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tPA modulation of the blood-brain barrier: a unifying explanation for the pleiotropic effects of tPA in the CNS?

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Abstract

The plasminogen activation (PA) system is best known for its role in fibrinolysis. However, it has also been shown to regulate many non-fibrinolytic functions in the central nervous system (CNS). In particular, tissue-type plasminogen activator (tPA) is reported to have pleiotropic activities in the CNS, regulating events such as neuronal plasticity, excitotoxicity and cerebrovascular barrier integrity, whereas urokinase-type plasminogen activator (uPA) is mainly associated with tissue remodeling and cell migration. It has been suggested that the role tPA plays in controlling barrier integrity may provide a unifying mechanism for the reported diverse, and often opposing, functions ascribed to tPA in the CNS. Here we will review the possibility that the pleiotropic effects reported for tPA in physiologic and pathologic processes in the CNS may be a consequence of its role in the neurovascular unit in regulation of cerebrovascular responses and subsequently parenchymal homeostasis. We propose that this might offer an explanation for the ongoing debate regarding the neurotoxic versus neuroprotective roles of tPA.

Keywords

tPA; PDGF-CC; PDGFRα; PAI-1; Neuroserpin; Blood-brain barrier

INTRODUCTION

In the vascular lumen the main function of the serine protease tissue-type plasminogen activator (tPA) is activation of the zymogen plasminogen to plasmin, and subsequent degradation of fibrin clots[1]. Because of its role in fibrinolysis, tPA is used as a thrombolytic agent for treatment of ischemic stroke, although its use is greatly limited due to concerns for

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hemorrhagic complications and the requirement that it is administered within a few hours of onset of symptoms[2]. The mechanism by which thrombolytic tPA might lead to hemorrhagic transformation of ischemic stroke is not completely understood, but it appears to be due to unique activities of tPA in the central nervous system (CNS), beyond its well established role in fibrinolysis. These unique CNS activities of tPA have over the past two decades been reported to include many diverse processes such as neuronal development[3], neuronal plasticity[4], axonal regeneration[5,6], neurovascular coupling[7], excitotoxicity[8–10], neuroprotection[11], microglial activation/inflammation[12,13], and blood-brain barrier (BBB) control[14–16]. In addition, these pleiotropic effects of tPA in the CNS have also been proposed to be mediated via multiple potential substrates and receptors[17,18]. How tPA mediates such diverse functions in the CNS is still a matter of debate but several hypotheses have been suggested, including tPA concentration and tPA conformation (single chain tPA versus two chain tPA) (reviewed in[19]). Recently we proposed an alternative explanation, suggesting that the neurovascular events regulated by tPA might provide a unifying pathway for many of the pleiotropic effects of tPA[20]. We reasoned that, instead of tPA directly performing all these diverse functions in the CNS, it might be that tPA's role in regulating cerebrovascular integrity, and thereby parenchymal homeostasis, could indirectly affect events such as neuronal signaling pathways and excitotoxicity through loss of precise control of the extracellular environment. In this review we will summarize what is known about the role of tPA in the CNS during physiology and disease in light of this cerebrovascular regulatory theory.

THE NEUROVASCULAR UNIT AND THE BBB

The cerebrovascular bed is unique in that normal CNS function requires a highly regulated extracellular environment to keep the concentrations of most molecules within a very narrow range[21]. Cerebrovascular homeostasis is maintained by the BBB, which forms a mechanical and functional barrier between the systemic circulation and the CNS that tightly controls trafficking of substances between the blood and the CNS[22]. The barrier properties are established by the brain endothelial cells, but it is widely recognized that the other cells in the neurovascular unit (NVU), including perivascular astrocytes in particular, as well as vascular mural cells (vascular smooth muscle cells and pericytes) and neurons, all work together in a coordinated way to regulate the extracellular environment of the brain parenchyma (Figure 1)[20,21,23,24]. Thus, the interaction between neurons, astrocytes and endothelial cells plays a central role coupling energy supply and changes in the CNS extracellular environment with neuronal activity[25–28].

IS tPA-MEDIATED REGULATION OF NEUROVASCULAR RESPONSES THE INITIAL EVENT DIRECTING LATER RESPONSES IN THE CNS?

It is well established that cerebrovascular responses to neuronal activity are critical for maintaining parenchymal homeostasis through three closely linked and related effects: 1) *neurovascular coupling*, which refers to increases in cerebral blood flow in response to neuronal activity; 2) *neurobarrier coupling*, which refers to changes in transport or the movement of molecules across the BBB; and 3) *neurometabolic coupling*, which refers to

changes in local metabolic factors, such as glucose and lactate, in response to neuronal activity (reviewed in[28]). The reports showing that tPA promotes neurovascular[7], neurobarrier[14], and possibly neurometabolic coupling[29] thus suggest that under physiologic conditions tPA is released into the NVU in response to neuronal activity where it exerts these neurovascular effects to accommodate the increased energy demand incurred by the increased firing rates occurring during routine processing in the neocortex. However, during pathologic conditions this is exaggerated leading to disproportionate opening of the BBB, which results in extravasation of harmful blood-borne substances into the CNS and the subsequent loss of the tight regulation of the CNS extracellular environment. Based on this we hypothesize that the neurovascular events regulated by tPA could provide a unifying pathway for many of the pleiotropic effects of tPA in the CNS. For example, studies have suggested that a primary role of tPA in the CNS is direct regulation of neuronal activity through its action on the N-methyl-D-aspartate (NMDA)-receptor and neuronal calcium signaling[10,30,31]. However, since the concentration of glutamate is 25–200-fold higher in plasma than in the CNS extracellular fluids[32], then it is conceivable that tPA-induced changes in BBB permeability could overwhelm the astrocyte-mediated glutamate shuttle resulting in the buildup of extracellular glutamate[33] which in turn, could promote dysregulation of the NMDA signaling pathways and lead to excitotoxicity[9]. Thus, we suggest a possible common pathway for tPA permitting modulation of CNS function through the regulation of the neurovascular unit.

PHYSIOLOGIC ROLES OF tPA IN THE CNS

Our understanding about the physiologic role of tPA in the CNS is still very limited and most of our knowledge is based on studies utilizing genetically modified mice in various experimental models of disease as well as *in vitro* systems. However, in recent years significant efforts and advancements have been made to delineate the function and mechanism of action of tPA in the CNS (summarized in table 1).

Expression, regulation and downstream mediators

Expression: tPA is widely expressed in the CNS, but until recently little has been reported on its cellular and subcellular distribution *in vivo* in healthy brain. Among the highest expression of tPA in the murine CNS is seen within the vascular endothelial cells[34], however it is believed that this source of tPA is nearly exclusively released into the blood stream upon stimuli and not into the parenchyma of the CNS[35]. High expression of tPA in the murine brain has also been reported in the cortex, amygdala and the mossy fibers of the hippocampus[36,37], in a variety of different cells including astrocytes[38], microglia[12], and neurons[39]. This expression pattern was confirmed by studies in transgenic mice expressing a LacZ reporter gene under the human tPA promoter showing remarkable tPA promoter directed expression to the hippocampus, dentate gyrus and to various layers within the cortex[40]. Whilst the expression in astrocytes and microglia cells in the healthy murine brain has been questioned by some researchers[41], the neuronal expression has been confirmed by many studies in mice[12,20,37,41] and in humans[42]. A recent study claims that the neuronal expression of tPA is restricted to a subset of excitatory neurons in mice and rats (after blockage of axo-dendritic transport)[41], however this is not supported by our own findings

indicating that tPA is also expressed in a subset of perivascular interneurons in the naïve murine CNS[20]. Nevertheless, the neuronal expression of tPA and the fact that it has been shown to be released in an activity-dependent manner via exocytosis[43,44], in combination with the reports that tPA can be found in close proximity to arterioles on the brain parenchymal side of the cerebral vessels (Figure 2A)[15,20,37], has led to the suggestion that tPA is involved in regulation of cerebrovascular responses in physiologic settings (discussed in further detail below)[7]. This is supported by our recent findings that the perivascular neurons expressing tPA are VIP (vasoactive intestinal peptide)-positive interneurons, which are known to regulate vascular responses such as vascular tone[22,45,46].

Regulation: The main inhibitor of tPA activity in the blood is plasminogen activator inhibitor 1 (PAI-1)[1], however, this *ser*ine *p*rotease *in*hibitor (serpin) is only weakly expressed in the healthy murine[20,47] and human[48,49] brain. It was therefore considered unlikely that PAI-1 is a major regulator of tPA activity in the CNS, at least in regulation of physiologic events. Nevertheless, in pathologic conditions, such as cerebral ischemia and traumatic brain injury (TBI), PAI-1 has been shown to play a role in regulation of tPA activity, which is further supported by the increased levels of PAI-1 found in the CNS during pathologic conditions[50,51]. The identification of another member of the serpin family, neuroserpin, that is largely specific for the CNS[48,49,52–54] led to the hypothesis that this is the physiologic relevant inhibitor regulating tPA activity in the brain[55]. This notion was supported by expression analysis and biochemical evidence showing strong inhibition of tPA by neuroserpin and considerably less efficient inhibition of other serine proteases[52,54]. However, it was not until recently that we were able to demonstrate that neuroserpin was not only blocking tPA activity *in vitro*, it was also a relevant regulator of tPA activity in the murine CNS[20]. Interestingly, confocal microscopy studies revealed that perivascular neuroserpin is expressed in specific somatostatin-positive interneurons, which, like tPA-expressing VIP-positive interneurons, are known to regulate cerebrovascular responses[22,45,46]. It has been postulated that neuroserpin may also have other protease targets[56] and non-inhibitory functions[57] *in vivo* although this requires further exploration (reviewed in[58]).

Downstream mediators: The mechanism by which tPA exerts its actions in the CNS is controversial. Both plasminogen-dependent and plasminogen-independent pathways have been postulated to act through several potential downstream mediators in the CNS including matrix metalloprotease 9 (MMP-9)[59], activated protein C (APC)[60,61], neurotrophic factors (pro-NGF and pro-BDNF)[62,63] and platelet-derived growth factor CC (PDGF-CC)[15,64]. tPA is also known to bind and/or activate a set of receptors such as low density lipoprotein receptor-related protein (LRP)[65,66], NMDA-receptor[10], annexin-II[67], and epidermal growth factor receptors (EGFRs)[68]. These downstream mediators and co-receptors of tPA have been shown to control a wide array of biological functions in the CNS during normal physiology and in disease models, including neurotoxic and neuroprotective roles as well as regulation of cerebrovascular permeability (discussed in further detail below).

Function in the CNS

To investigate the role of tPA in the CNS many studies have utilized mice where the gene for tPA, *Plat*, has been deleted[69]. These mice have been said to express the non-proteolytic domains of tPA with the potential to influence experimental outcomes[70], however there is no experimental evidence in the literature supporting this claim. It has also been reported that the existing strain of tPA deficient mice ($tPA^{-/-}$) do retain significant segments of 129 DNA associated with the *Plat* allele, even after extensive backcrossing onto C57BL/6J background[71]. This study also showed that this region of chromosome 8 carries a significant number of mutations unique to the 129 strain that co-segregate with the *Plat* allele, including several potential null mutations. This may confound the interpretation of experiments performed utilizing the original $tPA^{-/-}$ mice[69], and is why a novel "passenger mutation"-free tPA deficient mouse strain ($tPA^{-/-\ NIH}$) was generated as a useful community resource for further exploration of tPA function in physiology and disease[71].

CNS development—Very little is known about the role of tPA in CNS development, although genetic deletion studies indicate that tPA does not play an essential role during embryonic development[69,71]. Expression studies in the developing mouse CNS have shown that tPA is synthesized by a variety of neuronal cells, with the highest expression reported in areas of extensive neuronal migration and tissue remodeling[72]. Based on these findings it has been hypothesized that tPA facilitates neuronal migration or neurite outgrowth through degradation of cell-cell or cell-matrix adhesions. Since then a number of studies, mainly *in vitro* cell culture experiments but also some *in vivo* studies, have been performed supporting a role for tPA in neuronal remodeling and migration during CNS development. For example, tPA was found to be released at the neuronal growth cone[73], and to mediate neurite outgrowth and remodeling[74]. Further it was shown that migration of cerebellar granule neurons is perturbed in $tPA^{-/-}$ mice[75]. With regard to the BBB hypothesis it is tempting to speculate that increased metabolic demand during neuronal migration might lead to tPA-mediated changes in neurovascular, and/or neurometabolic, coupling responses.

The $tPA^{-/-}$ mice were recently reported to display congenital brain defects including abnormal cerebrovascularization indicating a previously unrecognized role of tPA in cerebrovascular development[76]. Interestingly, the $tPA^{-/-}$ mice were also found to display mild cerebral ventricular malformations, a feature previously associated with ablation of PDGF-C[77], thereby providing a potential *in vivo* link between tPA and PDGF signaling in CNS development. However, considering the novel report of the "passenger-mutations" in these $tPA^{-/-}$ mice it will be interesting to determine whether these congenital cerebrovascular and ventricular abnormalities are a true effect of tPA ablation also in the novel $tPA^{-/-\ NIH}$ strain[71]. If so, it will be interesting to determine whether this is a plasminogen-dependent or plasminogen-independent process through studies in plasminogen deficient mice. It is interesting in this regard that obstructive hydrocephalus, associated with cerebroventricular enlargement, has been reported to be associated with plasminogen deficiency in humans[78]. It should be noted that these congenital defects might have unforeseen effects on the experimental outcome and thus needs to be taken into account when interpreting data achieved utilizing genetically modified mice.

Adult CNS physiology

Learning and memory: The idea that tPA is involved in the process of learning and memory came from the findings that tPA was highly expressed in the mossy fibers of the hippocampus and that neuronal activity induced mRNA expression of tPA in hippocampal pyramidal neurons during late phase long-term potentiation (L-LTP)[36]. L-LTP is a well-studied model system of learning[79] and has been shown to be significantly decreased in tPA $^{-/-}$ mice[80,81]. In addition, tPA has been implicated in other models of increased neuronal activity including models of learning[4,82,83], visual cortex plasticity[84] and memory[85]. Studies using transgenic mice over-expressing tPA[86] or intrahippocampal infusion of tPA[82] confirms that tPA facilitates L-LTP and learning.

The underlying mechanism by which tPA might facilitate L-LTP has been shown to be mediated by LRP[87], potentially via plasmin-mediated cleavage of proBDNF to the mature form of BDNF[63]. Interestingly though, L-LTP has been shown to be associated with increased BBB permeability[88] and the large pyramidal neurons within the hippocampus are known to have particularly high metabolic demand, thus making them especially sensitive to damage from a variety of environmental and biological insults[89]. Thus, in line with our hypothesis, it might be that tPA-induced changes in the NVU and the subsequent increase in cerebrovascular permeability during learning, through an LRP-dependent process, leads to altered parenchymal homeostasis which in turn promotes cleavage of proBDNF.

Neurovascular and neurometabolic coupling: Local cerebral blood flow increases rapidly in response to neuronal activity, a phenomenon termed functional hyperemia or neurovascular coupling. This is believed to be critical for the maintenance of substrate and energy supply to the activated neurons as well as for clearance of metabolic by-products[22]. Although the process of neurovascular coupling is still not completely understood, perivascular astrocytes and various vasoactive mediators, including tPA[7], have been proposed to be of importance[22,90]. The prospect that tPA has a role in normal neurovascular coupling, as suggested by Park et al. utilizing tPA$^{-/-}$ mice in a model of whisker evoked neuronal activation[7], is supported by several lines of evidence. First, coupling of local neuronal activity to local blood flow has been shown to be controlled to a large extent by penetrating arterioles and tPA has been shown to primarily be associated with arterioles in the CNS (Figure 2A)[15,20,91]. Second, our recent data illustrate perivascular tPA to be expressed by VIP-positive interneurons, whereas its inhibitor neuroserpin was found in somatostatin-positive interneurons[20]. This is interesting since VIP-expressing ‘vasomotor’ interneurons have been reported to induce dilation of local microvessels, while somatostatin-expressing neurons induce contraction[22,45,46], suggesting that neuroserpin/tPA may form a regulatory circuit that regulates cerebrovascular responses. Third, tPA has been reported to reduce vessel reactivity to increased luminal pressure and vasoactive mediators, suggesting that tPA may be involved in regulating vascular tone[92,93], and finally, systemic delivery of tPA at low concentrations can directly reduce cerebral vascular resistance and systemic blood pressure[93]. However, given the recent observations that tPA$^{-/-}$ mice display a decreased number of large diameter ASMA-positive vessels[76], and that neurovascular coupling was found to take place exclusively at ASMA-positive, SMC-covered, arterioles[94], it might be that the cerebrovascular rearrangements associated with tPA deficiency may

explain the attenuated neurovascular coupling response in these mice[7]. This warrants further investigation.

In addition, tPA has been implicated in neurometabolic coupling[29,95], which refers to changes in local metabolic factors, such as glucose and lactate, in response to neuronal activity[28,96]. Using *in vitro* neuronal cultures under conditions of oxygen-glucose deprivation it was shown that tPA, via a plasminogen-independent mechanism, increase neuronal uptake of glucose through induction of the glucose transporter GLUT3. Although intriguing, and in line with the idea that tPA facilitates neuronal function through coupling energy supply to demand, this needs to be confirmed *in vivo*, in non-pathologic conditions.

Vascular permeability: The first indications that tPA plays a role in the regulation of vascular permeability came from animal studies of embolic stroke, where thrombolytic treatment with tPA was associated with evidence of increased vascular permeability[97,98]. Since then the association of tPA with the NVU and its correlation with BBB regulation has been well established, although the mechanism underlying this capacity of tPA remains controversial[18]. Some reports have suggested a plasmin-independent role for tPA[14,15] whereas others have reported tPA-mediated plasmin generation as crucial for tPA activity on the BBB[16,99]. Nevertheless, tPA's action on the BBB has been shown to require interaction with the CNS side of the NVU, as tPA administered intravenously in unchallenged wild type mice does not elicit opening of the BBB whereas tPA administered on the CNS side of the NVU does[15].

Our previous data show that platelet-derived growth factor CC (PDGF-CC) is acting directly downstream of tPA in the CNS where it regulates BBB integrity through PDGF receptor α (PDGFRα) signaling on perivascular astrocytes (Figure 2B)[15]. PDGF-CC is expressed as a latent factor that is cleaved by tPA in a plasmin-independent manner to generate active PDGF-CC capable of binding PDGFRα[64]. Injection of active PDGF-CC protein into the CSF of mice was reported, like tPA, to rapidly increase BBB permeability and neutralizing antibodies against PDGF-CC inhibited tPA-induced opening of the BBB[15]. No gross changes of vascular structures were reported within this time frame, even though the extent of Evans Blue extravasation into the brain parenchyma was significant, suggesting that the activation of PDGF-CC/PDGFRα may represent a regulated physiological process that controls the BBB. The activation of PDGF-CC by tPA depends on interactions between the kringle-2 domain of the protease with the two domains in the PDGF-C polypeptide chain[100] as well as on binding to the co-receptor LRP[15].

This presumably ensures correct positioning of tPA and PDGF-CC enabling activation in close proximity to the PDGFRα receptor in the NVU and stimulation of increased cerebrovascular permeability. This is supported by the findings that desmoteplase, vampire bat-derived plasminogen activator, which lacks the kringle-2 domain[101], and thus presumably interaction with PDGF-CC, has been shown to have no effect on inducing BBB permeability[16]. Taken together we propose that in physiologic conditions tPA is released in response to neuronal activity into the perivascular space where it activates PDGF-CC, and subsequently PDGFRα signaling on perivascular astrocytes, through interaction with LRP, which leads to cerebrovascular changes associated with neuronal activity (Figure 3A)[18].

It should be noted that the action of tPA on BBB regulation has also been reported to be mediated by plasmin and downstream mediators other than PDGF-CC, including plasmin-induced truncation of MCP1[16,99]. In the case with MCP1 it was found that BBB integrity was compromised much later than that induced by PDGF-CC. Prolonged opening of the barrier will lead to changes in parenchymal homeostasis where blood-borne substances such as plasmin can enter the CSF and in turn cleave MCP1. In support of this hypothesis are our findings that early after barrier breach induced by PDGF-CC, no gross changes of vascular structures were noted[15], whereas MCP1-induced opening was associated with disruption of tight junction proteins (occludin and ZO-1) and reorganization of the actin cytoskeleton[99].

Further, it has been postulated that the controversy regarding mechanisms mediating the role of tPA in BBB regulation may, at least in part, be due to the concentration of tPA[70]. Through a number of elaborate calculations and assumptions the overall concentration of tPA in the brain three hours after an ischemic event has been proposed to be ~1 nM, and even lower during physiologic conditions[70]. Therefore, since the amounts of tPA often used in experimental settings could potentially result in tPA concentrations that are higher than this, it has been suggested that these potentially supraphysiologic concentrations might lead to non-physiologic observations[70]. However, the actual endogenous concentration of tPA in the NVU is not known, and may in fact be significantly higher than the 1nM suggested above. Indeed, the release of tPA from activated neurons and subsequent binding to co-factors such as LRP, could generate very high local concentrations of tPA in the NVU which could be underestimated when evaluating gross brain tissue extracts.

PATHOPHYSIOLOGIC ROLES OF tPA IN THE CNS

It was quickly recognized that the physiologic roles of tPA in the CNS may also be involved in pathophysiological events observed in several neurological diseases including cerebral ischemia, head trauma and seizures[58]. This led to a large number of *in vitro* and *in vivo* studies, but the role of tPA in pathology is still intensely debated and the literature is conflicted. The main controversy concerns the potential neuroprotective or neurotoxic functions of tPA in the CNS and what downstream mediators facilitates the response. Here we discuss how these controversies might be explained by the early effect of tPA on cerebrovascular permeability. Interestingly, the role of tPA in BBB regulation highlights a commonality in neurovascular signaling events between diverse neurologic disorders and may potentially represent a previously overlooked therapeutic target for seemingly unrelated brain diseases.

Ischemic stroke

Stroke is a leading cause of adult morbidity and mortality[102]. The most common form of stroke is ischemic stroke, which occurs when there is an abrupt interruption of blood flow to the brain. The finding that tPA specifically binds to, and is stimulated by fibrin[103,104] led to the hypothesis that clot lysis (thrombolysis) using tPA would facilitate a localized activation of plasmin at the site of occlusion. This was successfully demonstrated early on[105] and today tPA is the only approved thrombolytic drug for treatment of acute ischemic stroke[2]. However, early animal studies advised against indiscriminate use of thrombolytic tPA in

ischemic stroke as it was reported to mediate neuronal damage or cerebral haemorrhage[8,106–109]. Consistent with these preclinical studies, thrombolysis with tPA in ischemic stroke patients carries a significant risk of intracerebral hemorrhage[2,110–113], and due in part to this increased risk of hemorrhagic conversion it is estimated that only 5–7% of ischemic stroke patients receive intravenous tPA, with another 1–2% receiving intra-arterial therapy[2,114–119]. The mechanism by which thrombolytic tPA might lead to increased hemorrhagic transformation is not completely understood, but it appears to be due in part to unique activities of tPA in the CNS. This is supported by the findings that tPA activity rapidly increases in ischemic tissue in experimental models of ischemic stroke[14,107,120].

One of the first studies demonstrating that tPA can negatively affect outcome in an experimental model of middle cerebral artery occlusion (MCAO) showed that $tPA^{-/-}$ mice had significantly smaller cerebral infarcts than wild type mice, and that intravenous administration of tPA to $tPA^{-/-}$ mice increased infarct volume to levels comparable to wild type controls[107]. In support of this it has been shown that neuroserpin, the primary inhibitor of tPA in the CNS[20], provided neuronal protection and reduced infarct volume during MCAO[120,121]. Based on these studies, it was concluded that tPA has neurotoxic effects in the ischemic brain. It should however be noted that a number of conflicting studies have been published showing that loss of tPA is associated with increased lesion volume[122], and conversely, that increased levels of tPA, through intravenous administration of tPA to wild type mice or transgenic overexpression of tPA in neurons of mice (T4), decreased infarct volume following MCAO[29]. This proposed a neuroprotective role of tPA during MCAO and the mechanism of action was ascribed to tPA-induced increase in GLUT3 expression followed by increased glucose uptake to meet the increased metabolic demand of cerebral cortical neurons. In line with this hypothesis it was reported that the T4 transgenic mice that neuronally overexpress tPA displayed significant upregulation of GLUT3 protein expression in ischemic brain tissue and higher glucose uptake after MCAO[29]. However, it is somewhat difficult to reconcile these data since GLUT3 expression and glucose uptake was found to be decreased in wild type mice after MCAO, despite an increased endogenous release of tPA during ischemia[14,107,120]. Thus, it is possible that, similar to the $tPA^{-/-}$ mice, overexpression of tPA in the neurons of T4 mice is associated with some congenital cerebrovascular anomaly that has yet to be discovered.

The mechanism by which tPA exerts neurotoxic effects has been postulated to be mediated through plasmin-independent cleavage of the NR1 subunit in NMDA-receptor followed by enhanced signaling[10]. However, the direct cleavage of NR1 has later been questioned by other research groups who have suggested that the NMDA-receptor is not a direct target of tPA proteolysis, although plasmin was found to be able to mediate cleavage of the NMDA-receptor[123,124]. Instead, as noted above an alternative mechanism has emerged, suggesting that tPA-mediated changes in cerebrovascular permeability might be the underlying cause of the neurotoxic effects reported for tPA in the CNS. This was based on the discoveries that tPA increases permeability of the BBB both in rodents[14,98] and in humans[125] following ischemic stroke. This is particularly interesting since BBB dysfunction is a hallmark of many neurologic diseases, including ischemic stroke[126] and it has been proposed that pathologic disruption of the barrier will lead to extravasation of blood-borne molecules such

as fibrin(ogen) into the brain parenchyma where it can trigger different cellular and molecular responses including a delayed inflammatory response[127].

In one study[14] it was demonstrated that tPA$^{-/-}$ mice were protected from early loss of BBB integrity after MCAO. This study further showed that the tPA-induced opening of the BBB was dependent on the proteolytic activity of tPA, but was independent of plasmin, thus indicating the existence of another tPA substrate. Since MMP-9 had been implicated as a downstream mediator of tPA in the CNS[59] and MMP-9 deletion in mice had been shown to reduce BBB permeability and infarct volume 24h after stroke[128], this was initially thought to be responsible for the effect of tPA on the BBB. However, MMP-9 deficient mice were found to display BBB dysfunction similar to wild type mice early (6h) after MCAO[14]. Furthermore, depletion of circulating leukocytes was found to completely block the rise in MMP-9 activity within the first 24h after ischemic stroke[129], suggesting that the increased MMP-9 activity seen after ischemic stroke is likely due to infiltrating leukocytes. This is consistent with the findings that MMP-9 is not expressed in either neurons or astrocytes in the first 24h after ischemic stroke[130]. Finally, it was also shown that treatment with recombinant tPA after transient middle cerebral artery occlusion exacerbates BBB disruption 24 hours later in both wild-type and MMP-9 knockout mice[131]. Collectively these data suggest that MMP-9-mediated events occur on the luminal side of the NVU and are not involved in the early effects on BBB permeability mediated by tPA after ischemic stroke.

The findings that tPA regulation of the BBB is mediated through plasmin-independent catalysis of PDGF-CC and subsequent activation of PDGFRα on perivascular astrocytes (discussed above), and that blocking this pathway, either by neutralizing antibodies against PDGF-CC or with the PDGFRα inhibitor imatinib, significantly reduced BBB permeability and hemorrhagic complications associated with thrombolytic tPA treatment suggested a potential treatment strategy to reduce the complications associated with thrombolytic tPA by inhibiting PDGFRα signaling[15]. Support for the potential role of PDGF-CC in thrombolysis associated complications in humans was provided by a study showing that PDGF-CC levels are increased in the plasma of ischemic stroke patients after thrombolytic tPA treatment and higher levels of PDGF-CC were associated with an increased risk of hemorrhagic transformation[132]. On the basis of these findings a randomized controlled clinical trial has been conducted at the Karolinska University Hospital to assess the possibility of using imatinib as an adjuvant therapy with thrombolytic tPA and the outcome of this study is currently being evaluated (Istrokepilot, EudraCT Number: 2010-019014-25).

It is thus possible that decreased energy and metabolic supply to neurons during ischemia leads to increased release of tPA into the NVU, where it, through activation of PDGF-CC/PDGFRα signaling, leads to increased BBB permeability (Figure 3B). However, excessive signaling leads to further opening of the barrier and subsequent extravasation of blood-borne substances from the vascular space into the brain parenchyma. Following intravenous treatment with thrombolytic tPA it is plausible that exogenous tPA enters the brain through the breached BBB thereby exacerbating the PDGF-CC/PDGFRα signaling in the NVU. This might eventually cause complete disruption of the BBB consequently resulting in hemorrhagic complications. It should be noted that thrombolytic therapy with tPA in patients with pulmonary embolism, deep vein thrombosis or myocardial infarction also carries a ~1

to 1.5 % risk of symptomatic intracerebral hemorrhage[133,134], suggesting that even without cerebral ischemia exogenous tPA can induce BBB opening and promote hemorrhage. This is also supported by the fact that intravenous administered tPA can cross the intact barrier and slightly increase BBB permeability in naïve rats[135].

Interestingly, tPA-catalyzed activation of PDGF-CC/PDGFRα signaling can also induce BBB permeability and contribute to disease progression in a number of other experimental models of neurologic disease including TBI[136], seizures[20] and amyotrophic lateral sclerosis (ALS)[137] (discussed below).

Traumatic brain injury (TBI)

The broadening role of tPA in regulation of BBB permeability in ischemic stroke raised the question of its role in other CNS pathologies. In the case of TBI BBB disruption is a serious consequence which plays a major role in the promotion of cerebral edema and increase in intracranial pressure; two devastating clinical manifestations of TBI that contribute to the high level of mortality and morbidity post injury, and for which there are scant therapeutic options[138]. Although ischemic stroke and TBI have vastly different initiating stimuli, there was a clear rationale to explore the role of tPA in the promotion of BBB permeability following TBI.

Endogenous tPA, as well as most other components of the plasminogen activating system including plasminogen and PAI-1, are not only expressed in various compartments of the brain, they can be induced following various stimuli, including glutamate analogues[51,139,140]. As levels of excitotoxic amino acids are known to increase in the damaged brain following TBI[141], it stood to reason that this would promote increases in the levels of endogenous tPA following TBI and that this in turn would influence outcome and recovery. Initial support for a detrimental role for tPA post-TBI was provided in a study in 2001 where it was shown that tPA deficient mice had less edema and improved recovery in a model of TBI[109]. It was also shown in separate publications that administration of tPA to pigs subjected to TBI resulted in an increase in brain water content[142]. A later publication from the same group linked this effect of tPA to increased vasodilatation and activation of mitogen activated protein kinase[143]. Although none of these studies specifically investigated tPA mediated changes in BBB permeability following TBI, these results were certainly consistent with this possibility.

Direct evidence that levels of tPA activity were indeed upregulated following TBI, and was having a major influence on BBB permeability following TBI, was provided in 2011 and 2012. Sashindranath et al. used a modified amidolytic assay to show that endogenous level of tPA activity were transiently increased in the cortex following TBI[144]. tPA activity increased ~25% within 1h of TBI and this increase was maintained at 3h post injury but reduced to pre-injury levels by 24h. A subsequent study from the same group compared the extent of BBB permeability in wild type and $tPA^{-/-}$ mice following TBI using the Evan's Blue brain extravasation assay[145]. Wild type mice were shown to have marked increase in BBB permeability when assessed 3h post-TBI, however this was not seen in $tPA^{-/-}$ mice, thereby implicating endogenous tPA in this process. Importantly, $tPA^{-/-}$ mice also had improved neurological outcome following TBI. On the other hand, transgenic mice with

neuronal overexpression of tPA (T4 mice, described above) displayed enhanced BBB permeability and a more severe neurological phenotype post-TBI. Hence, tPA expression was causally linked with BBB permeability following TBI, with the absence of tPA being protective, and increased levels being deleterious in TBI, reminiscent of the results seen in earlier studies in mouse models of ischemic stroke.

An interesting observation arising from this study concerned the mechanism by which tPA was promoting BBB permeability following TBI. It was assumed that tPA was promoting parenchymal extravasation following TBI via its proteolytic capacity, either via plasmin generation or via different substrates (i.e. PDGF-CC). It was anticipated that inhibition of endogenous tPA would recapitulate the protective phenotype seen when $tPA^{-/-}$ mice subjected to TBI. The approach used by these authors was to inhibit local tPA activity within the lesion of the brain immediately following TBI by stereotactic injection of PAI-1. This approach indeed inhibited levels of endogenous tPA activity greater than 90% as determined using an amidolytic assay[145]. However, in stark contrast to expectations, the blocking effect of PAI-1 against tPA actually resulted in an increase in extravasation 3h post injury. This puzzling result was eventually explained when the authors investigated the downstream consequences of complexes formed between tPA and PAI-1. It has been known for decades that tPA:PAI-1 complexes (and indeed many other serpin:protease inhibitor complexes) are cleared from plasma and the extravascular space by LDL receptors[146,147]. It had also been reported that LDL receptors could modulate activity of integrins and receptor tyrosine kinases that in turn can initiate intracellular signaling[148]. Speculation therefore arose that the introduction of PAI-1 into the damaged brain following TBI, despite blocking endogenous tPA activity, was initiating an unanticipated signal through LDL receptors via the formation of tPA:PAI-1 complexes. Support for this notion was provided from a number of lines of evidence; first, co-injection of the LDL receptor antagonist, RAP with PAI-1 blocked the increase in extravasation; second, stereotactic injection of PAI-1 into $tPA^{-/-}$ mice had no effect at promoting extravasation post-TBI; third, co-injection of a PAI-1 mutant that poorly reacts with LDL receptors, failed to reproduce the effect, despite it being able to effectively inhibit tPA activity. Finally, injection of pre-formed tPA:PAI-1 complexes into the lesioned area of $tPA^{-/-}$ mice following TBI caused a significant increase in albumin extravasation.

It is possible that the discrepancy of this study, demonstrating that tPA-mediated opening of the BBB in TBI is through proteolytic inactive tPA in form of a tPA:PAI-1 complex[145], and the previous *in vivo* and *in vitro* studies of tPA-mediated BBB regulation discussed above, showing that opening of the barrier requires proteolytic active tPA[14,16] via activation of PDGF-CC signaling[15], might be explained by temporally distinct mechanisms regulating BBB integrity in TBI. To address this a recent study was conducted where imatinib, an inhibitor of PDGF-CC signaling, was evaluated in a mouse model of TBI[136]. It was shown that inhibition of PDGF signaling in TBI significantly attenuated the extent of neuronal injury and BBB opening when evaluated 24h post injury[136]. Of further note in this study was the finding of PDGF-CC in the cerebrospinal fluid of patients with TBI, with PDGF-CC levels positively correlating with injury severity.

Hence, although at first glance it appears that tPA modulates BBB permeability via two different mechanisms in TBI (i.e. active tPA or inactive tPA:PAI-1 complexes) it may as well

be that these differences relate to a temporal order of events. We speculate that the initial increase in BBB permeability during TBI is mediated by proteolytic active tPA, potentially in response to the increased energy and metabolic demand in neurons following insult, and that excessive signaling leads to further opening of the barrier and subsequent increase in PAI-1 levels (through upregulated expression in response to the injury as well as increased extravasation from the vascular space into the brain parenchyma). This in turn leads to formation of tPA:PAI-1 complexes that can exacerbate opening of the BBB, eventually causing disruption of the BBB and leakage of harmful blood-borne substances into the brain after TBI (Figure 3). It remains to be determined whether tPA:PAI-1 complex-mediated increase in BBB permeability also occurs in ischemic stroke, but this is worth investigating. Nonetheless, whatever the mechanism these findings have provided novel opportunities to minimize BBB opening following TBI and in doing so reduce the devastating complications of cerebral edema and the rise in intracranial pressure, thus demonstrating clear translational possibilities of these findings.

Seizures

The first evidence that tPA is important in the development of seizures came from studies in mice showing that tPA expression is increased early after seizures[36] and that tPA ablation leads to a higher threshold for seizures[8,149], whereas neuronal overexpression leads to a lower seizure threshold[150]. The relationship between tPA and seizures in humans is less well understood, but a recent study has described a positive correlation between increased serum tPA levels and epilepsy severity in children with idiopathic and intractable epilepsies[151]. The mechanism by which tPA affects seizures is not fully established, but tPA has been suggested to act directly on neuronal cells by cleavage of the NMDA-receptor[10], or indirectly by altering cerebrovascular permeability[20]. The latter is supported by the fact that impaired integrity of the BBB is a well-known feature of seizures, although it is debated whether impaired BBB function is just a consequence of seizure activity or a contributor to seizure progression (reviewed in[126]). However, since 30% of individuals with seizures fail to respond to existing treatments[152], recent studies have begun to consider the cerebrovasculature as a potential avenue for therapeutic intervention[20,153–156].

The seminal observation illustrating that $tPA^{-/-}$ mice are resistant to excitotoxin-induced neuronal death and seizures after intrahippocampal injection of excitotoxins, including the glutamate analog kanic acid (KA), led to the conclusion that, in pathologic conditions, tPA has neurotoxic properties[8]. Using a different experimental paradigm, where seizures were induced by injection of KA into the amygdala, other researchers were later able to confirm these results[149]. The fact that KA was not injected directly into the hippocampus allowed these researchers to dissect KA-induced, from seizure-induced, hippocampal neuronal death, yet genetic deficiency of tPA was associated with slower progression of KA-induced seizure activity throughout the limbic system and a decrease in seizures-induced hippocampal cell 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 of mice followed by intravenous treatment with tPA[157]. The advantage of this

model is that the injection of NMDA into the striatum does not induce seizures. Using this approach, these investigators found that intravenous administration of tPA increases the volume of the necrotic lesion caused by the injection of NMDA[157]. Interestingly, glutamate has been shown to induce BBB permeability[158] suggesting that following NMDA injection, the BBB may be more permeable thus enabling intravenous tPA to extravasate into the brain parenchyma and further disrupt the barrier integrity.

In line with this a recent study illustrated that tPA regulated seizure progression primarily through control of the neurovascular unit and BBB integrity, and not through direct effects of tPA on neuronal activity[20]. This was supported by multiple independent experimental results, including the observation that increasing BBB permeability in seizure-resistant tPA $^{-/-}$ mice dramatically enhanced the rate of seizure progression, while interventions that maintain BBB integrity delayed seizure propagation. In addition, the comparison of *in vivo* EEG recordings to *ex vivo* hippocampal electrophysiological recordings demonstrates that the phenotypic differences in seizure progression noticed between wild type (intermediate), tPA$^{-/-}$ (protected), and neuroserpin deficient mice (enhanced) *in vivo*, were absent in *ex vivo* studies where BBB regulation is no longer important for maintenance of the extracellular environment. As seen in ischemic stroke and TBI, the effect of tPA on BBB permeability in seizures was illustrated to be facilitated, at least in part, through PDGF-CC induced activation of PDGFRα signaling in perivascular astrocytes, which is of particular interest since earlier reports have suggested astrocytes to play a central role in the pathology of seizures[155,159].

CONCLUDING REMARKS

It is well appreciated that tPA is controlling unique functions within the CNS distinct from its role in fibrinolysis, although the downstream targets mediating these functions and the suggested outcome ascribed to tPA activity in the CNS are many and intensely debated. Here we summarize the existing evidence in support of the hypothesis that the neurovascular events regulated by tPA might provide a unifying pathway for many of the pleiotropic effects of tPA in the CNS.

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ESSENTIALS

- Tissue-type plasminogen activator (tPA) is highly expressed in the CNS
- The role of tPA in the CNS is very different from its role in fibrinolysis within the vascular space
- tPA is reported to have many pleiotropic activities in the CNS, mediated through a number of various downstream substrates
- Here we propose that the neurovascular events regulated by tPA might provide a unifying pathway for many of the pleiotropic effects of tPA in the CNS

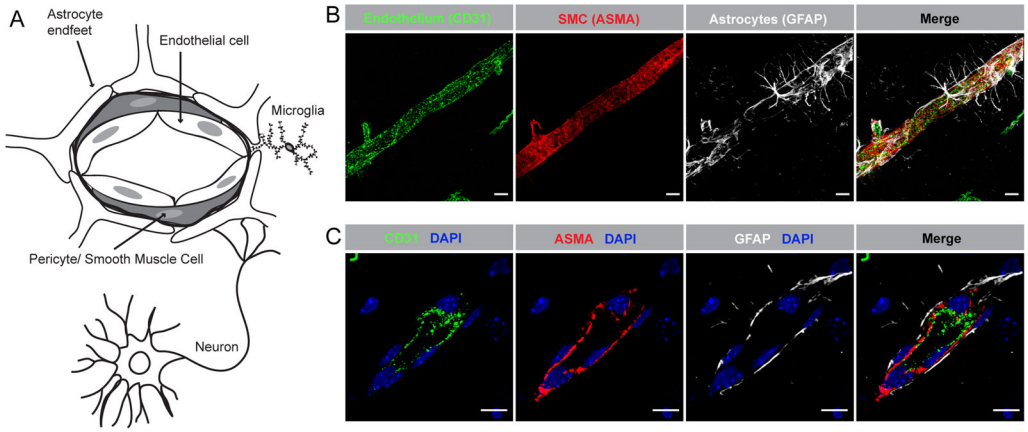

Figure 1.

The neurovascular unit (NVU). A) Schematic illustration of the structure and cellular components of the NVU. B) Confocal microscopy image showing the NVU in the naïve adult wild type murine brain. The perivascular astrocytic endfeet (white) completely ensheath the vascular smooth muscle cell (SMC) layer (red) and the endothelial tube (green). C) High magnification confocal image showing the order of cells in the NVU, with the endothelial cells (green) closest to the vessel lumen and the SMCs (red) tightly wrapping around the endothelial tube. Immunofluorescent staining of endothelial cells with anti-CD31 antibodies (CD31), SMC with anti-ASMA antibodies and perivascular astrocytes with anti-GFAP antibodies. Cell nuclei were visualized with DAPI (blue). The images in B display maximum intensity projections generated from confocal Z-stacks (25 μm). Scale bars in B and C 10 μm.

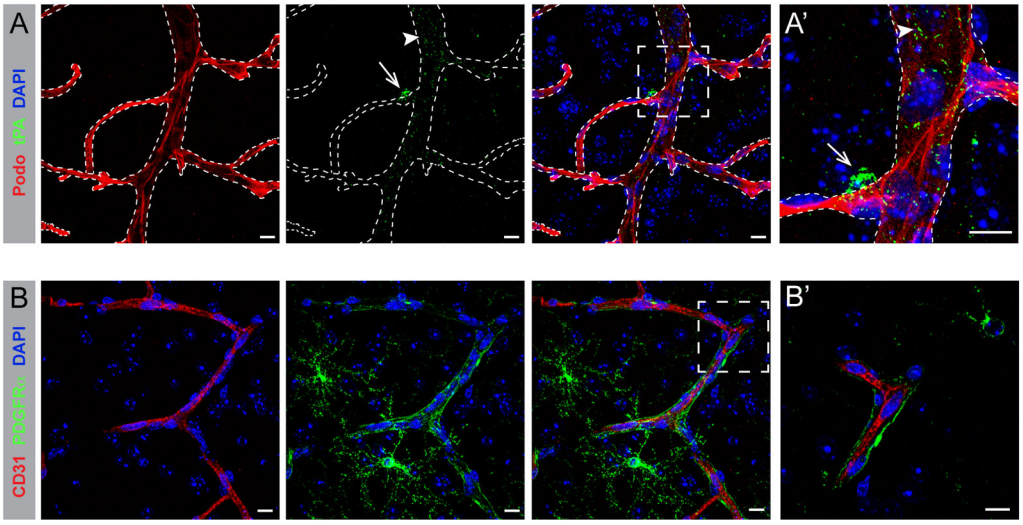

Figure 2.

Perivascular expression of tPA and PDGFRα in the NVU. A, A′) Confocal microscopy image showing expression of tPA (green) in the NVU of the naïve wild type murine brain. tPA is expressed as two distinct pools in the NVU; one within the endothelial cells (arrowheads) and another on the abluminal side of the vessels (arrows). Vessels were visualized by immunofluorescent staining using the endothelial cell marker podocalyxin (red, Podo). B, B′) Confocal image showing expression of PDGFRα (green) ensheathing (arrows) the vessel (red) in the NVU of the naïve wild type murine brain. Vessels were visualized by immunofluorescent staining using the endothelial cell markers in A, A′) podocalyxin (Podo) and in B, B′) CD31. Cell nuclei were visualized with DAPI (blue). The images display maximum intensity projections generated from confocal Z-stacks (A, A′ = 17 μm; B = 20 μm; B′ = 4 μm). Scale bars, 10 μm.

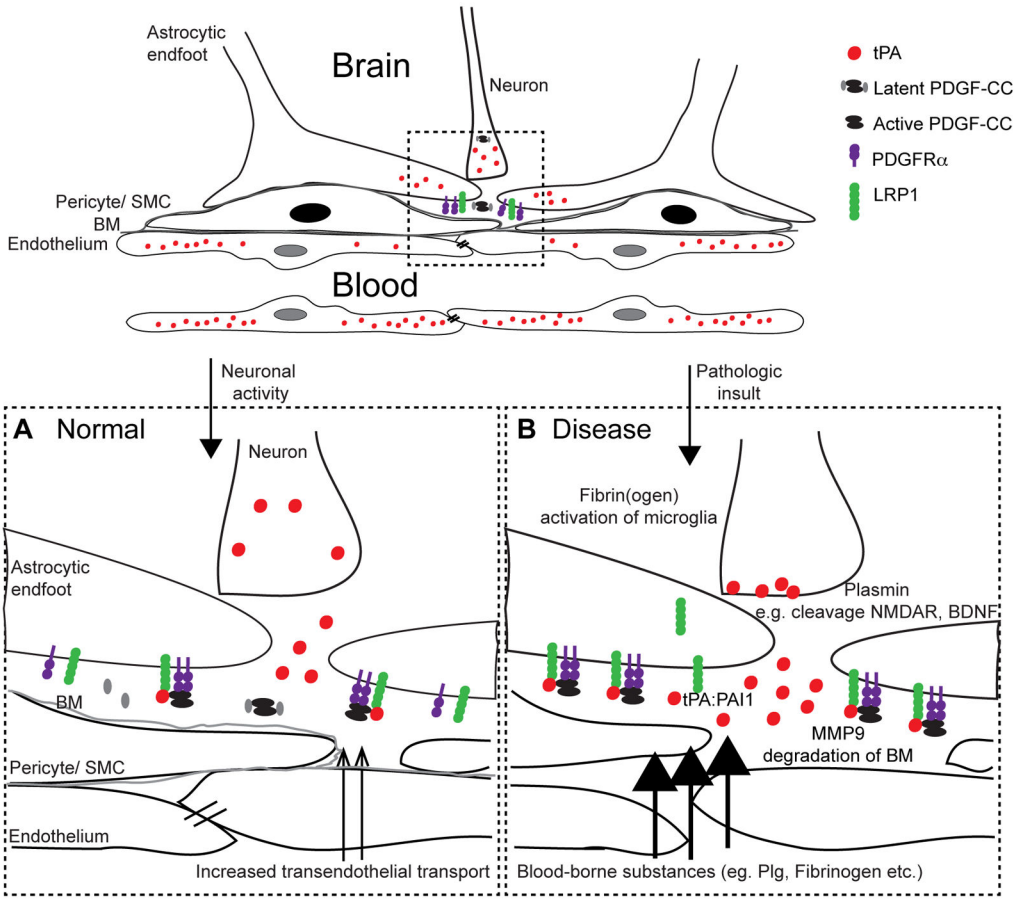

Figure 3.

tPA-mediated regulation of BBB integrity. A) Under physiologic conditions tPA is released by activated neurons into the perivascular space where it activates PDGF-CC, and subsequently PDGFRα signaling on perivascular astrocytes, through interaction with LRP, which leads to cerebrovascular changes associated with neuronal activity. B) In pathologic conditions initial increase in BBB permeability is mediated by proteolytic active tPA, potentially in response to the increased energy and metabolic demand in neurons following insult. Excessive signaling via either active tPA or via tPA:PAI-1 complex formation and LDLR signaling, leads to further opening of the barrier and subsequent extravasation of blood-borne substances from the vascular space into the brain parenchyma. Following intravenous treatment with thrombolytic tPA it is plausible that exogenous tPA enters the brain through the breached BBB thereby exacerbating the PDGF-CC/PDGFRα signaling in the NVU.

Table 1

Physiologic roles of tPA in the CNS

	Biologic process	Method	Effect	References
CNS development	Survival	Genetic deletion studies	Non essential role of tPA during embryonic development	69,71
	Neuronal migration	*Ex vivo* studies of $tPA^{-/-}$ mice and controls using cerebellar slice technology	Perturbed migration of cerebellar granule neurons in $tPA^{-/-}$ mice	75
	Neuronal remodeling	*In vitro* studies of neuroblastoma clonal cell lines and neural progenitor cell	tPA is released in the neuronal growth cone where it might affect neurite outgrowth and remodeling	73,74
	Cerebrovascular development	Immunohistochemical and confocal microscopy studies in $tPA^{-/-}$ and control murine brains	Reduced number of vascular smooth-muscle cell covered, large diameter vessels and increased junctional localization of tight junction protein ZO1 in $tPA^{-/-}$ mice	76
	Cerebroventricular development	*In vivo* MRI and immunohistochemical/confocal microscopy studies in $tPA^{-/-}$ and control mice	Mild cerebral ventricular malformations and distorted ependymal lining in $tPA^{-/-}$ mice	76
Adult CNS physiology	Learning and memory	Expression analysis and experimental *in vivo* models of learning/memory in $tPA^{-/-}$, tPA overexpressor and control mice	Learning and memory is significantly decreased in $tPA^{-/-}$ mice whereas overexpression of tPA facilitates learning	4,36,80,81,82,83,85,86
	Neurovascular coupling	*In vivo* models of whisker evoked neuronal activation in $tPA^{-/-}$ mice. Immunohistochemical/confocal microscopy studies and *in vitro*/*in vivo* effects of tPA on blood vessel tone	Neurovascular coupling is perturbed in $tPA^{-/-}$ mice. tPA is expressed in vasoactive interneurons and might regulate vascular tone.	7,20,93
	Neurometabolic effects	*In vitro* neuronal cultures	tPA increase neuronal uptake of glucose	29,95
	Vascular permeability	*In vitro* and *in vivo* studies	tPA increase permeability of the BBB through interaction with the NVU.	14,15,16,99,135