Identification of a neurovascular signaling pathway regulating seizures in mice

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Abstract

Objective: A growing body of evidence suggests that increased blood–brain barrier (BBB) permeability can contribute to the development of seizures. The protease tissue plasminogen activator (tPA) has been shown to promote BBB permeability and susceptibility to seizures. In this study, we examined the pathway regulated by tPA in seizures. Methods: An experimental model of kainate-induced seizures was used in genetically modified mice, including mice deficient in tPA (tPA−/−), its inhibitor neuroserpin (Nsp−/−), or both (Nsp:tPA−/−), and in mice conditionally deficient in the platelet-derived growth factor receptor alpha (PDGFRα). Results: Compared to wild-type (WT) mice, Nsp−/− mice have significantly reduced latency to seizure onset and generalization; whereas tPA−/− mice have the opposite phenotype, as do Nsp:tPA−/− mice. Furthermore, interventions that maintain BBB integrity delay seizure propagation, whereas osmotic disruption of the BBB in seizure-resistant tPA−/− mice dramatically reduces the time to seizure onset and accelerates seizure progression. The phenotypic differences in seizure progression between WT, tPA−/−, and Nsp−/− mice are also observed in electroencephalogram recordings in vivo, but absent in ex vivo electrophysiological recordings where regulation of the BBB is no longer necessary to maintain the extracellular environment. Finally, we demonstrate that these effects on seizure progression are mediated through signaling by PDGFRα on perivascular astrocytes. Interpretation: Together, these data identify a specific molecular pathway involving tPA-mediated PDGFRα signaling in perivascular astrocytes that regulates seizure progression through control of the BBB. Inhibition of PDGFRα signaling and maintenance of BBB integrity might therefore offer a novel clinical approach for managing seizures.

Introduction

The serine protease tissue-type plasminogen activator (tPA) is primarily known for its thrombolytic activity; however, within the central nervous system (CNS) tPA is reported to have pleiotropic effects, regulating events such as neuronal plasticity, neurovascular coupling, and neurovascular barrier control (reviewed in 1–3). In vitro studies have demonstrated that tPA activity can be regulated by neuroserpin, a member of the serine protease inhibitor (serpin) family that is primarily expressed in the brain. In humans, mutations in the neuroserpin gene have been linked to epilepsy and dementia, and it is thought that this is due to neurotoxic effects of neuroserpin inclusion bodies. However, it has been suggested that the epilepsy phenotype might actually be caused by...
the loss of regulation of tPA activity, but so far there is no direct in vivo evidence linking neuroserpin and tPA in the brain. The hypothesis that unregulated tPA activity contributes to epilepsy is supported by work in rodents. These studies showed that tPA expression is increased early after seizures and that tPA deficiency leads to a higher threshold for seizures, whereas neuronal overexpression leads to a lower seizure threshold. In addition, tPA and neuroserpin are rapidly released from neurons in response to neuronal depolarization. The relationship between tPA and seizures in humans is less well understood, but a recent study described a positive correlation between increased serum tPA levels and epilepsy severity in children with idiopathic and intractable epilepsies.

The mechanism by which tPA affects seizures is not known. It is possible that tPA acts on neuronal cells directly to affect neuronal excitability and survival, or indirectly by altering cerebrovascular permeability. We have previously demonstrated an effect of tPA on blood–brain barrier (BBB) integrity in stroke, which is mediated through cleavage of platelet-derived growth factor-CC (PDGF-CC) and activation of the PDGFRα on perivascular astrocytes. However, it is not known whether increases in tPA activity during seizure lead to loss of BBB control caused by enhanced PDGF signaling in the neurovascular unit, or if this affects seizure severity. While it is well-established that impaired integrity of the BBB is a feature of seizures, it is debated whether BBB dysfunction is just a consequence of seizure activity or a contributor to seizure progression. The therapeutic potential of restoring BBB function has therefore been largely overlooked. However, as 30% of individuals with seizures fail to respond to existing treatments, recent studies have begun to consider the cerebrovascular integrity as a potential avenue for therapeutic intervention.

In this study, we demonstrate that neuroserpin and tPA form a regulatory circuit in the murine CNS that influences seizure progression via tPA-mediated PDGFRα signaling and control of the BBB. These findings offer novel targets for anticonvulsant therapy and contribute to our understanding of the relationship between BBB dysregulation and seizures.

Materials and Methods

Animal strains

Age- and gender-matched tPA−/−, neuroserpin−/−, plasminogen activator inhibitor 1 (PAI-1)−/−, and double Nsp−/− deficient mice back-crossed at least 10 generations into C57BL/6J and their wild-type C57BL/6J (WT) controls were used. In addition, age- and gender-matched conditional PDGFRα-deficient mice (GFAP-Cre+;Rαfl/fl) and littermate controls (GFAP-Cre−;Rαfl/fl) 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.

Murine model of seizure and EB analysis

The animals were anesthetized with chloral hydrate (450 mg/kg, Sigma-Aldrich, St. Louis, MO, USA, intraperitoneal) and placed in a digital stereotactic frame. 0.5 μL of 16 mmol/L kainic acid (KA, Sigma-Aldrich) diluted in lactated Ringer’s solution was injected unilaterally using a 33G needle and Hamilton syringe into the basolateral amygdala; bregma, −1.6 mm; medial-lateral, 2.5 mm; dorso-ventral, −4.5 mm. The injection needle was removed after 4 min and the animals were observed for 240 min. Behavioral seizures were scored by a blinded investigator as follows: onset, myoclonic jerks involving the whisker pads; and generalization, bilateral myoclonic activity. For analysis of cerebrovascular permeability after KA injection, mice were injected with 100 μL of 4% Evans blue (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 KA injection. Each hemisphere was then homogenized in N,N-dimethylformamide (Sigma-Aldrich) and centrifuged for 45 min at 25,000 g. The supernatants were collected, absorbance determined and EB extravasation in each hemisphere quantified from the formula: (A620 nm − ((A500 nm + A740 nm)/2))/mg wet weight.

Hippocampal local field potential recording

For electroencephalogram (EEG) analysis mice were anesthetized with 2% isofluorane. Body temperature was maintained at 37°C by a temperature-controlled feedback system. An intraperitoneal catheter was implanted for administration of chloral hydrate. Mice were placed in a stereotactic frame and the skull prepared for electrode placement. Anesthesia was transitioned to chloral hydrate (450 mg/kg). A monopolar electrode was placed in the CA1 region of the hippocampus contralateral to the kainate injection site and a reference electrode was placed in the cerebellum. With this monopolar recording
arrangement, the EEG signal primarily reflects neuronal activity within the hippocampal tissue (hippocampal seizure activity). EEG activity was recorded using a Biopac 100B amplifier and a Biopac MP100 Data Acquisition System (Biopac Systems, Goleta, CA, USA). For EEG analysis, Acknowledge 3.9.1.6 software was used. After recording baseline EEG activity for 15 min, KA was injected into the left amygdala as above. EEG activity was recorded continuously for a minimum of 2 h post injection. Coordinates for electrode placement (relative to bregma) were as follows: Contralateral hippocampus: AP: −1.94 mm, ML: −1.0 mm, Depth: −1.5 mm. Cerebellum reference electrode: AP: −6.0 mm ML: −1.0 mm, Depth: −1.0 mm. For EEG analysis, the average time to sustained ictal activity was measured by two independent investigators blinded to study group. For each animal, the amplitude of normal baseline activity was determined as recurrent bursting of at least three bursts for any given minute of baseline. A threshold of two times the baseline amplitude was set and the number of peaks above this threshold was plotted. The inflection point of this plot was considered the onset of high amplitude electrical activity. Ictal activity was confirmed at these time points by the presence of continuous ictal spiking. If at the onset of increased electrical activity sustained ictal activity was not confirmed the first point of continuous spiking above threshold was taken.

Ex vivo synchronous activity was assessed using a no Mg2+/high K+ model.33 Hippocampal slices were prepared and extracellular field potential recording methods were used similar to that previously described.34,35 Briefly, coronal brain slices (350 μm) were cut on a vibratome under ice-cold (<1°C) oxygenated sucrose-based cutting solution containing the following (in mmol/L): 2.8 KCl, 1.25 MgCl2, 1.0 MgSO4, 1.25 NaH2PO4, 1.25 CaCl2, 26 sucrose, 26 NaHCO3, 10 d-glucose, 0.40 ascorbic acid. Slices were transferred to a holding chamber filled with artificial cerebrospinal fluid (aCSF) containing the following (in mmol/L): 124 NaCl, 2.8 KCl, 1 MgSO4, 1.25 NaH2PO4, 2.5 CaCl2, 26 NaHCO3, 10 d-glucose, 0.40 ascorbic acid at room temperature and remained there for at least 1 h before being individually transferred to a submersion chamber and continuously perfused (~1.5 mL/min) with oxygenated aCSF heated to 31°C. Extracellular field potential recordings were made using glass-pipettes filled with aCSF (tip resistances ~1 MΩ), placed in stratum oriens of region CA3 just adjacent to the cell body layer. Spontaneous seizure activity was induced by exchanging the normal aCSF perfusion with aCSF which contained 0 mmol/L Mg2+ and elevated (10 mmol/L) K+. The latency from the time of exchange until the first spontaneous high-frequency burst was documented.

Imatinib treatment and mannitol analysis

To block PDGFRα activation, mice were treated with the tyrosine kinase inhibitor imatinib (200 mg/kg, oral gavage) three times (morning-night-morning) before KA injection. Lactated Ringer’s solution was used as vehicle control. To temporarily induce opening of the BBB, mannitol (Sigma-Aldrich) 100 μL of a 1 mol/L solution in Lactated Ringer’s was infused through the internal carotid artery 30 min after KA injection. Saline-treated animals were used as controls. Seizure behavior for both the imatinib and mannitol experiments was scored as described above. To demonstrate mannitol-induced opening of the BBB, mannitol was administered to animals not undergoing craniotomy and immediately followed by intravenous injection of EB. Cerebrovascular permeability analysis was carried out as described above.

Immunostaining and confocal microscopy

For immunofluorescence, frozen sections (14 μm) or vibratome sections (50 μm) were used and antigen retrieval was performed in retrieval solution (S1700; DAKO, Glostrup, Denmark) by boiling. The sections were permeabilized in 0.5% TritonX-100 and nonspecific binding blocked using TNB blocking buffer (NEL700A001KT, Perkin Elmer, Waltham, MA, USA) or 1% Bovine Serum Albumin (BSA) in 0.5% TritonX-100/PBS. The sections were incubated with primary antibodies in blocking solution overnight at 4°C followed by incubation with fluorescent-conjugated secondary antibodies 45 min, room temperature (RT). The specific primary antibodies used were as follows: tPA (12 μg/mL, rabbit HTMTPA and goat SHTMTPA, Molecular Innovations, Novi, MI, USA), neuroserpin (15 μg/mL, rabbit HTMNs; Lawrence Lab), Podocalyxin (1:200, AF1556; R&D systems, Minneapolis, MN, USA), CD31 (rat anti PECAM, 1:50, 553370; BD Biosciences, Franklin Lakes, NJ, USA), somatostatin (rat anti SST, 1:200, 8330-0009; AbD Serotec, Oxford, UK), VPF (Guinea Pig anti VIP, 1:200, S-3048; Bachem, Bubendorf, Switzerland), PDGFRα (rabbit anti PDGFRα, 1:100, #3164; Cell Signaling Technology, Denver, MA, USA), glial fibrillary acidic protein (rat anti GFAP, 1:200, 13-0300; Life Technologies, Stockholm, Sweden), and α-smooth muscle actin-Cy3 (mouse anti ASMA-Cy3, 1:300, C6198; Sigma-Aldrich). To visualize the vessels with Lycopersicon esculentum (tomato) lectin (# B 1175; Vector Laboratories, Burlingame, CA, USA), biotinylated-lectin was dialysed against PBS overnight, and injected via the tail vein of anesthetized mice (100 μL of 0.7 mg/mL). After 5 min the mice were perfusion fixed, brains dissected, sectioned by vibratome and stained with fluorescent-conjugated streptavidin. DAPI (4’,6-diamidino-2-
the primary inhibitor of tPA activity in the CNS. To this end, it has been hypothesized that neuroserpin is the primary inhibitor of tPA activity in the CNS. Antibodies were specific for their respective antigen and used for antibody control and demonstrated that both antibodies were specific for their respective antigen and that tPA appeared to be normally expressed in the brains of Nsp−/− mice and vice versa. All images were acquired in RT with a Zeiss LSM510 alternatively LSM700 confocal microscope and the ZEN 2009 software (Carl Zeiss, Oberkochen, Germany). The images are representative of the respective stainings and were processed and analyzed using Volocity 3D image analysis software (PerkinElmer) and Photoshop CS5 (Adobe, San Jose, CA, USA). Brightness and contrast settings were changed to generate final image and were applied equally to entire image.

**Statistical analysis**

The data are presented as Kaplan–Meier plots in order to account for mice that did not generalize during the 240 min experimental period, or as mean ± SEM. Assistance with statistical analysis was obtained from the University of Michigan Center for Statistical Consultation and was performed using GraphPad Prism 6 statistical software (GraphPad Software, La Jolla, CA, USA). 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 and is summarized in Table S1. All 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**

**Neuroserpin and tPA form a regulatory circuit in the murine CNS**

The main inhibitor of tPA activity in blood is the serpin PAI-1; however, based on both in vitro studies and expression analysis it has been hypothesized that neuroserpin is the primary inhibitor of tPA activity in the CNS. To study the role and putative in vivo link between neuroserpin and tPA in seizure onset and progression we used a murine model of seizure where the chemo-convulsant KA was injected into the basolateral nucleus of the amygdala. This brain structure is part of the limbic system but distant from the hippocampus, thus this model permits the investigation of seizure activity within the limbic system without direct injection of excitotoxins into the hippocampus proper. The behavioral response to injection of KA into the amygdala is characterized by unilateral myoclonic contractions of the face (onset, Fig. 1A), which ultimately spread to full generalization involving...

Figure 1. Neuroserpin/tPA regulate severity of KA-induced seizures in mice. Seizures were induced with KA and convulsive behavior/severity was scored as: (A) Onset, seizures affecting one side of the face or neck; (B) Generalization, bilateral seizure activity involving four limbs; and (C) Survival. All data are presented as Kaplan–Meier plots in wild-type mice (WT, n = 24); neuroserpin-deficient mice (Nsp−/−, n = 15); PAI-1-deficient mice (PAI-1−/−, n = 16); tPA-deficient mice (tPA−/−, n = 14); neuroserpin and tPA double-deficient mice (Nsp:tPA−/−, n = 10). Median times (t) or survival proportions at 240 min (%) are indicated in the figure. The data are from 2 to 7 independent experiments. Statistical significance was determined by log-rank (Mantel-Cox) test and *P < 0.05; **P < 0.01; ***P < 0.001; ns = nonsignificant relative to controls. tPA, tissue plasminogen activator; KA, kainic acid; PAI, plasminogen activator inhibitor 1.
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that Nsp−/− mice experienced very severe seizures with significantly decreased latency to onset (P < 0.05) (Fig. 1A) and generalization compared to WT controls (P < 0.01) (Fig. 1B). In contrast, we found that tPA−/− mice showed a significantly delayed latency to onset and generalization compared to WT controls (onset, P < 0.05; generalization, P < 0.001) (Fig. 1A and B). This latter result is consistent with previously published data.14,15 We also found that seizure progression in PAI-1−/− mice phenocopied WT controls (onset, P = 0.22; generalization, P = 0.60), suggesting that PAI-1 is not regulating tPA activity in the brain during seizures (Fig. 1A and B). These findings are supported by quantitative real-time PCR experiments showing that neuroserpin expression is high, whereas PAI-1 expression is low in both the murine (data not shown) and human brain.7,37

To investigate if the opposing effects of neuroserpin and tPA on seizure progression are due to regulation of tPA activity by neuroserpin, we created mice lacking either protein. We reasoned that the phenotype of the double-deficient mice (Nsp:tPA−/−) would either mimic the severe phenotype of Nsp−/− mice, be intermediate to Nsp−/− and tPA−/− mice if the effect of neuroserpin is independent of tPA activity, or phenocopy the tPA−/− mice if neuroserpin acts upstream of tPA, regulating its activity. Comparing the Nsp:tPA−/− mice with each single-deficient strain revealed that the double-deficient mice phenocopied tPA−/− mice (onset, P = 0.47; generalization P = 0.49) and were largely protected from seizure progression to generalized seizures (Fig. 1A and B). In contrast, Nsp:tPA−/− mice had a significantly delayed latency to onset and generalization compared to Nsp−/− mice (onset and generalization, P < 0.001). The results from the scoring of behavioral manifestations were confirmed by analysis of survival of the various genotypes (Fig. 1C). Nsp−/− mice showed a significantly shorter mean time of survival than any of the other genotypes (P < 0.01 vs. WT) with a survival of only 20% at the 4-h time point. tPA−/− and double-deficient Nsp:tPA−/− mice were protected from seizure-induced death, with both strains showing a survival of 100% at 4 h after KA injection. WT and PAI-1−/− mice showed similar rates of survival (about 57%) at the endpoint, which is intermediate to the Nsp−/− mice, and the tPA−/− or Nsp:tPA−/− mice. Taken together, the opposing seizure phenotypes observed in tPA−/− and Nsp−/− mice, as well as the results with the Nsp:tPA−/− and PAI-1−/− mice, indicate that neuroserpin is primarily acting as an inhibitor of tPA in the murine brain during seizure.

Seizure severity correlates with BBB permeability

As vascular permeability has been shown to be increased during seizures27,38 and tPA has been shown to regulate the integrity of the BBB,22 we investigated whether neuroserpin and tPA affect seizures through control of vascular integrity. We first determined whether the integrity of the BBB was affected in our experimental model of seizure by measuring EB dye extravasation into the brain parenchyma at 2 and 4 h after KA injection in WT mice (n = 10), at 2 h in Nsp−/− mice (n = 11), and at 4 h in tPA−/− mice (n = 7) (Fig. 2A). Our results showed a significant, time-dependent increase in EB extravasation in the hemisphere ipsilateral to the KA injection in WT mice, demonstrating an increased loss of BBB control from 2–4 h (P < 0.001). Unlike WT mice, the Nsp−/− mice already showed a loss of BBB control at 2 h compared saline-injected controls (P < 0.01), and compared to WT mice there was a significant increase in EB leakage in the ipsilateral hemisphere of Nsp−/− mice 2 h after KA injection (P < 0.05). In contrast, the tPA−/− mice were protected from loss of BBB control. Even 4 h after KA injection they showed only a nonsignificant increase in EB leakage compared to saline-injected tPA−/− mice and significantly less EB leakage compared to WT mice at 4 h (P < 0.001) (Fig. 2A) (due to the low survival of the Nsp−/− mice we were not able to measure EB extravasation at 4 h in these mice). Interestingly, we also found a significant increase in EB extravasation in the contralateral hemisphere of WT mice at 4 h compared to saline-injected controls and to KA-injected WT mice at 2 h (P < 0.001) (Fig. 2B). No significant differences were detected in baseline EB extravasation between the three genotypes or between any of the sham operated saline-injected controls (Table S1). These data indicate that decreased BBB integrity accompanies the propagation of abnormal electrical activity as the seizure spreads from the ipsilateral to the contralateral hemisphere. Together they confirm that this seizure model is associated with vascular leakage, similar to what has been reported in seizure patients,25 and that the extent of BBB opening correlates with seizure severity. This is illustrated by the higher EB values recorded in Nsp−/− mice compared to WT controls at 2 h post-KA injection; at which time ~80% of the Nsp−/− mice, but only ~20% of the WT controls, had progressed into generalized seizures. In contrast, at the later time point only about half of the tPA−/− animals
had progressed into generalized seizures as compared to all of their WT controls, which correlated with lower EB leakage in tPA-/- mice.

**Neuroserpin and tPA are expressed in perivascular cells in the murine brain**

To investigate the potential association of neuroserpin and tPA with cerebral vessels we performed immunofluorescent staining on WT murine brain tissue sections and analyzed the expression of neuroserpin and tPA by confocal microscopy. Neuroserpin was detected on the brain parenchymal side of the cerebral vessels in perivascular cells with projections that appeared to interact with the cerebrovascular wall (arrowheads, Fig. 3A). Consistent with previous publications we found two distinct pools of tPA expression associated with cerebral vessels in the murine brain: one pool was found throughout the brain within vascular endothelial cells (arrowheads, Fig. 3B), whereas a second pool was found in close proximity to arterioles on the brain parenchymal side of the cerebral vessels (arrows, Fig. 3B). Costaining analysis of neuroserpin (green, arrowheads) and tPA (red, arrows) expression in the cerebral vasculature revealed that the protease and its inhibitor were expressed in close proximity to each other around cerebral vessels, but that they were localized to different cellular compartments (Fig. 3C).

The size of the perivascular cells expressing neuroserpin and tPA, and the localization of neuroserpin to cellular projections innervating the cerebrovascular wall, suggested that neuroserpin and tPA-positive cells might be neurons involved in regulation of vascular function. As GABAergic (gamma-aminobutyric acid) neurons have been reported to show the highest expression of neuroserpin in the human brain, and subsets of GABAergic interneurons have been reported to regulate vascular responses such as vascular tone, we next investigated whether neuroserpin and tPA in the murine brain are coexpressed with different markers of these cells, including somatostatin (SST) and vasoactive intestinal peptide.
VIP-expressing "vasomotor" interneurons induce dilation of local microvessels, whereas SST-expressing interneurons induce contraction. Our immunostainings of murine brain sections revealed that neuroserpin is expressed by perivascular SST-positive cells (Fig. 4A), whereas tPA is expressed by VIP-positive cells (Fig. 4B). This is supportive of a role of neuroserpin and tPA in control of cerebrovascular responses during seizure progression.

Protection of the BBB delays seizure onset and progression

Several studies have suggested that BBB disruption can promote seizures, and we have previously shown that tPA, via PDGFRz-activated signaling in the neurovascular unit, controls BBB integrity. Therefore, to investigate whether preservation of barrier integrity during seizures contributes to seizure resistance we used both pharmacologic and genetic strategies to inhibit tPA-mediated PDGFRz signaling. We hypothesized that inhibition of tPA-induced PDGFRz signaling would result in less severe seizures through protection of BBB integrity. Consistent with our hypothesis we found that pharmacologic inhibition of PDGFRz signaling with the receptor tyrosine kinase inhibitor imatinib resulted in a significant delay in both onset (Fig. 5A) and generalization (Fig. 5B) of KA-induced seizure in both WT (n = 27) and neuroserpin-deficient mice (Nsp^−/−, n = 14), but not in tPA-deficient mice (tPA^−/−, n = 10), as compared to vehicle-treated controls (WT, P < 0.001; Nsp^−/−, P < 0.05) and generalization was about 15–30% slower (WT, P = 0.05; Nsp^−/−, P < 0.05). In tPA^−/− mice, which demonstrate marginal BBB leakage of EB after seizure induction (see Fig. 2A) and low PDGFRz-activation after experimentally induced ischemic stroke, there was no effect from imatinib on seizure activity (onset, P = 0.88; generalized, P = 0.98). Our results imply that maintenance of BBB integrity...
delays seizure progression and that imatinib, possibly through inhibition of tPA-catalyzed PDGFRα signaling in the neurovascular unit, might offer a novel clinical approach for treating seizures.

To more precisely identify the molecular target of imatinib we further investigated the link between tPA-catalyzed activation of PDGFRα signaling in the neurovascular unit and experimentally induced seizures. Based on our previous findings that showed PDGFRα to be expressed on perivascular astrocytes in the neurovascular unit, we generated astrocyte-specific PDGFRα-deficient mice. For this, we utilized floxed PDGFRα mice and a transgenic mouse strain expressing Cre recombinase under the control of the human glial fibrillary acidic protein promoter (GFAP-Cre). Immunofluorescent staining of WT murine brain tissue sections confirmed expression of PDGFRα in GFAP-positive perivascular astrocytes in the neurovascular unit (Fig. 6A) and showed that the receptor is distributed in the border between the astrocytes and the vascular mural cells, here visualized by ASMA (Fig. 6B). Characterization of the conditional PDGFRα-deficient strain demonstrated that perivascular PDGFRα expression was very low in the conditional mice (Cre+;Rαfl/fl) as compared to floxed PDGFRα littermate controls (Cre−;Rαfl/fl) (Fig. 7A). Quantification of PDGFRα expression (n = 3 per genotype) revealed a significant reduction (~50% lower) in receptor levels in conditional Cre+;Rαfl/fl mice as compared to floxed Cre−;Rαfl/fl littermate controls (P < 0.05) (Fig. 7B). Supporting our data with pharmacologic inhibition of PDGFRα signaling using imatinib, we found that astrocyte-specific ablation of PDGFRα in Cre+;Rαfl/fl mice (n = 10) significantly delayed progression into generalized seizures following KA injection compared to floxed Cre−;Rαfl/fl controls (n = 10) (P < 0.05) (Fig. 7C). This delay was not due to the presence of GFAP-driven Cre recombinase per se as KA-induced seizure onset and progression in Cre−;Rαwt/wt mice (n = 5) phenocopied WT Cre−;Rαwt/wt littermate controls (n = 6) (Fig. 7D). We noted that floxed Cre−; Rαfl/fl mice had faster time to onset and generalization (Fig. 7C) than WT Cre−;Rαwt/wt mice (Fig. 7D) which is possibly due to a reduction in PDGFRα expression in nonperivascular cells in the murine brain of floxed Cre−; Rαfl/fl mice (Fig. 7E). Together our results suggest that tPA-catalyzed activation of PDGFRα signaling in perivascular astrocytes play a role in seizure spreading and that inhibition of this signaling pathway in the astrocytes of the neurovascular unit reduces seizure severity.
Although our data imply a correlation between BBB permeability and seizures, it is still possible that removing or inhibiting PDGFRα signaling in perivascular astrocytes affect seizures in an unanticipated way and that the opening of the BBB is just a consequence of the seizure activity. Therefore, to address the relationship between increased BBB permeability and seizure progression we used high molarity mannitol, administered through the internal carotid artery, to induce opening of the BBB. Mannitol has been shown to cause opening of the BBB through vasodilation and shrinkage of endothelial cells, which results in widening of the interendothelial tight junctions. If decreased integrity of the BBB promotes seizure progression we hypothesized that mannitol treatment would enhance the severity of seizures in otherwise protected tPA−/− mice. Conversely, if the role of tPA in seizure spreading is mediated in a vascular-independent manner then mannitol would not affect the protected phenotype seen in tPA−/− mice. For these studies, we first measured EB leakage into the brain parenchyma to confirm that the BBB in tPA−/− mice could be manipulated by mannitol (Fig. 8A). We found significantly increased EB extravasation in tPA−/− animals following mannitol injection (n = 6) as compared to controls (n = 4) (P < 0.05, Fig. 8B). Assessment of behavioral seizure manifestation in tPA−/− mice after mannitol-induced opening of the BBB (n = 8) revealed significantly faster onset time of seizures (P < 0.05) (Fig. 8C) and faster time to generalization (P < 0.001) (Fig. 7D) compared to Ringer’s infused controls (n = 7). This indicates that the tPA−/− mice are not inherently protected from severe seizures and that opening of the BBB enhances seizure progression. Interestingly, mannitol has been reported to be...
associated with seizures when administered as a means to lower intracranial pressure caused by brain edema following traumatic brain injury (TBI), or when administered as a way to enhance the entry of chemotherapeutic agents for treating brain tumors. Our results suggest that the mechanism behind the seizure side effects accompanying osmotherapy with mannitol might be that decreased BBB integrity lowers the threshold for neuronal synchronous activity.

Seizure onset/progression is regulated at the level of the neurovascular unit

To further investigate the neurovascular role of neuroserpin/tPA in seizure progression we carried out electrophysiological experiments. For this, we used in vivo hippocampal EEG recordings and an ex vivo seizure model. We reasoned that if the differences in seizure progression observed between the tPA\(^{-/-}\) and Nsp\(^{-/-}\) mice were driven by differences in BBB integrity, then these phenotypic differences in seizure progression would be absent in an ex vivo preparation where the BBB plays no role in regulation of the parenchymal extracellular environment. Alternatively, if the phenotypic differences were independent of BBB integrity then these differences should be maintained in ex vivo electrophysiological experiments. Consistent with the behavioral seizure scoring data, in vivo EEG recordings confirmed an increased latency to seizure activity in tPA\(^{-/-}\) mice (\(P < 0.05, n = 5\)) and decreased latency in Nsp\(^{-/-}\) mice (\(P < 0.05, n = 5\)) relative to WT control mice (\(n = 7\)) (Fig. 9A). In contrast, we found that in ex vivo hippocampal preparations the genotypic differences were lost or slightly opposed to the in vivo results (Fig. 9B). The ex vivo recordings revealed no significant difference between WT (\(n = 14\)) and Nsp\(^{-/-}\) mice (\(P = 0.22, n = 11\)) and a small decrease in latency to onset observed in the tPA\(^{-/-}\) (\(P < 0.05, n = 8\)) as compared to WT controls. Collectively, these results suggest that the effect of the neuroserpin/tPA regulatory circuit on seizure onset/progression is at the level of the neurovascular unit.

Discussion

The data presented here establish that, in vivo, endogenous neuroserpin and tPA form a regulatory circuit in the brain that significantly affects seizure progression. These results are consistent with previous reports indicating that tPA\(^{-/-}\) mice are seizure resistant, that neuronal overexpression of tPA in mice leads to a lower seizure threshold;
and that mutations in the human neuroserpin gene are associated with epilepsy.\textsuperscript{8,9,48–50} We propose that neuroserpin and tPA regulate seizure progression primarily through control of the neurovascular unit and BBB integrity, and not through direct effects of tPA on neuronal activity. This hypothesis is supported by multiple independent experimental results, including the observation that increasing BBB permeability in seizure-resistant tPA\textsuperscript{+/−} mice dramatically enhances the rate of seizure progression, whereas interventions that maintain BBB integrity delay seizure propagation in both WT and Nsp\textsuperscript{−/−} mice, but not in tPA\textsuperscript{−/−} mice. In addition, the comparison of in vivo EEG recordings demonstrates that the phenotypic differences in seizure progression between WT, tPA\textsuperscript{−/−}, and Nsp\textsuperscript{−/−} mice are maintained in vivo, but are absent in ex vivo studies where BBB regulation is no longer necessary for maintenance of the extracellular environment. Finally, we show that mice with a conditional reduction in PDGFR\textsubscript{x} expression in astrocytes have delayed seizure progression, which is consistent with the hypothesis that tPA regulates BBB integrity through PDGF-CC induced activation of PDGFR\textsubscript{x} signaling in perivascular astrocytes.\textsuperscript{25} The observation that PDGFR\textsubscript{x} signaling in astrocytes plays a role in regulating seizure progression is of particular interest as earlier reports have suggested that

Figure 7. Conditional ablation of PDGFR\textsubscript{x} in perivascular astrocytes improves seizure outcome. (A) Co-immunofluorescent staining of murine brain sections from astrocyte-specific PDGFR\textsubscript{x}-deficient mice (Cre\textsuperscript{−};R\textsuperscript{fl/fl}) and floxed littermate controls lacking GFAP-driven Cre recombinase expression (Cre\textsuperscript{−};R\textsuperscript{fl/fl}) show that perivascular PDGFR\textsubscript{x} expression (green, arrows) is decreased in the conditional mice (arrowheads). Vessels were visualized using the endothelial cell marker podocalyxin (white) and cell nuclei by DAPI (blue). The pictures are two-dimensional renderings of the three-dimensional reconstructions (left panel) and maximum intensity projections (right panels) generated from confocal Z stacks. (B) Quantification of the amount of PDGFR\textsubscript{x}-positive staining from five coronal brain sections per animal revealed a significant reduction in PDGFR\textsubscript{x} expression in Cre\textsuperscript{−};R\textsuperscript{fl/fl} brains (n = 3) as compared to floxed Cre\textsuperscript{−};R\textsuperscript{fl/fl} controls (n = 3). (C) Convulsive behavior following KA injection was classified and scored as in Figure 1 in floxed Cre\textsuperscript{−};R\textsuperscript{fl/fl} (n = 10) and conditional Cre\textsuperscript{−};R\textsuperscript{fl/fl} (n = 10) mice. (D) Seizure activity in Cre\textsuperscript{−};R\textsuperscript{wt/wt} (n = 5) mice phenocopied wild-type Cre\textsuperscript{−};R\textsuperscript{wt/wt} littermate controls (n = 6). (E) Quantification of the amount of PDGFR\textsubscript{x}-positive staining from three coronal brain sections per animal (n = 3) in floxed Cre\textsuperscript{−};R\textsuperscript{fl/fl} and conditional Cre\textsuperscript{−};R\textsuperscript{fl/fl} mice as compared to wild-type Cre\textsuperscript{−};R\textsuperscript{wt/wt} littermate controls. Data are presented as mean ± SEM (B and E) and Kaplan-Meier plots of seizure progression (C and D). Median times (t) are indicated in the figure. The data are from at least two independent experiments. Statistical significance was determined by Student’s paired t-test (B and E) and log-rank (Mantel-Cox) test (C and D) and \( *P < 0.05; **P < 0.01; \) ns = non significant relative to control. Scale bars, (A) left panel, 10 \( \mu \)m and right panels, 5 \( \mu \)m. PDGFR\textsubscript{x}, platelet-derived growth factor receptor alpha; GFAP, glial fibrillary acidic protein; DAPI, 4,6-diamidino-2-phenylindole, dihydrochloride; KA, kainic acid.
Figure 8. Opening of the BBB by mannitol exacerabtes seizure outcome. (A) Osmotic opening of the BBB was induced by infusion of high molality mannitol through the internal carotid artery in tPA−/− mice and visualized by Evans blue (EB) extravasion (arrow). (B) Vascular permeability was determined 1 h following mannitol treatment by quantitative analysis of EB extravasion in brain extracts. The results show significantly increased EB levels in tPA−/− brain extract following mannitol infusion (n = 6) compared to saline controls (n = 4). (C and D) Assessment of behavioral seizure manifestations in tPA−/− mice after mannitol-induced opening of the BBB (n = 8) revealed significantly faster onset (C) and generalization (D) compared to saline infused controls (n = 7). Data are presented as mean ± SEM (B) and Kaplan–Meier plots (C and D) from two independent experiments. Median times (t) are indicated in the figure. Statistical significance was determined by (B) unpaired Student’s t-test and (C and D) log-rank (Mantel-Cox) test. *P < 0.05, ***P < 0.001 significant relative to control. BBB, blood–brain barrier; tPA, tissue plasminogen activator.

Astrocytes play a central role in the pathology of seizures. Together, our data are consistent with the growing body of evidence showing that increased BBB permeability may constitute a significant pathologic factor in the development of seizures. Importantly, our results identify a specific molecular pathway involving tPA-mediated PDGFRα signaling in astrocytes that may regulate this process. We suggest that such a mechanism may contribute to the development of seizures as a common sequela of neuropathological events associated with significant disruption of the BBB, such as TBI and stroke. Indeed, in both ischemic and hemorrhagic stroke models, PDGFRα signaling has been shown to play a significant role in pathologic BBB dysfunction.

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 59). Confocal studies of comminostainings of neuroserpin and tPA with various neuronal markers revealed that neuroserpin is expressed in specific SST-positive interneurons, whereas tPA was found in VIP-positive neurons. These data are consistent with the idea of a neuroserpin/tPA regulatory circuit in the regulation of cerebrovascular responses as VIP-expressing “vasomotor” interneurons are reported to induce dilation of local microvessels, whereas SST-expressing interneurons are known to induce contraction. These data are also in agreement with the observation that tPA−/− mice have a diminished neurovascular coupling response, and with recent studies suggesting that tPA may be involved in neurometabolic coupling. We hypothesize that under normal conditions tPA is released in response to excitatory activity and promotes neurovascular and neurobarrier coupling (Fig. 2), and possibly neurometabolic coupling, to accommodate relatively high firing rates that occur during routine processing in the neocortex. However, during periods of aberrantly high firing rates, as occurs during recurring bouts of ictal activity, neuroserpin is released from inhibitory neurons to reduce coupling (via inhibition of tPA) which might act to augment GABA mediated feedforward inhibition that is thought to arise during seizure propagation.

The role of tPA in the CNS has been controversial. It has been proposed that tPA directly affects multiple physiologic processes, such as neuronal development, neuronal plasticity, axonal regeneration, as well as multiple pathologic processes, such as excitotoxicity, microglial activation/inflammation, and BBB dysfunction. Multiple potential substrates and receptors for tPA have also been proposed to mediate these diverse effects, including plasminogen, NMDA receptors, LRP1, and PDGF-CC. However, it is possible that the neurovascular events regulated by tPA and neuroserpin...
Figure 9. An intact BBB regulates seizure onset and progression. Neurophysiological experiments using (A) the in vivo KA seizure model and interhippocampal EEG recordings or (B) an ex vivo model of spontaneous synchronous activity induced in hippocampal slices by exclusion of Mg²⁺/elevation of K⁺ concentrations in aCSF. (A) Monopolar electrodes were positioned into the contralateral hippocampus and the time to onset of electrographic seizure activity was determined as described in Methods. The recordings mimic the clinical scorings presented in Figure 1 and show that in vivo onset of electrographic seizure activity in wild-type (WT) mice (n = 7) is intermediate between onset in Nsp⁻⁻ mice (n = 5, faster) and tPA⁻⁻ mice (n = 5, slower). (B) Extracellular field potential recordings were obtained in stratum oriens of the CA3 region in ex vivo brain slices from WT (n = 14), Nsp⁻⁻ (n = 11) and tPA⁻⁻ (n = 8) mice and the time to the first spontaneous high-frequency burst was recorded as described in Methods. Data are presented as Kaplan–Meier plots and median time (t) until electrographic event is indicated in the figure. The data are from 4 to 10 independent experiments. Statistical significance was determined by log-rank (Mantel–Cox) test and *P < 0.05; ns = non significant relative to WT control. BBB, blood–brain barrier; KA, kainic acid; EEG, electroencephalogram; aCSF, artificial cerebrospinal fluid; tPA, tissue plasminogen activator.

described here could provide a unifying pathway for many of the pleotropic effects of tPA. 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 NMDA receptor and neuronal calcium signaling. It is conceivable, however, that tPA-induced changes in BBB permeability could lead to a loss of precise control of the extracellular environment, which in turn, could promote dysregulation of neuronal signaling pathways such as the NMDA pathway. Likewise, tPA has been suggested to directly promote excitotoxicity. In this latter scenario, it may be that the loss of control of parenchymal homeostasis due to tPA-mediated changes in BBB permeability act to promote excitotoxic neuronal loss by allowing the buildup of parenchymal excitotoxins. Thus, our data suggest a possible common pathway for tPA and neuroserpin, permitting modulation of CNS function through the regulation of the neurovascular unit. However, it is important to note that although our data identify a critical neurovascular pathway regulated by neuroserpin/tPA, this does not rule out their potential role in the modulation of nonvascular CNS pathways, and the relative importance of these other potential interactions in different physiologic and pathologic pathways remains to be determined.

The association of mutations in the human neuroserpin gene with epilepsy has generally been thought to be due to the neurotoxic effects of neuroserpin inclusion bodies, although loss of regulation of tPA activity has also been proposed (reviewed in 75). Our results, demonstrating that Nsp⁻⁻ mice have a lower threshold for seizures, supports a potential “loss-of-tPA regulation” mechanism, and thus, suggests a potential pathway for therapeutic intervention in these patients by blocking tPA-induced PDGFRζ signaling. However, in a recent study by Tan et al., increased expression of tPA in the murine brain was found not to influence kindling-induced epileptogenesis, despite a lower threshold for electrically induced seizures. They also showed that the development of either early poststroke seizures or acquired epilepsy was not significantly increased following I.V. administration of thrombolytic tPA in humans. Together these studies suggest that tPA does not directly induce seizures or epilepsy, but instead lowers the threshold of other seizure provoking insults through its effect on cerebrovascular responses. It should, however, be noted that in a recent meta-analysis of stroke patients, hemorrhagic transformation of ischemic stroke was strongly correlated with early poststroke seizures. As hemorrhagic transformation is associated with severe BBB disruption and is one of the major complications of thrombolytic tPA therapy, it may be that in this subset of patients thrombolytic tPA contributes to an increase in early poststroke seizures. It will therefore be interesting to find out whether adjuvant imatinib therapy used with thrombolytic tPA in ischemic stroke patients leads to a decrease in the
incidence of poststroke seizures and epilepsy (ongoing I-STROKE clinical trial, European Stroke Network).

In conclusion, the data presented here support the hypothesis that loss of tPA regulation may contribute to the epilepsy phenotype seen in patients carrying neuroserpin mutations. As such, interventions aimed at controlling the effects of unregulated tPA activity may provide some benefit to these patients and possibly to patients suffering from seizures caused by other disorders. Furthermore, our data indicate that a critical feature controlling neuronal activity and seizure development is strict maintenance of the extracellular environment, as loss of BBB integrity correlates with loss of neuronal inhibitory control. Finally, this study suggests that there may be a common pathway responsible for many of the pleotropic effects of tPA in the CNS, and that these similar neurovascular signaling events may be common to different CNS disorders, including ischemic stroke and seizure. This commonality suggests that other diverse and seemingly unrelated CNS disorders where BBB dysfunction is involved, including neurodegenerative diseases, may also be influenced by tPA-mediated PDGFRα signaling.

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Conflict of Interest

Dr. Murphy reports grants from United Stated Department of Defense, during the conduct of the study. Dr. Su has a patent methods and compositions for modulation of bloodneural barrier issued. Dr. Lawrence reports grants from National Institutes of Health, during the conduct of the study; In addition, has a patent methods and compositions for modulation of bloodneural barrier issued. Dr. Fredriksson reports grants from Swedish research council, The Swedish Governmental Agency for Innovation Systems, during the conduct of the study; In addition, has a patent methods and compositions for modulation of bloodneural barrier issued. Dr. Stevenson reports grants from postdoctoral fellowship for foreign researchers from the Japan society for the promotion of science (JSPS), during the conduct of the study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of experimental settings, results, and statistics. The table summarizes the number of animals used (N), the results and the statistical analysis with P values for each of the experiments.