neutrophils are the source of MMP-9 (Yushchenko et al., 2000; Justicia et al., 2003; Gidday et al., 2005; Cuadrado et al., 2008; Gautier et al., 2009) which is contributing to hastening of the ischemic injury including infarct extension and the development of complications such as hemorrhagic transformation.

CONCLUSIONS

We propose a likely model to explain the sequential breakdown and repair of the BBB following stroke (**Figure 1**). Early after stroke (12–48 h) neutrophils adhere to the cerebrovascular endothelium where they release MMP-9 that degrades the basal lamina of the endothelial cells and astrocytes. This enables migration of neutrophils into the ischemic tissue and subsequent opening of the BBB results in the formation of cerebral edema. At the ischemic core, MMP-9 may be released by both infiltrating neutrophils and resident microglia. Neutrophil-derived MMP-9 degrades the BBB, whilst the microglia-derived MMP-9 acts in concert with other molecules to produce neuronal and glial cell death and microglia likely undergo apoptosis in this environment. Upon restoration of blood flow, repair of the BBB may be initiated at the margins of the infarction. At this stage, all cells, including endothelial cells, glia, and neurons, may release MMPs and other proteases at low levels in order to remodel the basement membrane and restore cell-cell contacts to allow for angiogenesis and gliogenesis to occur in order to re-establish the BBB. Therefore, manipulation of the MMP-TIMP system following stroke needs to be well orchestrated to prevent tissue injury early but also assist in tissue remodeling late after the event and the therapeutic window available to limit BBB destruction following stroke is quite short, after which reparative functions would need to predominate.

In conclusion, a number of experimental studies suggest that neutrophils are in fact the main cellular course of MMP-9 following stroke (not resident brain cells) and that these cells use MMP-9 to transmigrate into ischemic tissue, contribute to ischemic injury in the form of ECM degradation, BBB permeability changes, vasogenic edema, and HT, whilst also

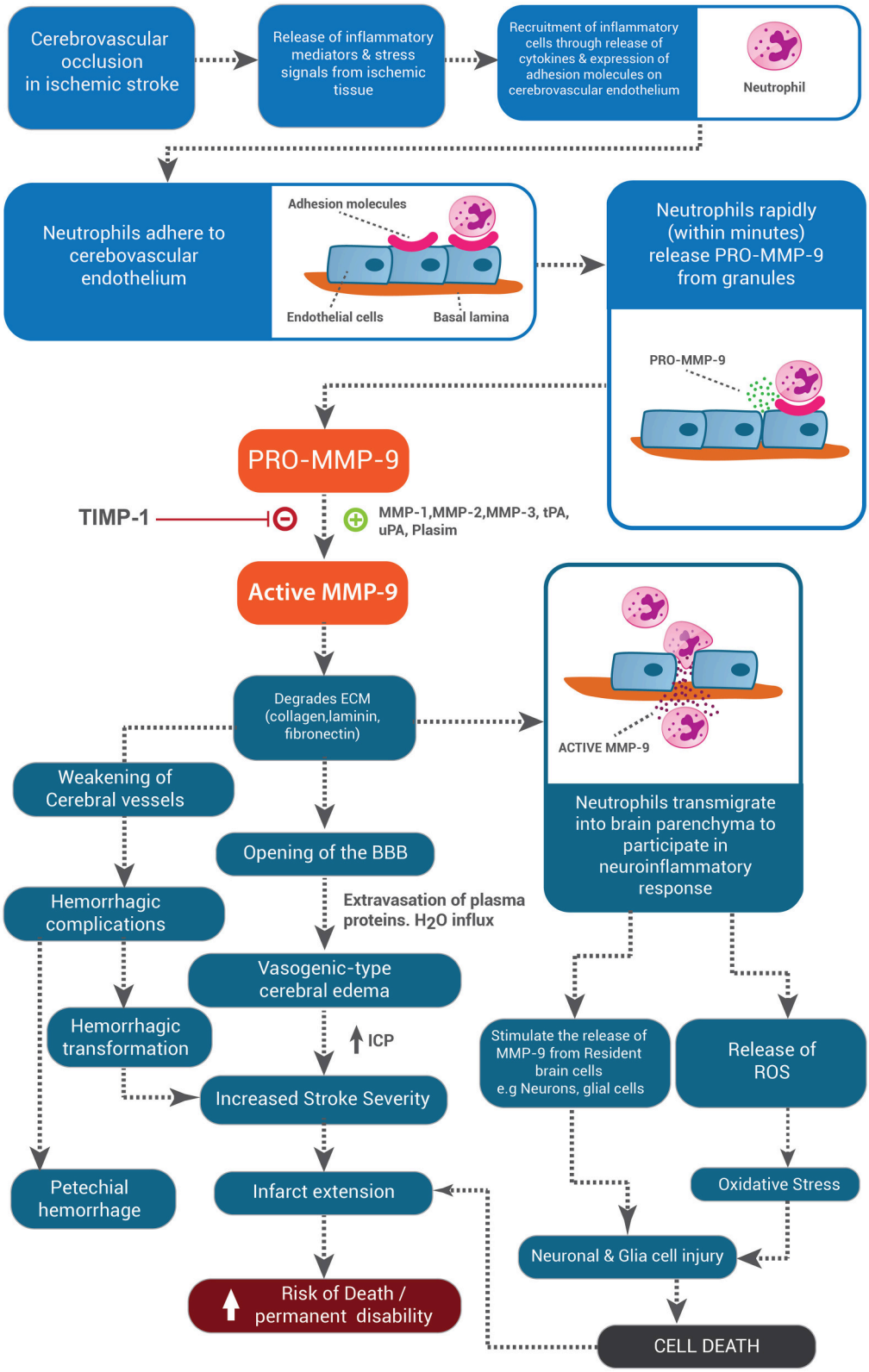

FIGURE 1 | The role of MMP-9 in exacerbating injury pathways in ischemic stroke. MMP-9 released from neutrophils allows these cells to transmigrate into the brain tissue, where they release MMP-9 and other deleterious agents such as ROS, thereby stimulating the release of MMP-9 from resident brain cells and contributing to the cell injury and cell death pathways. Neutrophil-derived MMP-9 actively degrades components of the BBB leading to the development of cerebral edema and hemorrhagic transformation, both of which worsen stroke severity, lead to infarct extension and increase the risk of death and disability post-stroke.

stimulating the release of MMP-9 from resident brain cells such as neurons and microglia. Clearly, the interaction of action of leukocytes at the BBB following stroke requires further investigation to elucidate the contribution of neutrophil-derived to ECM degradation, BBB disruption and subsequent vasogenic edema, hemorrhage and parenchymal injury.

Many studies have focused on the protection of neurons as a therapeutic target. However, given that such a small percentage of all cells in the brain are neurons it seems more appropriate to consider protection across many cell types. Accordingly, investigation of the neurovascular unit and the complex interplay of endothelial cells, pericytes, astrocytes, and infiltrating leukocytes is likely to yield more appropriate therapeutic targets. Information regarding the relationship between MMP-9 and neutrophils may facilitate understanding of the mechanisms involved in BBB breakdown following stroke and ultimately guide therapeutic intervention.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Cellular Neuroscience

MINI REVIEW
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Combination low-dose tissue-type plasminogen activator plus annexin A2 for improving thrombolytic stroke therapy

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Risk of hemorrhagic transformation, incomplete reperfusion, neurotoxicity, and a short treatment time window comprises major challenges for tissue plasminogen activator (tPA) thrombolytic stroke therapy. Improving tPA therapy has become one of the highest priorities in the stroke field. This mini review article focuses on our recent efforts aimed at evaluating a novel combination approach of low-dose tPA plus recombinant annexin A2 (rA2, a tPA, and plasminogen co-receptor), which might enhance tPA thrombolytic efficacy, while reducing its associated complications related to intracerebral hemorrhagic transformation. Results of our experimental studies using a focal embolic stroke model in rats support the feasibility of the combination approach and suggest the potential for successful clinical translation.

Keywords: cerebral ischemia, tissue-type plasminogen activator, annexin A2, thrombolysis, combination therapy, focal embolic stroke model, rats

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Limitations of tPA Thrombolytic Stroke Therapy

By stimulating thrombolysis and rescuing the ischemic brain via restoring blood flow, intravenous administration of recombinant tissue plasminogen activator (tPA) remains the most effective intervention with FDA approval for emergency treatment of stroke (Whiteley et al., 2014). However, a short treatment time window, hemorrhagic transformation, poor thrombolytic perfusion rate, and neurotoxicity comprise the major limitations to its application (Alexandrov and Grotta, 2002; Bambauer et al., 2006; Weintraub, 2006). Although other thrombolytic agents are being tested, none has been established as effective or as a replacement for tPA (Wang et al., 2004, 2008; Adams et al., 2007).

Recent clinical investigations have suggested the potential for improving tPA therapy. For example, perfusion-/diffusion-weighted imaging (PWI/DWI) mismatch and the diffusion/fluid attenuated inversion recovery (DWI/FLAIR) mismatch might have implications in selecting patients for reperfusion therapy (Shah et al., 2015; Wouters et al., 2015). A randomized phase III trial, ECASS III, designed to test treatment with tPA at 3–4.5 h, showed improved clinical outcomes for ischemic patients with thrombolytic treatment (Hacke et al., 2008). However, there

was a detectable large difference in odds-ratios between early reperfusion (approximately 2.8 OR) and delayed reperfusion (approximately 1.4 OR). The benefits of thrombolysis are, thus, still heavily dependent on the treatment time, and tPA remains associated with increased risk of intracranial hemorrhage and reperfusion injury (Hacke et al., 2008). It has been clinically prioritized to seek combination therapies that may extend the therapeutic window, reduce tPA-associated hemorrhagic transformation, and improve thrombolytic efficacy (Wang et al., 2004, 2008; Whiteley et al., 2014).

Pleiotropic Effects of Exogenous tPA for Stroke Reperfusion Therapy

Ischemic stroke is a thrombotic cerebrovascular event. For emergency treatment, intravenous tPA administration is intended to reopen occluded vessels by lysis of the thrombus, thereby improving clinical outcome through restoration of regional cerebral blood flow (CBF), thus salvaging the ischemic brain tissues (Fugate and Rabinstein, 2014). One limitation is that tPA thrombolysis is only partially or fully successful for re-canalization in about 50% of patients (Hacke et al., 2004). How does the ischemic brain respond to tPA in the non-responders within the context of weakened vessels and perturbed neurovascular homeostasis? Although unequivocal human data are lacking, experimental investigations in animal models suggest that tPA thrombolytic stroke therapy may have deleterious consequences due to the non-thrombolytic actions of tPA (Kaur et al., 2004). More investigational efforts are needed to dissect the molecular signaling mechanisms initiated by the infused exogenous tPA in the occluded vessel and ischemic brain. Accumulating experimental data suggest that exogenous tPA may have additional pleiotropic actions within the brain (Kaur et al., 2004), such as direct vasoactivity (Nassar et al., 2004; Armstead et al., 2009) enhanced excitotoxicity (Nicole et al., 2001), and activation of extracellular proteases (Nicole et al., 2001; Wang et al., 2003, 2004; Benchenane et al., 2004; Zeevi et al., 2007). These non-thrombolytic actions of tPA may exacerbate edema, increase ischemic neurotoxicity, damage the blood–brain barrier, and increase risk of cerebral hemorrhage, ultimately compromising its usefulness as a thrombolytic agent (Yepes et al., 2003, 2009; Su et al., 2008; Armstead et al., 2009).

Re-canalization has been well established as an important predictor of better stroke outcome, regardless of thrombolytic modality employed. However, a major remaining challenge is that exogenous tPA may potentiate ischemia-induced blood–brain-barrier disruption, increase the risk of symptomatic intracranial hemorrhage, which restricts prolonging the therapeutic time window (Tsivgoulis et al., 2014). One strategy may overcome these dose-dependent side effects of tPA by simply lowering the tPA dose, but this step would likely reduce the perfusion efficacy. Clearly, an optimization strategy for tPA thrombolytic therapy requires rebalancing the potential benefits of reperfusion against the detrimental effects of exogenous tPA (Wang et al., 2004, 2008).

tPA Receptor Annexin A2 and Fibrinolytic Assembly

In fibrinolysis, tPA plays a key role by enzymatically converting clot-bound plasminogen to active plasmin, which degrades cross-linked fibrin to break down fibrin-containing thrombi. This process is called fibrinogenolysis (Ranby and Brandstrom, 1988). Interestingly, recent vascular biology studies have demonstrated that tPA may interact with cellular receptors to activate specific signal transduction pathways (Wang et al., 2008). A new concept of fibrinolytic assembly for cell-surface fibrinolysis was proposed, in which the tPA conversion of plasminogen to active plasmin is precisely orchestrated through a multi-molecular complex, consisting of tPA, the annexin A2 heterotetramer, and plasminogen (Kim and Hajjar, 2002).

Annexin A2 is a 36-kDa cell-surface protein, a calcium-dependent phospholipid-binding protein. In complex with its binding partner p11, annexin A2 forms a heterotetrameric ($A2_2p11_2$) receptor for both plasminogen, the inactive precursor of plasmin, and its activator, tPA (Birnbaum et al., 1999). The assembled complex of tPA–annexin A2–plasminogen increases the catalytic efficiency of tPA in converting plasminogen to plasmin about 60-fold compared with the same amount of tPA alone (Hajjar and Menell, 1997; Birnbaum et al., 1999; Kim and Hajjar, 2002). More experimental evidence suggest that fibrinolytic assembly plays a critical role in maintaining blood and vascular homeostasis (Hajjar and Acharya, 2000). Additionally, annexin A2 exists in both membrane-bound and soluble forms of vascular endothelial cells (Siever and Erickson, 1997), and it can be transported to the cell surface in response to cellular stress (Deora et al., 2004). Complete deficiency of annexin A2 in mice leads to a loss of tPA cofactor activity, intravascular fibrin accumulation, and failure to clear arterial thrombi. In sum, these experimental findings support an important role of annexin A2 in fibrinolytic assembly (Ling et al., 2004).

Manipulating tPA Fibrinolytic Assembly for Improving Thrombolytic Stroke Therapy

Biologically, tPA efficiently converts plasminogen to clot-dissolving plasmin relying on the fibrinolytic assembly of a trimetric complex of tPA–annexin A2–plasminogen. However, clinically giving a large amount of tPA alone may lead to inefficient assembly of the tPA–annexin A2–plasminogen complex due to a limiting amount of annexin A2. This in turn would reduce the efficacy of the tPA in converting plasminogen to plasmin, which may be partially responsible for the shortcomings of tPA reperfusion stroke therapy. Thus, "High dose tPA required, high hemorrhage risk, low reperfusion efficiency, and short therapeutic time window," which is a major challenge in our field, can perhaps be avoided (Lo et al., 2002; Wang et al., 2004). It may revolutionize tPA-based stroke therapy if the tPA fibrinolytic assembly can be enhanced and utilized clinically. Because plasminogen (plasmin precursor) exists in circulation, binds to the endothelial cell surface and is enriched in the clot

(Sakharov and Rijken, 1995; Birnbaum et al., 1999; Hajjar and Krishnan, 1999), intravenous tPA combined with annexin A2 will locally form tPA–annexin A2–plasminogen complexes and consequently amplify plasmin generation, resulting in more effective and specific fibrinolysis (Birnbaum et al., 1999). By translating the tPA fibrinolytic assembly into tPA therapy development, we hypothesized that combining recombinant annexin A2 protein (rA2) will lower the required dose of tPA for reperfusion, while enhancing thrombolytic efficacy, and attenuating intracerebral hemorrhagic (ICH) transformation. By doing so, it will prolong therapeutic time windows and improve long-term outcomes (Fan et al., 2010).

Experimental Investigation of Low-Dose tPA Plus rA2 Combination

In the past few years, we have tested this hypothesis in a rat focal embolic stroke model. Our experimental findings support the feasibility of this approach and suggest clinical translation potential (Fan et al., 2010; Zhu et al., 2010; Walvick et al., 2011; Wang et al., 2014; Jiang et al., 2015). Consistent with previous reports (Cesarman et al., 1994; Kim and Hajjar, 2002), *in vitro* plasmin activity assays showed that rA2 significantly amplified tPA-mediated plasmin generation, and equivalent levels of *in vitro* plasmin activity can be reached by using high-dose tPA alone or lower-dose tPA in combination with rA2 (Zhu et al., 2010). Because of species-related differences in fibrin specificity, the equivalent effective dose of human recombinant tPA in the rat was about 10 times higher than the dose used in humans, or about 10 mg/kg (Korninger and Collen, 1981). In a focal embolic stroke model in rats, when animals were treated intravenously 2 h after initiation of ischemia, the 25–50% lower-dose tPA plus rA2 combination was as effective as the standard high-dose tPA alone in restoring perfusion and reducing infarct size (Zhu et al., 2010). This suggests that rA2 can make low-dose tPA more effective in an embolic stroke animal model. Improved reperfusion by the combination was confirmed by MRI analysis in focal embolic stroke of rats (Walvick et al., 2011). We extended these promising findings by asking whether the benefits of tPA plus rA2 combination therapy can be sustained for long-term neurological outcomes. We compared the effects of intravenous high-dose tPA alone (10 mg/kg) versus a combination of low-dose tPA (5 mg/kg) plus 10 mg/kg rA2 in a model of focal embolic cerebral ischemia in rats treated at 3 h after embolization. Compared with conventional high-dose tPA alone, the combination significantly decreased infarction (19.6% reduction, $P < 0.05$) and considerably improved neurological function ($P < 0.05$) at 1-month after stroke (Wang et al., 2014).

In the most recent experimental study, we asked whether the same dose regimen of the combination therapy is still more efficacious and safer when the treatment time window is delayed to 4 h after stroke (Jiang et al., 2015). Our experimental results showed the combination slightly reduced brain infarction compared to saline (9.2% reduction), and tPA (7.4% reduction) at 24 h after stroke, although the reductions did not reach statistical significance, whereas the combination significantly reduced (22.2% reduction, $P < 0.05$) the conventional tPA-elevated ICH transformation. At 7 days after stroke, the combination significantly diminished the conventional tPA alone-elevated iron deposition in peri-lesion areas (68.2% reduction, $P < 0.05$). At 1 month after stroke, the combination significantly improved sensorimotor function recovery ($P < 0.05$) accompanied by a higher microvessel density in the peri-infarct areas compared to rats treated with conventional tPA alone group ($P < 0.05$). Given at a 4-h delay time point after stroke, these experimental results suggest the low-dose tPA plus rA2 therapy combination provides a safer profile by lowering the risk of ICH transformation, accompanied by an improved neurological function recovery after stroke (Jiang et al., 2015). Clinically even within the 3-h time window, intravenous tPA only results in partial or complete reperfusion to about 50% stroke patients. Unfortunately, we are still unable to predict who are the tPA thrombolysis responders or non-responders before giving tPA to ischemic stroke patients. The 50% non-responders may face a higher risk of tPA thrombolytic therapy-associated ICH transformation, where the 3-month mortality was about 60% for ischemic stroke patients who had ICH after receiving intravenous tPA administration (Hacke et al., 2004). Therefore, the notably lower hemorrhagic transformation by the combination treatment might reduce mortality and improve long-term outcome clinically. Regarding the underlying molecular mechanisms of the better neurological function recovery by the delayed combination treatment, we might speculate that in addition to the reduction in tPA-associated ICH transformation, the lower tPA dose and resulting rA2–tPA complex might limit tPA brain penetration-associated neuronal excitotoxicity (Yepes et al., 2009), and that rA2 might bind and neutralize angiostatin-associated endothelial toxicity (Tuszynski et al., 2002), where angiostatin is one of the tPA–plasminogen converting products. Ultimately, the decreased hemorrhagic brain damage and fewer neurovascular side effects might translate into better vascular remodeling and improvement of long-term neurological outcome (Fan et al., 2010). We acknowledge that both tPA therapy-mediated hemorrhagic risk and functional recovery involves complex cascades of BBB, neurovascular and gliovascular responses (Jiang et al., 2015), and the full spectrum of these associated molecular mechanisms remains to be elucidated (Zhang and Chopp, 2009). Some of our ongoing experiments are aiming to address these questions.

Since annexin A2 accelerates the activation of plasmin by complexing with tPA and plasminogen, and this complex binds to the endothelial cell surface and is enriched in the clot (Sakharov and Rijken, 1995; Hajjar and Krishnan, 1999), the rA2–tPA combination may thus generate more plasmin locally at the clot site. Alternatively, within the thrombus, the fibrin-associated plasminogen could be activated by A2-associated tPA, resulting in more effective fibrinolysis. In addition, rA2-bound tPA and plasmin might be relatively protected from their circulating inhibitors, plasminogen activator inhibitor-1 (PAI-1), and alpha$_2$-antiplasmin (alpha$_2$-AP) (Fan et al., 2010). Although the underlying molecular mechanisms for the improved therapeutic efficacy by the combination needs to be further elucidated, from a clinical

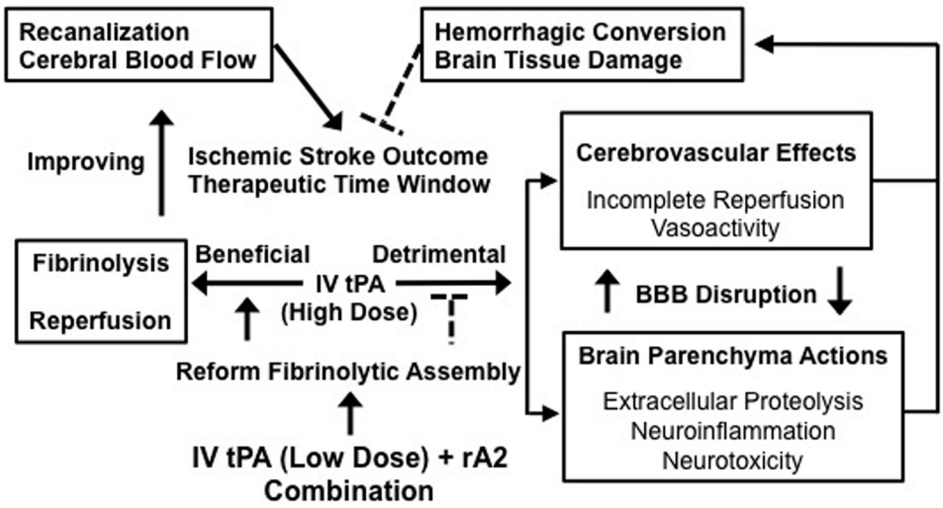

FIGURE 1 | Changing the balance of thrombolytic reperfusion benefits versus hemorrhagic side effects of exogenous tPA through the use of a low-dose tPA plus rA2 combination. A schematic outline for the potential mechanisms of the combination is to link multiple beneficial and detrimental effects of exogenous tPA acting intravascularly and extravascularly. By rebalancing the beneficial/detrimental effect ratio, our new combination approach may improve both therapeutic efficacy and safety of tPA-based thrombolytic stroke therapy.

perspective, all these possible mechanisms may ultimately yield a rebalancing of tPA thrombolytic reperfusion benefits against its detrimental side effects.

Summary

Our experimental investigations provide strong evidence in support of the hypothesis that the combination of low-dose tPA plus rA2 improves stroke thrombolytic therapy. By improving the fibrinolytic assembly to accelerate plasmin generation, thus enhancing thrombolytic reperfusion efficacy, the combination restores CBF, and rescues ischemic brain tissue more efficiently. This lowers the required dose of tPA, which minimizes both tPA-direct and -associated side effects of neurotoxicity, neuroinflammation, extracellular proteolytic dysfunction, and hemorrhagic conversion (Fan et al., 2010). Changing the rebalance by improving reperfusion benefits while reducing side effects through the combination may diminish the risk of ICH, prolong the therapeutic time window, and improve long-term outcome of ischemic stroke patients (**Figure 1**). We acknowledge that safety issues and all translational aspects of this potential treatment need to be carefully investigated for future preclinical evaluation.

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REVIEW
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Combined neurothrombectomy or thrombolysis with adjunctive delivery of 3K3A-activated protein C in acute ischemic stroke

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In the treatment of acute ischemic stroke (AIS), vessel recanalization correlates with improved functional status and reduced mortality. Mechanical neurothrombectomy achieves a higher likelihood of revascularization than intravenous thrombolysis (IVT), but there remains significant discrepancy between rates of recanalization and rates of favorable outcome. The poor neurological recovery among some stroke patients despite successful recanalization confirms the need for adjuvant therapy, such as pharmacological neuroprotection. Prior clinical trials of neuroprotectant drugs failed perhaps due to inability of the agent to reach the ischemic tissue beyond the occluded artery. A protocol that couples mechanical neurothrombectomy with concurrent delivery of a neuroprotectant overcomes this pitfall. Activated protein C (APC) exerts pleiotropic anti-inflammatory, anti-apoptotic, antithrombotic, cytoprotective, and neuroregenerative effects in stroke and appears a compelling candidate for this novel approach.

Keywords: activated protein C (APC), endovascular restorative neurosurgery, mechanical neurothrombectomy, neuroprotection, neurorestoration, stroke, thrombolysis

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Abbreviations: APC, Activated protein C; AIS, Acute ischemic stroke; BBB, Blood brain barrier; CNS, Central nervous system; ERN, Endovascular restorative neurosurgery; ESCAPE, Endovascular Treatment for Small Core and Anterior Circulation Proximal Occlusion with Emphasis on Minimizing CT to Recanalization Times; EXTEND IA, Extending the Time for Thrombolysis in Emergency Neurological Deficits—Intra-Arterial; FAST MAG, Field Administration; IMS, Interventional Management of Stroke; IAT, Intra-arterial therapy; IV, Intravenous; IVT, Intravenous thrombolysis; LVO, Large vessel occlusion; MMP, Matrix metalloproteinase; MERCI, Mechanical Embolus Removal in Cerebral Ischemia; MR RESCUE, Mechanical Retrieval and Recanalization of Stroke Clots Using Embolectomy; MCAO, Middle cerebral artery occlusion; mRS, Modified Rankin score; Multi-MERCI, Multi Mechanical Embolus Removal in Cerebral Ischemia; MR CLEAN, Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands; NINDS, National Institute of Neurological Disorders and Stroke; NIHSS, National Institutes of Health Stroke Scale; NR, Not reported; NF-κB, Nuclear factor kappa B; PROACT, Prolyse in Acute Cerebral Thromboembolism; PAR, Protease activated receptor; RCT, Randomized clinical trial; rpro-UK, Recombinant pro-urokinase; SWIFT, Solitaire With the Intention For Thrombectomy; SWIFT PRIME, Solitaire With the Intention For Thrombectomy as Primary Endovascular Treatment; STAR, Solitaire FR Thrombectomy for Acute Revascularization; SARIS, Stent assisted recanalization in acute ischemic stroke; STAIR, Stroke Therapy Academic Industry Roundtable; sICH, Symptomatic intracerebral hemorrhage; TICI, Thombolysis in cerebral infarction; TIMI, Thombolysis in myocardial infarction; tPA, Tissue plasminogen activator; FDA, United States Food and Drug Administration.

Stroke is the second leading cause of death worldwide and the number one cause of disability in the United States (Mozaffarian et al., 2015). Despite extensive research into the pathophysiology underlying acute ischemic stroke (AIS), intravenous tissue plasminogen activator (IV tPA) remains the only drug approved by the United States Food and Drug Administration (FDA) for its treatment. However, the time window for IV tPA eligibility is short, and there are contraindications for its use (Jauch et al., 2013). Furthermore, the (mis)perception of marginal utility, high risk of intracerebral bleeding, and/or high liability associated with its administration curtails the enthusiasm of many providers (SoRelle, 2013). As a result of these and other factors, only about 5% of AIS patients receive IV tPA (Jauch et al., 2013; Schwamm et al., 2013; Mozaffarian et al., 2015). Among those treated, rates of recanalization and good neurological outcome vary based on the site and size of the affected vessel, but can be as low as 5% for proximal occlusion of the internal carotid or basilar arteries (Bhatia et al., 2010). Clearly, a need exists for more effective reperfusion and neuroprotective strategies.

Mechanical neurothrombectomy has recently emerged as a promising approach to AIS therapy. The current generation of aspiration and stent retrieval devices achieves recanalization in the majority of patients (**Table 1**; Penumbra Pivotal Stroke Trial Investigators, 2009; Berkhemer et al., 2015; Nogueira et al., 2012; Saver et al., 2012, 2015a; Almekhlafi et al., 2014; Campbell et al., 2015; Goyal et al., 2015). Detailed analysis of adverse events and safety data confirms that neurothrombectomy procedures can be performed with minimal morbidity and mortality (Akins et al., 2014). Nonetheless, the likelihood of functional independence following neurothrombectomy (14–58%) remains poor compared with rates of recanalization (60–90%; **Table 1**).

The disparity between the rates of recanalization and rates of neurological recovery underscores the need for adjunctive therapy, such as pharmacological neuroprotection. Myriad preclinical studies and human trials with potential neuroprotective agents have been reported, yet none has proven unequivocally efficacious and none has achieved FDA approval (Ginsberg, 2009; Tymianski, 2013). Among the reasons cited for the failure of clinical trials in the face of encouraging animal data is the delayed time to administration, but even prehospital delivery of magnesium, given an average of just 45 min after symptom onset, failed to show benefit in a well-organized trial (Saver et al., 2015b). Another plausible explanation for the failed translation from bench to bedside is that the agent cannot reach the ischemic tissue due to lack of perfusion. When given systemically, neuroprotective agents must rely on collateral flow to ischemic tissue as they cannot traverse the occluded artery, but such collateral flow may be insufficient for adequate drug delivery. This provides impetus for a strategy coupling revascularization with the ancillary administration of a neuroprotective drug.

In this article, we review the foundation for an ongoing clinical trial coupling neurothrombectomy with adjunctive delivery of an activated protein C (APC) analog. APC confers pleiotropic benefits, such as stabilizing blood brain barrier (BBB) integrity, preventing thrombosis, enhancing fibrinolysis, promoting neuroprotection, attenuating inflammation, and facilitating neuroregeneration (Griffin et al., 2002, 2015; Zlokovic and Griffin, 2011). It represents a novel multiple-action multiple-target approach that ameliorates all facets of the pathogenic triad (consisting of vascular damage, neuronal injury, and neuroinflammation) that characterizes stroke and many other central nervous system (CNS) disorders (Zlokovic and Griffin, 2011). Since first report of the anti-inflammatory, cytoprotective, and antithrombotic properties of APC in stroke (Shibata et al., 2001), it has progressively fulfilled Stroke Therapy Academic Industry Roundtable (STAIR) criteria for drug development (Zlokovic and Griffin, 2011). The preclinical safety and pharmacokinetic profile of APC has been well characterized in mice and monkeys (Williams et al., 2012). A phase I safety study in normal human subjects has shown that high dose bolus regimens of modified APC are well-tolerated (Lyden et al., 2013), and a multicenter phase II dose-escalation clinical trial of intravenous administration for AIS (NCT02222714, NN104) is currently in progress (ZZ Biotech LLC, 2015).

Limitations of Pharmacologic Thrombolysis

Data from intravenous thrombolysis (IVT) trials serve as the benchmark against which recanalization therapies such as neurothrombectomy are measured. Several small randomized trials of IV tPA suggested its safety efficacy in AIS (Mori et al., 1992; Haley et al., 1993), but large, randomized trials of another thrombolytic drug, intravenous streptokinase, were stopped early because of unacceptable rates of symptomatic intracranial hemorrhage (sICH; Donnan et al., 1995; Hommel et al., 1995).

The seminal National Institute of Neurological Disorders and Stroke (1995) trial reported favorable results that formed the basis for FDA approval of IV tPA in AIS. In this study, subjects were randomly assigned to receive either 0.9 mg/kg IV tPA (maximum 90 mg) given within 3 h of symptom onset or placebo. Due to the Phase 2 design of this pilot study, there were numerous exclusion criteria such as systolic blood pressure above 185 mm Hg or diastolic blood pressure above 110 mm Hg, prior stroke or head trauma within 3 months, major surgery within 14 days, history of intracranial bleed, anticoagulant use, platelet counts below 100,000 mm^3, blood glucose concentration above 400 mg per deciliter, and others. The median National Institutes of Health Stroke Scale (NIHSS) score was 14 (range 1–37). There was a powerful and statistically significant benefit shown: at 3 months, the odds ratio for favorable outcome was 1.7 in the tPA group as compared with placebo. There was also a 12% absolute increase and 32% relative increase in the number of patients with minimal or no disability (Barthel Index 95–100) in the tPA group. Although there was no statistically significant difference between the group given tPA and that given placebo in the percentage of patients who showed a 4-point neurological improvement at 24 h (47 vs. 39% respectively, $p = 0.06$), using any other cut point yielded a highly significant benefit at 24 h (Haley et al., 1997). In addition, a long-term benefit 1 year later was observed for the tPA group using a global test statistic that represents a composite of other scales, including modified Rankin score

TABLE 1 | Summary of major clinical trials of revascularization therapies.

Trial name	Year published	Study type	Intra-arterial therapy	Adjunctive therapy	Recanalization (%)	mRS 0–2 (%)	sICH (%)	Mortality (%)	Comments
NINDS	1995	RCT	None	*IV tPA*	NR	39	6.4	17	Includes small vessel strokes and low NIHSS strokes
NINDS	1995	RCT	None	Placebo	NR	28	0.6	21	Includes small vessel strokes and low NIHSS strokes
PROACT I	1998	RCT	rpro-UK	IV heparin	57.7	30.8	15.4	26.9	Recanalization reported as TIMI 2 or more; outcome reported as mRS 0–1
PROACT I	1998	RCT	none	IV heparin	14.3	21.4	7.1	42.9	Recanalization reported as TIMI 2 or more; outcome reported as mRS 0–1
PROACT II	1999	RCT	rpro-UK	IV heparin	66	40	10	25	Recanalization reported as TIMI 2 or more
PROACT II	1999	RCT	none	IV heparin	18	25	2	27	Recanalization reported as TIMI 2 or more
IMS I	2004	Open label	tPA	IV tPA (low dose)	56	43	6.3	16	Recanalization reported as TIMI 2 or more
MERCI	2005	Single arm	Merci	None	46	27.7	7.8	43.5	Recanalization reported as TIMI 2 or more
IMS II	2007	Open label	tPA	IV tPA (low dose)	60	46	9.9	16	Recanalization reported as TIMI 2 or more
MULTI MERCI	2008	Single arm	Merci; +/– IA tPA	+/– IV tPA	68	36	9.8	34	Recanalization reported as TIMI 2 or more
Penumbra Pivotal	2009	Single arm	Penumbra	+/– IV tPA	81.6	25	11.2	32.8	Recanalization reported as TIMI 2 or more
SARIS	2009	Single arm	implanted stent	+/– IV tPA	100	60	5	25	Small series (n = 20)
SWIFT	2012	RCT	Solitaire	+/– IV tPA	61	58	2	17	Recanalization reported as TIMI 2 or more
SWIFT	2012	RCT	Merci	+/– IV tPA	24	33	11	34	Recanalization reported as TIMI 2 or more
Trevo 2	2012	RCT	Trevo	+/– IV tPA	86	40	7	33	Recanalization reported as TICI 2a or more
Trevo 2	2012	RCT	Merci	+/– IV tPA	60	21.8	9	24	Recanalization reported as TICI 2a or more

(Continued)

TABLE 1 | (Continued).

Trial name	Year published	Study type	Intra-arterial therapy	Adjunctive therapy	Recanalization (%)	mRS 0–2 (%)	sICH (%)	Mortality (%)	Comments
STAR	2013	Single arm	Solitaire	+/− IV tPA	79.2	57	1.5	6.9	
IMS III	2013	RCT	tPA and/or mechanical	IV tPA (variable dose)	38–44	40.8	6.2	19.1	Recanalization reported as TICI 2b or more
IMS III	2013	RCT	None	IV tPA	NR	38.7	8.9	21.6	
Synthesis	2013	RCT	tPA and/or mechanical	None	NR	42	6	8	
Synthesis	2013	RCT	None	IV tPA	NR	46	6	6	
MR Rescue	2013	RCT	IA tPA, Merci and/or Penumbra	+/− IV tPA	67	18.8	4.7	18.8	Recanalization reported as TICI 2a or more
MR Rescue	2013	RCT	None	+/− IV tPA	NR	20.4	3.7	24.1	
MR CLEAN	2014	RCT	Neurothrombectomy +/− IA tPA	+/− IV tPA	81.6	32.6	7.7	18.9	Recanalization reported as TICI 2a or more
MR CLEAN	2014	RCT	None	+/− IV tPA	NR	19.1	6.4	18.4	
ESCAPE	2015	RCT	Neurothrombectomy +/− IA tPA	+/− IV tPA	72.4	53	3.6	10.4	Recanalization reported as TICI 2b or more
ESCAPE	2015	RCT	None	+/− IV tPA	NR	29.3	2.7	19	
EXTEND IA	2015	RCT	Solitaire	IV tPA	100	71	0	9	Reperfusion assessed on CT imaging
EXTEND IA	2015	RCT	None	IV tPA	37	40	6	20	Reperfusion assessed on CT imaging
SWIFT PRIME	2015	RCT	Solitaire	IV tPA	88	61.2	1	9.2	Recanalization reported as TICI 2b or more
SWIFT PRIME	2015	RCT	None	IV tPA	NR	35.5	3.1	12.4	

The disparities between rates of recanalization and good clinical outcome are highlighted, emphasizing the need for adjunctive therapy such as infusion of 3K3A-APC. Modified after Jadhav and Jovin (2013) and other sources. NR, not reported; RCT, Randomized Clinical Trial. See text for details and accompanying list for other abbreviations.

(mRS). sICH within 36 h after stroke onset occurred in 6.4% of patients given tPA but only 0.6% of patients given placebo ($p < 0.001$). Mortality at 3 months was 17% in the tPA group and 21% in the placebo group ($p = 0.30$).

The benefit of IVT was confirmed in several subsequent, independent clinical trials (Hacke et al., 2008; Group et al., 2012). An ensuing meta-analysis of nine randomized trials confirms the robust benefit of IV tPA for AIS, with earlier administration associated with bigger proportional gain (Emberson et al., 2014). Among patients treated within 3 h, good outcome occurred in 259 (32.9%) of 787 patients given IV tPA vs. 176 (23.1%) of 762 who received control (OR 1.75, 95% CI 1.35–2.27). Treatment instituted after 3 h but before 4.5 h resulted in good outcome for 485 (35.3%) of 1375 patients vs. 432 (30.1%) of 1437 (OR 1.26, 95% CI 1.05–1.51). Delayed treatment beyond 4.5 h was not associated with statistically significant benefit.

In the NINDS trial, the size and location of the occluded vessel was not reported, as there was no time for vascular imaging. Stroke subtype (e.g., lacunar vs. large vessel) was determined using accepted clinical definitions. Similarly, there could be no data about recanalization. Subsequent studies have found that rates of recanalization with IV tPA alone can be as low as 5–10% for proximal large vessel occlusion (LVO), but much higher for more distal occlusions (Bhatia et al., 2010). Therefore, it is likely that the trials of IV tPA conducted without vessel imaging may have included patients with small vessel stroke and thus may overestimate any potential benefit to patients with LVO.

Due to these low rates of recanalization with LVO, various exclusion criteria, the short time window for benefit, the modest rates of good outcome, and other limitations of IV tPA, investigative efforts have focused on alternative revascularization strategies for LVO, including intra-arterial therapy (IAT). Such approaches include *in situ* delivery of thrombolytic drugs or other pharmacologic agents as well as neurothrombectomy. Currently, IAT is performed in patients who fail to recanalize after IV tPA or those who are ineligible for IV tPA on the basis of time or other exclusion criteria.

Local delivery of intra-arterial thrombolytics in animal models and small case series of human subjects suggested the safety and feasibility of this approach (Jadhav and Jovin, 2013), leading to subsequent randomized clinical trials (RCTs) such as the Prolyse in Acute Cerebral Thromboembolism (PROACT) I and II trials (del Zoppo et al., 1998; Furlan et al., 1999), which studied the intra-arterial delivery of recombinant pro-urokinase (rpro-UK) among patients with proximal middle cerebral artery occlusion (MCAOs: M1 or M2 segments) treated within 6 h of stroke onset. All patients also received adjunctive intravenous heparin, which likely contributed to rates of recanalization and/or reperfusion hemorrhage. Recanalization was assessed using the Thrombolysis in Myocardial Infarction (TIMI, 1985) score, which ranges from 0–3. Using an IAT dose of 6 mg rpro-UK and high vs. low doses of iv heparin, PROACT I proved superior rates of recanalization with thrombolytic (58%) than with control (15%; del Zoppo et al., 1998). Hemorrhagic transformation causing neurological deterioration within 24 h of treatment occurred in 15.4% of the rpro-UK group and 7.1% of the placebo group, but the difference was not statistically significant due to small sample size. Both recanalization and hemorrhage frequencies were influenced by heparin dose. Therefore, in an effort to increase recanalization while decreasing sICH, PROACT II used an IAT dose of 9 mg pro-UK and low dose IV heparin. This study also demonstrated superior rates of recanalization with thrombolytic (66%) than with control (18%; Furlan et al., 1999). Rates of functional independence (mRS 0–2) were also higher among the rpro-UK group (40%) than control (25%). Hemorrhagic transformation causing neurological deterioration within 24 h of treatment occurred in 10% of the rpro-UK group and 2% in the control group. Overall rates of hemorrhage, however, were as high as 68% at 10 days in the rpro-UK cohort, emphasizing that many hemorrhages within the infarcted territory do not produce incremental deficit. Ultimately, rpro-UK IAT was not pursued further for AIS.

Following on the promising results of PROACT, the IMS Study Investigators (2004) and IMS II Trial Investigators (2007) trials assessed a combined intravenous and intra-arterial approach to recanalization. In these prospective open label studies, reduced dose (0.6 mg/kg, 60 mg maximum) IV tPA was given within 3 h, followed by adjunctive IAT using up to 22 mg of tPA delivered at the site of occlusion. Compared with age- and severity-matched historical controls of IV tPA alone from the NINDS trial, the suggestion of improved outcomes with the combined IV and IA approach formed the rationale for IMS-III, a subsequent phase III RCT (Broderick et al., 2013).

Mechanical Neurothrombectomy: Lessons Learned

In 2004, the Merci device (Stryker Neurovascular, Kalamazoo, MI, USA) became the first mechanical clot retriever to receive FDA clearance for AIS. The Merci system consists of a nitinol wire with a helical terminus that is deployed distal to the occlusion and then withdrawn proximally after engaging thrombus within its corkscrew structure. A balloon integrated into the guiding catheter is inflated during this process in order to arrest anterograde flow, thereby mitigating against the dislodgement of distal emboli. The Mechanical Embolus Removal in Cerebral Ischemia (MERCI) trial was a single arm, prospective, multicenter study large vessel stroke treated within 8 h of symptom onset who were ineligible for IV tPA (Smith et al., 2005). Recanalization (TIMI 2–3) was achieved in 46% of patients. Favorable outcome at 90 days (mRS 0–2) occurred in 27.7% of subjects overall, but there was a significant difference between those with recanalization (46%) and those without (10.4%). Similarly, overall mortality at 90 days was 43.5% overall but was better for those with recanalization (31.8%) than those without (54.2%). sICH was observed in 7.8%.

The Multi-MERCI trial was also a single arm, prospective, multicenter study of AIS patients with LVO treated within 8 h of stroke onset (Smith et al., 2008). However, some patients also

received adjunctive IV tPA. In addition to the use of a newer generation Merci device, investigators could also perform salvage therapy with intra-arterial thrombolytic infusion. This approach increased the recanalization (TIMI 2–3) rate from 55% with the retriever alone to 68% overall. The rate of good outcome (mRS 0–2) at 90 days, however, remained disparately low at 36%. Mortality was 34% and sICH occurred in 9.8%.

In 2007, the Penumbra system (Penumbra Inc., Alameda, CA, USA) became the second FDA approved device for mechanical removal of thrombus in AIS. Unlike the Merci retriever, the Penumbra device does not require traversal of the clot and instead applies aspiration force to its proximal surface. Theoretically, this might reduce the incidence of distal emboli caused during the procedure and thus improve functional outcome. The Penumbra Pivotal Stroke Trial Investigators (2009) was a single-arm, prospective multicenter study of AIS patients with LVO who were either ineligible for or refractory to IV tPA. The device achieved an impressive revascularization rate of 81.6% using the thrombolysis in cerebral ischemia (TICI) scale, an analog to the TIMI rating (Higashida et al., 2003). However, only 25% had mRS 0–2 at 90 days, while another 33% died. The sICH rate was 11.2%.

Subsequent efforts at revascularization in AIS focused on stent technology, borrowing from the rich experience of these devices in the treatment of acute myocardial ischemia. Indeed, placement of implantable stents in AIS achieves high rates of recanalization, but it requires peri- and post-procedural antiplatelet therapy to prevent in-stent thrombosis, thus heightening the risk of reperfusion hemorrhage (Levy et al., 2009). In response, two new retrievable stents ("stentrievers") were developed to exploit their efficacy in revascularization while eliminating the need to implant them *in situ*. As these devices are deployed within the clot, their struts engage the thrombus. When the stentriever is withdrawn, the entrapped clot is extracted from the body.

The first of these devices to gain FDA approval in 2012 was the Solitaire stent (Medtronic Inc., Minneapolis, MN, USA). In a study that directly compared its performance with that of the Merci device, the Solitaire yielded superior rates of recanalization (61% vs. 24%), mRS 0–2 (58% vs. 33%), sICH (2% vs. 11%), and mortality (17% vs. 38%; Saver et al., 2012). Detailed analysis of adverse events and safety data showed that minimal morbidity and mortality were attributable to the neurothrombectomy procedure itself (Akins et al., 2014). A subsequent single-arm registry confirmed these favorable results (Almekhlafi et al., 2014). The Trevo stentriever (Stryker Inc., Kalamazoo, MI, USA) became the fourth (and currently last) device to gain FDA approval for mechanical clot removal in AIS. In a study that directly compared its performance with that of the Merci device (Nogueira et al., 2012), the Trevo also yielded superior rates of recanalization (86% vs. 60%), mRS 0–2 (40% vs. 21.8%), and sICH (7% vs. 9%), but not mortality (33% vs. 24%, NS).

Although these single arm studies or randomized comparisons of various neurothrombectomy devices suggested progress compared with historical controls from IV thrombolysis trials, direct comparisons were lacking, and it remained uncertain whether or not the higher rates of recanalization achieved with IAT would translate into improved clinical outcome. Three subsequent RCTs failed to support the superiority of IAT over IV tPA alone (Broderick et al., 2013; Ciccone et al., 2013; Kidwell et al., 2013). However, all of them were subject to methodological flaws that call their validity into question.

The SYNTHESIS trial randomly assigned AIS patients less than 4.5 h from symptom onset to endovascular therapy (intra-arterial thrombolysis with tPA, mechanical clot disruption or retrieval, or combinations thereof) or standard dose IV tPA (Ciccone et al., 2013). The rates of good outcome (mRS 0–2) were comparable between IAT and IVT (42% vs. 46%) as were rate of sICH (6% vs. 6%) and mortality (8% vs. 6%), respectively. Of note, vessel imaging and confirmation of LVO pre-enrollment were not reported, and almost half of the patients had NIHSS <11, making LVO less likely in those subjects. Similarly, the locations of vessel occlusion and rates of LVO or recanalization were also not reported. Few IAT patients actually received neurothrombectomy, and the newer generation devices were rarely employed. Furthermore, IAT was instituted 1 h later (3.75 vs. 2.75 h) than IVT on average. Lastly, the withholding of IV tPA in the IAT group represents a departure from real world practice and likely contributed to lower rates of good clinical outcome in this group.

The IMS III trial randomly assigned eligible patients who received IV tPA within 3 h of symptom onset to receive additional IAT or IVT alone (Broderick et al., 2013). The trial was terminated for reasons of futility when a prespecified stopping rule was triggered, after it was demonstrated that rates of mRS 0–2 at 90 days were similar in the IAT and IVT groups (40.8% vs. 38.7%, respectively). Rates of sICH (6.2% vs. 5.9%) and mortality at 90 days (19.1% vs. 21.6%) were also similar. As with the SYNTHESIS trial, however, IMS III suffers from several criticisms that call into question its relevance to contemporary practice. First of all, vessel imaging and confirmation of LVO were not required. In the IAT group, many received the lower dose of IV tPA as per the prior IMS trials (0.6 mg/kg, 60 mg max) while only some received full dose (0.9 mg/kg, 90 mg maximum). Since the trial was conducted over 6 years, the nature of IAT varied according to available technology but mostly consisted of intra-arterial thrombolysis (80%) followed by use of earlier generation neurothrombectomy devices such as Merci (28%) or Penumbra (16%), while only 1.5% of patients were treated with stentrievers. Thus, rates of reperfusion according to the standard metric of TICI 2b-3, ranged from 38–44% according to location. Lastly, as with SYNTHESIS, there was significant delay in the initiation of IAT of up to more than 1 h.

In retrospective analysis of the prior trials, outcomes were generally worse among patients with established infarction on initial imaging. The Mechanical Retrieval and Recanalization of Stroke Clots Using Embolectomy (MR RESCUE) sought to assess the benefit of IAT in the presence of a penumbra of vulnerable tissue surrounding a small core infarct, based on the premise that salvage of this area through reperfusion might improve clinical outcome (Kidwell et al., 2013). The

trial randomly assigned patients within 8 h of symptom onset to receive neurothrombectomy (with Merci or Penumbra) or standard care. Revascularization was achieved in 67% of the IAT group. However, at 3 months, rates of good outcome among the IAT and medical therapy groups were similarly poor (18.8% vs. 20.4% overall) whether or not a penumbral pattern was present (21% vs. 26%, 17% vs. 10%, respectively), as were those for sICH and death (**Table 1**). Criticisms of this trial include the long delay to initiation of IAT and the use of first generation neurothrombectomy devices.

Seeking to redress many of the problems of the trials showing no benefit of IAT over IVT alone, 4 RCTs were recently conducted, all proving the superiority of IAT when LVO is confirmed, newer generation devices are employed, appropriate adjunctive therapy is administered, and attention is focused on timely intervention (Berkhemer et al., 2015; Campbell et al., 2015; Goyal et al., 2015; Saver et al., 2015a).

The Multicenter Randomized Clinical Trial of Endovascular Treatment for AIS in the Netherlands (MR CLEAN) trial randomly assigned patients to intra-arterial treatment plus usual care or usual care alone (Berkhemer et al., 2015). All patients had proximal arterial occlusion in the anterior circulation confirmed by vessel imaging. Prior to enrollment, 89% of patients received IV tPA. Retrievable stents were used in 81.5% of patients assigned to interventional treatment, and TICI 2a or greater recanalization was achieved in 81.6% of patients. The rate of functional independence (mRS 0–2) with intervention (32.6%) was greater than that of control (19.1%) but was still comparatively low, reinforcing the unmet needs in AIS care. There were no significant differences in the occurrence of symptomatic intracerebral hemorrhage (7.7% vs. 6.4%) or 30-day morality (18.4% vs. 18.9%) in the interventional and control arms, respectively.

The Endovascular Treatment for Small Core and Anterior Circulation Proximal Occlusion with Emphasis on Minimizing CT to Recanalization Times (ESCAPE) trial compared IAT plus standard care vs. standard care alone in AIS patients with a small infarct core, proximal intracranial arterial occlusion, and moderate-to-good collateral circulation (Goyal et al., 2015). Importantly, patients up to 12 h after symptom onset were included. The study was terminated early due to the favorable rates of good outcome (mRS 0–2) in the interventional group (53%) vs. control (29.3%). Recanalization (TICI 2b or greater) occurred in 72.4%. sICH occurred in 3.6% of the intervention group and 2.7% of control, and mortality was reduced in the interventional group compared with control (10.4%, vs. 19.0%, respectively).

The Extending the Time for Thrombolysis in Emergency Neurological Deficits—Intra-Arterial (EXTEND-IA) trial studied AIS patients in whom CT perfusion confirmed the presence of salvageable brain and small infarct core (Campbell et al., 2015). All received standard dose IV tPA. Half were randomized to IVT alone while the other half was randomized to IAT using the Solitaire device. Reperfusion (assessed on CT imaging and thus on a different scale than TICI) was 100% in the Solitaire arm compared with 37% in the tPA-only group. Good functional outcome of mRS 0–2 was also more likely in the IAT group (71%) than the control (40%). Rates of sICH (0% vs. 6%) and mortality (9% vs. 20%) were also better for the IAT group.

The Solitaire With the Intention For Thrombectomy as Primary Endovascular Treatment (SWIFT PRIME) Trial is the most recently completed RCT of IAT vs. IVT alone for AIS (Saver et al., 2015a). All patients received full dose IV tPA. Using the Solitaire device, the IAT arm demonstrated an 88% rate of recanalization. Good functional outcome (mRS 0–2) occurred in 60.2% of the interventional group compared with 35.5% in the IV tPA arm. The rate of sICH (1%) in the Solitaire group was exceedingly low.

Collectively, these studies confirm that the newest generation of neurothrombectomy devices can achieve recanalization in the vast majority of patients. Even when neurothrombectomy is performed expeditiously among AIS patients with small infarct cores, however, the rate of good clinical outcomes is comparatively poor (**Table 1**). In real world settings outside the idealized circumstances of a clinical trial, clinical outcomes are likely to be even worse, thus reinforcing the need for adjunctive neuroprotective therapy, such as APC infusion.

Overview of Activated Protein C

APC is an endogenous serine protease that favorably regulates multiple pathways within different cell types comprising the neurovascular unit, including neurons, vascular cells (endothelium, pericytes, and vascular smooth muscle cells), and glia (astrocytes, microglia, and oligodendroglia), all of which contribute to disease initiation and/or progression in AIS (Zlokovic and Griffin, 2011). APC is generated as part of the physiologic protective response to cerebral ischemia (Griffin et al., 2002). Further evidence of the importance of APC in stroke comes from prospective observational data suggesting that circulating levels of its zymogen precursor, protein C, are inversely related to the incidence of ischemic stroke (Folsom et al., 1999).

Protein C is a plasma zymogen that is synthesized in the liver and proteolytically activated by thrombin when bound to the endothelial protein C receptor (EPCR). The EPCR mediates many subsequent actions of APC (Domotor et al., 2003; Zlokovic and Griffin, 2011; Griffin et al., 2015) including transport across the BBB into the extravascular tissue (Deane et al., 2009) and activation of several cell signaling pathways, which ultimately affects the expression of hundreds of proteins (Griffin et al., 2015).

Conversely, dissociation of APC from EPCR allows its interaction with circulating clotting factors. By cleaving activated cofactors Va and VIIIa to yield the inactivated factors Vi and VIIIi, APC exerts potent antithrombotic effects. Replacement of a cluster of positively charged residues with neutral amino acids on the top surface of the APC protease domain near the C-terminus generates variants with reduced anticoagulant activity, but preserves the cell-signaling actions mediated by its N-terminus. For instance, substitution of three consecutive lysine residues 191–193 by alanine (3K3A-APC) causes loss of >92% of anticoagulant activity in human plasma and in

monkeys compared with wild type APC (Zlokovic and Griffin, 2011; Williams et al., 2012). Such analogs are likely to prove favorable for therapeutic application in conditions where the cytoprotective properties are maintained, while the risk of serious bleeding is diminished (Williams et al., 2012).

EPCR-bound APC activates a family of G-protein coupled protease-activated receptors (PARs), including PAR1 (Domotor et al., 2003). The latter can also be activated by thrombin, but each ligand triggers divergent intracellular signaling cascades (biased agonism; Griffin et al., 2015). Whereas thrombin-mediated activation of PAR 1 leads to disruption of the BBB, vascular leakage, neurotoxicity, apoptosis, and neuroinflammation, APC-mediated activation of PAR1 produces the opposite effects. APC-induced biased signaling following PAR-1 activation is required for neuroprotective actions of APC (see **Figure 1**). These multiple downstream effects of APC have been reviewed elsewhere (Griffin et al., 2002, 2015; Zlokovic and Griffin, 2011) and are summarized below.

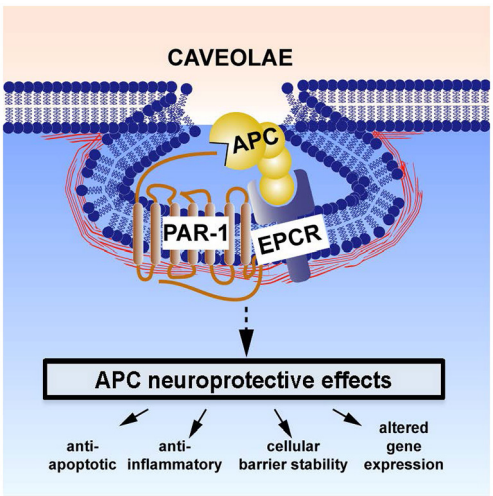

FIGURE 1 | Neuroprotective direct effects of APC on cells involves receptors endothelial protein C receptor (EPCR) and PAR-1. The cellular receptors EPCR and PAR1 are required for APC's beneficial effects on many types of brain cells. These activities include APC-mediated anti-apoptotic activities, anti-inflammatory activities, protection of endothelial barrier functions, and alterations of gene expression profiles. One or more of these activities plus other yet to be defined signaling actions are required for APC's multiple neuroprotective activities (Zlokovic and Griffin, 2011). This paradigm in which EPCR-bound APC activates PAR-1 to initiate biased signaling (Griffin et al., 2015) is supported by many *in vitro* and *in vivo* data (Zlokovic and Griffin, 2011; Griffin et al., 2015). Localization of APC signaling in the caveolin-1 rich microdomains (caveolae) may help differentiate mechanisms for cytoprotective APC signaling vs. proinflammatory thrombin signaling (Zlokovic and Griffin, 2011; Griffin et al., 2015). Additional mechanisms for APC effects on cells may also involve other receptors, such as PAR-3, sphingosine-1-phosphate receptor 1, apolipoprotein E Receptor 2, and/or Mac1 (CD11b/CD18). For example, the beneficial actions of APC or 3K3A-APC on middle cerebral artery occlusion (MCAO) injury required PAR-1, EPCR, and PAR-3 (Cheng et al., 2003, 2006; Guo et al., 2004, 2013; Thiyagarajan et al., 2008; Gorbacheva et al., 2010; Petraglia et al., 2010; Zlokovic and Griffin, 2011) and stimulation of development of neurons within human neuroprogenitor cell populations required PAR-1, PAR-3, and sphingosine-1-phosphate receptor 1 (Guo et al., 2013).

BBB Preservation

The vasculoprotective effects of APC include PAR1-mediated activation of sphingosine kinase-1 which leads to activation of Rac1, a member of the Rho family of GTPases, and the rearrangement of the endothelial cytoskeleton. By stabilizing BBB integrity and attenuating post-ischemic BBB breakdown, APC prevents secondary neuronal injury due to the accumulation of blood-derived neurotoxic and vasculotoxic molecules such as fibrin, hemosiderin, thrombin, and plasmin (Zlokovic and Griffin, 2011).

APC also downregulates nuclear translocation of nuclear factor kappa B (NF-κB), thus blocking NF-κB dependent transcriptional activation of matrix metalloproteinase-9 (MMP-9). This molecule and other proteinases are responsible for post-ischemic degradation of the basement membrane proteins of the BBB, leading to intracerebral hemorrhage, an effect that is potentiated by tPA. In murine ischemic stroke models of MCAO, APC reduced tPA-induced intracerebral hemorrhage through its actions on PAR1 and reduced MMP9 expression (Cheng et al., 2006).

Additionally, APC blocks apoptosis of endothelial cells by inhibiting mitochondria-mediated caspase-9-dependent pathways, upregulating anti-apoptotic genes, suppressing p53 pro-apoptotic pathways, and other mechanisms (Cheng et al., 2003). Lastly, APC stimulates angiogenesis from endothelial cells in both *in vivo* and *in vitro* models, leading to the formation of new capillaries that can provide the nourishment for an environment conducive to neuroregeneration (Zlokovic and Griffin, 2011).

Suppression of Neuroinflammation

APC inhibits transport of neutrophils and monocytes across the BBB, thereby blocking early post-ischemic infiltration of the brain by leukocytes. It also suppresses microglia activation. These effects are mediated by suppressing NF-κB dependent expression of proinflammatory cytokines such as tumor necrosis factor–α, interleukins, and vascular adhesion molecules (Zlokovic and Griffin, 2011).

Neuroprotection

APC and its analogs cross the BBB via EPCR-dependent transport to reach neuronal targets and exert direct neuronal protection (Deane et al., 2009), as demonstrated in murine N-Methyl-D-aspartate (NMDA) excitotoxic injury models *in vivo* and in cultured neurons *in vitro* (Guo et al., 2004; Gorbacheva et al., 2010). Furthermore, as it does in endothelial cells, APC inhibits the intrinsic, caspase-9 dependent and p-53 mediated proapoptotic pathways and upregulates anti-apoptotic genes in neurons, too (Guo et al., 2004). Importantly, the therapeutic window of APC for stroke is much wider than that for tPA. Preclinical models demonstrate that APC is neuroprotective even when first administered 12 h after permanent MCAO or 24 h after transient MCAO (Guo et al., 2004; Wang et al., 2009). One key element for APC's neuroprotection is its ability to signal activation of the Akt-survival pathways.

Neuroregeneration

In addition to its neuroprotective effects, APC has been shown to promote post-ischemic neurogenesis in the mouse brain and in human embryo-derived neuroprogenitor cell cultures where activation of the Akt pathway is required (Thiyagarajan et al., 2008; Guo et al., 2013). These effects involve increased proliferation of neuronal progenitor cells of the subventricular zone and increased migration of neuroblasts from this area towards the ischemic border. Similar effects on neurogenesis were shown when APC was used in preclinical models of traumatic brain injury (Petraglia et al., 2010).

Rationale for Combining Neurothrombectomy and Adjunctive APC

Untreated cerebral thrombosis is associated with high rates of morbidity and mortality. For instance, among patients with persistent proximal vessel in the anterior circulation, up to 80% die within 90 days of stroke onset or fail to regain functional independence (Goyal et al., 2015). Timely restoration of blood flow to the ischemic territory improves clinical outcome by salvaging the hypoperfused tissue at risk of infarction. Indeed, meta-analysis of several studies confirms the strong correlation between recanalization and outcome in AIS; the odds ratio of functional independence or death for those with recanalization compared to those without is 4.43 and 0.24, respectively (Rha and Saver, 2007). Similarly, for every 30 min delay in reperfusion, the likelihood of favorable outcome decreases by 10% (Khatri et al., 2009), as it is estimated that for each minute during acute stroke, 1.9 million neurons, 14 billion synapses, and 12 km (7.5 miles) of myelinated fibers are destroyed (Saver, 2006).

However, the premise that underlies this notion, the "recanalization hypothesis", has been repeatedly contested (von Kummer et al., 1995; Rha and Saver, 2007). Analysis of the reasons behind this challenge reinforces the benefit of a strategy combining neurothrombectomy with adjunctive delivery of APC (**Table 2**). Adding APC to neurothrombectomy should strengthen the biological relationship between recanalization and outcome.

Firstly, recanalization of upstream large arteries is not always tantamount to tissue reperfusion distal to the occlusion. Rethrombosis, migration of emboli, secondary thrombosis of downstream arteries, or microcirculatory occlusion may produce a no-reflow phenomenon despite proximal vessel opening (Bai and Lyden, 2015). The inherent antithrombotic activity of APC might mitigate the deleterious clotting that underlies no-reflow. Conversely, excessive anticoagulation might promote intracerebral bleeding. APC variants such as 3K3A-APC, which have reduced anticoagulant activity compared with wild type, might represent a favorable compromise between these prothrombotic and anticoagulant forces, but this remains to be proven in human subjects.

Secondly, restoration of flow to ischemic brain tissue may risk reperfusion injury, hemorrhagic transformation, or cerebral edema, which could also counteract the theoretical benefit of recanalization. By repairing the integrity of the damaged BBB within the ischemic tissue, adjunctive APC confers vasculoprotective benefits.

Next, some recanalization therapies such as tPA have intrinsic neuorotoxicity through induction of caspases and other proapoptotic pathways, as well as or through breakdown of the BBB leading to the toxic accumulation of serum proteins that effect secondary neuronal injury (del Zoppo, 1998; Liu et al., 2004; Zlokovic and Griffin, 2011). The neuroprotective actions of APC might overcome this damage.

Lastly, recanalization may occur too late to benefit ischemic tissue that has already progressed to infarction. The neurogenic and angiogenic properties of APC, confirmed in both *in vitro* and *in vivo* models, might contribute to functional recovery and improved clinical outcome in such scenarios.

Current Protocols and Future Directions

The "Safety Evaluation of 3K3A-APC in Ischemic Stroke (RHAPSODY)" trial (NCT02222714, NN104) is a multicenter, prospective, double-blinded, dose-escalation Phase 2 RCT. It intends to assess the safety, pharmacokinetics and efficacy of 3K3A-APC following treatment with tPA, mechanical neurothrombectomy, or both (for subjects undergoing neurothrombectomy, onset time to arterial puncture must be <6 h). Four different doses of 3K3A-APC are being tested to establish the maximum tolerated dose. Eligibility criteria include age 18–90 and NIHSS ≥ 5. The trial started in October 2014 and will enroll up to 100 subjects.

Prior studies in sepsis have shown that low-dose continuous infusion of APC is not optimally suited to harness its cell signaling actions and that bolus dosing more effectively promotes the receptor activation that leads to altered gene expression

TABLE 2 | Challenges to the "recanalization hypothesis" and rationale for adjunctive APC.

Recanalization problem	APC solution
No reflow phenomenon due to rethrombosis, migration of emboli, secondary thrombosis of downstream arteries, or microcirculatory occlusion	Antithrombotic activity
Reperfusion injury, hemorrhagic transformation, or cerebral edema,	Vasculoprotective effects on BBB integrity
Neurotoxicity	Neuroprotection
Recanalization occurs too late	Neurogenesis and Angiogenesis

The potential explanations for poor clinical outcome despite reopening of occluded vessels and the beneficial properties of APC that redress each of these pitfalls are summarized.

profiles and the salutary effects of BBB stabilization and of anti-apoptic and anti-inflammatory activities (Griffin et al., 2015). For this reason, the RHAPSODY protocol employs a regimen of intravenous APC bolus doses every 12 h, up to a total of five doses. The previous Phase 1 safety study in normal subjects confirmed that high dose bolus regimens using 3K3A-APC are safe (Lyden et al., 2013).

In the future, consideration can be given to direct intra-arterial administration of APC at the time of neurothrombectomy. When given through a microcatheter that has been navigated past the occlusion, intra-arterial injection can achieve better and more reliable delivery to the affected territory, even if the proximal vessel subsequently re-occludes and there is no recanalization. The intra-arterial delivery of APC after neurothrombectomy exemplifies the concept of endovascular restorative neurosurgery (ERN) that we previously advanced (Amar et al., 2003). Theoretic advantages of ERN with intra-arterial drug delivery include the possibility of widespread distribution, the capability to deliver large volumes and doses to target tissue relative to IV infusion, the ability to bypass the occlusive lesion and access territory not perfused by collateral flow, limited perturbation of neural tissue, and the feasibility of repeated administration (Amar et al., 2003). The relatively high dose of drugs like APC that can be delivered intra-arterially via ERN strategies support the biological rationale for this approach (Amar et al., 2003).

Conclusion

An approach that couples mechanical neurothrombectomy with adjunctive delivery of a multiple action—multiple target drug offers many advantages over conventional treatment of AIS. The anti-inflammatory, anti-apoptotic, neuroprotective, and neuroregenerative properties of APC make this agent a compelling candidate for this strategy. While current protocols employ intravenous delivery of this agent, future studies of intra-arterial delivery are warranted.

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Author Contributions

All authors (AA, JH, and BZ) fulfill the following criteria:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Final approval of the version to be published; and
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tissue plasminogen activator inhibits NMDA-receptor-mediated increases in calcium levels in cultured hippocampal neurons

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NMDA receptors (NMDARs) play a critical role in neurotransmission, acting as essential mediators of many forms of synaptic plasticity, and also modulating aspects of development, synaptic transmission and cell death. NMDAR-induced responses are dependent on a range of factors including subunit composition and receptor location. Tissue-type plasminogen activator (tPA) is a serine protease that has been reported to interact with NMDARs and modulate NMDAR activity. In this study we report that tPA inhibits NMDAR-mediated changes in intracellular calcium levels in cultures of primary hippocampal neurons stimulated by low (5 μM) but not high (50 μM) concentrations of NMDA. tPA also inhibited changes in calcium levels stimulated by presynaptic release of glutamate following treatment with bicucculine/4-aminopyridine (4-AP). Inhibition was dependent on the proteolytic activity of tPA but was unaffected by α_2-antiplasmin, an inhibitor of the tPA substrate plasmin, and receptor-associated protein (RAP), a pan-ligand blocker of the low-density lipoprotein receptor, two proteins previously reported to modulate NMDAR activity. These findings suggest that tPA can modulate changes in intracellular calcium levels in a subset of NMDARs expressed in cultured embryonic hippocampal neurons through a mechanism that involves the proteolytic activity of tPA and synaptic NMDARs.

Keywords: tissue plasminogen activator, NMDA receptor, calcium signaling, hippocampal neurons, synapse

Introduction

NMDA receptors (NMDARs) play an essential role in the regulation of synaptic strength in the brain (Lau and Zukin, 2007; Traynelis et al., 2010; Paoletti, 2011). These gated cation channels are activated by the excitatory neurotransmitter glutamate and are essential mediators of brain plasticity, impacting synaptic structure and function. NMDA receptor activation leads to rapid alterations in synaptic strength that contribute to long-term potentiation (LTP) and long-term depression as well as longer term changes that are important for maintaining neuronal network function. Stimulation of NMDARs leads to activation of calcium-dependent signaling pathways and changes in expression of plasticity-related genes. It is becoming increasingly clear that these signaling properties are dependent on receptor localization and subunit composition. NMDARs are mobile and move laterally between synaptic and extrasynaptic pools (Lau and Zukin, 2007; Bard and Groc, 2011). Synaptic and extrasynaptic receptors can stimulate different signaling

pathways resulting in different neuronal responses. These may be mediated by differences in NMDA receptor subunit composition at the different sites, enabling synaptic and extrasynaptic receptors to associate with different signaling molecules (Rao and Finkbeiner, 2007). NMDARs form tetrameric complexes. The subunit composition of these complexes is diverse and plastic, resulting in a large number of receptor subtypes that varies during development and at adult synapses (Paoletti, 2011).

Tissue-type plasminogen activator (tPA) is a member of the serine protease family most well known for its role in vascular thrombolysis where it activates the zymogen plasminogen to form plasmin, which degrades fibrin and remove blood clots (Cesarman-Maus and Hajjar, 2005). tPA has been identified in both the developing and adult nervous system where a number of distinct roles have been proposed (Sappino et al., 1993; Friedman and Seeds, 1994; Ware et al., 1995; Teesalu et al., 2004). tPA is released from neurons following membrane depolarization (Lochner et al., 2006) and regulates LTP and synaptic plasticity. A genetic deficiency of tPA or inhibition of tPA activity leads to a loss of LTP (Frey et al., 1996; Huang et al., 1996; Calabresi et al., 2000) while overexpression of tPA or addition of recombinant tPA leads to prolonged LTP (Baranes et al., 1998; Madani et al., 1999). These and other observations are consistent with roles for tPA in learning and memory (Centonze et al., 2002; Pawlak and Strickland, 2002; Fernández-Monreal et al., 2004b; Benchenane et al., 2007). Several mechanisms may underpin these effects. At the cellular level tPA has been linked to the formation of perforated synapses (Neuhoff et al., 1999) and presynaptic varicosities (Baranes et al., 1998) as well as changes in dendritic spines (Mataga et al., 2004; Pawlak et al., 2005). Mechanistically, the proposed roles for tPA in synaptic plasticity mainly focus on changes in the proteolytic microenvironment impacting the remodeling of extracellular matrix (Wu et al., 2000; Bukhari et al., 2011) and synaptic connectivity, including cleavage of neurotrophins (Pang et al., 2004; Barnes and Thomas, 2008) and neurotransmitter receptors (Samson et al., 2008a; Macrez et al., 2010; Ng et al., 2012).

In this study we have investigated the effects of tPA on NMDA-mediated changes in intracellular calcium levels using a primary embryonic rat hippocampal culture model (Banker and Cowan, 1977). Calcium flux was stimulated directly with varying concentrations of the glutamate receptor agonist NMDA. We also stimulated presynaptic release of glutamate using a γ-aminobutyric acid A ($GABA_A$) receptor antagonist and potassium channel blocker, 4-aminopyridine (4-AP; Hardingham et al., 2002). Possible roles for plasmin and low density lipoprotein receptor-related protein 1 (LRP-1), an endocytic and signaling receptor that interacts with tPA (Zhuo et al., 2000) were also examined.

Materials and Methods

Materials

Recombinant human tPA (Actilyse®) was a generous gift from Boehringer Ingelheim (Auckland, New Zealand) or purchased from Biopur (Reinach, Switzerland). For some experiments the excipients in Actilyse® tPA were removed by dialysis against HEPES (Samson et al., 2008b). NMDA, MK-801 and amino-5-phosphonovalerate (APV) were purchased from Sigma Aldrich (Auckland, New Zealand). Nimodipine, bicuculline and 4-AP were purchased from Tocris (MO, USA). 2,7-Bis-(4-aminobenzylidene)-cycloheptan-1-one dihydrochloride (tPA-STOP) was purchased from American Diagnostica (Greenwich, CT, USA). Human α_2-antiplasmin was purchased from MyBioSource (San Diego, CA, USA). Receptor-associated protein (RAP) was produced as a glutathione S-transferase fusion protein and purified by glutathione-affinity chromatography. The glutathione S-transferase tag was removed by thrombin cleavage prior to use. The active site mutant human tPA (S478A) was purchased from Molecular Innovations (MI, USA).

Primary Cell Culture

The use of animals in this research was approved by the University of Auckland Animal Ethics Committee. Primary hippocampal cultures were prepared from embryonic (E18) Wistar rats as described previously (Borges et al., 2010; Lee et al., 2015). Briefly, dissected hippocampi were dissociated using papain and plated at 20,000 cells/well in clear-bottom, black-walled 96-well amine plates (BD Biosciences, Auckland, New Zealand). Cultures were maintained for 14–17 days *in vitro*, in Neurobasal medium containing 1 × B27 and 1 × Glutamax (all from Invitrogen, Carlsbad, CA, USA), with half medium changes 24 h following plating and then at 7 day intervals.

Calcium Assays

All experiments were performed on cultures maintained for 14–17 days *in vitro*. Cells were loaded with Fluo-4 AM (Life Technologies) according to the manufacturer's instructions. Calcium responses were recorded on an Envision plate reader (Perkin Elmer, MA, USA) using the following settings: excitation filter, FITC 485 nm; emission filter, 520 nm. Each well was recorded individually, with 15 s of baseline recording, followed by injection of agonist and recording of the response for a further 45 s. Antagonists were added manually 5 min (tPA) or 15 min (MK-801, APV, nimodipine, tPA-STOP, α_2-antiplasmin, RAP), prior to recording. Raw fluorescence data were converted to $\Delta F/F_0$; where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max} - F_0$.

Statistics

The area under the curve (AUC) from 0 to 45 s was determined and used as the dependent variable for all statistical analyses. These data were first transformed using a square root transform to ensure that there was homogeneity of variance and an approximately normal distribution of residuals in the fitted statistical models. The data for all NMDA treatments were analyzed as a complete set. This data set contained 24 individual plates of cells, 121 treatment by plate biological replicates and 351 individual data points. The data for the bicuculline and 4-AP treatments were analyzed separately,

consisting of 4 plates of cells, 19 treatment by plate replicates and 53 individual data points. Statistical analysis was conducted in R 3.0.2 (R Development Core Team, 2013) using linear mixed models of the transformed data. Data were modeled with a one-factor randomized block design containing a fixed effect for treatment and each plate considered a block. This ensured that each experimental unit (block) was a replicate plate of cells, with the technical replicates within a plate functioning as sampling units. As the data contained a nested structure (technical replicates nested within biological replicates) and were not balanced (not all treatments were in all plates, differing number of technical replicates), the mixed models were fitted by restricted maximum likelihood (REML) instead of general linear model/ANOVA (Littell et al., 2002; Lazic, 2010). The statistical model equation (Littell et al., 2002) was $y_{ijk} = \mu + \alpha_i + b_j + (\alpha b)_{ij} + e_{ijk}$ where y_{ijk} is the transformed AUC measurement for the kth technical replicate for treatment i in plate j, $\mu + \alpha_i$ is the mean transformed AUC measurement for treatment i, b_j is the random effect associated with plate j, $(\alpha b)_{ij}$ is the plate by treatment random effect (biological replicates) and e_{ijk} is the random error associated with technical replicate k for treatment i in plate j. The R command *lmer* from the package *lme4* (Bates et al., 2013) was used to fit the linear mixed models using the following model specification: *transformed_AUC*$\sim$ *treatment* + (1|*plate_id*) + (1|*plate_id*:*treatment*). All statistical tests were planned comparisons between a treatment and the appropriate control and were calculated as contrasts from the fitted models with p-values obtained using degrees of freedom determined by the Kenward-Roger method using packages *lsmeans* (Lenth, 2013) and *pbkrtest* (Højsgaard, 2014). As these were decided *a priori* no correction for multiple tests was applied (Ruxton and Beauchamp, 2008). Data are plotted as backtransformed means and SEM.

Results

tPA Inhibits the Calcium Response of Hippocampal Neurons Activated with Low but not High Concentrations of NMDA

To study the effect of recombinant tPA on NMDA-mediated calcium flux, intracellular calcium levels were monitored in embryonic hippocampal neurons cultured between 14 and 17 DIV (days *in vitro*), using a Fluo-4-based calcium assay and a high speed fluorometric plate reader. Treatment with the NMDAR agonist NMDA alone (5–100 μM) resulted in a rapid and concentration-dependent increase in intracellular calcium levels (**Figures 1A,B**). The fluorescent profiles indicated differences in handling between lower (<10 μM) and higher (>10 μM) concentrations of NMDA. Both responses were characterized by a rapid influx of calcium (the amplitude of which was concentration-dependent) which in the former gradually returned towards baseline, but in the latter plateaued, resulting in sustained calcium levels. In some instances small oscillations were observed at the lower NMDA concentrations. To investigate the effect of tPA on the calcium response,

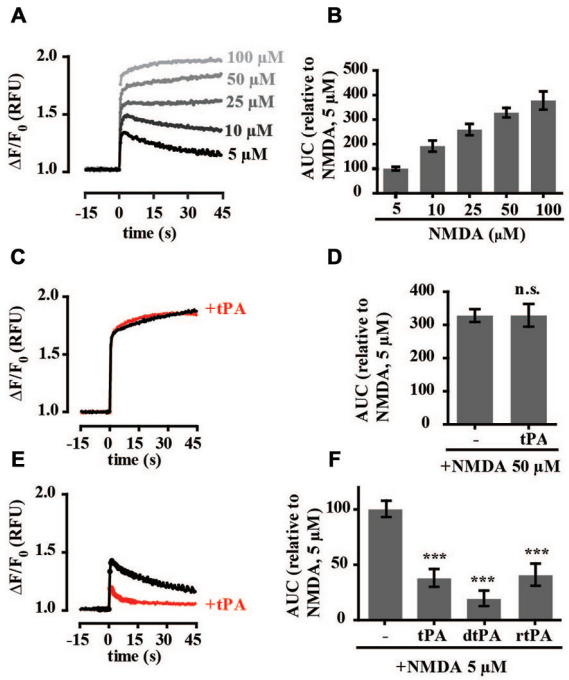

FIGURE 1 | Tissue-type plasminogen activator (tPA) inhibits increases in intracellular calcium in cultured rat hippocampal neurons stimulated with low (5 μM) but not high (50 μM) concentrations of NMDA. **(A)** Baseline Fluo-4 fluorescence was monitored in hippocampal cultures for 15 s prior to the addition of NMDA, at time = 0, to final concentrations ranging between 5 and 100 μM. Fluo-4 fluorescence was monitored for a further 45 s. Raw fluorescence values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max} - F_0$. **(B)** The responses in A were quantitated by measuring the area under the curve (AUC) and are presented relative to the AUC for 5 μM NMDA (100%). Results are from three independent experiments. Error bar, SEM. **(C,D)** Hippocampal cultures were preincubated with tPA (40 μg/ml) for 5 min and Fluo-4 fluorescence monitored before and after addition of NMDA to a final concentration of 50 μM. Data was collected as in A and quantitated as in B. n.s, not significant. **(E)** Hippocampal cultures were preincubated with tPA (40 μg/ml) for 5 min and Fluo-4 fluorescence monitored before and after addition of NMDA to a final concentration of 5 μM. **(F)** Quantitation of responses to tPA (Actilyse, sourced from Boehringer Ingelheim), rtPA (rtPA sourced from Biopur) and dtPA (dialysed Actilyse). RFU, Relative Fluorescent Units, ***$p < 0.001$. Error bar, SEM.

Fluo-4-loaded neurons were treated with tPA. Addition of tPA alone (40 μg/ml) did not produce any detectable change in Fluo-4 fluorescence (Supplementary Figure 1) and the calcium response to 50 μM NMDA was unaffected by pre-incubation with tPA (**Figures 1C,D**). However, the calcium response to 5 μM NMDA was significantly reduced to 38 ± 8% of control (**Figures 1E,F**). The tPA used in these experiments (Actilyse®) contains a number of excipients, including L-arginine, which has been suggested to be toxic to neurons (Oh et al., 2005; Samson et al., 2008b). To examine for any effects of the excipients in the Actilyse® tPA on the calcium response we repeated the experiments with a dialysed preparation of Actilyse® tPA (dtPA) and a second commercial recombinant tPA, Biopur tPA, which does not contain excipients. Both produced

similar inhibition of the 5 μM NMDA-mediated calcium response (**Figure 1F**), supporting the designation of tPA as the active agent. For all subsequent experiments Actilyse® tPA, which will be referred to from now simply as tPA, was used.

tPA Modulates Trans-Synaptic Stimulation of NMDA Receptors

To investigate the NMDAR responses in more detail we assessed the effects of glutamate and calcium channel antagonists on tPA-sensitive calcium flux. The selective competitive NMDAR antagonist APV (50 μM) and the use-dependent NMDAR open channel blocker MK-801 (10 μM) both significantly inhibited the calcium response of neurons treated with 50 μM NMDA while the L-type voltage-gated calcium channel blocker nimodipine (10 μM) had no significant effect on calcium levels (**Figures 2A–D**). The calcium response of hippocampal neurons to 5 μM NMDA was also blocked by APV and MK-801. However, in contrast to stimulation with 50 μM NMDA, nimodipine also inhibited the response (**Figures 2E–H**). These results are consistent with previously published data (Jensen and Wang, 1996; Soriano et al., 2006) and support trans-synaptic activation of the postsynaptic cell by glutamate at 5 μM NMDA, and direct stimulation of postsynaptic NMDARs at 50 μM NMDA. We further investigated an effect of tPA on the calcium response stimulated by presynaptic release of glutamate using the $GABA_A$ receptor antagonist bicuculline (50 μM) and the potassium channel blocker 4-AP (250 μM; Hardingham et al., 2001b). Treatment resulted in synchronous spontaneous calcium oscillations suggestive of synaptic coupling between neurons (**Figures 3A–C**). These oscillations were markedly reduced by MK-801 and nimodipine (**Figures 3A–C,D**). Importantly, the calcium oscillations were also markedly inhibited by tPA (**Figure 3D**).

The Proteolytic Activity of tPA is Required for its Inhibitory Effect on NMDA-Mediated Changes in Intracellular Calcium Levels

As proteolytic and non-proteolytic mechanisms have been reported to modulate NMDA-mediated calcium levels, we tested the effects of tPA-STOP, a reversible competitive inhibitor of trypsin-like serine proteases, as well as an enzymatically inactive tPA mutant. Unexpectedly, preincubation of cultures with 1 μM tPA-STOP alone almost completely inhibited intracellular calcium changes activated by 5 μM NMDA (Supplementary Figure 2). This result suggested an off-target effect of tPA-STOP and so it was not used in any further experiments. In a second approach we tested the effect of an enzymatically-inactive tPA mutant, with the active site Ser_{478} residue mutated to alanine. In contrast to tPA, the enzymatically-inactive tPA_{S478A} had no effect on 5 μM NMDA-mediated changes in calcium levels (**Figures 4A,B**).

Inhibition of NMDA-Induced Calcium Influx by tPA is Independent of Plasmin and LRP-1

To investigate if tPA's effects on intracellular calcium levels involved tPA-mediated activation of plasmin, cultures were preincubated with α_2-antiplasmin. α_2-antiplasmin (140 nM) alone did not affect 5 μM NMDA-induced calcium flux and did not block the effect on tPA on 5 μM NMDA-induced calcium flux (**Figures 5A,B**). This suggests that plasmin was not responsible for the observed tPA response. It does not rule out the possibility that tPA may be converting plasminogen to plasmin in our cultures, only that this conversion is not necessary for the observed effect of tPA on 5 μM NMDA-induced changes in calcium levels. As tPA has also been proposed to modulate NMDA-mediated calcium flux through interaction with LRP as a complex with a specific tPA inhibitor (Martin et al., 2008; Samson et al., 2008a) experiments were undertaken with the

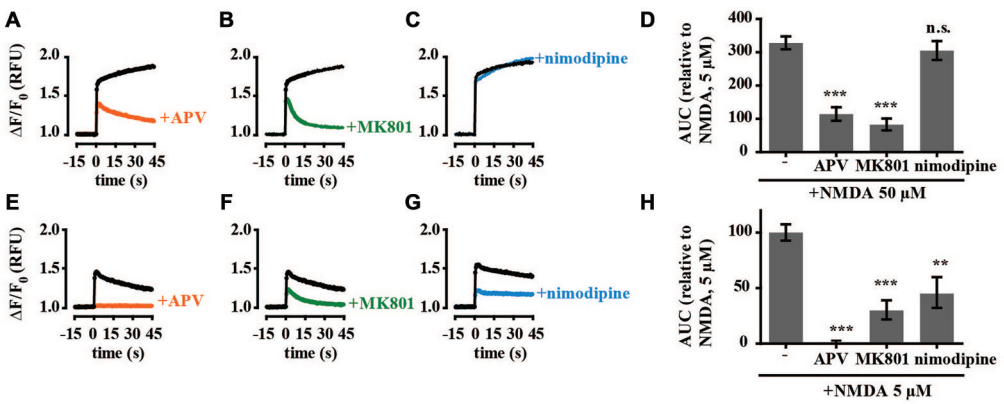

FIGURE 2 | Calcium responses of cultured hippocampal neurons to high (50 μM) and low (5 μM) concentrations of NMDA are pharmacologically distinct. Hippocampal cultures were preincubated with antagonists for 15 min before recording calcium responses. Baseline Fluo-4 fluorescence was monitored for 15 s prior to the addition of NMDA (**A–C**, 50 μM NMDA; **E–G**, 5 μM NMDA) at time = 0, then monitored for a further 45 s. Raw fluorescence values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max}-F_0$. Antagonists tested were amino-5-phosphonovalerate (APV) (**A,E**; 50 μM), MK-801 (**B,F**; 10 μM); nimodipine (**C,G**; 10 μM). **(D,H)** Responses were quantitated by measuring the AUC and are presented relative to the AUC for 5 μM NMDA (100%). RFU, Relative Fluorescent Units; $^{**}p < 0.005$, $^{***}p < 0.001$. Error bar, SEM.

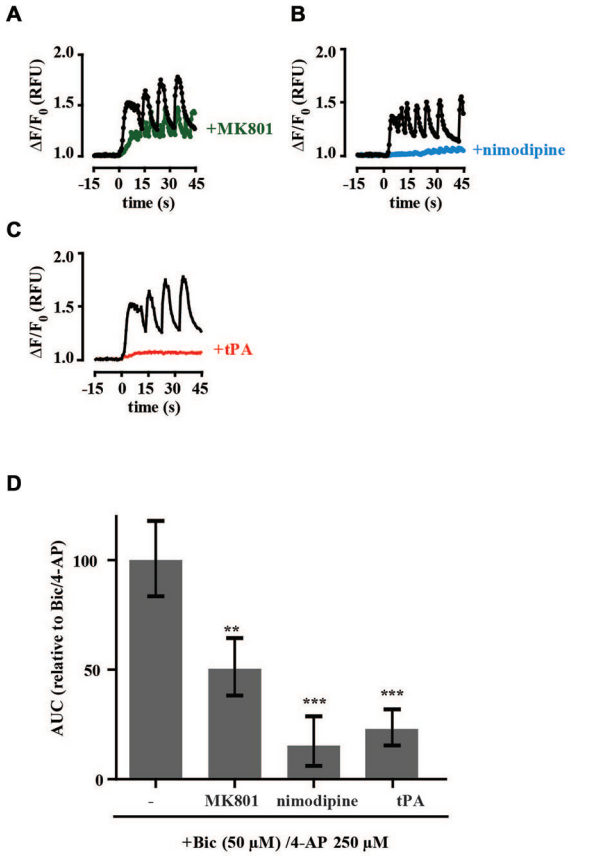

FIGURE 3 | tPA inhibits the calcium response of cultured hippocampal neurons stimulated by presynaptic release of glutamate. Hippocampal cultures were preincubated with antagonists (15 min) or tPA (5 min). Baseline Fluo-4 fluorescence was monitored for 15 s prior to the addition of bicucculine (50 μM) and 4-aminopyridine (4-AP, 250 μM) at time = 0. Fluorescence was monitored for a further 45 s and values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of baseline recording. Agents tested were **(A,B)** nimodipine (10 μM), **(C)** tPA (40 μg/ml). **(D)** Responses were quantitated by measuring the AUC and are presented relative to the AUC for 5 μM NMDA (100%). RFU, Relative Fluorescent Units; $^{**}p < 0.005$, $^{***}p < 0.001$. Error bar, SEM.

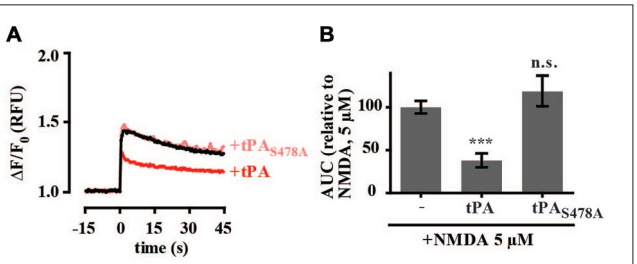

FIGURE 4 | The proteolytic activity of tPA is required for inhibition of NMDA-induced calcium influx. (A) Hippocampal cultures were preincubated with tPA or the enzymatically inactive tPA mutant tPA_{S478A} (40 μg/ml) for 5 min. Baseline Fluo-4 fluorescence was monitored for 15 s prior to the addition of NMDA (5 μM), at time = 0. Fluo-4 fluorescence was monitored for a further 45 s. Raw fluorescence values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max} - F_0$. **(B)** The responses in A were quantitated by measuring the AUC and are presented relative to the AUC for 5 μM NMDA (100%). RFU, Relative Fluorescent Units; n.s, not significant; $^{***}p < 0.001$. Error bar, SEM.

competitive LRP-1 receptor antagonist RAP. RAP alone had no effect on NMDA-mediated calcium flux (**Figures 5C,D**). RAP also failed to block the inhibitory effect of tPA on NMDA-mediated calcium flux (**Figures 5C,D**) suggesting that the tPA-mediated inhibition of 5 μM NMDA-induced calcium flux does not involve an interaction with LRP-1 or a similar RAP-sensitive receptor.

Discussion

In this study we have explored the effect of the proteolytic enzyme tPA on NMDA receptor-induced calcium flux in primary cultures of rat hippocampal neurons. Our results reveal that tPA's effects on NMDA-mediated changes in intracellular calcium levels vary with the concentration of NMDA. They support an inhibitory effect of tPA on calcium flux activated by low concentrations of NMDA or synaptic action potentials, through a mechanism that requires the enzymatic activity of tPA.

We found that pre-treatment of cultured hippocampal neurons with tPA had no effect on intracellular free calcium levels following stimulation with 50 μM NMDA. This relatively high concentration of NMDA has been reported to directly activate post-synaptic NMDARs (Bading et al., 1993; Hardingham et al., 2002). Our own results support this view with NMDA-mediated calcium flux activated by 50 μM NMDA inhibited by NMDA receptor antagonists but not by an antagonist of voltage-gated calcium channels. In contrast, tPA pre-treatment inhibited NMDA-stimulated calcium flux following activation of cultured hippocampal neurons with 5 μM NMDA. While all NMDARs are expected to be activated by this lower concentration of NMDA, the majority of calcium influx in hippocampal neuronal cultures has been attributed to an NMDA-induced increase in neuronal firing. Such action potential-induced intracellular calcium influx is mediated mainly by synaptic NMDARs (Hardingham et al., 2001a, 2002; Soriano et al., 2006). Our results support this view with significant inhibition of the calcium response following antagonism of L-type voltage-gated calcium channels, which have an established role in synaptically-stimulated calcium entry (Jensen and Wang, 1996; Wang and Gruenstein, 1997). We further investigated a role for tPA at synaptic NMDARs by examining its effect on synaptically-evoked bursts of action potentials. Hippocampal cultures typically contain approximately 10% inhibitory interneurons that tonically inhibit the neuronal network. We treated cultures with the $GABA_A$ receptor antagonist bicuculline to relieve this inhibition, leading to bursts of action potentials and activity-dependent calcium transients mediated largely by calcium influx through synaptic NMDA receptor (Hardingham et al., 2001b). Co-treatment with 4-AP, a weak potassium-channel blocker led to elevated calcium oscillations with "plateau-type" calcium signal (Hardingham et al., 2002). Pretreatment of these cultures with tPA resulted in

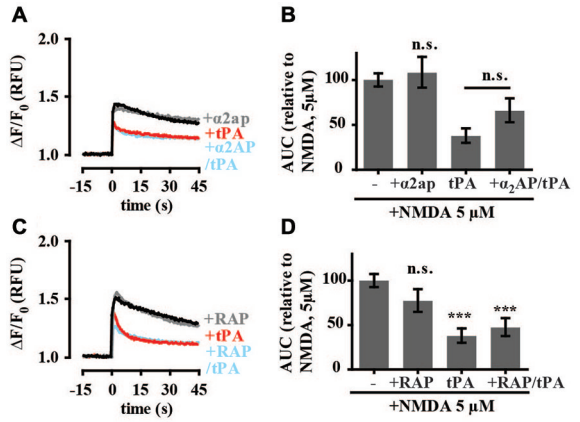

FIGURE 5 | tPA inhibition of NMDA-induced calcium influx is independent of plasmin and lipoprotein receptor-related protein 1 (LRP-1). (A) Hippocampal cultures were preincubated with tPA (40 μg/ml; for 5 min) or α_2-antiplasmin (140 nM; for 15 min) and tPA (for 5 min). Baseline Fluo-4 fluorescence was monitored for 15 s prior to the addition of NMDA (5 μM), at time = 0. Fluo-4 fluorescence was monitored for a further 45 s. Raw fluorescence values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max}-F_0$. **(B)** The responses in A were quantitated by measuring the AUC and are presented relative to the AUC for 5 μM NMDA (100%). **(C)** Hippocampal cultures were preincubated with tPA (40 μg/ml) for 5 min or receptor-associated protein (RAP) (500 nM; for 15 min) and tPA (for 5 min). Baseline Fluo-4 fluorescence was monitored for 15 s prior to the addition of NMDA (5 μM), at time = 0. Fluo-4 fluorescence was monitored for a further 45 s. Raw fluorescence values were converted to $\Delta F/F_0$, where F_0 is the average fluorescence over the first 15 s of recording prior to addition of agonist (baseline) and ΔF is $F_{max}-F_0$. **(D)** The responses in C were quantitated by measuring the AUC and are presented relative to the AUC for 5 μM NMDA (100%). RFU, Relative Fluorescent Units; n.s, not significant; ***$p < 0.001$. Error bar, SEM.

a marked decrease in calcium oscillations, further supporting an inhibitory effect of tPA on synaptic NMDARs.

To the best of our knowledge this is the first study to report an inhibitory effect of tPA on NMDA receptor-mediated calcium flux in neurons. Several other groups have investigated the effect of tPA on intracellular calcium levels (Nicole et al., 2001; Reddrop et al., 2005; Samson et al., 2008b). However in contrast to our results, these studies reported that tPA potentiated NMDA-mediated calcium levels. While these studies all involved analyses of mouse cortical cultures, either as a mixed population of neurons and glial cells (Nicole et al., 2001) or enriched in neuronal cells (Reddrop et al., 2005; Samson et al., 2008b), our inhibitory responses were seen in rat hippocampal cultures cultured under conditions to enrich for neuronal cells. Glial cells may influence tPA-mediated responses with tPA recently been described as a gliotransmitter (Cassé et al., 2012). Another obvious difference is the concentration of NMDA used across the different experiments. The tPA inhibitory effects we report were seen using 5 μM NMDA while the published studies used concentrations ranging between 25 μM and 100 μM. The higher concentrations of NMDA would be expected to result in cellular responses dominated by direct stimulation of NMDARs whereas the inhibitory effects we observe appear to be due to trans-synaptic stimulation of synaptic NMDARs. However, this suggestion alone does not explain why we do not see a potentiation of NMDA-mediated calcium levels at the higher NMDA concentrations. The differential effects of tPA on NMDA-mediated calcium flux could also involve differences in NMDA receptor subunit composition and differences in NMDAR responses reflecting association with regulatory proteins.

Our data support tPA inhibition of NMDA-mediated changes in calcium levels through a proteolytic mechanism. The changes could involve direct effects of tPA on the NMDA receptor to inhibit calcium entry but we cannot exclude changes in intracellular calcium also involving differential release of calcium from intracellular stores. Treatment of cultures with a catalytically inactive tPA mutant did not inhibit NMDA-mediated calcium flux. tPA has been proposed to cleave the GluN1 subunit of the NMDA receptor and potentiate NMDA-induced calcium influx (Nicole et al., 2001; Fernández-Monreal et al., 2004a; Reddrop et al., 2005; Benchenane et al., 2007). However, as all NMDARs contain the GluN1 subunit and as cleavage of this subunit potentiates NMDA-activated levels of intracellular calcium in neurons, it seems unlikely that the effects we are seeing are mediated through this subunit. Others have failed to detect cleavage of the GluN1 subunit of the NMDA receptor by tPA (Matys and Strickland, 2003; Kvajo et al., 2004; Liu et al., 2004). There is also evidence that plasmin can modulate NMDA receptor function directly (Samson et al., 2008a) or indirectly (Mannaioni et al., 2008). If plasmin were the functional unit in our assay conditions we would expect to see relief of the tPA-mediated inhibition of intracellular calcium levels when cultures were pre-incubated with the plasmin-specific inhibitor α_2-antiplasmin. While, no such change was observed (**Figures 4A,B**), these experiments do not completely exclude a direct role for plasmin. Future experiments should investigate the effects of exogenous plasmin on NMDA-mediated calcium levels.

tPA has also been shown to interact with the NMDA receptor via the low-density LRP1. Several studies have implicated LRP1 as the major tPA receptor in the brain facilitation subsequent downstream signaling (Zhuo et al., 2000) and engagement of the LDLR has been reported to be required for tPA to influence NMDAR function, including potentiation of NMDA-mediated calcium influx (Samson et al., 2008a). In these studies the calcium responses of primary cortical cultures to 25 μM NMDA were measured before and after a 5 min perfusion with tPA by video microscopy. tPA was found to enhance NMDA-mediated calcium influx and the effect was blocked by the LDLR pan-ligand blocker RAP. Rather than this response reflecting direct binding of tPA to the LRP receptor, the authors suggest a model where tPA initially interacts with a substrate, which they suggest is protease nexin-1 (PN-1), and a member of the serine protease inhibitor family, and it is this complex that interacts with the LDLR to activate NMDA signaling. We saw no significant effect of RAP alone on NMDA-mediated changes in calcium levels. Moreover, in our assay conditions, RAP did not block tPA's inhibitory effect. This may

suggest that LRP's main influence on NMDAR function, as previously reported, is through an interaction with extrasynaptic NMDARs.

Another potential target of tPA is GluN2D-containing NMDARs. tPA has been proposed to potentiate GluN2D-containing NMDA receptor-dependent activation in cortical neurons activated with 50 μM NMDA with receptor activation monitored by quantitation of ERK signaling or increased neuronal death (Baron et al., 2010; Jullienne et al., 2011; Parcq et al., 2012). Interestingly, tPA was unable to potentiate NMDA-mediated cell death in hippocampal cultures in this study, even though NR2D expression is seen in the CA2 region of the hippocampus. Investigations of tPA's effect on NMDA-mediated changes in intracellular calcium levels in cortical cultures should shed further light on the role of GluN2D-containing NMDARs and is an important area for future study. Another consideration is the molecular form of tPA. tPA is secreted as single-chain tPA but can then undergo cleavage into a two-chain form by plasmin or kallikrein (Rijken et al., 1982; Rajapakse et al., 2005). Parcq et al. (2012) recently reported that single-chain tPA selectively cleaves the NMDA receptor to promote NMDA-induced calcium influx in mouse embryonic cortical cultures with two-chain tPA having no effect. Our experiments used human recombinant tPA (Actilyse) which is 90–95% single-chain tPA and a recombinant human tPA supplied by Biopur that is also the single-chain form. They further support an important role for single-chain tPA as a modulator of NMDAR responses. Whether two-chain tPA is inactive in our model remains to be determined.

In conclusion, our study has found that tPA can inhibit NMDA receptor-mediated changes in intracellular calcium levels in cultured primary rat embryonic hippocampal neurons when NMDARs are activated with either low concentrations of NMDA or through activation of synaptic NMDARs by blocking $GABA_A$ receptor function. These effects require tPA to be proteolytically active and appear not to involve plasminogen as a substrate or LRP as part of a receptor-mediated mechanism. Our data provide additional evidence for the involvement of tPA in modulating NMDA receptor function and suggest a further level of complexity to the way that tPA may influence neuronal physiology and pathology. Further research is needed to determine which NMDA receptor subtypes are affected and how the effects relate to hippocampal synaptic plasticity *in vivo* (Liu et al., 2004).

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00404/abstract

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frontiers in Cellular Neuroscience

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Neuroserpin Differentiates Between Forms of Tissue Type Plasminogen Activator via pH Dependent Deacylation

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Tissue-type plasminogen activator (t-PA), initially characterized for its critical role in fibrinolysis, also has key functions in both physiologic and pathologic processes in the CNS. Neuroserpin (NSP) is a t-PA specific serine protease inhibitor (serpin) found almost exclusively in the CNS that regulates t-PA's proteolytic activity and protects against t-PA mediated seizure propagation and blood–brain barrier disruption. This report demonstrates that NSP inhibition of t-PA varies profoundly as a function of pH within the biologically relevant pH range for the CNS, and reflects the stability, rather than the formation of NSP: t-PA acyl-enzyme complexes. Moreover, NSP differentiates between the zymogen-like single chain form (single chain t-PA, sct-PA) and the mature protease form (two chain t-PA, tct-PA) of t-PA, demonstrating different pH profiles for protease inhibition, different pH ranges over which catalytic deacylation occurs, and different pH dependent profiles of deacylation rates for each form of t-PA. NSP's pH dependent inhibition of t-PA is not accounted for by differential acylation, and is specific for the NSP-t-PA serpin-protease pair. These results demonstrate a novel mechanism for the differential regulation of the two forms of t-PA in the CNS, and suggest a potential specific regulatory role for CNS pH in controlling t-PA proteolytic activity.

Keywords: neuroserpin, tissue plasminogen activator, serpin, serine protease, decacylation

INTRODUCTION

Tissue t-PA was initially described as an intravascular protease capable of initiating fibrinolysis by activation of plasminogen to plasmin (Collen, 1980). However, t-PA is also an important extravascular protease in the central nervous system (CNS). T-PA participates in axonal remodeling, neuronal plasticity, long-term potentiation (Seeds et al., 1995), propagation of excitotoxin-induced seizures (Qian et al., 1993; Tsirka et al., 1995; Endo et al., 1999; Wu et al., 2000; Yepes et al., 2002; Pawlak et al., 2005; Fredriksson et al., 2015), progression of cerebral infarct volume during ischemic stroke (Wang et al., 1998; Kano et al., 2000; Yepes et al., 2000), and regulation of blood brain barrier permeability in a number of pathologic conditions

Abbreviations: NSP, neuroserpin; PAI-1, plasminogen activator inhibitor type-1; sct-PA, single chain t-PA; t-PA, tissue-plasminogen activator; tct-PA, two-chain t-PA; u-PA, urokinase plasminogen activator.

(Yepes et al., 2003; Su et al., 2008; Fredriksson et al., 2015; Lewandowski et al., 2016). The molecular mechanisms for these physiologic processes include both proteolytic and non-enzymatic t-PA properties.

Similar to other serine proteases, t-PA is synthesized and secreted as a zymogen-like molecule, sct-PA. However, in contrast to other serine protease zymogens, sct-PA exhibits appreciable proteolytic activity, by some measures demonstrating 25% of the activity of the fully mature protease, two chain t-PA (tct-PA) (Boose et al., 1989; Tachias and Madison, 1997). The primary substrates of t-PA in the CNS are plasminogen and PDGF-CC (Su et al., 2008; Fredriksson et al., 2015). Studies have also suggested that the GluN1 subunit of the NMDA receptor can be cleaved by t-PA. However, the direct cleavage of the NMDA receptor by t-PA is controversial (Matys and Strickland, 2003; Vivien et al., 2003; Samson et al., 2008; Yuan et al., 2009). It has also been proposed that NMDA receptor mediated excitotoxicity is triggered by sct-PA, and not tct-PA (Parcq et al., 2012; Bertrand et al., 2015), identifying a potential proteolytic regulatory mechanism based on the state of the t-PA molecule.

The control of t-PA enzymatic activity is important for regulation of tPA protease-dependent processes. There are two primary inhibitors of t-PA in the CNS, PAI-1 (Colucci et al., 1986) and NSP (Osterwalder et al., 1996; Hastings et al., 1997; Krueger et al., 1997; Osterwalder et al., 1998; Fredriksson et al., 2015). Although the amount of PAI-1 in the CNS is small (Yamamoto et al., 1994; Fredriksson et al., 2015), the molecular chemistry by which it inhibits t-PA enzymatic activity is well characterized and is consistent with the current understanding of serine protease inhibition by a cognate serpin.

Plasminogen activator inhibitor type-1 functions via the established serpin mechanism, which involves presentation of a specific peptide bond within the serpin reactive center loop as a pseudosubstrate for the protease. The protease initiates cleavage as it would with a substrate, forming an acyl-enzyme intermediate with the P_1 residue of the serpin (Lawrence et al., 1995; Olson et al., 1995; Wilczynska et al., 1995). However, before the acyl-enzyme can undergo deacylation to complete cleavage of the scissile bond, the serpin undergoes structural re-arrangement, translocating the protease, still covalently linked to the P_1 residue, 70 Å to the opposite pole of the serpin (Stratikos and Gettins, 1997; Stratikos and Gettins, 1999; Huntington et al., 2000; Dementiev et al., 2006). Within the acyl-enzyme complex, the structure of the protease active site geometry is distorted such that catalytic deacylation can no longer take occur (Huntington et al., 2000; Dementiev et al., 2006). The rearranged acyl-enzyme complex exhibits novel molecular determinants that subsequently mediate specific cell-surface receptor mediated internalization and degradation of the protease-serpin complex (Horn et al., 1998; Stefansson et al., 1998). Hence, protease inhibition by a serpin results in clearance and degradation of both molecules, constituting effectively irreversible inhibition.

Our earlier studies noted a potential mechanistic difference between NSP and PAI-1 inhibition of tPA (Barker-Carlson et al., 2002). *In vitro* biochemical studies demonstrated only transient inhibition of t-PA by NSP due to increased efficiency of catalytic NSP-t-PA deacylation compared to PAI-1-t-PA acyl-enzyme complexes (Barker-Carlson et al., 2002). The shorter period of stable t-PA inhibition by NSP appears to be biologically meaningful however, as NSP interaction with t-PA *in vivo* has been identified as a significant negative-regulator of t-PA mediated effects on ischemic stroke (Yepes et al., 2000), seizure propagation (Yepes et al., 2002) and seizure induced blood brain barrier dysregulation (Fredriksson et al., 2015). Interestingly, in both the above noted pathologic states, ischemic stroke and seizure, the pH of the cerebral spinal fluid decreases to levels that affect the function of human serine proteases (Siesjo, 1985; von Hanwehr et al., 1986).

To determine whether the observed differences in NSP inhibition of t-PA between the *in vitro* and *in vivo* systems might reflect an effect of pH (regulated at 7.2–7.4 *in vitro*, and decreased to <6.8 *in vivo*) we investigated whether the pH in which NSP-tPA interactions were studied might be a differentiating factor in these assay systems. The data presented in this paper demonstrate that although NSP mediated inhibition of t-PA is less stable than PAI-1 inhibition of t-PA at physiologically neutral pH, NSP mediated inhibition is modulated by biologically relevant changes in pH, which result in changes in the rate of catalytic deacylation of NSP-t-PA complexes. Moreover, pH differences also allow NSP to differentiate between sct-PA and tct-PA. This leads us to hypothesize that variations in pH may play a heretofore unrecognized regulatory role in CNS processes that involve t-PA.

MATERIALS AND METHODS

Proteins and Reagents

Tissue-t-PA was purchased from Calbiochem (Leola, CA, USA) and Biopool (Sweden) (>95%, and >99% sct-PA, respectively, as assessed by SDS-PAGE under reducing conditions followed by silver staining); tct-PA was generated by treatment of sct-PA with plasmin-linked sepharose for a time determined to yield complete conversion of sct-PA to tct-PA as assessed by SDS-PAGE as above (Schwartz and Espana, 1999). Plasmin, spectrozyme t-PA, and traysolol were purchased from American Diagnostica (Greenwich, CT, USA), and two-chain urokinase (tcu-PA) was obtained from Dr. Gene Murano (Monsanto, St. Louis, USA). Cell culture grade bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Recombinant human PAI-1 (14-1b stabilized mutant) was produced in a bacterial expression system as previously described (Berkenpas et al., 1995). Polyclonal rabbit antibody against PAI-1 was a generous gift of Dr. Peter Andreasen (Aarhus University, Denmark) (Zeheb et al., 1987). Polyclonal antibody against NSP was generated in rabbits (Hastings et al., 1997) and HRP-conjugated goat antibody against rabbit IgG was purchased from Pierce (Rockford, IL, USA) and Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Human NSP cDNA was obtained from Human Genome Sciences (Rockville, MD, USA) and NSP was expressed in a bacculovirus system (Hastings et al., 1997).

Electrophoresis and Western Blot Analysis

Both the mini-protean three apparatus used for electrophoresis and protein transfer, as well as electrophoresis reagents were from BioRad (Hercules, CA, USA). Polyacrylamide gels were cast with 3% stacking and 10% separating gels and Western blotting for NSP or PAI-1 was carried out as previously described (Barker-Carlson et al., 2002; Li et al., 2008). Analysis of immunoblots using known amounts of NSP that was intact, cleaved, or in complex with sct-PA demonstrated no detectable difference in epitope detection among the states of NSP (Barker-Carlson et al., 2002). As noted in our previous paper (Barker-Carlson et al., 2002) these reagents were validating as demonstrating no detectable difference in epitope detection among the states of NSP. This information is included in the "Methods" section. A standard curve for NSP-sc-tPA complex detection using this methodology is also included in Supplemental Figures S1A,B. Kodak 1D Software was used to image all films, and Prism 3.0 or 4.0 software were used for data analysis as indicated.

Assessment of Serpin-PA Complex Stability as a Function of pH

Seventy nanometer NSP, or 140 nM PAI-1 was incubated at 37°C for 5 and 15 min, respectively, with equimolar amounts of the indicated forms of t-PA, or tcu-PA in 0.1 M NaCl, 0.001 M sodium phosphate, pH 7.2, and 100 μg/mL BSA. Reactions with NSP included 0.1% Triton X-100, and those with PAI-1 included 1,000 U/mL trasylol. Preliminary experiments demonstrated that Triton X-100 had no influence on the function of NSP. Reactions were then diluted fivefold into a series of 0.1 M sodium phosphate buffers at the indicated pH values, from 6.0 to 8.0, and incubated for 30 min (NSP) or 90 min (PAI-1) at 37°C. Reactions were quenched by addition of SDS-sample buffer, boiled and subjected to SDS-PAGE under reducing conditions, followed by Western blot analysis for NSP or PAI-1. The resulting immunoblot images were analyzed to quantify remaining acyl-enzyme serpin-protease complexes as a fraction of the complexes at time zero.

Effect of pH on Serpin Inhibition of t-PA Activity

One hundred and thirty-seven nanometer sct-PA or tct-PA was pre-incubated with 302 nM NSP or 200 nM PAI-1 for 5 (sct-PA) or 2 min (tct-PA), at 37°C in 0.15 M NaCl, 0.001 M Tris, pH 7.2 with 100 μg/mL BSA. Reactions were then diluted fivefold into buffers containing 0.05 M NaCl, 0.1 M Tris, 100 μg/mL BSA at the pH's indicated, and incubated a further 50 min (sct-PA) or (tct-PA), or 30 min at 37°C. Reactions with NSP contained 0.1% Triton X-100. Residual t-PA activity was determined by adding spectrozyme t-PA at a final concentration of 1.5 mM using the kinetic analysis module in a Beckman DU-640 spectrophotometer. Inhibition of t-PA enzymatic activity was calculated as shown in Equation 1.

Equation 1:

$$100 - 100(\text{t-PA} - \text{serpin}_{\text{pHx}}/\text{t-PA}_{\text{pHx}}) \;=\; \%\text{t-PA inhibition}$$

Where t-PA-serpin$_{pHx}$ represents the rate of chromogenic substrate cleavage in reactions containing t-PA and the indicated serpin at a specific pH between 6.0 and 8.0, and t-PA$_{pHx}$ represents the rate of chromogenic substrate cleavage by t-PA at the same pH in the absence of serpin.

Neuroserpin-t-PA (either form of t-PA) acyl-enzyme complexes demonstrated no catalytic deacylation at pH 6.0, thus inhibition of t-PA activity at pH 6.0 was designated 100%, and inhibition at other pH's was expressed relative to that at pH 6.0, as shown in Equation 2.

Equation 2:

$$\%\,\text{t-PAinhibition}_{\text{pHx}}/\%\,\text{t-PAinhibition}_{\text{pH6.0}} \;=\; \text{t-PA inhibition relative to that at pH 6.0}$$

Where % t-PA inhibition pH_x represents the percent inhibition of t-PA at the indicated pH, and % t-PA inhibition $_{pH6.0}$ is the percent inhibition at pH 6.0, both determined as in Equation 1. The pH profile for the relative inhibition of t-PA by NSP was generated and fit to a sigmoidal curve with variable slope non-linear regression using Prism 4.0 software.

Effect of pH on the Rate of NSP-t-PA Acyl-Enzyme Complex Deacylation

Formation of acyl-enzyme complexes between each form of t-PA and NSP progresses more rapidly than does deacylation (Barker-Carlson et al., 2002). Preliminary experiments defined conditions wherein NSP-t-PA acyl-enzyme complex formation had gone to completion, and therefore the decrement in NSP within those complexes over time could be quantified. Equimolar concentrations (70 nM) of NSP and sct-PA, or tct-PA were incubated for 10 or 2 min, respectively, at 37°C in 0.1 M NaCl, 0.001 M sodium phosphate, pH 7.2, with 100 μg/mL BSA and 0.1% Triton X-100. Reactions were then diluted fivefold (final concentration of t-PA and NSP, 14 mM) into 0.1 M sodium phosphate buffer, at the indicated pH between 6.0 and 7.6, containing 100 μg/mL BSA and 0.1% Triton X-100, and incubated at 37°C. At sequential times, aliquots were removed and quenched by addition of SDS-sample buffer, boiled, and analyzed by SDS-PAGE under reducing conditions, followed by Western blot analysis of NSP antigen. The deacylation rate constant at each pH was determined by fitting the data for the decay of NSP-t-PA acyl-enzyme complexes, which was linear with respect to time at each pH tested, to the equation for unimolecular decay (Equation 3).

Equation 3:

$$\ln[\text{NSP-t-PA}]_t \;=\; \ln[\text{NSP-t-PA}]_o - k_3 t$$

Where $[NSP\text{-}t\text{-}PA]_t$ is the concentration of NSP-t-PA acyl-enzyme complexes at time t, $[NSP\text{-}t\text{-}PA]_o$ is the initial concentration of complexes, and k_3 is the deacylation rate constant (Schechter et al., 1997; Plotnick et al., 2002a,b; Schechter and Plotnick, 2004).

RESULTS

Previous work from our labs revealed that the interaction between t-PA and NSP did not form the prototypically stable serpin-protease acyl-enzyme complexes as seen with t-PA and its other cognate serpin, PAI-1. Rather, formation of acyl-enzyme complexes between t-PA and NSP occurred readily, but was followed by rapid deacylation yielding cleaved serpin and active enzyme (Barker-Carlson et al., 2002). In contrast to these unstable *in vitro* NSP-t-PA complexes, administration of NSP to animals undergoing experimental stroke or seizure yielded evidence of t-PA inhibition (Osterwalder et al., 1998; Yepes et al., 2000, 2002). Unlike *in vitro* biochemical experiments which are generally performed at pH 7.2–7.4, the CNS is relatively poorly buffered and its pH drops to as low as 6.0 during pathologies wherein t-PA plays a role (Meldrum and Brierley, 1973; Meldrum and Horton, 1973; Aminoff and Simon, 1980; Siesjo, 1985, 1986, 1992; von Hanwehr et al., 1986; Bereczki and Csiba, 1993; Meric et al., 1994). We hypothesized that the rapid drop in CNS pH documented to occur following the onset of stroke or seizure might influence the stability of NSP-t-PA acyl-enzyme complexes, and the accompanying inhibition of t-PA enzymatic activity.

To test this hypothesis, NSP was allowed to complex with either sct-PA or tct-PA at pH 7.2, aliquots were then brought to different pH's across the range of 6.0–8.0 and incubated a further 30 min at 37°C. The reactions were then analyzed by SDS-PAGE and Western blotting for NSP antigen, and the amount of NSP that remained in complex was compared to the amount initially present in acyl-enzyme complex. **Figure 1** demonstrates several findings. First, the effect

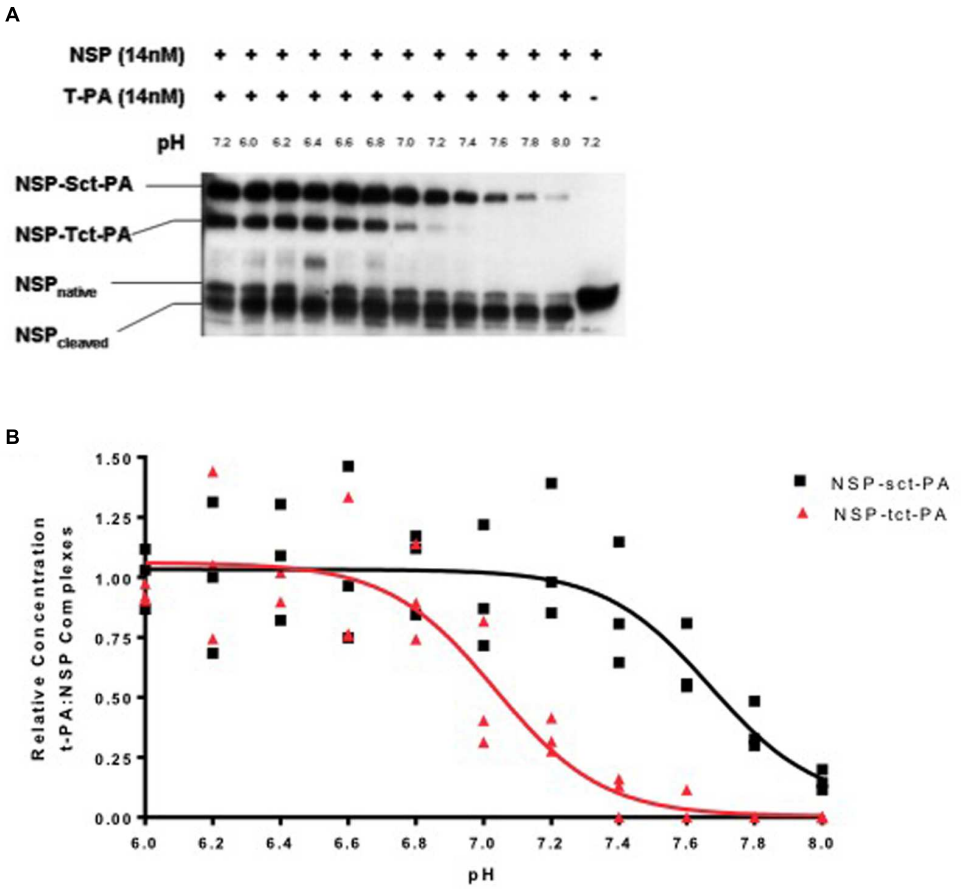

FIGURE 1 | Stability of t-PA-NSP acyl-enzyme complexes is pH dependent. (A) Following initial formation of NSP t-PA complexes with NSP at pH 7.2, reactions were diluted into buffers between 6.0 and 8.0 and incubated at 37°C for an additional 30 min as detailed in Methods, then analyzed by SDS-PAGE and Western blotting for NSP. The far left lane illustrates the quantity of NSP-t-PA complexes present at the beginning of the 30-min incubation (maximal complex). The far right lane contains NSP that was not reacted with t-PA. The remaining lanes show the reaction products at the end of the 30-min incubation at each noted pH. The identities of the individual bands are indicated on the left side of the gel. For ease of comparison, reactions with sct-PA and tct-PA were run in the same wells, since the NSP-t-PA acyl-enzyme complexes involving each form of t-PA are easily resolved. Identical results were obtained when reactions between NSP and each form of NSP were analyzed separately. **(B)** The ratio of t-PA-NSP complex remaining at the end of the 30-min incubation at the indicated pH, to the amount of complex present immediately before dilution into various pH buffers was graphed as a function of the pH into which the reactions were diluted. Triangles: NSP-tct-PA complexes; Squares: NSP-sct-PA complexes. Data are presented as points that represent the individual values, from 3 independent experiments, and lines that represent the means of those values.

of pH on acyl-enzyme stability is clearly different for NSP in complex with sct-PA compared to NSP in complex with tct-PA. Second, the difference in acyl-enzyme stability between NSP in complex with the two forms of t-PA is greatest at physiologic pH, 7.4. Third, although acyl-enzyme complexes between NSP and tct-PA are unstable at pH 7.4 suggesting tct-PA is free to exert proteolytic activity at physiologic pH, slight acidic shifts result in significant stabilization of these complexes.

The loss of acyl-enzyme complexes does indeed signify catalytic deacylation, as the appearance of cleaved NSP accounts for the reduction in acyl-enzyme-complex with t-PA (**Figure 1A**), and the pH range in which this occurs is not compatible with hydroxide mediated deacylation (Calugaru et al., 2001). These findings are consistent with the mechanism for catalytic deacylation of an acyl-enzyme intermediate of a serine proteinase, which requires that the active site histidine within the catalytic triad accept a proton (Plotnick et al., 2002a). At lower pH values, the histidine is likely already protonated, and thus catalytic deacylation cannot proceed. These data are also consistent with previous observations that at pH 7.2, deacylation readily occurs (Barker-Carlson et al., 2002).

These effects do not seem to be general characteristics of t-PA-serpin interactions, as PAI-1-t-PA acyl-enzyme complexes are stable across the pH range in which NSP-t-PA complexes undergo catalytic deacylation, and no differential stability between PAI-1-sct-PA complexes and PAI-1-tct-PA complexes was evident (**Figures 2A,B**). In addition, this pattern of acyl-enzyme instability does not characterize NSP interactions with other proteases closely related to t-PA. Under no circumstances tested were acyl-enzyme complexes between plasmin and NSP detected, but instead NSP acted as a pure plasmin substrate across the pH range tested (data not shown). Two-chain u-PA cleaved NSP with no detectable acyl-enzyme intermediate at physiologic pH, essentially treating the serpin as a substrate. However, rapid adjustment of conditions to very acidic pH allowed detection of stable tcu-PA-NSP acyl-enzyme complexes (**Figure 2C**). This is consistent with the pattern observed for non-cognate protease-serpin pairs

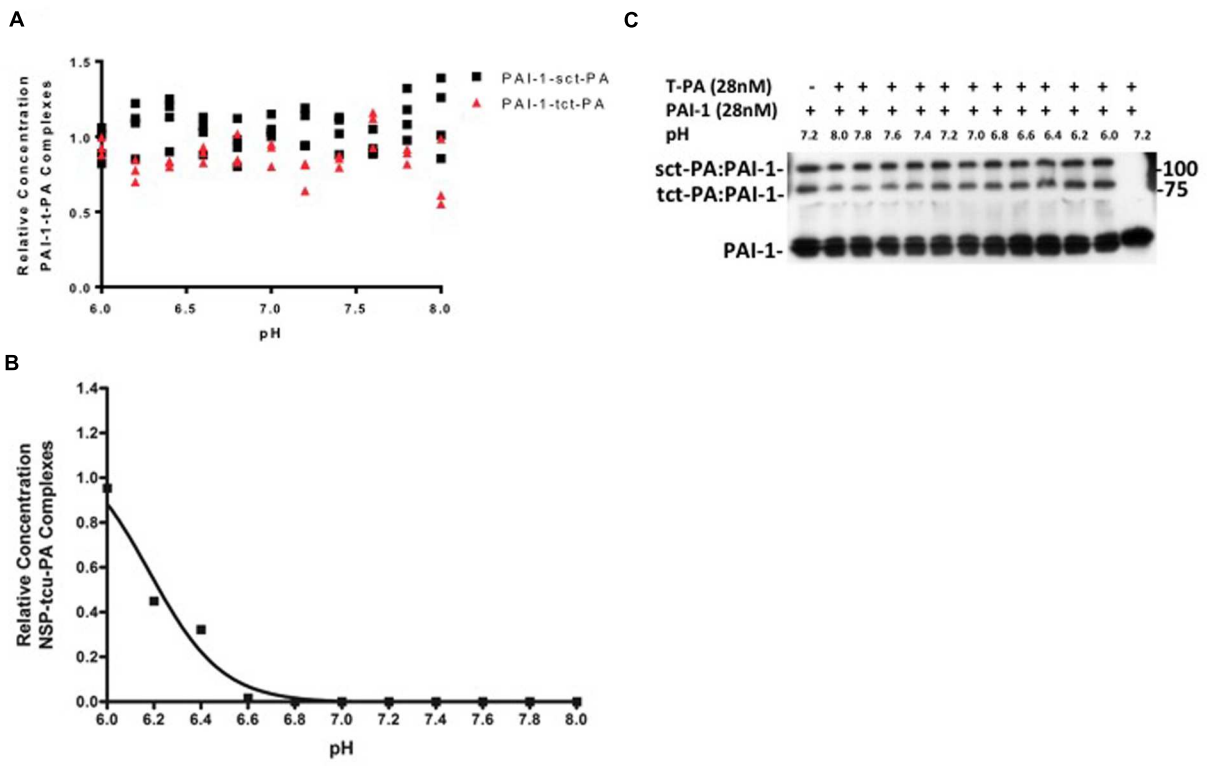

FIGURE 2 | (A,B) pH dependent stability of acyl-enzyme complexes is specific for NSP-t-PA complexes. PAI-1-t-PA complex stability was assayed in a manner analogous to NSP-t-PA complex stability, as described in Methods. The ratio of PAI-1-t-PA complex remaining at the end of the 90-min incubation to the amount of complex present immediately before dilution into various pH buffers, was graphed as a function of the pH into which the reactions were diluted. Triangles: PAI-1-tct-PA complexes; Squares: PAI-1-sct-PA complexes. Data are presented as points that represent the individual values, from 3 independent experiments Note that incubation of PAI-1-t-PA complexes at various pH's was for 90 min, compared to 30 min for NSP-t-PA complexes, further illustrating the difference in stability between the acyl-enzyme complexes. Panel **(B)** is a representative western blot from which the data were derived and quantified for **(A)**. **(C)** tcu-PA and NSP were incubated in buffer pH 7.2 for 1 min at 37°C, diluted fivefold into buffers at the indicated pH, and incubated at 37°C for 3 h. Samples were then analyzed by SDS-PAGE and western blotting for NSP as described in Methods. The ratio of the concentration of the tcu-PA-NSP complexes remaining after the 3-h incubation at each pH to the concentration of complex present immediately before dilution into various pH buffers was graphed against the pH into which the reactions were diluted.

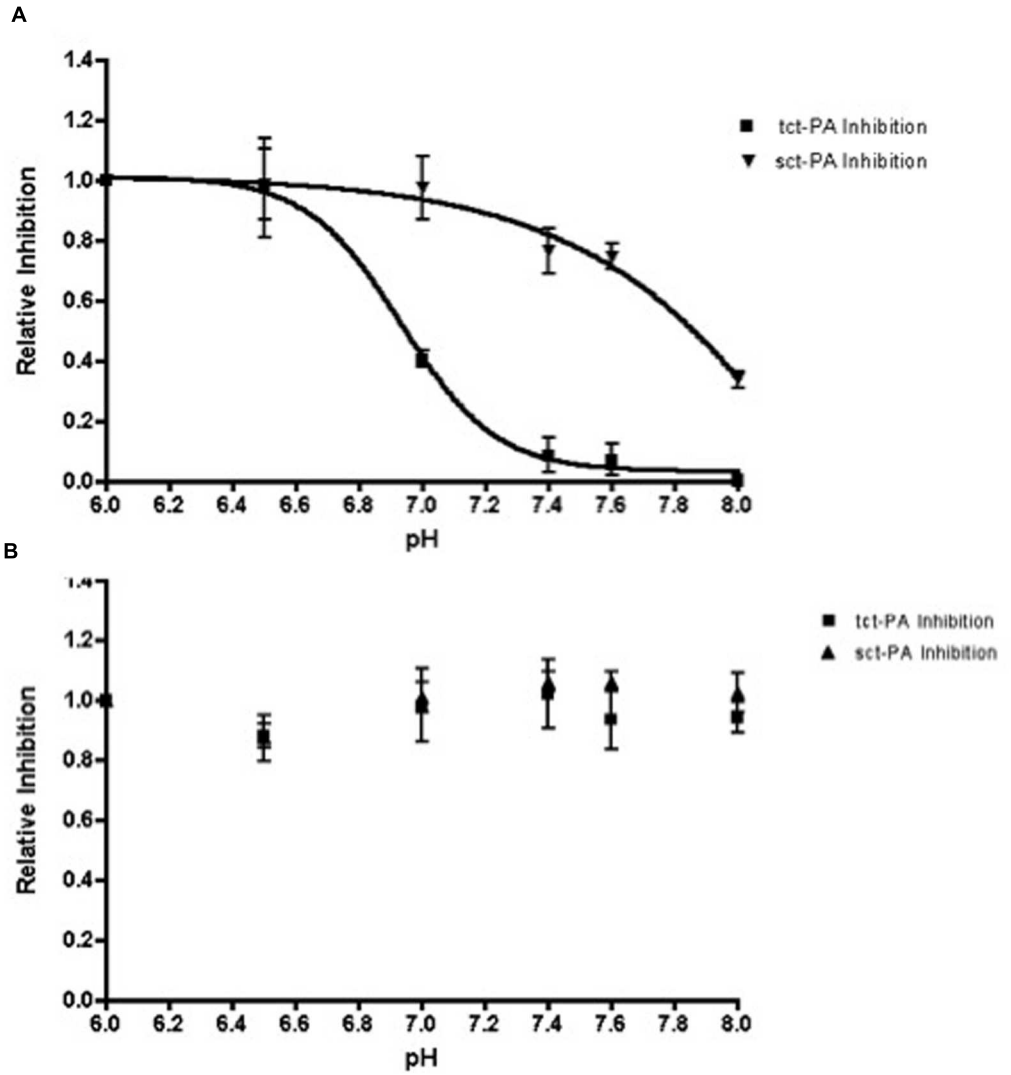

FIGURE 3 | Inhibition of t-PA enzymatic activity by NSP, but not PAI-1, is dependent on pH. Sct-PA or tct-PA were pre-incubated with either **(A)** NSP, or **(B)** PAI-1 as detailed in Methods. Reactions were then diluted into buffers with the indicated pH's as in Methods, and the residual t-PA activity determined by quantifying cleavage of a chromogenic substrate. The per cent t-PA inhibition at a given pH was compared to the maximum inhibition (observed at pH 6.0) to yield the relative inhibition of each form of t-PA, which is graphed as a function of pH. Triangles: Serpin-sct-PA complexes; Squares: Serpin-tct-PA complexes. Data represent the mean and SEM from 3 independent experiments.

(Schechter et al., 1997; Plotnick et al., 2002a,b; Schechter and Plotnick, 2004). Although single chain u-PA forms acyl-enzyme complexes with PAI-1 (Manchanda and Schwartz, 1995), no complexes between single chain u-PA and NSP were detected (data not shown). Hence, the regulation of NSP inhibition of t-PA over the narrow pH range known to occur in physiologic and pathologic processes in the mammalian CNS seems selective for this protease-serpin pair. This is in marked contrast to t-PA and u-PA reacting with the serpin PAI-1, and with the substrate plasminogen (Colucci et al., 1986), and is consistent with the findings of Fredriksson et. al., which suggests that NSP regulates a t-PA mediated process (Fredriksson et al., 2015). The intriguing possibility raised by the present data is that there may be selectivity for regulating the different forms of t-PA under certain biological conditions.

Stability of an acyl-enzyme complex suggests the active site architecture of the protease remains distorted within the complex, preventing catalytic deacylation (Lawrence et al., 1990; Huntington et al., 2000; Dementiev et al., 2006). Therefore, the pH dependent change in NSP-t-PA acyl-enzyme complex stability should be paralleled by a pH dependent change in t-PA inhibition. To test this, NSP and both forms of t-PA (sct-PA or tct-PA) were incubated at pH 7.2 to allow acyl-enzyme complexes to form. The complexes were then diluted into buffers with pH's between 6.0 and 8.0, incubated further, and then added to a t-PA sensitive chromogenic substrate to determine residual t-PA catalytic activity, and thus the degree

of t-PA inhibition. As seen in **Figure 3**, t-PA inhibition as a function of pH closely paralleled the persistence of NSP-t-PA acyl-enzyme complexes as a function of pH (compare **Figures 1B** and **3A**). In addition, the surprising difference in pH effect on acyl-enzyme complex stability between sct-PA and tct-PA was also seen in pH dependent inhibition of the two forms of t-PA (**Figure 3A**). As was seen with acyl-enzyme complex persistence, this pattern of inhibition was unique to the interaction between t-PA and NSP, as PAI-1 showed no difference in t-PA inhibitory efficiency across the pH range tested, nor did PAI-1 differentiate between the two forms of the enzyme (**Figure 3B**). These data also suggest that the pH dependent difference in acyl-enzyme complex persistence was not a function of an SDS-PAGE based assay system, as the experiments determining inhibition of enzymatic activity contained no denaturants.

The above data suggest that NSP may have the capacity to differentially inhibit sct-PA and tct-PA via distinct rates of deacylation of the acyl-enzyme complexes once they are formed. To test this hypothesis, NSP-sct-PA and NSP-tct-PA acyl-enzyme complexes were pre-formed, transferred to the indicated pH, and rates of acyl-enzyme complex deacylation determined. To ensure the data were not confounded by the formation of new NSP-t-PA acyl-enzyme complexes, measurement of NSP-t-PA complex decay was only performed at times after all NSP had been incorporated into acyl-enzyme complexes, as assessed by SDS-PAGE and western blotting for NSP. Hence, the reduction in NSP-t-PA complex intensity was an accurate measure of deacylation. As seen in **Figure 4**, the plots of the deacylation rate constants as a function of pH differed between NSP in complex with sct-PA, and tct-PA. In addition, the rate constants were consistent with the pH dependent acyl-enzyme persistence and enzyme inhibition with each form of t-PA (**Figures 1** and **3**). NSP-tct-PA deacylation rates remained low below pH 7.0, the range where acyl-enzyme complexes persisted and tct-PA enzymatic activity was inhibited. As the pH increased above 7.0, the deacylation rate increased (**Figure 4**), with parallel decreases in acyl-enzyme complex persistence (**Figure 1B**) and enzymatic inhibition (**Figure 3A**). For NSP in complex with sct-PA, the increase in deacylation rate occurred at a significantly higher pH, 7.4, again with corresponding changes in acyl-enzyme complex persistence and enzyme inhibition (**Figures 1B** and **3A,** respectively).

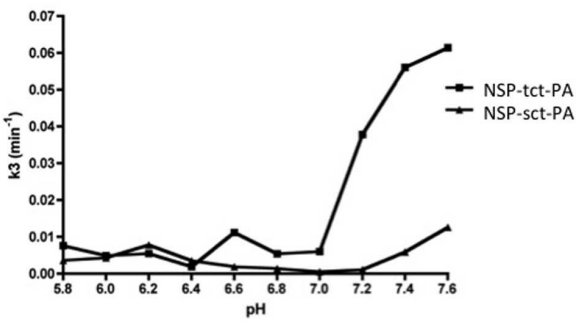

FIGURE 4 | Differential effects of pH on the deacylation rates of NSP-sct-PA and NSP-tct-PA acyl-enzyme complexes. Acyl-enzyme complex formation between sct-PA or tct-PA and NSP was allowed to progress to completion at pH 7.2, as determined in preliminary experiments. Individual reactions were then transferred to the indicated pH and the decay of acyl-enzyme complexes followed over time by subjecting aliquots to SDS-PAGE and western blotting for NSP antigen as detailed in Methods. The deacylation rate constant (k_3) was determined for each pH as described in Methods. Triangles, NSP-sct-PA complexes; Squares, NSP-tct-PA complexes. Data are representative of two independent sets of experiments.

DISCUSSION

Given the relatively steep slope of the pH effect on NSP inhibition of t-PA, and the similar slopes of pH dependent stability for acyl-enzyme complexes containing either form of t-PA (**Figure 1B**), it seems that this pH effect is due to a limited number of residues. The pH range across which NSP-t-PA catalytic deacylation is regulated is consistent with titration of the catalytic triad histidine (HIS 57, chymotrypsin numbering), and suggests significantly different molecular environments for HIS 57 of sct-PA versus tct-PA in complex with NSP, with a different H^+ ion concentration required to allow initiation of catalytic deacylation. However, once that threshold has been crossed, the similar titration curves for deacylation of NSP in complex with sct-PA or tct-PA suggests a similar process for each form of t-PA.

It is also possible that the distinct pH profiles of deacylation signify differences in a non-active site residue on either molecule important for maintaining t-PA in the deformed conformation (i.e., at a contact point between protease and serpin). For instance, it is possible that as a residue at the interface between t-PA and NSP is deprotonated, the ability of NSP to maintain misalignment of t-PA's active site architecture is lost, resulting in recovery of the capacity for catalytic deacylation of the acyl-enzyme complex. If so, the pH sensitivity of this residue in NSP-sct-PA complexes must differ from that in NSP-tct-PA complexes. Such a scenario would also yield a difference primarily in the threshold at which deacylation is initiated, with subsequent events being similar between forms of t-PA. It will be important to test these and other hypotheses of the molecular mechanism for the differential pH effects on NSP-t-PA deacylation in future experiments.

This model is consistent with the findings of Calugaru et al. (2001), who observed that in certain non-cognate serpin-protease pairs, Ca^{++} served as an allosteric ligand which restored partial proteolytic capability to the serpin-complexed protease active site, allowing for catalytic deacylation of the serpin-protease intermediate. In the case of NSP-t-PA complexes, there does not appear to be a requirement for an allosteric ligand to bring about a pH responsive conformation; such a conformation appears to have evolved specifically in NSP-t-PA complexes.

An intriguing correlation may be made with recent findings by Lee et al. (2015), who showed that NSP-t-PA complex

stability is dependent on evolutionarily conserved residues in NSP, and their findings will inform design of future NSP-mutants to identify the amino acid residues that are ultimately responsible for the pH dependency of NSP-t-PA complex deacylation.

The findings in this report are also consistent with a population of NSP-complexed t-PA molecules that retain some degree of catalytic function in equilibrium with NSP- complexed t-PA molecules exhibiting little or no catalytic function, as hypothesized for unstable serpin-enzyme pairs (Calugaru et al., 2001; Plotnick et al., 2002b). As the somewhat functional t-PA molecules complete the catalytic cleavage of NSP, an essentially irreversible event, the equilibrium model suggests that some molecules in the non-functional conformation then shift to the somewhat functional state, and further catalytic deacylation of complexed NSP occurs. This is depicted in Scheme 1.

Scheme 1:

$$\text{t-PA}_\text{D} - \text{NSP} \leftrightarrows \text{t-PA}_\text{F} - \text{NSP} \rightarrow \text{t-PA} + \text{NSP}_\text{cleaved}$$

where t-PA_D is deformed and catalytically inactive t-PA in complex with NSP, t-PA_F is t-PA that has retained some catalytic function in complex with NSP, and $\text{NSP}_\text{cleaved}$ is NSP that has had the cleavage of P_1–P_1' completed. Therefore, the catalytic activity of the t-PA_F form of the protease might be pH sensitive (presumably due to titration of HIS 57), or the stabilization of the t-PA_D-NSP complex might be pH dependent (presumably via optimization of intermolecular contacts).

It is interesting that despite the metabolic fragility of the CNS, the CSF has relatively poor buffering capacity. This suggests that allowing changes in CNS pH may have been preserved through evolution, a concept that is consistent with pH having an important regulatory role in the in this anatomic compartment. The data in this paper suggest that specific regulation of t-PA activity by NSP may be an example of a process regulated by shifts in CNS pH. For instance, neuronal depolarization is accompanied by a flux of hydrogen ions at the synapse, and similarly, secretion of NSP-containing dense core secretory vesicles, which have a pH of 5.0–6.0, would also be expected to transiently lower the local synaptic pH (Loh et al., 1984; Chuang et al., 1999; Parmar et al., 2002; Ishigami et al., 2007). Additional mechanisms of synaptic pH modulation include the Ca+2/H+-ATPase and carbonic anhydrases that are all functional within the microdomain of the synapse (Sinning and Hubner, 2013). A shift in pH within a single synapse could be sufficient to modulate NSP inhibitory stability, resultant t-PA activity, and t-PA-dependent neuronal function for that specific synapse (Qian et al., 1993; Seeds et al., 1995; Frey et al., 1996; Calabresi et al., 2000; Zhuo et al., 2000). These same pH alterations can also be hypothesized to modify NMDA receptor activity (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Sinning and Hubner, 2013), with relatively little effect on AMPA- and kainite receptor function (Lei et al., 2001). Although beyond the scope of this manuscript, it is tempting to speculate that the inhibition of NMDA receptor activity noted with decreased extracellular pH may in part be related to persistence of NSP-t-PA acyl-enzyme complexes, thus altering t-PA interaction with the NR2B subunit of the NMDA receptor (Norris and Strickland, 2007; Parcq et al., 2012).

It is also intriguing to speculate that pH dependent regulation of t-PA proteolytic activity may be operative in pathologic instances such as seizures, where depolarization goes unchecked. The spread of seizures in mice is t-PA dependent (Yepes et al., 2002), and in a murine model of neonatal febrile seizures, hyperventilation driven alkalinization of the CSF was shown to be the trigger for seizures (Schuchmann et al., 2006). This is consistent with the use of hyperventilation to induce seizures during video EEG monitoring to determine seizure focus in the CNS (Guaranha et al., 2005). Perhaps hyperventilation-induced CNS alkalinization results in loss of NSP inhibitory activity, releasing unopposed t-PA activity, thus facilitating seizure spread.

NSP polymerization has also been shown to be dependent on pH (Belorgey et al., 2010, 2011), and NSP is more resistant to polymerization at low pH than is PAI-1 (Ishigami et al., 2007; Takehara et al., 2009; Belorgey et al., 2010). Because available NSP or PAI-1 is a function of the balance between polymerized and free serpin, with only free serpin being able to complex with protease, it is possible that the pH effect described in the present report reflects differences in NSP polymerization, and thus differences in the serpin's availability to inhibit t-PA. However, deacylation was determined starting with NSP that was already in complex with each form of t-PA (**Figure 4**), something that requires non-polymerized NSP. Hence, the regulatory step described here appears to be separate from the pH effect on NSP polymerization.

It is also worth considering whether differential pH-dependent regulation of sct-PA and tct-PA reflects different functions for each form of the protease in the CNS. This differentiation may reflect one reason t-PA evolved uniquely as a protease with such a remarkably active zymogen, and is consistent with the distinct role of sct-PA in activating NMDA receptor-dependent neurotoxicity (Parcq et al., 2012; Bertrand et al., 2015) Moreover, Parmar et al.'s finding that tct-PA forms acyl-enzyme complexes with NSP at lower pH than does sct-PA (Parmar et al., 2002) is consistent with different molecular environments in the active sites of the two forms of t-PA during acyl-enzyme formation. Those data, combined with the findings in this paper regarding acyl-enzyme stability, strengthen the hypothesis that changes in H+ ion concentrations are important in regulating the interaction of NSP and t-PA.

Importantly, the properties of NSP that make it uniquely suited as a differential regulator for two forms of t-PA in the pH-sensitive environment of the CNS support the conclusion of Fredriksson et al. (2015) that t-PA is a physiologic target of NSP in the CNS. This is further supported by recent findings that there is very little PAI-1 in neuronal tissue of the normal brain, further supporting the important regulatory function of NSP for t-PA inhibition in the extravascular compartment of the CNS (Yamamoto et al., 1994; Fredriksson et al., 2015).

Further studies will be required to define the molecular mechanisms and physiologic import of this novel form of protease regulation.

AUTHOR CONTRIBUTIONS

KS-C and LN contributed equally to this manuscript. KS-C, LN, KS, DL, and BS designed and implemented the experiments, and co-wrote this manuscript.

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frontiers in Cellular Neuroscience

MINI REVIEW
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Physiological and pathological roles of tissue plasminogen activator and its inhibitor neuroserpin in the nervous system

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Although its roles in the vascular space are most well-known, tissue plasminogen activator (tPA) is widely expressed in the developing and adult nervous system, where its activity is believed to be regulated by neuroserpin, a predominantly brain-specific member of the serpin family of protease inhibitors. In the normal physiological state, tPA has been shown to play roles in the development and plasticity of the nervous system. Ischemic damage, however, may lead to excess tPA activity in the brain and this is believed to contribute to neurodegeneration. In this article, we briefly review the physiological and pathological roles of tPA in the nervous system, which includes neuronal migration, axonal growth, synaptic plasticity, neuroprotection and neurodegeneration, as well as a contribution to neurological disease. We summarize tPA's multiple mechanisms of action and also highlight the contributions of the inhibitor neuroserpin to these processes.

Keywords: serine protease, serpin, neuronal migration, neurite growth, synaptic plasticity, neurodegeneration and neuroprotection, Alzheimer's disease, neurovascular unit

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Introduction

Research of tissue plasminogen activator (tPA) in the nervous system has linked this protease to a number of functions, including cell migration, axonal growth, and synaptic plasticity, as well as a contribution to neurodegeneration in pathological states. The main inhibitor of plasminogen activator proteolytic activity in the vascular space is the serpin plasminogen activator inhibitor 1 (PAI-1; SERPINE1). This serpin, however, is only weakly expressed in the brain (Sawdey and Loskutoff, 1991; Masos and Miskin, 1997). Another serpin, protease nexin-1 (PN-1; SERPINE2) is expressed throughout the brain (Sappino et al., 1993; Reinhard et al., 1994). Although PN-1 may play some role in regulating tPA activity (Kvajo et al., 2004; Samson et al., 2008), its inhibitory kinetics suggest that it mainly functions as inhibitor of thrombin (Scott et al., 1985). Instead, the predominant inhibitor of neuronal tPA activity is believed to be the neuroserpin (SERPINI1), a serpin that is largely specific to the nervous system (Osterwalder et al., 1996; Hastings et al., 1997; Krueger et al., 1997). This review will focus on the interplay of these two players in the nervous system (**Figure 1**).

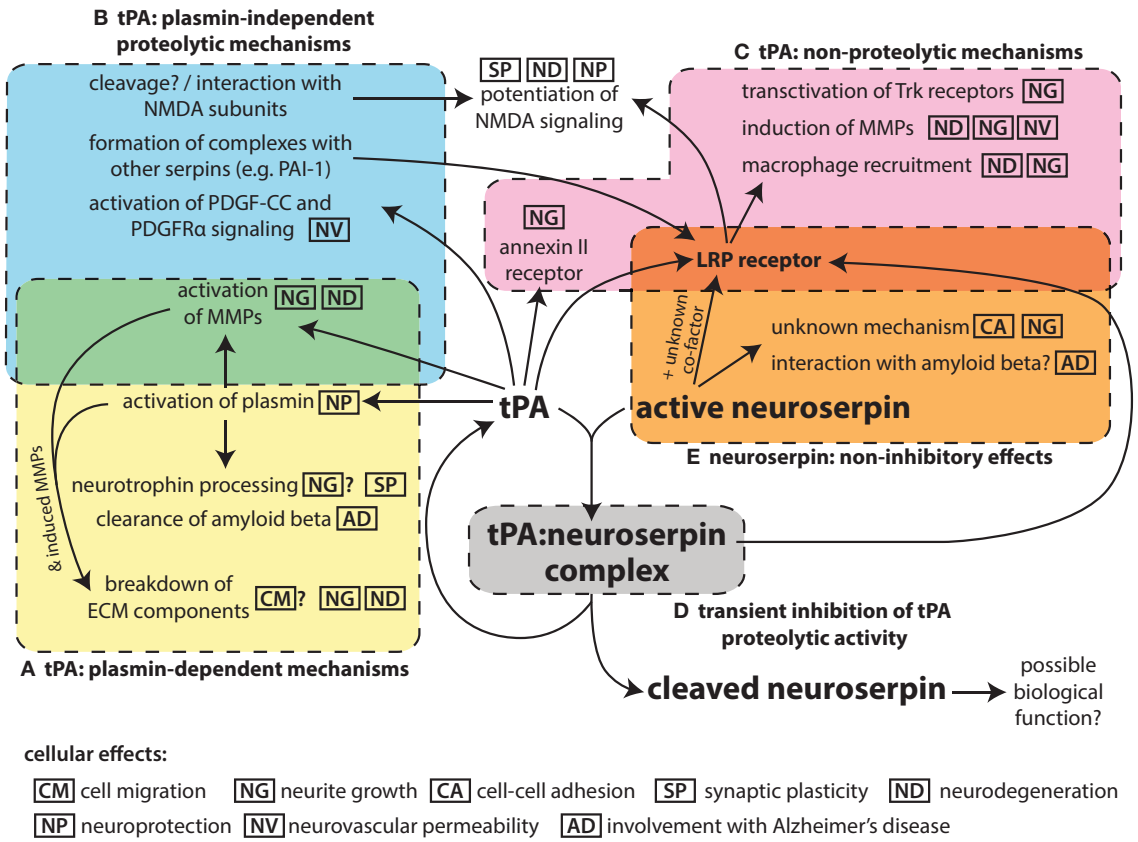

FIGURE 1 | Schematic summarizing the pleotropic roles of tPA in the nervous system and its interaction with the inhibitor neuroserpin. While tPA is named for its function to proteolytically activate the zymogen plasminogen to plasmin **(A)**, it can also act on other substrates in a plasmin-independent manner **(B)**. In addition, tPA can bind to cell-surface receptors and act via non-proteolytic mechanisms **(C)**, although binding to the LRP receptor in particular is affected by formation of complexes of tPA with serpin partners, which requires the proteolytic activity of tPA. The proteolytic activity of tPA can be inhibited by neuroserpin **(D)**, although tPA:neuroserpin complexes are unstable and this inhibition is only transient. There is also evidence of neuroserpin having non-inhibitory effects **(E)** although the mechanism of these is unknown. The main molecular events for each mechanism are indicated; the icons indicate the cellular effects associated with these molecular events. Effects for which there is only limited evidence are shown with a question mark.

Neuroserpin as an Inhibitor of tPA

Analysis of neuroserpin sequence indicated that it was likely to be an inhibitor of trypsin-like serine proteases (Osterwalder et al., 1996). Biochemical evidence subsequently showed strong inhibition of tPA by neuroserpin and considerably less efficient inhibition of urokinase plasminogen activator (uPA), trypsin, NGF-γ, plasmin, and thrombin (Hastings et al., 1997; Osterwalder et al., 1998). The function of neuroserpin as an inhibitor of tPA is supported by their similar expression patterns in the nervous system (Hastings et al., 1997; Krueger et al., 1997; Teesalu et al., 2004) and data showing that tPA activity levels are decreased by over-expression of neuroserpin in the brain (Cinelli et al., 2001).

Other results, however, indicate that neuroserpin does not behave as a classical inhibitory serpin toward tPA. Unlike most covalent serpin:protease complexes, tPA:neuroserpin is unstable and dissociates within minutes to release cleaved neuroserpin and active tPA (Barker-Carlson et al., 2002; Ricagno et al., 2009; Lee et al., 2015). As complex dissociation is expected to occur prior to clearance (Barker-Carlson et al., 2002), these data suggest that neuroserpin is likely to function as a transient inhibitor of tPA *in vivo*. Interestingly, evolutionarily conserved residues in neuroserpin regulate the half-life of tPA:neuroserpin complexes, suggesting that the precise half-life of the complexes may be physiologically important (Lee et al., 2015). tPA:neuroserpin interactions may involve other players, such as an unknown co-factor that stabilizes the complex (Barker-Carlson et al., 2002). Neuroserpin's weak inhibition of other proteases, such as plasmin, may also be physiological important (Wu et al., 2010) and it is also possible that neuroserpin may have other protease targets that have not yet been determined. There is evidence of non-inhibitory functions of neuroserpin (Lee et al., 2008), raising the alternate possibility that tPA modulates neuroserpin activity by cleaving neuroserpin to produce a form with distinct (non-inhibitory) biological activity.

Expression of Neuroserpin and tPA in the Nervous System

Both tPA and neuroserpin are expressed in neurons throughout the developing and the adult nervous systems (Sappino et al., 1993; Friedman and Seeds, 1994; Ware et al., 1995; Osterwalder et al., 1996; Krueger et al., 1997; Teesalu et al., 2004), with spatial and temporal expression patterns suggesting roles in neuronal migration, axonal growth, synaptic development, neuronal plasticity and regulation of neurovascular responses. High neuroserpin expression has been shown in post-mitotic cells undergoing neurogenesis in the adult (Yamada et al., 2010), also suggesting a function in neuronal maturation.

Neuroserpin and tPA are targeted to the regulated secretory pathway, being sorted to dense core secretory granules and released in response to stimulation (Parmer et al., 1997; Lochner et al., 1998; Hill et al., 2000; Parmar et al., 2002; Silverman et al., 2005; Ishigami et al., 2007; Miranda et al., 2008). At a subcellular level, tPA has been localized to neuronal growth cones (Lochner et al., 1998; Silverman et al., 2005) and dendritic spines (Lochner et al., 2006), while neuroserpin has been localized to the neurite tips of differentiated PC12 cells (Parmar et al., 2002; Miranda et al., 2008), as well as axons, dendrites and presynaptic terminals of cultured neurons (Ishigami et al., 2007; Borges et al., 2010). Two unique features of neuroserpin that are important for regulated secretion are a targeting sequence at the C-terminus (Ishigami et al., 2007) and a resistance to polymerization at low pH (Belorgey et al., 2010).

In addition to activity-dependent secretion, it has been shown that the expression of tPA is regulated by several forms of neuronal activity including long-term potentiation (LTP) (Qian et al., 1993) and long term depression (LTD) (Napolitano et al., 1999; Calabresi et al., 2000). The expression of neuroserpin has also been shown to be regulated by neuronal depolarization (Berger et al., 1999), neuronal activity during visual cortex development (Wannier-Morino et al., 2003), and several signaling factors and hormones including nerve growth factor (NGF), anti-Müllerian hormone (AMH), thyroid hormone, and progesterone (Berger et al., 1999; Navarro-Yubero et al., 2004; Lebeurrier et al., 2008; Vanlandingham et al., 2008).

Functions of tPA and Neuroserpin in Neuronal Migration and Axonal Growth

A role for tPA in neuronal migration is supported by results showing that migration of cerebellar granule neurons is perturbed in tPA-deficient mice (Seeds et al., 1999). While it has been hypothesized that tPA regulates neuronal migration by activating plasmin to break down cell adhesions or extracellular matrix (ECM) (Seeds et al., 1999; Basham and Seeds, 2001), there is no direct evidence to support this role.

Evidence for a function of tPA and neuroserpin in regulating axonal growth has come from studies of cultured cells. Inhibition of tPA activity or tPA knockout have been shown to block axonal growth in cultured neurons (Pittman et al., 1989; Baranes et al., 1998; Minor et al., 2009), while exogenous tPA or tPA over-expression causes increased neurite outgrowth (Pittman and Dibenedetto, 1995; Baranes et al., 1998; Lee et al., 2007a). Similarly, altered expression of neuroserpin has been shown to trigger changes in the extension of neurite-like processes of AtT-20 cells (Hill et al., 2000) and NGF-mediated neurite outgrowth in PC12 cells (Parmar et al., 2002; Navarro-Yubero et al., 2004).

A role of tPA in axonal growth has also been shown *in vivo*. In one study, tPA-knockout mice show abnormal growth of mossy fiber axons in the dentate gyrus following seizure (Wu et al., 2000). Other reports have focused on the role of tPA in axonal regeneration following damage. In studies using the sciatic nerve crush model of peripheral nervous system regeneration, tPA is induced in the neurons and supporting cells of the nerve following crush damage, while axonal regeneration and functional recovery is reduced in tPA or plasminogen knockout animals and improved with exogenous tPA or tPA/plasminogen (Akassoglou et al., 2000; Siconolfi and Seeds, 2001, 2003; Zou et al., 2006).

Multiple mechanisms have been suggested to mediate the effects of tPA on axonal growth. Proteolysis of ECM components may create channels for neurites to extend through (Pittman and Dibenedetto, 1995) and/or remove the inhibitory effects of these components (Wu et al., 2000; Bukhari et al., 2011). This is likely to involve activation or induction of additional downstream proteases such as matrix metalloproteinases (MMPs) (Siconolfi and Seeds, 2003; Wang et al., 2003; Hu et al., 2006; Zou et al., 2006). During axonal regeneration, the removal of fibrin deposits by tPA/plasmin also appears to be important (Akassoglou et al., 2000; Zou et al., 2006), as well as macrophage recruitment to remove cellular debris (Zou et al., 2006), which may involve tPA binding to the LDL-related protein (LRP) receptor (Cao et al., 2006). Binding of tPA to the LRP receptor and the annexin II receptor has also been shown to mediate non-proteolytic effects of tPA on neurite outgrowth (Lee et al., 2007a; Shi et al., 2009). Finally, tPA may regulate neurite growth via proteolytic processing of neurotrophins (Pang et al., 2004; Bruno and Cuello, 2006).

The mechanism of neuroserpin's effects on neurite outgrowth are largely undetermined. Neuroserpin may act by modulating tPA activity, for example, neuroserpin has been shown to regulate proteolytic processing of the neurotrophin NGF (Bruno and Cuello, 2006). Interestingly, the neurite outgrowth effects of neuroserpin could be triggered by non-inhibitory mutant forms of neuroserpin (Lee et al., 2008), suggesting neuroserpin may also act independently of tPA, possibly by binding to a cell surface receptor such as LRP (Makarova et al., 2003).

Effects of tPA and Neuroserpin on Neuronal Plasticity

Deficits in hippocampal late phase LTP are seen in tPA-knockout mice (Frey et al., 1996; Huang et al., 1996; Calabresi et al., 2000). Conversely, LTP is increased by exogenous tPA or tPA overexpression (Baranes et al., 1998; Madani et al., 1999). Knockout of the tPA gene also leads to defects in both LTP and LTD in the striatum (Calabresi et al., 2000; Centonze et al.,

2002). Numerous studies have also shown a role of tPA in memory and learning. For example, tPA-knockout mice exhibit deficits in hippocampal-dependent and striatum-dependent tasks (Huang et al., 1996; Calabresi et al., 2000; Pawlak et al., 2002; Benchenane et al., 2007) and cerebellar motor learning (Seeds et al., 2003), while transgenic mice over-expressing tPA were found to have improved spatial learning (Madani et al., 1999). tPA is also required for altered amygdala- and hippocampal-dependent behavioral responses that occur in mice subjected to restraint-stress (Pawlak et al., 2003, 2005b; Norris and Strickland, 2007).

At the cellular level, tPA's involvement in LTP has been associated with the formation of new presynaptic varicosities (Baranes et al., 1998), while activity-dependent formation of perforated synapses in cultured neurons can be blocked by tPA inhibitors (Neuhoff et al., 1999). In animals subjected to restraint stress, induction of the plasticity-related gene GAP43 (Pawlak et al., 2003) and changes in dendritic spine number were absent in tPA knockout mice (Pawlak et al., 2005b). During visual cortex development, experience-dependent plasticity and pruning of dendritic spines is also reduced in tPA-knockout mice and can be partly restored by exogenous tPA (Mataga et al., 2002, 2004).

A number of different mechanism underlying tPA's effects on synaptic plasticity have been proposed. Firstly, tPA may contribute to LTP by regulating plasmin-mediated processing of BDNF from its precursor proBDNF to mature BDNF (mBDNF) (Pang et al., 2004; Barnes and Thomas, 2008). It has been shown that tPA is secreted from neurons in response to high-frequency, but not low-frequency, stimulation of neurons, leading to changes in the proBDNF/mBDNF ratio (Nagappan et al., 2009). Since proBDNF has been linked to LTD (Woo et al., 2005) while mBDNF has been linked to LTP, these results suggest that tPA may mediate the differing cellular responses to different patterns of neuronal activity. Secondly, there is general agreement in the literature that tPA can potentiate NMDA-receptor signaling. The manner in which it does so, however, remains unclear. Results from the Vivien group suggest that tPA may act by interacting with the GluN1 subunit of NMDA receptors, particularly in GluN2D-containing receptors (Benchenane et al., 2007; Macrez et al., 2010; Obiang et al., 2012). Other results suggest that tPA modulates NMDA signaling through GluN2B subunits (Pawlak et al., 2005a; Norris and Strickland, 2007; Noel et al., 2011; Ng et al., 2012) or by a mechanism involving LRP (Martin et al., 2008; Samson et al., 2008). The importance of LRP in tPA-mediated neuronal plasticity was also reported in an earlier study on LTP (Zhuo et al., 2000).

Experiments in culture systems have provided some evidence that neuroserpin is involved in cellular plasticity. Firstly, altered neuroserpin expression in PC12 cells has been linked to changes in cell-cell adhesion mediated by the synaptic adhesion molecule N-cadherin (Lee et al., 2008). In addition, overexpression of neuroserpin in cultured neurons has been found to lead to changes in the number and shape of dendritic spines (Borges et al., 2010). Altered neuroserpin expression *in vivo* has also been shown to lead to behavioral changes, with both neuroserpin overexpression and neuroserpin-knockout leading to increased phobic and anxiety-like responses (Madani et al., 2003). Localized overexpression of neuroserpin in the adult rat hippocampus did not cause any changes in learning and memory, but it altered the expression of postsynaptic scaffolding protein PSD-95 (Tsang et al., 2014). Overall, little is known about neuroserpin's mechanism of action for these effects, however, the results from the PC12 studies show that inhibition of tPA was not required (Lee et al., 2008), and the changes in behavior in neuroserpin-knockout animals were not correlated with altered tPA activity (Madani et al., 2003).

Neuroserpin and tPA in Neurodegeneration and Neuroprotection

Initial evidence for a contribution of tPA to neuronal death came some years ago, when it was shown that tPA knockout mice were resistant to excitotoxin-induced neuronal degeneration (Tsirka et al., 1995) and had reduced ischemic damage in a stroke model (Wang et al., 1998). These results have been independently confirmed by a number of other groups (Strickland, 2001; Kaur et al., 2004).

Three main mechanisms for tPA's effects on neuronal death have been proposed. Firstly, tPA may cause ECM breakdown by proteolytically activating plasmin and/or MMPs (Chen and Strickland, 1997; Tsirka et al., 1997; Sumii and Lo, 2002; Wang et al., 2003). Secondly, the ability of tPA to potentiate NMDA receptor-mediated calcium influx may also contribute by promoting excitotoxic neuronal death (Nicole et al., 2001). In support of this, immunotherapy to block interaction of tPA with NMDA receptors has been shown to reduce neuronal damage in stroke models (Benchenane et al., 2007; Gaberel et al., 2013). Thirdly, tPA may signal through the LRP receptor to trigger a number of inter-related effects including induction of MMP expression (Wang et al., 2003, 2004; Lee et al., 2007b; Sashindranath et al., 2012), opening of the blood-brain barrier (Yepes et al., 2003; Sashindranath et al., 2012) and recruitment and activation of microglia (Rogove and Tsirka, 1998; Rogove et al., 1999; Siao and Tsirka, 2002; Zhang et al., 2009). Paradoxically, the tPA inhibitor PAI-1 has been shown to exacerbate, rather than reduce, some of these effects of tPA, as tPA:PAI-1 complexes bind more strongly to LRP than tPA itself (Sashindranath et al., 2012). The instability of tPA:neuroserpin complexes could therefore be a mechanism to temporarily reduce tPA activity without excessive LRP activation.

There is also evidence of neuroprotective effects of tPA, first shown some time ago (Kim et al., 1999; Yi et al., 2004; Liot et al., 2006) but highlighted by a series of recent results from the *in vitro* oxygen and glucose deprivation (OGD) model of ischemic death, as well as in *in vivo* models of excitotoxic neuronal death (Haile et al., 2012; Wu et al., 2012, 2013). These studies have suggested that lower concentrations of tPA mediate survival instead of neuronal death, through both plasmin-dependent and LRP-dependent/plasmin-independent mechanisms involving NMDA signaling.

In animal models of stroke, administration of exogenous neuroserpin alone, neuroserpin in combination with tPA and neuroserpin overexpression have been shown to reduce ischemic

damage *in vivo* (Yepes et al., 2000; Cinelli et al., 2001; Zhang et al., 2002). In these studies, the effects of neuroserpin were associated with reductions in tPA and uPA activity, ECM degradation, microglia activation and blood brain barrier leakage. Conversely, neuroserpin-knockout mice have worse ischemic damage and neurological outcomes than controls, with the effects attributed to tPA-mediated activation of microglia (Gelderblom et al., 2013). Similarly, studies in the OGD model and a mouse model of motoneuropathy have also shown neuroprotective effects of neuroserpin with results suggesting a mechanism involving tPA inhibition (Simonin et al., 2006; Rodríguez-González et al., 2011). However, neuroserpin has been shown to promote neuronal survival in tPA knockout mice, indicating it can also act through a tPA-independent mechanism, possibly through inhibition of uPA or plasmin (Wu et al., 2010).

Functions of tPA and Neuroserpin in the Neurovascular Unit

There is considerable evidence that tPA in the central nervous system side of the neurovascular unit increases the permeability of the blood-brain barrier (e.g., Yepes et al., 2003; Su et al., 2008; Sashindranath et al., 2012). This effect of tPA may contribute to neurodegeneration following stroke, and recent results also suggest a contribution to seizure propagation (Fredriksson et al., 2015). tPA has also been shown to regulate functional hyperemia (Park et al., 2008). A number of downstream events have been identified for the neurovascular effects of tPA including activation of neuronal nitric oxide synthase (Parathath et al., 2006; Park et al., 2008), proteolytic activation of platelet-derived growth factor-CC (PDGF-CC) and platelet-derived growth factor receptor alpha (PDGFRα) signaling (Su et al., 2008; Fredriksson et al., 2015), LRP signaling and induction of MMPs (Sashindranath et al., 2012). While most of the neurovascular effects of tPA are considered to be plasmin-independent (Yepes et al., 2003), there is also evidence for an involvement of plasmin (Freeman et al., 2014; Niego and Medcalf, 2014). As an inhibitor of tPA, neuroserpin can act as an antagonist of tPA in the neurovascular unit (Yepes et al., 2003; Fredriksson et al., 2015).

Contributions of tPA and Neuroserpin to Neurological Disease

Mutations in the neuroserpin gene cause a rare autosomal-dominant dementia accompanied by epilepsy called Familial Encephalopathy with Neuroserpin Inclusion Bodies (FENIB), characterized by polymerization of neuroserpin, formation of inclusion bodies and subsequent neuronal degeneration (Davis et al., 1999, 2002; Takao et al., 2000; Gourfinkel-An et al., 2007; Coutelier et al., 2008; Hagen et al., 2011). Other studies have suggested a role for neuroserpin in Alzheimer's disease, with neuroserpin hypothesized to be either beneficial by interacting with amyloid-beta peptides and altering their oligomerization (Kinghorn et al., 2006) or detrimental by reducing tPA-mediated clearance of amyloid-beta (Fabbro and Seeds, 2009; Fabbro et al., 2011). Changes in the expression of neuroserpin have also been linked to schizophrenia (Hakak et al., 2001; Vawter et al., 2004; Brennand et al., 2011). A recent study also suggests that the expression of neuroserpin by tumor cells may inhibit plasmin-mediated death signals and allow metastasis into the brain (Valiente et al., 2014).

Conclusion

Research over the years has shown that tPA has pleiotropic effects in the nervous system and can act through multiple mechanisms. It is also clear that neuroserpin does not function as a classical serpin inhibitor for tPA and this must be considered when making inferences regarding its function and mode of action. Future research should take a broad view and consider all possible mechanisms of these two players to provide a more complete understanding of their roles in the nervous system.

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REVIEW
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Impacts of tissue-type plasminogen activator (tPA) on neuronal survival

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Tissue-type plasminogen activator (tPA) a serine protease is constituted of five functional domains through which it interacts with different substrates, binding proteins, and receptors. In the last years, great interest has been given to the clinical relevance of targeting tPA in different diseases of the central nervous system, in particular stroke. Among its reported functions in the central nervous system, tPA displays both neurotrophic and neurotoxic effects. How can the protease mediate such opposite functions remain unclear but several hypotheses have been proposed. These include an influence of the degree of maturity and/or the type of neurons, of the level of tPA, of its origin (endogenous or exogenous) or of its form (single chain tPA versus two chain tPA). In this review, we will provide a synthetic snapshot of our current knowledge regarding the natural history of tPA and discuss how it sustains its pleiotropic functions with focus on excitotoxic/ischemic neuronal death and neuronal survival.

Keywords: tissue-type plasminogen activator, excitotoxicity, apoptosis, NMDA receptors, differential effects

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THE NATURAL HISTORY OF tPA

Morgagni (1761) noted that the blood of patients who died suddenly was not completely coagulated. Denis (1838) observed the spontaneous dissolution of blood clots. Fifty years later, Denys and de Marbaix (1889) postulated the existence of an endogenous fibrinolytic enzyme. Accordingly, Hedin (1903) revealed a proteolytic activity in serum globulin fraction, later identified as the fraction containing a precursor of plasmin. Christensen and Macleod (1945) proposed that this inactive circulating precursor, named plasminogen, could be activated by bacterial extracts like streptokinase. Macfarlane and Biggs (1948) completed the description of the plasminogen activation cascade. In parallel, Conradi (1902) identified tPA, at this time named fibrikinase, in different organs), later characterized to mediate fibrinolysis (Fleisher and Loeb, 1915; Astrup and Permin, 1947; Astrup and Stage, 1952). tPA was then purified from human vessels and uterus in Binder et al. (1979), Rijken et al. (1979) and in larger amounts from Bowes melanoma cell line allowing its biochemical characterization (Collen et al., 1982; Collen and Lijnen, 2009). Pennica et al. (1983) succeeded in cloning and expressing recombinant tPA, providing the primary structure of tPA. tPA is a protein of 527 amino-acids including three glycosylation sites and 17 disulfide bridges (Pennica et al., 1983). Collen and Lijnen (1991) then provided evidence that tPA could facilitate the dissolution of blood clots by inducing the degradation of fibrin in a plasminogen-dependent manner. tPA is now used in the clinic to promote fibrinolysis, especially at the acute phase of ischemic stroke either alone (NINDS, 1995) or combined with thrombectomy (Campbell et al., 2015; Goyal et al., 2015).

In addition to this fibrinolytic function at the origin of its discovery, an increasing number of studies have since the mid-90s, discovered functions of tPA within the brain parenchyma. In particular, tPA is believed to control neuronal fate during several CNS disorders, including multiple sclerosis, Alzheimer's disease, and stroke. The aim of this review is to summarize and discuss structure-function studies related to the influence of tPA on neuronal death and survival.

tPA OR tPAS?

The mature form of tPA is a mosaic protein of five distinct modules, which, from its N-terminal end to its C-terminal end, are: a finger domain (F), an epidermal growth factor-like domain (EGF), two kringle domains (K1 and K2), and a serine protease proteolytic domain (SP). The finger domain is involved in tPA binding to fibrin and is necessary to promote fibrinolytic activity at low plasminogen activator concentrations (Larsen et al., 1988). In the brain, other functions attributed to the finger domain include its ability to cross the blood–brain barrier (Benchenane et al., 2005), its astrocytic clearance (Cassé et al., 2012) and some of its signaling pathways (Siao and Tsirka, 2002; Pineda et al., 2012). The EGF-like domain shows homology with EGF. Both the trophic and mitogenic functions of tPA have been attributed to this domain (Liot et al., 2006; Ortiz-Zapater et al., 2007; Correa et al., 2011; Haile et al., 2012). The EGF-like domain has been also reported to contribute to the hepatic recapture of tPA (Hajjar and Reynolds, 1994). The kringle domains fold into large loops stabilized by three disulfide bridges. Because of the high-mannose-type glycosylation at Asn117, K1 is of major importance in the uptake of tPA by mannose receptors on liver endothelial cells *in vivo* and *in vitro* (Kuiper et al., 1996). The K2 domain and more specifically its lysine binding site (LBS) is involved in the capacity of tPA to bind and activate substrates and/or receptors such as plasminogen, PDGF-CC (platelet derived growth factor-CC; Fredriksson et al., 2004) and NMDAR (*N*-methyl-D-aspartate receptor; López-Atalaya et al., 2008). The K1 of tPA does not possess a LBS (Kim et al., 2003). The C-terminal domain supports the catalytic activity of tPA and forms the catalytic triad (*His 322, Asp 371, and Ser 478)* involving an aspartic acid residue (Asp371) hydrogen-bonded to a histidine (His322), which itself is hydrogen-bonded to a serine (Ser478).

As detailed here after, the literature suggests that there is not one but several forms of tPAs.

Long and Short Variants

The pro-form of tPA is a molecule of 562 amino acids. The signal peptide and a pro-peptide of, respectively, 22 and 10 amino acids should be removed before storage in vesicles and release. Three additional amino acids (Gly–Ala–Arg) at the N-terminal end of the molecule can be also removed leading to the release of either the long variant (L-tPA) or the short variant (S-tPA) of 530 and 527 amino acids, respectively (Jörnvall et al., 1983; Berg and Grinnell, 1991). These tPAs include 17 disulfide bridges.

sc-tPA vs. tc-tPA

In contrast to the other members of the chymotrypsin family, tPA is not synthesized and secreted as a "true" zymogen (Madison et al., 1993). Like other members of the family, the secreted single-chain tPA (sc-tPA; **Figure 1A**) can be processed into a two-chain form tPA (tc-tPA; **Figure 1B**) by plasmin or kallikrein (Wallén et al., 1982; Ichinose et al., 1984). However, sc-tPA is an unusually active zymogen (high intrinsic proteolytic activity, low zymogenicity) that does not require proteolytic processing to be active but relies on the presence of an allosteric regulator, such as fibrin (Thelwell and Longstaff, 2007). The passage from the sc-tPA to the tc-tPA form results from the hydrolysis of the peptide bond linking the Arg275 and the Ile276, both parts of the protein remaining connected by a disulfide bridge between Cys299 (heavy chain A) and Cys430

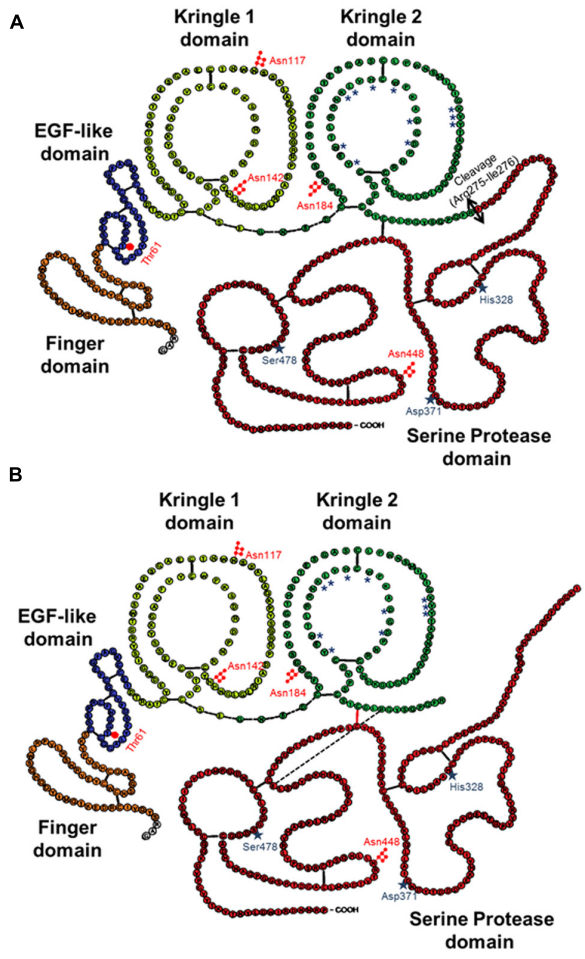

FIGURE 1 | Schematic representations of the primary structure of sc-tPA (A) and tc-tPA (B). Each amino acid is represented by its single letter symbol. Sites of N- (#) or O-glycosylation (●) are showed. The active site residues His322, Asp371, and Ser478 are marked by stars. The amino acids involved in the structure of the lysine binding site are noted with asterisks. The black bars indicate disulfide bonds. The black bar dotted indicate salt bond. The double-arrow indicates the cleavage site for conversion of sc-tPA to tc-tPA.

(light chain B) and a novel salt bridge between Arg302 and Glu445 (Lamba et al., 1996). In the absence of an allosteric regulator such as fibrin, tc-tPA is fivefold catalytically more active than sc-tPA (Rånby et al., 1982; Wallén et al., 1982; Tate et al., 1987; Petersen et al., 1988; Boose et al., 1989). However, in the presence of fibrin, both sc-tPA and tc-tPA display the same catalytic activity (Thelwell and Longstaff, 2007).

Type I vs. Type II tPA

Type plasminogen activator is a glycoprotein containing three major N-glycosylation sites. Two glycosylations are constitutives at Asn117 within the kringle 1 domain and at Asn448 within the serine protease domain. A third one is alternative at Asn184 within the kringle 2 domain. Type I tPA is glycosylated at Asn117, Asn184, and Asn448, while type II tPA is glycosylated only at Asn117 and Asn448 (Pohl et al., 1984; Spellman et al., 1989; Mori et al., 1995; Jaques et al., 1996). Asn184 acts as a switch that enables long-distance communication between fibrin-binding residues (achieved by the finger domain) and the catalytic site in the protease domain (Rathore et al., 2012). Glycosylation of Asn184 (i.e., type I) reduces the ability of tPA to activate plasminogen as well as its binding to fibrin (Einarsson et al., 1985; Wittwer et al., 1989; Berg et al., 1993). Type I sc-tPA seems to be more stable than type II sc-tPA regarding its conversion to tc-tPA (Wittwer and Howard, 1990; Berg et al., 1993; **Figure 2**). tPA also contains a O-linked fucose at Thr61 (occupancy 100%) within the EGF domain (Harris et al., 1991) and potentially an additional N-glycosylation site at Asn142 within the K1 domain (occupancy 1%; Borisov et al., 2009).

IS tPA GOOD OR BAD FOR NEURONAL SURVIVAL?

The Facts

The group of Sidney Strickland was the first to demonstrate that tPA deficient mice were more sensitive to hippocampal neuronal death induced by both NMDAR- and non-NMDAR-agonists (Tsirka et al., 1995), an effect dependent of the ability of tPA to activate plasminogen into plasmin (Tsirka et al., 1997a,b; **Figure 3A**). Accordingly, several studies have reported that inhibitors of tPA, such as neuroserpin and type 1 plasminogen activator inhibitor (PAI-1) protect neurons against toxicity induced by the over-activation of NMDARs (Buisson et al., 1998; Zhang et al., 2002; Gabriel et al., 2003; Lebeurrier et al., 2005). Exogenous tPA was then reported pro-neurotoxic, on cortical neurons, in paradigms of *in vitro* or *in vivo* excitotoxicity mediated by over-activation of NMDAR (Nicole et al., 2001; Liberatore et al., 2003; Reddrop et al., 2005; Park et al., 2008; **Figure 3B**). The tPA was also reported to promote damages on Purkinje cells (Lu and Tsirka, 2002; Li et al., 2006, 2013; Cops et al., 2013; **Figure 3C**), especially by altering the neurotrophic mechanisms that control their postnatal development (Li et al., 2006, 2013).

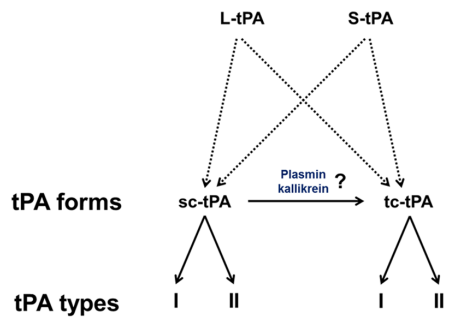

FIGURE 2 | The diversity of tPAs. L-tPA and S-tPA are released under their single chain form (sc-tPA), possibly cleaved into their two-chain form (tc-tPA) by plasmin or kalikrein. Each form of tPA exists in two glycosylated states, types I or II.

Both plasmin-dependent and plasmin-independent mechanisms have been proposed to explain the potentiation of NMDAR signaling by tPA (Nicole et al., 2001; Pawlak et al., 2002; Matys and Strickland, 2003), but several recent studies agree that it can occur independently of plasminogen activation (Samson et al., 2008; Echeverry et al., 2010; Parcq et al., 2012). For instance, tPA can interact with the GluN1 subunit of NMDAR involving the LBS of its K2 domain (Nicole et al., 2001; Fernández-Monreal et al., 2004; Kvajo et al., 2004; López-Atalaya et al., 2008; Parcq et al., 2012). Our group reported that the cleavage of the amino-terminal domain of GluN1 subunit is necessary for enhancement of NMDAR signaling by tPA (Nicole et al., 2001; Fernández-Monreal et al., 2004). In the brain of protease nexin-1 (PN-1, an inhibitor of tPA) deficient mice, Kvajo et al. (2004), demonstrated an increase in the proteolytic activity of tPA, correlated with a decrease in the amount of the GluN1 subunit of the NMDA receptor. However, no cleavage of GluN1 was observed despite the interaction of tPA with the GluN1 subunits of NMDAR (Kvajo et al., 2004). Other groups did not detect tPA-dependent cleavage of GluN1, despite enhancement of NMDAR function by exogenous tPA in cortical cultures (Samson et al., 2008). In a more recent study, it was reported that sc-tPA, but not tc-tPA can promote NMDAR signaling and neurotoxicity in cortical neurons (Parcq et al., 2012; Bertrand et al., 2015). These data were the first to describe a differential function of sc-tPA and tc-tPA. tPA would also act on neuronal death by engaging Low density lipoprotein related protein (LRP) receptors, which in turn would enhance Ca^{2+} downstream of NMDAR (Samson et al., 2008). More recent data obtained from Schwann cells showed that tPA can promote NMDAR signaling independently of LRP1 (Mantuano et al., 2015). Similarly, in PC12 and N2a neuron-like cells, tPA may signal through a complex containing NMDAR, LPR1, and Trk receptors (Mantuano et al., 2013). Plasmin, which is generated by the tPA-dependent conversion of plasminogen, has also been reported to cleave NMDARs, specifically the GluN2 subunit. This cleavage can occur at two sites: Lys317 on GluN2A, which relieves Zn^{2+} inhibition and thereby increases

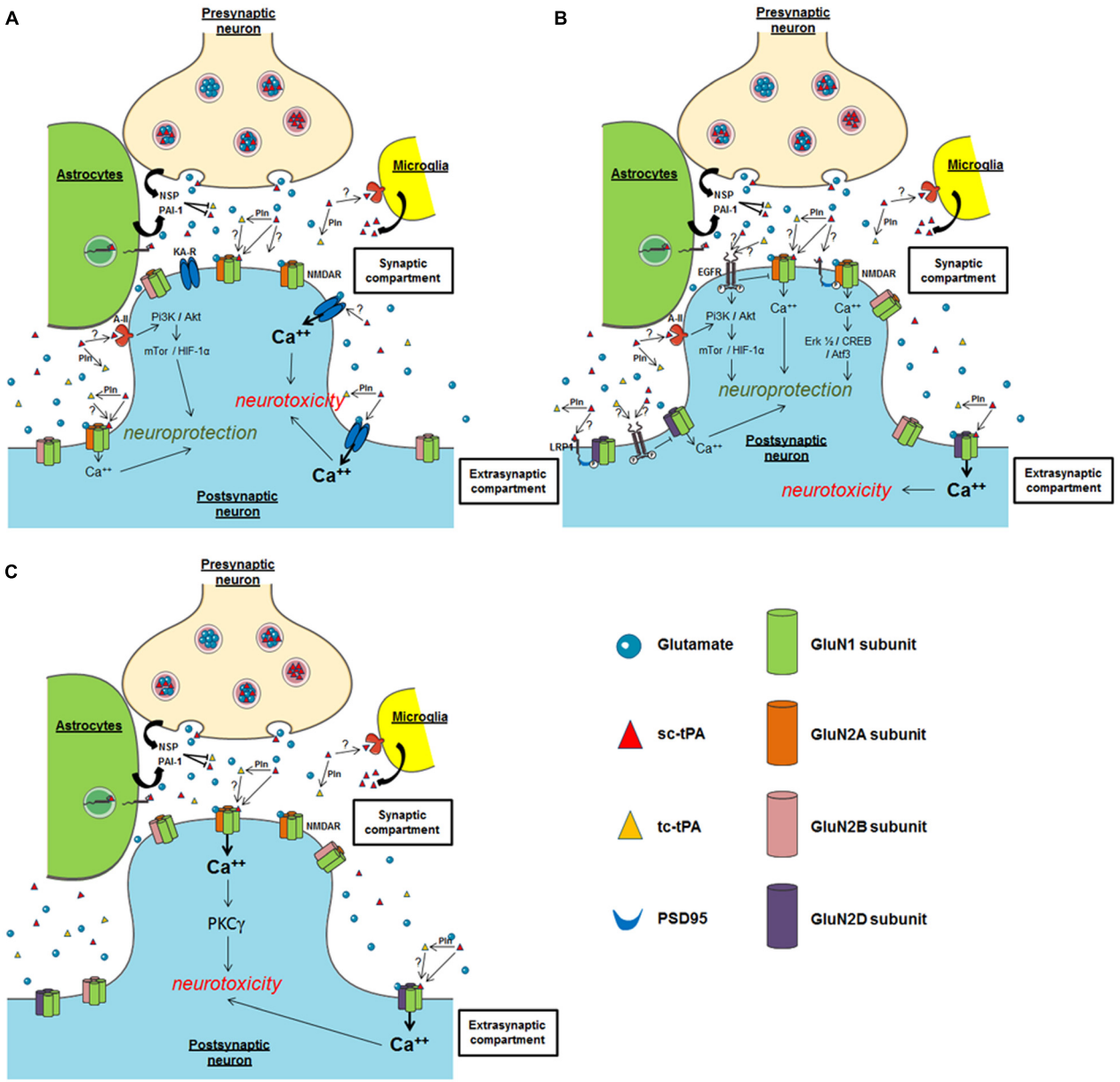

FIGURE 3 | Possible mechanisms of tPA on neuronal survival. (A) Hippocampal neurons; **(B)** cortical neurons; **(C)** cerebellar neurons. NSP, neuroserpin; Pln, plasmin; PAI-1, plasminogen activator inhibitor-1; NMDAR, *N*-methyl-D-aspartate receptor; EGFR, epidermal growth factor receptor; KA-R, kainate receptor; A-II, annexin II receptor.

NMDAR function (Yuan et al., 2009), and Arg67 on GluN2B, which increases sensitivity of the NMDAR to glycine (Ng et al., 2012). Whether tPA-dependent plasmin formation counteracts or interferes with tPA-dependent NMDAR activation is still under debate. Whatever the mechanism, all these studies showed that tPA can increase NMDAR signaling.

By contrast, other studies, in particular using transgenic mice over-expressing tPA in neurons (T4 transgenic mice) or tPA KO mice, suggested that tPA can also have neuroprotective effects (Haile et al., 2012; Wu et al., 2012). These two studies also proposed a mechanism dependent on the activation of NMDAR and independent on plasmin. *In vitro* and *ex vivo* studies also reported pro-survival effects of tPA on neurons (Liot et al., 2006; Lee et al., 2007; Polavarapu et al., 2007; Bertrand et al., 2015; Lemarchand et al., 2015), mainly anti-apoptotic effects. Also interesting, tPA was reported to attenuate zinc-induced neuronal cell death independently of its proteolytic action (Kim et al., 1999; Siddiq and Tsirka, 2004). Despite the heterogeneity of the paradigms used in these different studies, they all showed that this effect of tPA occurs independently of its proteolytic activity, with the activation of either PI3K/Akt,

AMPK- or mTor-HIF-1alpha-dependent signaling pathways needed (Correa et al., 2011; Wu et al., 2012; **Figure 3B**). Two candidates have been proposed as the receptors mediating the pro-survival effects of tPA: Annexin II and EGF receptor (Siao and Tsirka, 2002; Wu et al., 2012; Bertrand et al., 2015; Lemarchand et al., 2015). The ability of tPA to convert the pro-neurotrophins (BDNF, NGF) to their active forms (Pang et al., 2004) is also a possible explanation to the pro-survival effects of tPA.

WHAT ARE THE POSSIBLE EXPLANATIONS OF THE DIFFERENTIAL EFFECTS OF tPA ON NEURONAL SURVIVAL? (TABLE 1)

Are Target Receptors the Explanations?

In the brain parenchyma, pro-survival and pro-neurotoxic effects of tPA have been shown to involve key receptors/pathways, including NMDAR (Nicole et al., 2001), LRP-mediated PSD95 activation (Martin et al., 2008), annexin-II (Siao and Tsirka, 2002), and EGF receptor (Liot et al., 2006; Lemarchand et al., 2015). Focusing on NMDARs, the fact that tPA induces toxic or protective effects could also depend on the different subtypes of GluN subunits involved, and/or their location (synaptic versus extrasynaptic; Paoletti et al., 2013). For instance, based on the current literature, it could be postulated that exogenous tPA could promote neurotoxicity on cortical neurons by activating extrasynaptic GluN2D-containing NMDARs (Baron et al., 2010; Jullienne et al., 2011; Montagne et al., 2012), but could lead to a neuroprotective effect by activating synaptic GluN2A-containing NMDARs (Wu et al., 2013a; **Figure 3B**). Several studies also propose that the neuroprotective activity of tPA, even in a paradigm involving NMDARs, is NMDAR-independent (Correa et al., 2011), independent of its proteolytic activity (Liot et al., 2006). In a model of apoptosis induced by serum deprivation (Liot et al., 2006) or when subjected to OGD, the neuroprotective effect of tPA is mediated by an activation of either EGFR (Correa et al., 2011; Bertrand et al., 2015; Lemarchand et al., 2015) or annexin II (Lee et al., 2007). Whether LRP is also involved is still under debate, again dependent on the paradigm used (Martin et al., 2008). Up to now, it is not clear how these different receptors contribute to the differential effects of tPA in neuronal survival. Additional studies are needed including investigations about possible crosstalks between these different receptors.

Are Protocols of Neuronal Injury the Explanation?

Type plasminogen activator-dependent over-activation of NMDARs has been proposed as a mechanism that could mediate both neuroprotective (Wu et al., 2013b) and neurotoxic (Baron et al., 2010) effects of tPA (**Figure 3B**). This discrepancy may be explained by the use of different models to induce neuronal death, either pure NMDAR-mediated excitotoxicity (Baron et al., 2010) or oxygen glucose deprivation (OGD; Wu et al., 2013b). Whether OGD induces excitotoxicity and/or apoptosis is not well documented and might depend on the severity/duration of the stress. Pathways such as autophagy or endoplasmic reticulum stress may also occur (Badiola et al., 2011; Shi et al., 2012). Another explanation could be the use of differential strategies to block tPA-induced potentiation of NMDAR signaling, MK-801 as a broad irreversible antagonist of NMDARs on one hand (Terro et al., 2000) and an antibody previously characterized to specifically prevent the tPA-dependent potentiation of NMDARs signaling without affecting their basal activity (Benchenane et al., 2007; Macrez et al., 2010) on the other hand. It is interesting to note that either over-activation and blockage of NMDARs are neurotoxic, the first one leading to excitotoxic neuronal death (Nicole et al., 2001), the second one inducing apoptosis (Mattson and Duan, 1999; Henry et al., 2013).

Is Neuronal Maturity an Explanation?

To discuss the differential impact of tPA on neuronal survival, how neurons are mature is also an important issue including whether experiments were performed *in vitro* (neuronal cultures performed from E16 embryo and maintained different times *in vitro*, 5–14 days (Buisson et al., 1998; Samson et al., 2008), *ex vivo* (hippocampal slices harvested at P3 and maintained different times *in vitro*; Lemarchand et al., 2015) or *in vivo* (young versus aged animals; Roussel et al., 2009). For example, it was well-demonstrated that mouse primary cultures of cortical neurons become sensitive to NMDA-induced neuronal death only after 10 days *in vitro*, an effect potentiated by exogenous tPA (Launay et al., 2008). At early times (days *in vitro*), they require trophic factors contained in the culture media (serum) to survive (Hetman et al., 2000; Terro et al., 2000). When removed, serum deprivation led to neuronal apoptosis with a protective effect of exogenous tPA (Liot et al., 2006). Type of neurons may also be critical, with neurotoxic effects of tPA mainly described in cortical neurons (Nicole et al., 2001) or Purkinje neurons (Cops et al., 2013; Li et al., 2013; **Figures 3B,C**). The protective effect of tPA was described on hippocampal neurons (Flavin and Zhao, 2001; Echeverry et al., 2010; Lemarchand et al., 2015; **Figure 3A**) and on cortical neurons (Liot et al., 2006; Wu et al., 2013a; **Figure 3B**).

Does the Origin of tPA (Endogenous vs. Exogenous) make the Difference?

Another important point of discussion is to know whether exogenous and endogenous tPA have differential effects on neuronal survival. The most recent literature in this field demonstrates that endogenous tPA displays neuroprotective activities (Wu et al., 2013a; Lemarchand et al., 2015) and exogenous tPA is neurotoxic (Parcq et al., 2012). Nevertheless, using tPA deficient mice, exogenous tPA may also protect hippocampal neurons subjected to OGD (Lemarchand et al., 2015). These data suggest that tPA (exogenous or endogenous) may have either pro-neurotoxic or pro-survival effects depending of the type of

TABLE 1 | Reported effects of tPA on challenged neurons.

Reference	Model(s)	tPA	Mechanism(s)
Beneficial			
Kim et al., 1999	*In vitro*: cortical cultures exposure to 300 mM zinc (mice) *In vivo*: kainate injection (10 mg/kg) in rats	Exogenous 10 μg/ml Intracerebroventricular tPA 1 mg/ml	Independently of its proteolytic action, tPA attenuated zinc-induced cell death tPA attenuated kainate seizure-induced neuronal death in the hippocampus
Flavin and Zhao, 2001	*In vitro*: OGD, 2.5 h Cultured hippocampal neurons from rats (DIV 7–10)	Exogenous 1,000 IU	tPA protects neurons from oxygen glucose deprivation (OGD) by a non-proteolytic action
Centonze et al., 2002	*Ex vivo*: striatal neurons WT and tPA –/– mice subjected to OGD	Endogenous	tPA enhanced ischemia-induced neuronal damage by facilitating apoptosis rather than necrosis
Yi et al., 2004	*In vitro*: mixed cortical cell cultures (mice) Treatment: zinc (35 μmol/l)	Exogenous 10 μg/ml	tPA attenuated zinc-induced neuronal death, independently of its proteolytic activity
Head et al., 2009	*In vitro*: primary cultures of neurons (DIV 5–21) exposed to 1.4% isoflurane for 4 h *In vivo*: 1.4% isoflurane (anesthetic mediated neurotoxicity in mice)	Exogenous 0.03–3 μg/ml	Isoflurane induced apoptosis at DIV 5 (but not DIV 14 or DIV 21) in cultured neurons tPA decreases isoflurane-induced cell death in primary cultures of neurons (DIV 5) Isoflurane-induced neurotoxicity in the developing rodent brain is mediated by reduced tPA synaptic release and enhanced proBDNF/p75NTR-mediated apoptosis
Echeverry et al., 2010	*In vitro*: cultures of hippocampal neurons (OGD conditions for 30 min (preconditioning) or not, followed 24 h later by incubation under OGD conditions for 55 min)	Endogenous (tPA KO mice) and exogenous (0–1 μM)	Treatment after OGD (early preconditionning). Beneficial effect of tPA involving a LRP1 dependent signaling pathway and independent of its proteolytic activity. Treatment 24 h after OGD (delayed preconditioning): beneficial effect of tPA via a NMDA-dependent signaling pathway (activation of pAkt), and activation of plasmin
Wu et al., 2012	*In vitro:* cultures of cortical neurons (55 min OGD and then exposed 10 min later to a second episode of hypoxia (10 min OGD, post-conditioning))	Endogenous (transgenic mice T4)	Decrease of the activation of mTor- HIFα, involving NMDAR
Wu et al., 2013a	*In vivo*: excitotoxin-induced neuronal death T4 mice and WT Intrastriatal injection of NMDA (50 mM) *In vitro:* cerebral cortical neurons (mice) NMDA induced neuronal death (50 M)	T4 mice or IV 1 mg/Kg on WT mice Exogenous 5–500 nM	tPA protected the brain from excitotoxin-induced cell death Dose-dependent effect of tPA on NMDA-induced neuronal death – 5 and 10 nM beneficial – 100 at 500 nM deleterious (1) The neuroprotective effect of tPA was mediated by activation of synaptic GluN2A containing NMDAR via a plasminogen-independent mechanism (2) ERK ½ activation mediated the protective effect of tPA against excitotoxin-induced neuronal death (3) tPA activated the ERK ½-CREB-Atf3 pathway (4) Atf3-mediated the protective effect of tPA against excitotoxin-induced neuronal death
Wu et al., 2013b	*In vitro*: cultures of cortical neurons (OGD 55 min)	Endogenous (transgenic mice T4)	Adaptation to metabolic stress – AMPK activation involving NMDAR
Henry et al., 2013	*Ex vivo*: cortical brain slices from postnatal P10 mice	Exogenous (20 μg/mL)	tPA significantly reduced caspase-3 activity In superficial layers (less mature), tPA alone inhibited apoptosis via EGFR

(Continued)

TABLE 1 | Continued

Reference	Model(s)		tPA	Mechanism(s)
No effects				
Vandenberghe et al., 1998	*In vitro:* spinal cords cultures of mice tPA –/– and WT (DIV 10–12) Kainate-induced death of motoneurons (20 and 100 μM for 24 h)		Endogenous	tPA did not affect the vulnerability of cultured neurons to kainite
Tucker et al., 2000	*In vivo:* primary cultures of rat cortical neurons Treatment: Aβ (16 or 25 μM) and plasminogen (30 nM)		Exogenous 10 μg/ml	tPA required plasminogen to inhibit Aβ toxicity and to block Aβ deposition Degradation of Aβ fibrils is dependent on tPA and Plg proteolytic activity
Flavin and Zhao, 2001	*In vitro:* cultured hippocampal neurons from rats (DIV 7–10) ± NMDA 10 μM		Exogenous 1,000 IU	tPA resulted in a modest exaggeration of this injury
Yi et al., 2004	*In vitro:* mixed cortical cell cultures (mice) Treatment: NMDA (30 μmol/l)		Exogenous 10 μg/ml	Calcium-mediated neuronal death was not attenuated by tPA
Deleterious				
Tsirka et al., 1995	*In vivo:* kainate induced neuronal death	Mouse tPA –/– Mouse WT	Endogenous 120 μg tPA for 3 days (intra-parenchymal)	tPA is required to promote neuronal degeneration
Wang et al., 1999	*In vitro:* PC12 cells and primary cultures of cortical neurons (rats; DIV 12–14)		Exogenous 50 μg/ml	tPA significantly increased hemoglobin-induced cell death
Flavin and Zhao, 2001	*In vitro:* cultured hippocampal neurons on rats (DIV 7–10) ± plasminogen		Exogenous 100 IU	Proteolytic action
Nicole et al., 2001	*In vitro*: mixed cortical cultures or near-pure neuronal cultures (mice) Excitotoxicity: NMDA (10 or 12.5 μM) or 50 μM kainate Calcium imaging		Exogenous 0.2–20 μg/ml	tPA failed to modify the neurotoxicity induced by the exposure to a non-NMDA agonist (kainate) The catalytic activity of tPA enhanced neuronal death induced by exposure to NMDA tPA cleaves the GluN1 subunit of the NMDAR
	In vivo: NMDA induced excitotoxic lesions (rats) (50 nmol)		Exogenous 3.0 μg (intra-parenchymal)	
Gabriel et al., 2003	*In vitro:* cultured cortical neurons (mice) Mixed cortical cultures of neurons and astrocytes (mice)	Apoptosis: serum deprivation (DIV 7) Nifedipine (50 μM, DIV 14) Excitotoxicity (DIV 13–14) 12.5 μM of NMDA	Endogenous	TGF-α rescued neurons from NMDA-induced excitotoxicity in mixed cultures through inhibition of tPA activity, involving PAI-1 overexpression by an ERK-dependent pathway in astrocytes
Liberatore et al., 2003	*In vivo*: kainate-induced excitotoxicity on tPA –/– and WT mice (1.5 nmol of kainate)		Exogenous 1.85 μmol/L	Infusion of tPA into tPA –/– mice restored sensitivity to kainate-mediated neurotoxicity and activation of microglia
	In vivo: NMDA-induced excitotoxicity in mice (50 mmol/L NMDA)		Exogenous 46 μmol/L	tPA increased the lesion volumes induced by NMDA injection into the striatum
Liot et al., 2004	*In vitro:* pure cultures of mouse cortical neurons exposed to NMDA (12.5 μmol/L)		Exogenous 20 μg/ml	Proteolytic activity

(Continued)

TABLE 1 | Continued

Reference	Model(s)	tPA	Mechanism(s)
Liu et al., 2004	*In vitro*: primary neuronal cultures (mice; DIV 14) NMDA treatment–induced apoptosis in neurons	Exogenous 20 μg/ml	tPA potentiated apoptosis in mouse cortical neurons treated with *N*-methyl-D-aspartate (NMDA) by shifting the apoptotic pathway
Benchenane et al., 2005	*In vivo:* striatal excitotoxic lesions (rats; NMDA 50 nmol)	Exogenous IV 1 mg/kg	tPA potentiated excitotoxic lesions
Lebeurrier et al., 2005	*In vivo*: excitotoxic lesions in mice induced by NMDA (10 nmol in striatum or 20 nmol in cortex) *In vitro*: neuronal cortical cultures from mice Treatment: neuroserpin (0.5–1 μM) Serum deprivation (DIV 7) Excitotoxic paradigms (DIV 13–14) NMDA (12.5 μmol/l) AMPA (10 μmol/l) Calcium videomicroscopy	Endogenous	Overexpression of neuroserpin in the brain parenchyma might limit the deleterious effect of tPA on NMDAR-mediated neuronal death
Medina et al., 2005	*In vitro*: mouse neuroblastoma N2a cells; primary cultures of hippocampal neurons tPA –/– or WT (mouse)	Exogenous 20 μg/ml	tPA induced Erk1/2 activation in neurons (independently of plasmin), tau phosphorylation and promoted A-beta mediated apoptosis tPA treatments induced GSK3 activation, tau hyperphosphorylation, microtubule destabilization and apoptosis in hippocampal neurons
Benchenane et al., 2007	*In vivo* studies: Excitotoxic lesions in mice performed by injection of NMDA (10 nmol) into the striatum *In vivo* studies: permanent MCAO in mice	Exogenous 1 mg/kg	Immunization against the NTD of the GluN1 subunit of NMDAR prevented the neurotoxic effect of endogenous and exogenous tPA
López-Atalaya et al., 2007	*In vivo:* striatal excitotoxic lesions (rats; 50 nmol)	Exogenous IV 1 mg/kg	tPA increased lesion volumes induced by NMDA (+40%)
López-Atalaya et al., 2008	*In vitro:* pure neuronal cultures (mice) Excitotoxicity (NMDA 10 μmol/L) Calcium videomicroscopy (NMDA 12.5–100 μmol/L)	Exogenous 0.3 μmol/L	Interaction of tPA with GluN1 led to a subsequent potentiation of NMDA-induced calcium influx and neurotoxicity
Wiegler et al., 2008	*In vitro*: hippocampal slices from P12 rats (OGD 30 min) Treatment: c-Jun N-terminal kinase inhibitor (XG-102; 12 nM 6 h after OGD)	Exogenous 0.9 μg/ml	Addition of tPA after OGD enhanced neuronal death in CA1 and XG-102 administration reduced neuronal death, alone or in the presence of tPA
Sun et al., 2009	*In vitro:* cultured dopaminergic neuroblasts (rat; N27 line) Treatments: aprotinine (200 KIU/ml), ε-aminocaproic acid (2 mM), EGRck (Glu–Gly–Arg–CH2Cl, 100 mg/ml), FPRck (Phe–Pro–Arg–CH2Cl, 100 mg/ml), bivalirudin (20 mg/ml)	Exogenous 10–20 μg/ml	tPA induced N27 neuroblast cell death. Aprotinin and other protease inhibitors led to an inhibition of tPA-mediated neurotoxicity Aprotinin, FPRck, and EGRck directly antagonized the proteolytic activity of tPA, whereas ε-aminocaproic acid inhibited the binding of tPA to lysine residues on the cell surface
Baron et al., 2010	*In vitro* study: cortical and hippocampal neurons from mice (DIV 7 or DIV 12–14). Excitotoxic neuronal death (NMDA 50 μM) *In vivo*: excitotoxic lesions. Male Swiss mice Hippocampal or cortical bilateral injections of NMDA	Exogenous 20 μg/ml Exogenous IV 10 mg/kg	Catalytic tPA promoted NMDAR-induced Erk(1/2) MAPK activation tPA failed to potentiate excitotoxicity of hippocampal neurons lacking GluN2D tPA exacerbated neurotoxicity through GluN2D-containing NMDAR via Erk 1/2
Guo et al., 2011	*In vitro:* mouse cortical neurons (DIV14) Neuronal apoptosis model	Exogenous 20 μg/ml	The anticoagulant factor protein S (PS) protects mouse cortical neurons from tPA/NMDA induced injury. PS blocks the extrinsic apoptotic cascade

(Continued)

TABLE 1 | Continued

Reference	Model(s)		tPA	Mechanism(s)
Jullienne et al., 2011	*In vitro*: cortical and hippocampal neurons (mice; DIV 12–13) Excitotoxic neuronal death: NMDA (10 μM) Treatment: UBP145 (0.2 μM)		Exogenous 20 μg/mL	tPA increased NMDA-mediated neurotoxicity in cortical neuronal cultures but not in hippocampal neuronal cultures UBP145 had no effect on NMDA-mediated neurotoxicity in hippocampal neurons but prevented tPA-induced potentiation of NMDA-mediated neurotoxicity in cortical neurons
	In vivo: cortical excitotoxic lesions NMDA (mice; 2.5 nmol) Treatment: UBP145 (0.05 nmol)		Exogenous IV 10 mg/kg	Inhibition of GluN2D-containing NMDAR with UBP145 can fully prevent the pro-excitotoxic effect of intravenously administered tPA
Rodríguez-González et al., 2011	*In vitro:* primary mixed cortical cell cultures from rats (OGD 150 min)		Exogenous 5 mg/mL	Treatment with tPA after OGD increased LDH release, active MMP-9, MCP-1, and MIP-2 Treatment with neuroserpin after OGD decreased LDH release and active MMP-9
Roussel et al., 2011	*In vitro:* primary cultures of cortical neurons (mice; DIV 10) Excitotoxicity induced by 10 μM NMDA Treatment: HMGB-1 0.3 μM		Exogenous 0.3 μM	HMGB-1 reversed the pro-neurotoxic effect of tPA HMGB-1 prevented tPA from potentiating NMDA-evoked Ca^{2+} influx
Ma et al., 2012	*In vitro:* cultures of cortical neurons (rats; OGD/R) Treatment: neuroserpin		Endogenous	Neuroserpin protected neurons against OGD/R. mainly by inhibiting tPA-mediated acute neuronal excitotoxicity
Montagne et al., 2012	*In vitro:* cortical cultures of neurons from mice (DIV 12–13) Treatment: memantine (1–10 μmol/L)	Excitotoxicity NMDA (10 μmol/L) OGD (30 min) Calcium videomicroscopy NMDA (50 μmol/L)	Exogenous 0.3 μmol/L	Memantine prevented the potentiation of excitotoxic neuronal death induced by rtPA Memantine prevented rtPA-exacerbated calcium influx through activated NMDAR
	In vitro: cultures of cortical neurons from mice (DIV 15–16) Excitotoxic neuronal death: NMDA 50 μM		Exogenous 0.3 μM	In contrast to WT tPA, tPA mutants including deletion of the kringle 2 domain and point mutation of the LBS-containing kringle 2 domain did not promote NMDAR-mediated neurotoxicity
Parcq et al., 2012	*In vitro*	Excitotoxicity induced by exposure of cortical neurons to NMDA (mice; 50 μM) at DIV 14 NMDA-induced calcium influx recorded from cultured cortical neurons (mice; DIV 12–14) exposed to NMDA (50 μM)	Exogenous 0.3 μM	sc-tPA promoted NMDAR-mediated neurotoxicity through its proteolytic activity, tc-tPA did not sc-tPA promoted both NMDA-induced calcium influx and Erk (1/2) activation, tc-tPA did not
	In vivo	NMDA-induced excitotoxic brain lesions (NMDA 10 mM)	Exogenous 45 μM	
Henry et al., 2013	*Ex vivo:* cortical brain slices from postnatal P10 mice		Exogenous 20 μg/mL	In deeper layers (more mature), tPA was associated with glutamate-promoted neuronal necrosis
Omouendze et al., 2013	*In vivo:* excitotoxic insult by intra-cortical injection of Ibotenate in rats PAI-1 or tPA –/– or WT *Ex vivo*: brain sections		Endogenous or exogenous 20 μg/ml	Neonatal brain lesions

stress paradigms used and/or the type of neurons. Thus, whether experiments are performed on wild type neurons, tPA deficient neurons, tPA over-expressing neurons, *in vitro* and *in vivo*, is important to understand the impacts of tPA on neuronal survival (Tsirka et al., 1995; Wang et al., 1998; Nicole et al., 2001; Liot et al., 2006; Echeverry et al., 2010; Wu et al., 2013a).

WHAT ABOUT THE LEVEL OF tPA?

Some authors suggest that low levels of tPA are neuroprotective (Wu et al., 2013a), either exogenous (Baron et al., 2010) or produced by stressed cells (Lemarchand et al., 2015). In contrast, high levels of tPA (mainly exogenous) are neurotoxic (Nicole et al., 2001; Parcq et al., 2012).

Finally, Why not the Form of tPA?

There is so far only one study which discriminated tPA isoforms in the context of neuronal survival, with a clear evidence that sc-tPA is the only one capable to activate NMDAR and to promote excitotoxicity (in mouse cortical neurons subjected to NMDA exposure) both *in vitro* and *in vivo* (Parcq et al., 2012; Bertrand et al., 2015). It is thus interesting to note, that complexes formed between sc-tPA and neuroserpin (NSP) were reported more stable than those formed between tc-tPA and NSP, with no differences when complexes are formed with PAI-1 (Barker-Carlson et al., 2002). Whether conversion of sc-tPA into tc-tPA (by plasmin like activity) may influence the functions of tPA on neuronal survival, especially in the context of brain injuries, need to be investigated.

CONCLUSION

Depending on the study, endogenous tPA was reported as deleterious or beneficial for neurons. Although it is difficult to reconcile these findings, some propose that tPA is neuroprotective at low levels, but neurotoxic at higher levels. Assays of extracellular levels of tPA under specific conditions should be provided to support this hypothesis. Undoubtedly, the target involved is also a key trigger in the effect of tPA. In general, the pro-survival effects of tPA are independent on its proteolytic activity involving, interconnected or independently, EGF receptors, annexin II, PI-3 kinase-, AMPK-, mTor-HIF-1alpha-dependent signaling pathways. In the adult, the neurotoxic effects of tPA seem to be dependent on its proteolytic activity, targeting either plasminogen, NMDARs, components of the extracellular matrix, inflammatory mediators, and/or other proteases. However, indirect neurotoxicity might also occur via a non-proteolytic activation of microglia (Siao and Tsirka, 2002). For now, there is no clear clinical data to determine, in human, whether tPA is neurotrophic or neurotoxic and in what conditions. Additional studies are needed to understand further the possible differential functions of tPA on neuronal survival. To address this question, we should consider the different isoforms of tPA (type I sc-tPA, type I tc-tPA, type II sc-tPA, and type II tc-tPA), the possibility that tPA may activate its substrates and/or receptors with differential affinities and that these substrates and/or receptors could be differentially expressed in cortical versus hippocampal neurons depending on their maturity.

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frontiers in Cellular Neuroscience

REVIEW
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Tissue-type plasminogen activator is a neuroprotectant in the central nervous system

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Tissue-type plasminogen activator (tPA) is a serine proteinase found not only in the intravascular space but also in a well-defined sub-set of neurons in the brain. tPA is rapidly released from neurons after either exposure to hypoxia or hypoglycemia *in vitro*, or the induction of cerebral ischemia *in vivo*. It has been proposed that tPA has a neurotoxic effect in the ischemic brain. However, recent evidence indicate that once released into the synaptic cleft tPA activates specific cell signaling pathways that promote the detection and adaptation to metabolic stress. More specifically, the non-proteolytic interaction of tPA with N-methyl-D-aspartate receptors (NMDARs) and a member of the low-density lipoprotein receptor (LDLR) family in dendritic spines activates the mammalian target of rapamycin (mTOR) pathway that adapts cellular processes to the availability of energy and metabolic resources. TPA-induced mTOR activation in neurons leads to hypoxia-inducible factor 1α (HIF-1α) accumulation, HIF-1α-induced expression and membrane recruitment of the neuronal transporter of glucose GLUT3, and GLUT3-mediated uptake of glucose. These and other data discussed in this Review suggest that the postulated neurotoxic effect of tPA needs to be reconsidered and instead indicate the emergence of a new paradigm: that tPA is an endogenous neuroprotectant in the central nervous system (CNS).

Keywords: cerebral ischemia, neurovascular unit (NVU), middle cerebral artery occlusion (MCAo), tissue-type plasminogen activator (tPA), neuroprotection, excitotoxicity, plasminogen

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Introduction

Tissue-type plasminogen activator (tPA) is a serine proteinase of 527 residues. It has an A chain with a finger, an EGF and two kringle domains, and a B chain with a protease domain. tPA is secreted as a single chain molecule (sctPA), but cleavage by plasmin at its Arg275-Ile276 peptide bond generates two-chain tPA (tctPA). sctPA is not a zymogen and in the presence of fibrin is almost as active as tctPA (Rijken et al., 1982).

Our understanding of tPA's function in the brain has evolved rapidly. Indeed, although it was initially believed that endothelial cells (ECs) were the sole source of tPA and that its unique role was to catalyze the conversion of plasminogen into plasmin in the intravascular space, today we know that tPA is expressed in the brain parenchyma, where it has several functions, many of them independent of its ability to catalyze the conversion of plasminogen into plasmin.

tPA in the Brain

To better understand tPA's effect on neuronal survival is pivotal to consider its concentration in the brain parenchyma and to study its expression in the context of the neurovascular unit (NVU).

Concentrations of tPA

The concentration of tPA in the intravascular space is 5 μM/L which correspond to approximately 70 pM (Nicoloso et al., 1988). In contrast, tPA's concentration in the extracellular space of the brain is more difficult to quantify. However, taking into consideration the volume of brain tissue supplied by the middle cerebral artery (MCA) in the C57BL/6 mouse, the release of tPA from neuronal cultures (Echeverry et al., 2010), the area of the synaptic cleft, the concentration of tPA in the brain tissue under ischemic conditions and following the intravenous (IV) administration of recombinant tPA (rtPA; Haile et al., 2012), and a 60% contraction of the interstitial space in the ischemic brain (Hansen and Olsen, 1980), the extracellular concentration of tPA in the brain 3 h after MCA occlusion (MCAo) and IV treatment with either saline solution or 0.9 mg/Kg of rtPA is not superior to 1 nM and 2–4.5 nM, respectively. Although in all likelihood this is an overestimation and many other factors need to be considered to improve the accuracy of these calculations, it is clear that an experimental design with concentrations of tPA superior to 5 nM does not resemble tPA's concentrations in an *in vivo* system, even after the administration of rtPA. This point is of key importance for the translatability to the clinic of results obtained in basic sciences laboratories dedicated to the study of tPA's effect on neuronal survival.

tPA in the Neurovascular Unit (NVU)

The NVU is a dynamic structure assembled by ECs surrounded by a basement membrane (BM) encased by perivascular astrocytes (PVA). Synapses are found approximately 13 μm away from the ECs (Zhang et al., 2005). This concept is highly relevant because following its IV administration rtPA permeates the brain parenchyma (Haile et al., 2012) potentially entering in contact with the synaptic space. Although microglia and pericytes should also be considered part of the NVU, a discussion of tPA's expression and function in these cells is beyond the scope of this Review.

tPA in Endothelial Cells

Early studies in the non-human primate brain detected tPA antigen in a small fraction of ECs of the microvasculature, 90% of them pre-capillary arterioles and post-capillary veins (Levin and del Zoppo, 1994). Despite the importance of these findings, since the publication of this report more than 20 years ago, no further attempts have been made to study with new antibodies the expression of tPA in the brain vasculature. Nevertheless, it has been proposed that PVA regulate the expression of tPA mRNA in ECs (Tran et al., 1998) and that a number of stimuli including membrane depolarization induce its release into the intravascular space.

tPA in the Basement Membrane-Astrocyte (BM-A) Interface

tPA is abundantly expressed in PVA and its release into the BM-A interface has multiple effects including increase in the permeability of the blood-brain barrier (BBB; Yepes et al., 2003), detachment of astrocytic end-feet processes from the BM (Polavarapu et al., 2007), and induction of an NF-κB-regulated pro-inflammatory response (Zhang et al., 2007).

Neuronal tPA

Neurons are a major source of tPA synthesis *in vivo*. Studies using *in situ* zymography assays (Sappino et al., 1993) have detected tPA-catalyzed proteolysis in well-defined areas of the adult brain, namely hippocampus, hypothalamus, thalamus, amygdala, cerebellum and meningeal blood vessels. Intriguingly, tPA mRNA is found in several areas of the brain that do not display tPA-catalyzed enzymatic activity. That is the case of the cerebral cortex and the hippocampus. In the latter structure although tPA mRNA is widely expressed throughout all cell layers, only the CA2 and CA3 layers and the dentate gyrus (DG) show tPA-catalyzed proteolysis. It has been postulated that this discrepancy between tPA catalyzed-proteolysis and tPA mRNA expression may be due either to the localized expression of tPA inhibitors in those areas where tPA-catalyzed proteolysis is not observed, or to the accumulation of tPA protein at distant places of its synthesis, or to posttranslational regulation of tPA expression, or to the possibility that in some areas of the brain tPA has functions that do not require the generation of plasmin. Independently of the reason for this divergence, a substantial body of experimental evidence indicates that tPA is released at the neuronal growth cone (Krystosek and Seeds, 1981) and mediates neuronal migration (Seeds et al., 1999) and neurite outgrowth and remodeling during development (Lee et al., 2014) and in the ischemic brain (Shen et al., 2011). Likewise, tPA promotes the development of synaptic plasticity in *in vitro* and *in vivo* models of long-term potentiation (Qian et al., 1993), learning (Seeds et al., 1995, 2003), stress-induced anxiety (Pawlak et al., 2003), and visual cortex plasticity (Müller and Griesinger, 1998). Moreover, it has also been proposed that tPA plays a role on axonal remodeling after stroke (Liu et al., 2012).

The Neurotoxic Effect of tPA

History

One of the first achievements in our understanding of tPA's role in the brain was provided by the observation that it is induced as an immediate-early gene during seizures and long-term potentiation, and to the identification of a functional link between tPA and N-methyl-D-aspartate receptors (NMDAR; Qian et al., 1993). Shortly thereafter it was found that membrane depolarization induces the rapid release of neuronal tPA by a mechanism that does not involve tPA mRNA or protein synthesis but requires the influx of Ca^{+2} (Gualandris et al., 1996). It was quickly recognized that the conditions associated with the release of neuronal tPA (i.e., membrane depolarization, increase in the intracellular concentrations of Ca^{+2} and NMDAR

activation) also underlie the pathophysiological events observed in several neurological diseases such as cerebral ischemia, head trauma and seizures. This led to study in animal models of experimental cerebral ischemia changes in tPA activity in the brain tissue following MCAo. Although the interpretation of the initial studies was confusing, soon was evident that tPA activity increases rapidly in the ischemic tissue after MCAo (Wang et al., 1998; Yepes et al., 2003), and that this surge disappears at later time points when plasminogen activator inhibitor-1 (PAI-1) antigen is detected (Hosomi et al., 2001).

These reports were rapidly followed by the seminal observation that mice genetically deficient in tPA ($tPA^{-/-}$) in all the cellular components of the NVU have a ∼41% decrease in the volume of the ischemic lesion following MCAo (Wang et al., 1998; Nagai et al., 1999). The impact of these findings was contrasted by a later report from a different group of investigators using a similar experimental design that found just the opposite: an increase in the volume of the ischemic lesion in $tPA^{-/-}$ mice (Tabrizi et al., 1999). Shortly thereafter a group of researchers discovered neuroserpin (Osterwalder et al., 1996), an axonally secreted serine proteinase inhibitor preferentially expressed in neurons (Hastings et al., 1997). This finding was followed by biochemical studies that revealed that although neuroserpin is an efficient inhibitor of plasminogen activators and plasmin, it has a higher affinity for tctPA (k_i: 6.2×10^5 M^{-1} S^{-1}), than either sctPA (k_i: 8.0×10^4 M^{-1} S^{-1}), or high molecular weight uPA (k_i: 2.5×10^4 M^{-1} S^{-1}), or low molecular weight uPA (k_i: 9.2×10^3 M^{-1} S^{-1}), or plasmin (k_i: 3.6×10^2 M^{-1} S^{-1}; Hastings et al., 1997). These observations led to postulate neuroserpin as the inhibitor of tPA in the brain (Yepes and Lawrence, 2004). With this premise in mind, later studies with animal models of ischemic stroke revealed that either treatment with recombinant neuroserpin (Yepes et al., 2000) or genetic overexpression of neuroserpin (Cinelli et al., 2001) decreases the volume of the ischemic lesion following MCAo. Despite the discrepancies between animal studies with $tPA^{-/-}$ mice, these results helped to advance the concept that tPA has a neurotoxic effect in the ischemic brain. Since then several research groups have reported potential mechanisms for tPA's harmful effects and some have attempted to develop therapeutic strategies to antagonize it.

Clinical Relevance

The idea borne from basic sciences laboratories that tPA has a neurotoxic effect in the ischemic brain was in striking opposition to an almost simultaneous publication by the National Institute of Neurological Disorders and Stroke (NINDS) of a clinical study indicating that IV treatment with rtPA leads to complete or nearly complete neurological recovery in a significant number of acute ischemic stroke patients (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995). Notably, although since then several clinical studies have shown that besides improving neurological outcome rtPA also increases the risk of intracerebral hemorrhage (Hacke et al., 2004) and augments the permeability of the BBB (Kidwell et al., 2008), to this date and after more than 100,000 rtPA-treated acute ischemic stroke patients, no clinical study has shown a neurotoxic effect for rtPA. The translational impact of this disagreement between basic and clinical research has been heightened by the observation that following its IV administration rtPA crosses the BBB and permeates the ischemic tissue (Haile et al., 2012). In conclusion, while basic researchers suggest that clinicians are treating acute ischemic stroke patients with a neurotoxic agent, clinicians are developing protocols to increase the number of rtPA-treated patients. This discrepancy needs to be resolved promptly because it has questioned the translatability into the clinic of tPA's basic research.

A New Look at Old Data

The translational importance of the findings described above warrants a second examination of the published data with the help of information that was not available when the original results were published.

Does Genetic Deficiency of tPA Decrease the Volume of the Ischemic Lesion after Experimental Induction of Ischemic Stroke?

There is a growing awareness among basic science stroke researchers that a large number of factors besides the actual MCAo influence the quantification of the volume of the ischemic lesion in experimental models of ischemic stroke (Sena et al., 2010). With this in mind, it is not surprising to find that while some studies have reported a decrease (Wang et al., 1998), others have found an increase (Tabrizi et al., 1999) in the volume of the ischemic lesion in $tPA^{-/-}$ mice following MCAo. A similar reasoning applies to studies reporting either a deleterious (Wang et al., 1998), or beneficial (Wu et al., 2012), or lack of effect (Klein et al., 1999) on the volume of the ischemic lesion in animals treated with rtPA after MCAo.

In the analysis of these reports is important to consider that the earlier studies (Wang et al., 1998; Tabrizi et al., 1999) were criticized by their use of mice with two different genetic backgrounds: C57BL/6 and SV129. However, the validity of these concerns is difficult to evaluate because although it was initially believed that these two strains of mice have different vulnerability to the harmful effects of cerebral ischemia and excitotoxicity (Carmichael, 2005), later studies using magnetic resonance imaging (MRI) indicated that they are equally susceptible to both forms of injury (Pham et al., 2010). Thus it is problematic to invoke this factor to explain the opposite results observed in these studies. It also should be kept in mind that although the currently available $tPA^{-/-}$ mice lack the protease domain they still harbor tPA's finger, EFG and kringle domains. This fact has gained significant relevance over the last 10 years with the identification of several functions of tPA in the brain that do not require the conversion of plasminogen into plasmin. Therefore, it is difficult to assure that the finger and kringle domains do not have an effect in the final outcome of the ischemic injury in $tPA^{-/-}$ mice.

It is also relevant to consider that $tPA^{-/-}$ mice are deficient on tPA in all the cellular components of the NVU. Because tPA plays an unique and different role in ECs, PVA and neurons, it is difficult to determine whether the final outcome observed

in $tPA^{-/-}$ mice after MCAo is due to the effect of the lack of tPA on either the permeability of the BBB, or neuronal survival, or microglial activation. These are highly dynamic and variable processes that need to be dissected to have a better understanding of tPA's role in the ischemic brain. A step to address this problem was recently taken by a group of investigators that reported that mice overexpressing tPA only in neurons have ∼40% decrease in the volume of the ischemic lesion following MCAo (Wu et al., 2012). Based on these data and our knowledge that tPA increases the permeability of the BBB in rodents (Yepes et al., 2003) and humans (Kidwell et al., 2008), is plausible to postulate that the decrease in the volume of the ischemic lesion following MCAo observed by some investigators in $tPA^{-/-}$ mice may be due to abrogation tPA-induced increase in the permeability of the BBB instead of a direct effect on neuronal survival.

The data obtained from mice overexpressing neuroserpin and rats treated with recombinant neuroserpin after MCAo also deserves careful attention. Certainly, the observation of unchanged tPA activity in animals genetically deficient in neuroserpin (Madani et al., 2003) and the report that neuroserpin also decreases the volume of the ischemic lesion in $tPA^{-/-}$ mice (Wu et al., 2010), argue against tPA inhibition as a mechanism whereby neuroserpin decreases the volume of the ischemic lesion in these animals.

In summary, a new look at the available data suggests that the determination of the volume of the ischemic lesion in $tPA^{-/-}$ mice or in animals either overexpressing neuroserpin or treated with recombinant neuroserpin after MCAo may not be sufficient evidence to conclude that tPA has a neurotoxic effect in the ischemic brain. Instead, data obtained from studies using animals overexpressing tPA only in neurons suggest that tPA may have a neuroprotective effect.

Does tPA Induce Neuronal Death?

A strategy to examine the effect of tPA on neuronal survival without the confounding effects of tPA derived from other cellular components of the NVU is to study cell death in neuronal cultures incubated with rtPA. Data obtained with this experimental design unambiguously indicate that rtPA, even at very high concentrations, does not induce neuronal death (Wu et al., 2013). To reconcile these observations with the harmful effect of tPA reported by some studies with $tPA^{-/-}$ mice subjected to MCAo, some investigators postulated the hypothesis that while tPA does not directly cause neuronal death, it could act as a mediator of the deleterious effects of other agents known to cause neuronal death in the ischemic brain. This hypothesis is supported by studies performed with an animal model of neurodegeneration, namely the nervous (nr) mutant mouse, indicating that Purkinje cell degeneration correlates with high levels of tPA activity in the cerebellum (Li et al., 2006, 2013), and that mice overexpressing tPA in neurons develop Purkinje cell damage and ataxia (Cops et al., 2013). Although these studies do not indicate that tPA directly induces neuronal death, they suggest that under certain conditions tPA may facilitate the neurotoxic effect of other agents. Because the excitotoxic release of neurotransmitters underlies neuronal death in both, the ischemic brain and neurodegenerative diseases, then the obvious question was whether tPA mediates excitotoxin-induced neuronal death.

Does tPA Mediate Excitotoxin-Induced Neuronal Death?

The term excitotoxicity was coined in 1969 to describe the harmful effect on neuronal survival caused by prolonged and excessive activation of receptors of excitatory neurotransmitters (Olney, 1969). Since then it has been demonstrated that glutamate-induced excitotoxicity activates signaling pathways that lead to neuronal death in several neurological diseases including cerebral ischemia, seizures and head trauma (Choi, 1988). To study whether tPA mediates excitotoxin-induced neuronal death a group of researchers quantified neuronal demise in the hippocampus of $tPA^{-/-}$ mice injected into the CA1 layer with kainic acid (KA), an excitatotoxic glutamate analog. Using this experimental paradigm these investigators reported extensive neuronal loss in the CA1 region of Wt but not $tPA^{-/-}$ mice (Tsirka et al., 1995), and subsequent studies led them to propose that this effect was mediated by plasmin-induced proteolysis of laminin in the extracellular matrix (Chen and Strickland, 1997). These studies originated the idea that although under non-ischemic conditions tPA is pivotal for neurophysiological processes required for normal brain function such as neurite outgrowth (Krystosek and Seeds, 1981), learning (Seeds et al., 2003) and memory (Baranes et al., 1998), during cerebral ischemia it plays a completely opposite role as a mediator of excitotoxin-induced neuronal death.

In the murine hippocampus tPA protein is abundantly expressed in the CA2 and CA3 but not the CA1 layer (Salles and Strickland, 2002), which is known to have a high vulnerability to KA-induced cell death (Tsirka et al., 1995). This concept acquired special relevance when the same group of investigators found that the injection of KA causes a transient increase in tPA activity in some cells of the CA1 layer (Salles and Strickland, 2002). The striking observation was that those cells that exhibited an increase in tPA activity also survived the excitotoxic injury. Thus, when analyzed together with a previous report by a different research group (Kim et al., 1999) indicating that tPA protects hippocampal cells from the harmful effects of KA, it is clear that a casual link between tPA and excitotoxin-induced neuronal death needs was not clear yet. Importantly, this group of investigators also observed $tPA^{-/-}$ mice are resistant to KA-induced seizures (Tsirka et al., 1995). To analyze these observations is important to keep in mind that the onset and spreading of seizures throughout the brain requires the development of structural and functional changes in the synapse that allow the spread of the abnormal electrical activity throughout neuronal circuits. Thus, the higher resistance to pharmacologically-induced seizures in $tPA^{-/-}$ mice could also be explained by the lack of tPA-induced synaptic changes needed for the onset and spread of a seizure.

To gain perspective for the analysis of these data is important to keep in mind that the intrahipocampal injection of KA also induces prolonged seizures, that although very often remain clinically unrecognized, cause widespread hippocampal

cell death, particularly in the CA1 layer (Meldrum, 2002). Thus, with this experimental paradigm is difficult to analyze whether tPA mediates KA- or seizures-induced neuronal death. To answer this question, a research group used two different experimental approaches. In the first, they studied the clinical and electrophysiological spread of seizures throughout the limbic system in $tPA^{-/-}$ and Wt mice injected with KA into the amygdala (Yepes et al., 2002). The fact that KA was not injected directly into the hippocampus allowed these researchers to dissect KA- from seizures-induced hippocampal neuronal death. These experiments indicated that genetic deficiency of tPA is associated with slower progression of KA-induced seizure activity throughout the limbic system and a decrease in seizures-induced hippocampal cell death. In the second approach, they used an animal model in which transient occlusion of both common carotid arteries causes hippocampal cell death (Echeverry et al., 2010). With this paradigm these investigators were able to evaluate the effect on neuronal survival of endogenous glutamate released from hippocampal neurons in response to an ischemic insult. These studies showed that, as it was demonstrated years before in animals treated with KA (Salles and Strickland, 2002), hippocampal ischemia also causes an increase in tPA activity in the hippocampal CA1 layer. Remarkably, these investigators found that, as was also reported by the earlier studies with KA-injected mice, CA1 neurons that exhibit an increase in tPA activity also survive the ischemic injury. In agreement with these observations, it was found that hippocampal neurons of $tPA^{-/-}$ mice are more vulnerable to the ischemic injury than those of their Wt littermate controls. Together, these data suggested a neuroprotective role of tPA against ischemia-induced excitotoxic neuronal death.

To study the effect of tPA on excitotoxin-induced neuronal death without the confounding effect of seizures, a group of researchers injected NMDA, another glutamate analog, into the striatum followed by IV treatment with rtPA. The advantage of this model is that the injection of NMDA into the striatum causes neuronal death but not seizures. Using this approach, these investigators (Reddrop et al., 2005) found that 10 mg/Kg/IV of rtPA increases the volume of the necrotic lesion caused by the injection of NMDA. Moreover, the same group of investigators measured the volume of the necrotic area induced by the direct intrastriatal injection of NMDA alone or in the presence of 3 μg of either rtPA or desmoteplase, the plasminogen activator from the vampire bat *Demodus rotundus* (Liberatore et al., 2003), and found that tPA but not desmoteplase increased NMDA-induced neuronal death.

Together, these studies were interpreted as another demonstration that tPA enhances excitotoxin-induced neuronal death. Intriguingly, the dose of tPA used for the intracerebral injections was unusually large (3 μg) and at this moment is difficult to elucidate why desmoteplase did not potentiate NMDA's effect. More importantly, the dose of IV rtPA administered by these researchers is widely used by many investigators because it is believed that rodent plasminogen is 10-fold more resistant than human plasminogen to the catalytic action of rtPA. However, this concept has been challenged, and later studies with animal models of embolic stroke indicate that the dose of rtPA used to treat acute ischemic stroke patients (0.9 mg/Kg/IV) is also effective in rodents (Haelewyn et al., 2010). This has proved to be a very important fact because a different group of investigators using the same experimental paradigm demonstrated that treatment with 0.9 mg/Kg/IV of rtPA has just the opposite effect: a decrease in the volume of the necrotic lesion induced by the intrastraiatal injection of NMDA (Wu et al., 2013). In line with these observations, the damage induced by the intrastrial injection of NMDA was significantly attenuated in mice overexpressing tPA in neurons.

These studies underscored the need to investigate the effect of different doses of tPA on excitotoxin-induced neuronal death. With this concept in mind, this group of investigators quantified neuronal survival in Wt cerebral cortical neurons incubated with NMDA in the presence of 0–500 nM of either proteolytically active tPA or a mutant of tPA with an alanine for serine substitution at the active site Ser481 that renders it unable to catalyze the conversion of plasminogen into plasmin (proteolytically inactive tPA; Wu et al., 2013). These experiments indicated that tPA causes a modest increase in NMDA-induced neuronal death only at doses greater than 100 nM which, as it was discussed above, result in a concentration of tPA not found in an *in vivo* system, even after treatment with rtPA. In sharp contrast with these findings, the most important observation was that at concentrations found in the ischemic brain, tPA attenuates NMDA-induced neuronal death by a mechanism that does not entail plasmin generation but requires a co-receptor function by a member of the low-density lipoprotein receptor (LDLR) family, most likely LRP1.

What is the Nature of tPA's Interaction with NMDA Receptors (NMDARs)?

Despite abundant experimental evidence indicating that NMDARs mediate excitotoxin-induced neuronal death, clinical studies with ischemic stroke patients treated with NMDAR blockers were disappointing, showing either lack of or, in some cases, harmful effects that precluded their use (Davis et al., 2000). To understand this divergence between basic and clinical research is important to take into account that NMDARs are assembled by obligatory NR1 sub-units that interact with NR2A—D sub-units, generating a specific sub-unit composition that determines the function of the receptor. Hence, while NR2A-containing NMDARs are located in the synapse and their activation has been coupled to neuronal survival (Liu et al., 2007), most of the NR2B- and NR2D-containing NMDARs are extrasynaptic and linked to the activation of cell-death pathways (Hardingham and Bading, 2010).

Although a functional association between tPA and NMDARs has been identified by several researchers, it was not until 2001 when a group of investigators reported that at ∼280 nM of tPA enhances NMDA-induced neuronal death via a plasminogen-independent proteolytic cleavage of the NR1 sub-unit of the NMDAR that leads to increase in Ca^{+2} permeability (Nicole et al., 2001). These striking results led to postulate the NR1 sub-unit as a therapeutic target to antagonize the purported deleterious effect of tPA on excitotoxin-induced neuronal death. Unfortunately, since then a number of investigators

have been unable to replicate these findings (Matys and Strickland, 2003; Samson et al., 2008; Wu et al., 2013). The controversy has been further enhanced by an elegant study indicating that the interaction between tPA and NMDARs does not involve the cleavage of the NR1 sub-unit but instead that it requires a co-receptor function of a member of the LDLR family (Samson et al., 2008). Additionally, several investigators have convincingly demonstrated that NMDARs are a discrete proteolytic target for plasmin but not for tPA (Matys and Strickland, 2003; Yuan et al., 2009; Echeverry et al., 2010).

A further advance in our understanding of tPA's interaction with the NMDAR was provided by subsequent studies indicating that at concentrations likely to be found in an *in vivo* system tPA protects neurons against excitotoxin-induced cell death (Wu et al., 2013). Furthermore, the same group demonstrated that 1–10 nM of tPA induces phosphorylation but not cleavage of the NR2A sub-unit of the NMDAR at Y1325, and that this event leads to ERK 1/2-mediated activation of the cAMP response element binding protein (CREB) and induction of the neuroprotective effect of the CREB-regulated activating transcription factor 3 (Atf3). In line with these observations Atf3's down-regulation abrogated tPA's protective effect against excitotoxin-induced neuronal death. In this study tPA induced phosphorylation, but not cleavage, of extrasynaptic NR2B sub-units at Tyr1472 only at concentrations greater than 200 nM. These findings were also observed in *in vivo* studies with mice overexpressing neuronal tPA. This work also indicated that the non-proteolytic interaction between tPA and NMDARs requires the co-receptor function of a member of the LDLR family. An important point brought to light by these studies is the existence of a dose-dependent effect of tPA on NMDAR phosphorylation, where a protective NR2A-mediated response is activated by concentrations of tPA expected in an *in vivo* system, while a harmful NR2B-mediated neurotoxic pathway is triggered by concentrations of tPA unlikely to be found in the synapse.

In summary, a link between tPA and excitotoxin-induced neuronal death is not clear yet. Furthermore, although the functional association between tPA and NMDARs has been substantiated by a large number of studies, the mechanism underlying this interaction and its impact on cell survival remain contentious. The resolution of this controversy if of the utmost importance not only for the clinical implications of these findings but also for their obvious repercussions on the conceptual advancement of the field of tPA research.

The Emergence of a New Paradigm: tPA is a Neuroprotectant in the Central Nervous System (CNS)

Several lines of evidence suggest that tPA could instead be a neuroprotectant in the central nervous system (CNS). This concept, that was already suggested by earlier studies indicating that tPA protects neurons from excitotoxin-, seizures- (Kim et al., 1999) and serum deprivation-induced cell death (Liot et al., 2006), has been further advanced by more recent work with lower doses of rtPA and mice overexpressing tPA only in neurons.

tPA Promotes Survival in Neurons Exposed to Oxygen and Glucose Deprivation (OGD)

To study tPA's effect on neuronal survival we designed an *in vitro* model of ischemic stroke in which we quantified cell survival in Wt cerebral cortical neurons treated with 5 nM of rtPA between 5 min and 6 h after exposure to 55 min of oxygen and glucose deprivation (OGD). Our data indicated that treatment with rtPA protects cerebral cortical neurons from OGD-induced cell death. This effect is independent of tPA's proteolytic activity and although is abrogated by NMDAR and LRP1 antagonism with MK-801 and the receptor-associated protein (RAP), respectively (Echeverry et al., 2010), it remains unchanged by inhibition of the tyrosine kinase receptor B (TrkB), the cognate receptor of brain-derived neurotrophic factor (BDNF). Intriguingly, the detection of a maximal neuroprotective effect in cells treated within the first 3 h after OGD, and still present albeit with less intensity in cells treated 6 h after the hypoxic insult, has a notable resemblance with the probability of neurological recovery observed in acute ischemic stroke patients treated with rtPA (Hacke et al., 2004).

The obvious lack of a clot in our *in vitro* system indicated that a mechanism other than thrombolysis mediates tPA's neuroprotective effect. To better characterize these results Wt mice underwent transient MCAo followed immediately after reperfusion by treatment with 0.9 mg/Kg/IV of rtPA or a comparable volume of saline solution, and quantification of the volume of the ischemic lesion 24 h later. We found that treatment with rtPA decreases the volume of the ischemic lesion. However, because MCAo was performed with a nylon suture instead of a clot, it was evident that, as suggested by our *in vitro* studies, the mechanism of this effect is independent of tPA's ability to generate plasmin. This was further confirmed by our finding that treatment with rtPA also decreases the volume of the ischemic lesion in mice genetically deficient on plasminogen ($Plg^{-/-}$). These data indicated that tPA has a neuroprotective effect in the ischemic brain that is not mediated by the generation of plasmin and instead requires the co-receptor function of the NMDAR and a member of the LDLR family.

tPA-induced Neuroprotection: A Proteomics Approach

These results implied the activation of a neuroprotective cell signaling pathway by tPA. 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 with 5 nm of either proteolytically active or inactive rtPA. We found that compared to vehicle (control)-treated neurons, treatment with rtPA causes a >50% change in abundance in 589 unique neuronal proteins, by a mechanism that does not involve plasmin generation. Further studies with the Ingenuity Pathway Analysis tool showed that most of these proteins belong either to the oxidative phosphorylation (72 out of 160 proteins) or the

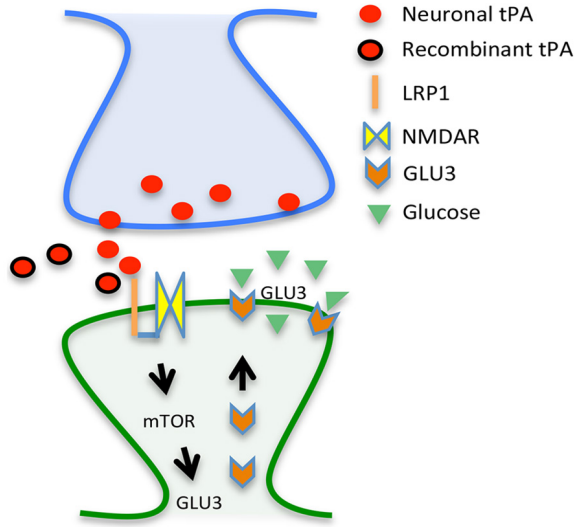

FIGURE 1 | Proposed mechanism for tPA-induced neuroprotection. Tissue-type plasminogen activator (tPA) either released from the presynaptic terminal (red circles) or given as a treatment (rtPA; red circles with a black margin) interact with a co-receptor formed by the low density lipoprotein receptor-related protein (LRP1; orange line) and the NMDA receptor (yellow squares) on the surface of the dendritic spine (post-synaptic compartment), leading to mTOR activation and mTOR-induced synthesis of the glucose transporter GLU3 (orange arrows) with the resultant increase in the uptake of glucose (green triangles).

mammalian target of rapamycin pathways (mTOR; 53 of 201 proteins) pathways. In agreement with these results, Western blot analysis indicated that either treatment of Wt cerebral cortical neurons with rtPA, or the IV administration of rtPA after the onset of cerebral ischemia, induces mTOR activation. More importantly, mTOR inhibition with rapamycin abrogated tPA-induced neuroprotection *in vitro* in cerebral cortical neurons exposed to OGD conditions and *in vivo* in Wt mice subjected to MCAo (Wu et al., 2012). Together, these data indicated that tPA's neuroprotective effect is mediated by its ability to activate the mTOR pathway.

tPA Promotes the Detection and Adaptation to Metabolic Stress

Hypoxia-inducible factor-1α (HIF-1α) is a trascription factor that plays a central role in hypoxia sensing and adaptation. HIF-1α expression is regulated by mTOR, and its accumulation has a neuroprotective effect in the ischemic brain (Shi, 2009). We found that either incubation with 5 nM of rtPA or the IV administration of 0.9 mg/Kg of rtPA induces HIF-1α accumulation in cultured neurons and in the ischemic brain, respectively. Furthermore, shRNA-induced HIF-1α down-regulation abrogated the neuroprotective effect of rtPA in neurons exposed to OGD conditions (Wu et al., 2012). More importantly, we found that tPA-induced mTOR-mediated HIF-1α accumulation leads to the recruitment of the neuronal transporter of glucose GLU3 to the neuronal plasma membrane. In line with these observations, tPA induced the uptake of glucose both in Wt cerebral cortical neurons *in vitro* and the ischemic brain *in vivo*.

Proposed Mechanistic Model for tPA-Induced Neuroprotection

Based on these data and other results not discussed here (An et al., 2014), we propose a model whereby tPA either released in the synaptic cleft following the onset of cerebral ischemia or intravenously administered interacts with LRP1, leading to NMDAR-mediated mTOR activation, mTOR-induced HIF-1α accumulation, HIF-1α-induced recruitment of the neuronal transporter of glucose GLUT3 to the neuronal plasma membrane, and GLUT3-mediated uptake of glucose by neurons in the ischemic brain (**Figure 1**). In summary, here we propose a mechanistic model whereby tPA is a neuroprotectant in the ischemic brain by its ability to promote the detection and adaptation to the metabolic stress triggered by the lack of oxygen and glucose.

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