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Yena Woo March 23, 2023

Hijacking the Plasminogen Activator System for Possible Therapeutic Potential for Neurovascular and Neurodegenerative Diseases

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a thesis submitted to the Faculty of Emory College of Arts and Sciences

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Abstract

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By Yena Woo

With the earlier onset of neurovascular and neurodegenerative diseases along with the increasing worldwide life expectancy, the need for therapeutics continues to grow[1]. In the last two decades, however, the hyperfixation on certain biomarkers and uni-modal treatments continues to abrogate the development of our understanding of these diseases. Aware of this need, this research aims to explore an ischemic stroke system, the Plasminogen Activator System (PAS), and enumerate the contributions to the developments regarding the possible therapeutic potential of this system. Here, the main activators and inhibitors of the PAS are investigated in relation to the astrocytic and neuronal components of the neurovascular unit (NVU). The data presented shows the role of urokinase-plasminogen activator (uPA) in astrocytic wound healing via astrocytic activation, in absence of an injury or pathological state, of the Wingless (WNT) pathway. Additionally, tissue-type plasminogen activator (tPA) is able to contribute not only to astrocytic activation but also to the modulation of cerebral ischemia via the inducement of aquaporin-4 (AQP4) in the astrocytic end feet. Finally, Plasminogen Activator Inhibitor-1 (PAI-1) is shown to increase the morphological complexity of neurons. The demonstrated role of the PAS in astrocytic activation and neuron arborization in this study highlights the future potential of hijacking the PAS in therapeutic target development for neurovascular and neurodegenerative diseases.

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Table of Contents

Introduction

Neurovascular Disorders and Neurodegenerative Disease

Neurodegenerative diseases, such as Alzheimer's Disease (AD), have a widespread worldwide impact, with the World Health Organization (WHO) noting that over 55 million people worldwide have dementia, with AD accounting for 60-70% of it.[2] Along with the terrible social and personal ramifications of AD, there is a large social and economic burden associated with AD. Within the United States alone, it is projected that yearly costs associated with AD will increase from $321 billion in 2022 to over $1 trillion in 2050, not to mention the burden on the healthcare system with the increasing patient population for long-term care.[3] Neurodegenerative diseases do not exist within a bubble and are associated with changes in the neurovasculature such as the permeability of the blood-brain barrier (BBB).[4] As there is a link between neurodegenerative diseases and neurovascular disorders, this study aims to explore a system, commonly studied in the vascular space, for its possible effect on different components of the neurovascular unit (NVU), with the hope of future applications for therapeutic development for neurovascular and neurodegenerative diseases.

Plasminogen Activator System

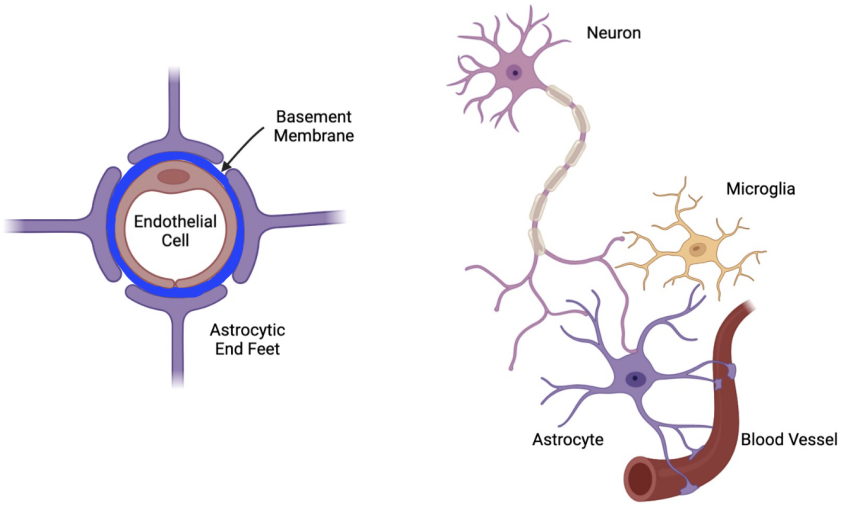
The Plasminogen Activator System (PAS) is an enzymatic cascade that is most popularly known for its anti-clotting role in the intravascular cascade. Plasminogen is a zymogen that must be cleaved to form active plasmin, which breaks down fibrin, the main structural unit of blood clots[5]. The main components of the PAS of interest for this paper are the two main mammalian activators: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), and one of three main inhibitors: Plasminogen Activator Inhibitor - 1 (PAI-1).

uPA consists of three regions: an amino-terminal growth factor domain, a Kringle domain, and a carboxy-terminal catalytic domain.[5] It is expressed in an inactive single-chain protein form, pro-uPA, which is activated into a two-chain uPA.[5,6] uPA has been associated with angiogenesis, tumorigenesis, cell adhesion, and cell migration.[7] tPA consists of 5 structural domains: a fibronectin finger domain, two Kringle domains, an epidermal growth factor analog, and a serine protease domain.[5] It is initially expressed as a single chain but is cleaved into two chains via a positive feedback mechanism from the activation of plasmin.[8] tPA is currently the gold standard of treatment for ischemic stroke, within 3.5 hours of onset.[9] PAI-1 is a single-chain glycoprotein of 45 to 50 - kDa and can interact with uPA, uPA and receptor complexes, and tPA.[10] These interactions modulate signal transduction pathways as PAI-1 is localized to the cell surface.[11] Additionally, PAI-1 induces microglial migration.[12]

Although the PAS has been commonly known for its peripheral proteolytic activity in the intravascular space, in the past two decades there has been a growing area of research in considering the role of the PAS in the central nervous system (CNS).[5]

The Neurovascular Unit

The neurovascular unit (NVU) is a concept that was first described in 2001, and describes the maintenance of cerebrovascular integrity and hemostasis through a cohesive system of neurons, astrocytes, microglia, endothelial cells, and the extracellular matrix.[13,14] This paper specifically focuses on the effect of the PAS on neuronal and astrocytic components of the NVU.



Scheme 1: Scheme of the Neurovascular Unit (NVU). Created in BioRender.com.[15]

Connecting the PAS to the NVU and neurodegenerative diseases

Neurodegenerative diseases, such as AD, are characterized by the loss of synaptic contacts via mechanisms that are still trying to be elucidated.[16] The formation of new synapses is known as synaptogenesis and it is mediated by other support cells such as astrocytes as highlighted by concepts such as the tripartite synapse.[17,18] Although the work presented here does not investigate synaptogenesis, elucidating the effect of the PAS on astrocytic neuronal components of the NVU could be integral research in neuro-repair. Astrocytes can modulate the strength and rate of synapse communication, play a role in synapse formation, and release different neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), in its normal, activated, and reactive states.[19] Previous work has shown that uPA[20] and tPA[21] induce astrocytic activation, and thus based on this knowledge, the aim is to investigate the mechanisms and effects of this astrocytic activation. Specifically, the aim is to investigate the role of uPA in astrocytic wound healing, the role of tPA on Aquaporin-4 (AQP4), a water channel associated with astrocytic motility[22], expression in astrocytes, and PAI-1's effect on neuronal complexity. Based on this knowledge

and previous work regarding the PAS in the CNS, it is hypothesized that uPA would increase astrocytic wound healing and tPA would increase AQP4 expression, and PAI-1 would increase neuronal complexity.

Materials and Methods

1. Astrocytic and Neuronal Primary Culture

All animal care was conducted by the lab managers, Dr. Enrique Torre and Zakia Sathi, in accordance with the approved protocol by the Institutional Animal Care & Use Committee (IACUC) of Emory University, Atlanta, GA. All mice used were C57BL/6J mice. Astrocytes (WT, $uPA^{-/-}$) were cultured from 1-day-old mice and then seeded on glass coverslips. As per lab protocol, primary cultures were handled by post-doctoral students. The following procedure was conducted by post-doctoral fellow, Dr. Ariel Diaz, as described in his protocol[23,24]. In brief cerebral cortices from the mice were harvested and placed in balanced Hank's salt solution supplemented with 1000 units/ml penicillin (Gibco, Thermofisher; Grand Island, NY; 15140-122), 100 µg/ml streptomycin (Gibco, Thermofisher; Grand Island, NY; 15140-122), and 10 mM HEPES.[24] After trypsinization and trituration, the tissue was resuspended and passed through a 70 mm pore filter and cultured on poly-L-lysine (MillPore; Burlington, MA; P1399) surfaces in T75 flasks for 10-15 days before use in individual experiments.[24] Neuronal cultures (using embryonic day 16 mice) followed the same methods with a difference in solution.[20] Cerebral cortices for neuronal cultures were incubated in 0.02% DNase in trypsin (Quality Biological; Gaithersburg, MD; 118-093-721) at 37℃ for 15 minutes prior to trituration. Prior to plating, the triturated tissue was resuspended in 2 mM L-glutamine in GS21-supplemented neurobasal medium.[20]

2. Astrocytic Wound Healing Model

Astrocytes from primary cultures (as described above), were plated onto glass coverslips with poly-L-lysine (Millipore; Burlington, MA, P1399) and were cultured until confluent in supplemented media. A sterile 20 μL pipette tip was used to inflict a scratch wound on the confluent monolayer of astrocytes. After wound infliction, slides were treated with the control and 5 nM of uPA for 24 hours. This was followed using live cell microscopy over 24 hours using live cell microscopy DP80 camera using the 10X objective magnification (Olympus, Center Valley, PA), while maintained at 37℃ in an incubator (Tokai Hit, Japan) atmosphere supplemented with 5% CO_2, 21% O_2, and 74% N_2. Images were taken at 10X objective magnification at t=0h and t=24h of each of the samples. Area growth was quantified by drawing at 500 μm line at the border the wound at t=0h, copying and pasting into the same XYZ coordinates for t=24h. Quantification was completed using Olympus CellSens Dimension 1.17 software, and percent growth was normalized to the control group.

3. Western Blot for Aquaporin 4 (AQP4) Quantification

Astrocytic cultures were treated with 5 nM of tPA for 3 hours, and then subsequently homogenized using RIPA buffer (TEKNOVA; Hollister, CA; R3792) and centrifugation at 21000g for 20 minutes at 4℃. Using the Pierce BCA Protein Assay (ThermoFisher; Grand Island, NY; 23225), equal amounts of protein were loaded onto a mini-protean TGX stain free 4-15% gel, transferred onto a 0.2 μm, nitrocellulose membrane (Bio-Rad; Hercules, CA; 1620112) and blocked with Odyssey Blocking Buffer (TBS) (Li-Cor; Lincoln, NE, 927-50000) for 1h. Membranes stained overnight at 4℃ for APQ4 using primary antibody rabbit anti-AQP4 (1:1000) (Abcam; Cambridge, UK; Cat # ab46182) and mouse anti-B-actin (1:10000) (ThermoFisher; Grand Island, NY; H3570). Subsequently, membranes were thoroughly washed and reacted with the following secondary antibodies: IRDye 800CW donkey anti-rabbit

(1:10000) (Li-Cor; Lincoln, NE; 926-32213) and IRDye 680RD donkey anti-mouse (1:10000) (Li-Cor; Lincoln, NE; 926-68072). Membranes were imaged using LI-COR Odyssey Fc reader and IR-signal was quantified and analyzed using IMAGE STUDIO 5.2. IR readings were normalized to control.

4. Immunocytochemistry and Imaging

Astrocytic cultures were fixed using 4% paraformaldehyde (Electron Microscope Sciences; Hatfield, PA; 15714) in TBS for 15 minutes. Cells were permeabilized for 10 minutes using 0.1% triton and 0.5% casein in TBS-T and blocked for 1 hour using goat and donkey serum in TBS. Cells were then incubated at 4℃ overnight in the primary antibody, rabbit polyclonal antibody for the intracellular domain of N-Cadherin (NCAD-ICD) (Abcam; Cambridge, MA; cat. # ab76057) (1:400) and mouse anti-β-catenin (ThermoFisher; Grand Island, NY; Cat. # A1978) (1:400), and then reacted with Alexa Fluor 594-conjugated donkey anti-mouse-IgG antibody (ThermoFisher; Grand Island, Ny; Cat. # A21203) (1:500) and goat anti-rabbit 33342 Alex Fluor 488 antibody (ThermoFisher; Grand Island, NY; Cat. # A11008) (1:5000), along with HOECHST (1:4000) stain. After thorough washing in TBS, glass coverslips were mounted with Prolong gold antifade mounting media (ThermoFisher; Grand Island, NY; P36930). Slides were imaged using the Fluoview FV10i automated confocal laser-scanning microscope (Olympus, Center Valley, PA) at 60X lens NA 1.35 oil, pinhole 1UA (50mm), resolution 1024 x 1024 pixels and 16 bit.

The same processes were repeated for neuronal cultures, but different primary and secondary antibodies were used. A mouse anti-MAP2 monoclonal primary antibody (Sigma-Aldrich; St. Louis, MO; M9942) (1:1000) along with Fluorescent Dye 488-l Phalloidin (Abnova; Jhongli, Taiwan; U0281) was used to mark the dendritic components of the neuron.

Images were taken using fluorescence microscopy at 20x magnification using (Olympus Microscope BX51).

5. Sholl Analysis vs. Fractal Analysis

Image J PlugIns "Sholl Analysis" and "Fractal Analysis" were used to quantify neuronal complexity. The basis of Sholl analysis is to determine dendritic arborization by counting the number of intersections of concentric circles at intervals from the fixed distance from the neuron soma. A unified basis of 50 μm from the center of the neuronal soma was determined based on control analysis. Concentric circles ranged every 10 μm starting from 50 μm to 200 μm away from the cell soma. A thresholding parameter was used to deal with the heterogenous neuronal population that was used for this experiment: neurons with less than three main dendritic branches were excluded from the analyses.. Fractal analysis, which quantifies the 3D area of the neuron by assigning a pixel to each part of the neuron.

7. Statistical Analysis

Astrocytic wound healing quantification: Wound healing of $uPA^{-/-}$ treatment was normalized as percentages to the WT untreated group. An unpaired student's t-test was conducted for statistical significance. In comparing control and uPA treatment to both genotypes (WT and $uPA^{-/-}$) a two-way ANOVA with Tukey's Multiple Comparison Test was conducted. The threshold as $p < 0.05$ was used for both analyses.

Quantification of Beta-Catenin Localization: Mander's coefficient was used as the metric for quantifying colocalization between beta-catenin and the intracellular domain of N-Cadherin. An unpaired student's t-test was conducted for statistical significance with the threshold as $p < 0.05$ for statistical significance.

tPA expression in astrocytes: Western blot analysis was conducted using IR signaling. All values were normalized to β-actin and then subsequently normalized to the control. A two-tailed student's t-test was conducted for statistical significance with the threshold as $p < 0.05$ for statistical significance.

Neuronal complexity: A two-tailed, unpaired student's t-test was conducted for statistical significance with the threshold as $p < 0.05$ for statistical significance.

Levene and Shapiro-Wilk tests were completed to test the normal distributions of all data prior to all statistical analyses.

Results

Wound Healing Potential of uPA

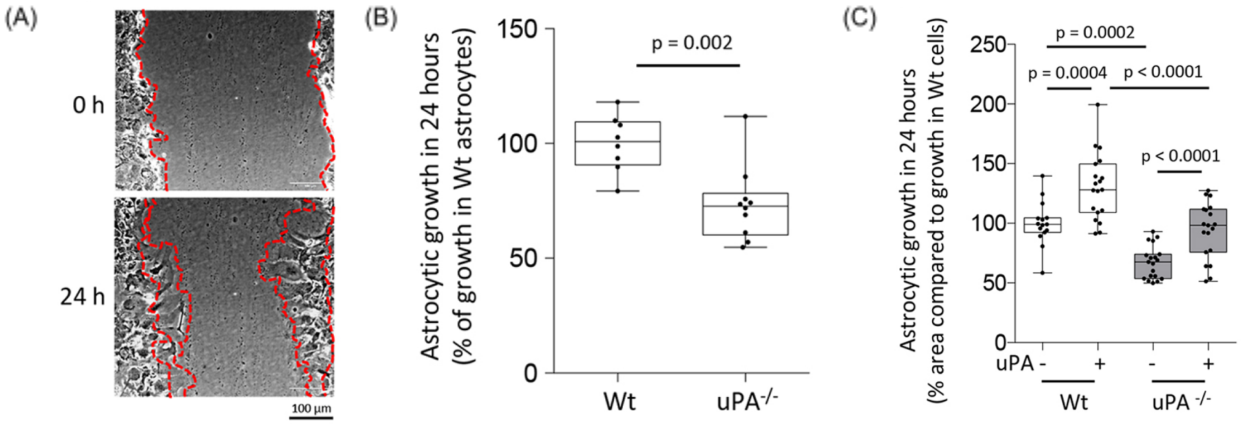


Figure 1: uPA treatment promotes astrocytic wound healing. (A) Representative image of the process of quantifying wound healing 24h post wound infliction. (B) Astrocytic growth of $uPA^{-/-}$ (n=10) astrocytes shown as percent of WT (n=8) astrocytes. Statistical analysis: unpaired, student's t-test (C) Astrocytic growth following treatment with vehicle (control) [WT, n=15; $uPA^{-/-}$, n=19] or 5 nM uPA [WT: n=15, $uPA^{-/-}$: n=19] post 24 hours of mechanical injury. Statistical analysis: Two-way ANOVA with Tukey's Multiple Comparison Test. Modified from Diaz et al., 2021.[24]

To investigate the effects of uPA on astrocytes, a wound-healing model was used. After growing a monolayer of astrocytes, a mechanical wound was inflicted to investigate the wound-healing possibilities of astrocytes. A 500 µm was drawn at t=0 and copied to the same

XYZ coordinates after 24h of growth, as seen in panel 1A. First uPA knockout (KO) mice were used to determine if there was any possible effect of uPA on wound healing. Figure 1B shows that uPA$^{-/-}$ had decreased astrocytic growth (73.47±16.38%, mean±s.d., P=0.002, two-tailed, student's t-test) as compared to WT genotype. To test if this effect is dependent on whether uPA is endogenous or exogenous, both genotypes were treated with recombinant uPA. Treatment with rt 5 nM uPA resulted in increased astrocytic growth in both genotype groups (WT/uPA$^{-/-}$) (Wt: 100% ± 18.56 %; Wt+uPA: 130.3% ± 27.82%; uPA$^{-/-}$: 67.21% ± 13.79%; uPA$^{-/-}$ + uPA: 93.72% ± 23.54%; mean±s.d.). It is important to note that the WT group had more astrocytic growth in both control and treatment groups as compared to its uPA$^{-/-}$ counterparts, suggesting the dual effect of both endogenous and rt uPA.

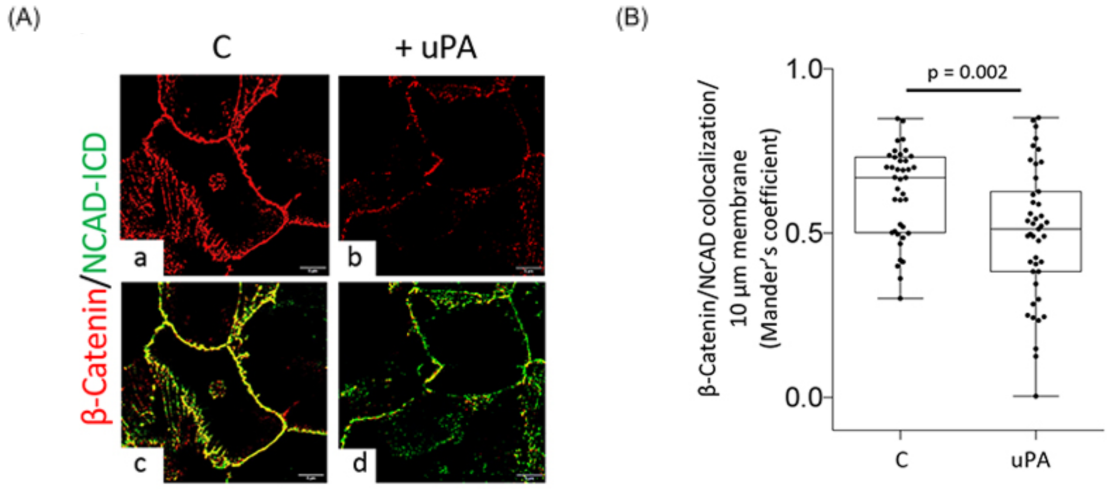


Figure 2: uPA treatment induces detachment of β-catenin from NCAD-ICD. (A) Representative confocal images of co-localization of β-catenin and NCAD-ICD in astrocytic membranes. Panels a,c controls. Panels b,d 30 min 5 nm uPA treatment. (B) Quantification of colocalization per 10 µm of membrane, control (n= 39) vs 5 nm uPA treatment (n=43). Statistical test: unpaired, student's t-test. Modified from Diaz et al., 2021.[24]

As previous research suggested the involvement of the Wnt pathway, the effect of uPA on the intracellular localization of both β-catenin and the intracellular domain of N-Cadherin

(NCAD-ICD) was investigated. An uninjured monolayer of cultured astrocytes was treated with 5 nM of uPA for 30 minutes, and then subsequently stained and imaged for co-localization of β-catenin and NCAD-ICD to see changes in intracellular localization, specifically in regards to changes in membrane localization. As seen in figure 2B, treatment of WT monolayer of astrocytes resulted in decreased colocalization of β-catenin and NCAD-ICD, suggesting a change in β-catenin localization. As seen in figure 2A, β-Catenin was stained red, NCAD-ICD green, with the overlap shown in yellow. The Mander's coefficient was used as a metric of colocalization of fluorescence with mean mander's coefficient of uPA treatment 0.4981 ± 0.2033; mean ± s.d.) being lower than control (0.6228 ± 0.1379; mean ± s.d.).

tPA increases AQP4 in neuronal astrocytes

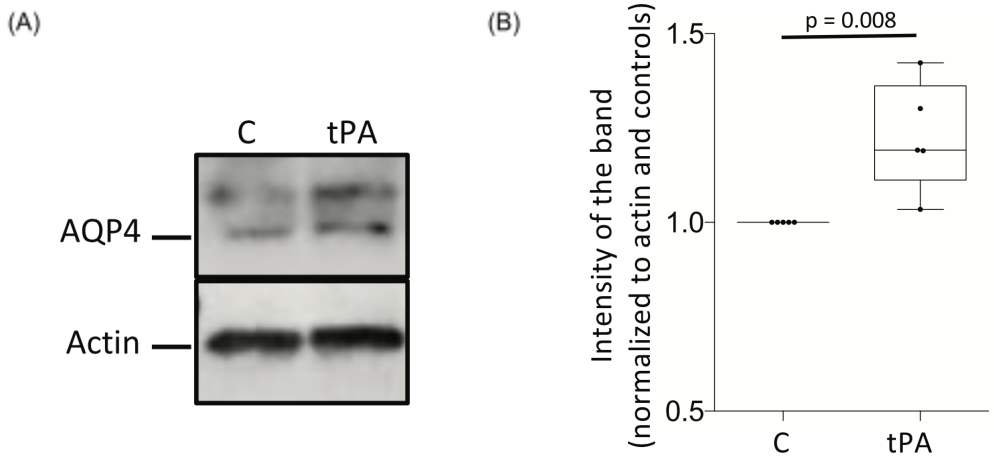


Figure 3: Preconditioning with tPA increased Aquaporin-4 in neuronal astrocytes (A) Representative image of western blot (WB) stained for AQP4 and B-actin. (B) Intensity of WB band shown as tPA treatment group (n=5) thresholded to control (n=5). Statistical Test: Two-tailed student's t-test. Modified from Diaz et al., 2022.[21]

To consider the PAS in the CNS, it was important to also consider the effect of 5 nM tPA for 3 hours, another activator, on astrocytes. Previous research in the laboratory showed that tPA induced astrocytic activation and modulated the permeability of the BBB.[21] To explore the mechanism by which this modulation may happen, the effect of tPA on Aquaporin-4 (AQP4), a known regulator of water and permeability in the NVU was studied. It was shown that preconditioning astrocytes increased AQP4 expression as normalized to the control (intensity of tPA treated 1.228 ± 0.1445, mean ± s.d., P=0.008, unpaired student's t-test, n=5). Following 5nM conditioning treatment with tPA for three hours before injury shows increased aquaporin 4 expression in neuronal astrocytes. Western Blot analysis of AQP4 (34 kD) was controlled to B-actin (42 kD) for all cells. This suggests that the pretreatment of tPA increased the expression of AQP4 in neuronal astrocytes.

PAI-1 on Neuronal Complexity

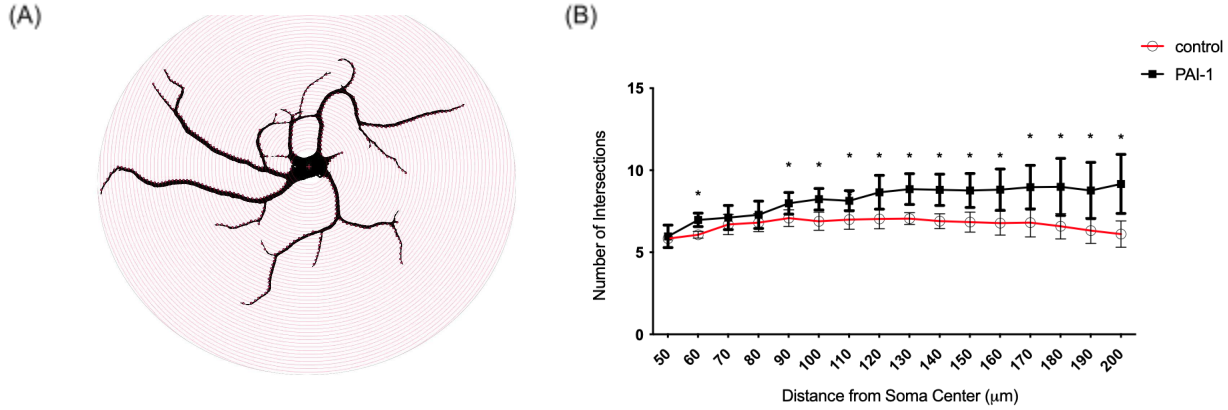


Figure 4: PAI-1 increases neuronal complexity (A) Representative Image of Sholl Analysis. (B) Sholl analysis of intersections at various distances from the neuron's soma center from 50 to 200 μm. n=4. Statistical analysis: unpaired, student t-test.

In investigating the effect of the PAS in the CNS, neurons must be studied. It has been shown that morphogenesis of neurons plays an integral part in neuronal communication, thus has been studied in regard to neurodegenerative disorders.[25] Previous work has shown that PAI-1

modulates the effect of uPA on astrocytes, and thus the question remains whether this is true for the neuronal component as well.[20] Low-density neuronal cultures were used to determine the effect of PAI on neuronal complexity, specifically dendritic components. The dendrites were stained with MAP2 and checked using a phalloidin stain for manual thresholding. These low-density cultures were treated with 10 nM PAI-1 for 24 hrs and analyzed via Sholl analysis. The basis of Sholl analysis is measuring the dendritic arborization by counting the number of intersections with concentric circles at fixed distances (see figure 5A). As seen in figure 5B, there was increased neuronal complexity by Sholl analysis at multiple distances from the neuron soma. Specifically, there was an increase in intersections at 60 and 100-200 μm from the soma with PAI-1 treatment (100 μm: 6.90 ± 0.55 intersections vs 8.24 ± 0.66 intersections , n=4, p=0.02; 200 μm: 6.11 ± 0.80 intersections vs 9.17 ± 1.80 intersections, n=4, p= 0.02). This was corroborated by fractal analysis done by Dr. Cynthia Martin, seen in supplemental information (see SI, Supplemental Figure 1).

Discussion

The PAS has only started to be studied in CNS in the past two decades.[5] The purpose of this investigation is to study the PAS effect in the CNS considering the NVU and tripartite synapse as the structural framework.

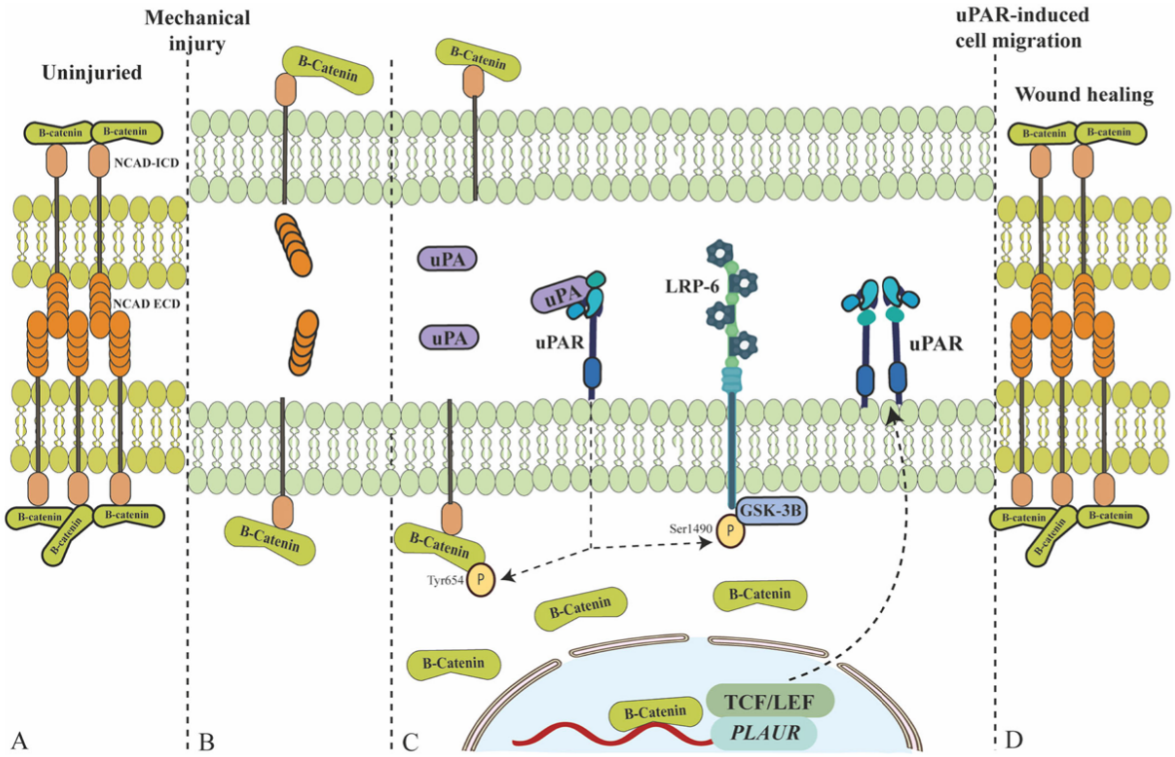
In considering astrocytic-mediated neuro-repair, it is imperative to consider the effect of activators on astrocytes, especially considering the vast literature regarding the “double-edged” nature of astrocytic activation.[19] Both uPA and tPA have effects on astrocytes in promoting astrocytic activation in absence of pathogenesis.[20,21] Here, it is shown that uPA has wound-healing properties after a mechanical injury. Not only did uPA treatment increase the area of healing, but $uPA^{-/-}$ showed decreased healing as compared to the treated WT genotype,

highlighting the role of both endogenous and exogenous uPA. Work continued by others in the lab has shown that this astrocytic wound healing is via astrocytic migration, rather than astrocytic proliferation.[24]

Reactive astrocytes, as caused by pathological changes, are found in many neurodegenerative diseases, such as Alzheimer's Disease, and prolonged astrocytic reactivity has been associated with the formation of glial scars and fibrosis that prevents axonal regeneration.[26–28] Despite this, astrocytic reactivity has also been associated with supporting axonal growth and directionality, and multiple ischemic stroke drugs work to activate and inhibit pathways that would balance the protective and destructive functions of reactive astrocytes.[19] It is important to note that there is a difference between astrocytic reactivity and astrocytic activation; astrocytic reactivity is in the presence of a pathological state.[29] Thus, it is incredibly valuable and important to be able to identify mechanisms in which astrocytic activation can be induced in absence of pathological change, and thus the inducement of astrocytic migration by uPA is integral.

The mechanism that was considered was the WNT pathway as it is known to be integral in cell migration and neural patterning.[30] uPA was shown to increase the detachment of β-catenin from the intracellular domain of N-Cadherin, suggesting translocation or degradation of β-catenin. Other work in the laboratory has shown that this detached β-catenin was not degraded by actually being promoted to have a nuclear translocation.[24] It is suggested that the Wingless pathway is triggered by uPA binding to uPAR as supported by the lab data that there is phosphorylation of β-catenin at the known important Tyr654 residue. It is known that phosphorylation at Tyr654 targets β-catenin for nuclear translocation, and phosphorylation at other sites by GSK-3 differently targets β-catenin for proteasomal degradation via

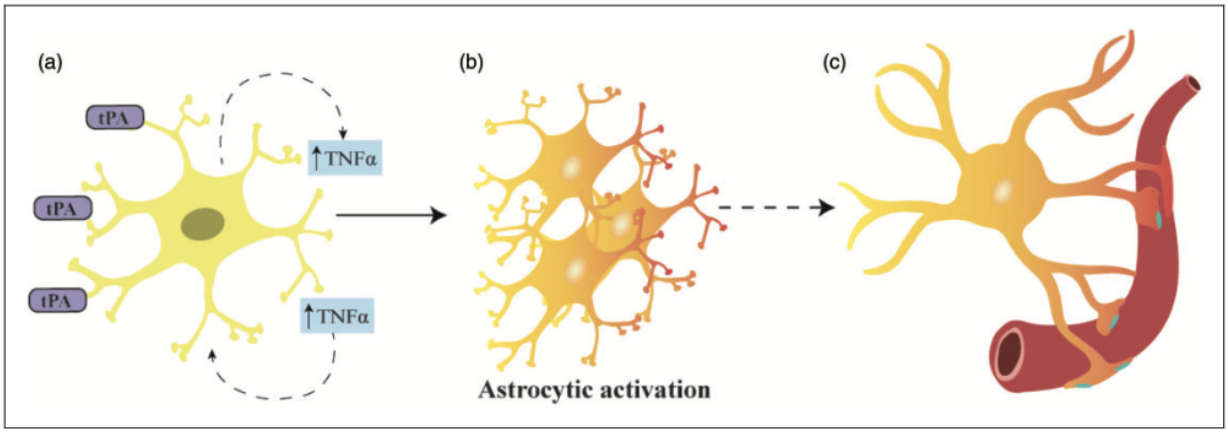
ubiquitination.[30,31] Additionally, other work in the lab has shown that uPA/uPAR binding also phosphorylated LRP6, which then abrogated GSK-3, preventing it from tagging β-catenin for degradation. The translocation of β-catenin to the nucleus has been associated with increased wound healing, as seen with the results of this paper.[24]



Scheme 2: Proposed mechanism of uPA-mediated β-catenin inducement of the WNT pathway for astrocytic wound healing. Adapted from Diaz et al., 2021.[21]

tPA, the other Plasminogen Activator, was also studied in regard to astrocytic activation. AQP4 became a question of interest due to other work highlighting the role of AQP4 in astrocytic motility.[22] tPA was shown to not only increase the production of AQP4 in astrocytes, but further work also showed the increase in the intracellular localization of AQP4 in the astrocytic end feet (see SI, Supplemental Figure 2). This served as the basis for others in the lab to determine the in-vivo effect of this, and a preconditioning effect on the blood-brain barrier

was seen, with increased cerebral edema in tPA knockouts.[21] The proposed mechanism is the activation of the TNF-α pathway in which TNF-α released by activated astrocytes (scheme 3, panel A) induce astrocytic activation with the effect of increased production of AQP4 (scheme 3, panel B), protecting the neurons from the deleterious effects of cerebral ischemia by maintaining the BBB (scheme 3, panel C). This modulation of the BBB with increased AQP4 is integral due to the growing body of evidence of the link between neurodegenerative diseases, such as AD, and cerebrovascular degeneration.[32]



Scheme 3: Proposed mechanism of astrocytic activation by tPA. Adapted from Diaz et al., 2022.[21]

In considering the effect of PAS on neuro-repair, an integral part is the neurons themselves. Thus, the effect of PAI-1 on neurons was investigated in line with a growing body of evidence suggesting the importance of this. The morphological complexity of the neurons, specifically the dendritic component, was used as a proxy for the potential of future investigation in synaptogenesis. The number and structure of dendritic spines, otherwise known as dendritic arborization, is important for the transmission of signals and for processes such as long-term potentiation[33]. Sholl analysis suggests an increase in the morphological complexity of the neurons, with statistically significant increases in the intersections from 100 to 200 μm from the

center of the neuronal soma. This bears the question of why there was no noted difference in the dendritic arborization at distances 50 to 100 μm from the center of the soma. This could be attributed to the differential soma sizes of the neurons but also could be due to the limitations of the Sholl analysis itself. Sholl analysis counts the number of branches at different points from the soma center, thus serving as a quantified metric for dendritic branching geometry.[34] However, this does not account for the more local changes (i.e. changes between those checkpoints; for this study determined to be every 10 μm from 50 to 200 μm from the neuronal soma), and thus may not fully capture the effect of PAI-1 on increasing morphological complexity of neurons.[35] It is also important to note that the basis of this particular Sholl analysis was conducted under the assumption of homogeneity across samples regarding the heterogeneity of the neuronal population. Aware of the criticism of Sholl analysis[36], another method of analysis called Fractal Analysis was also conducted by Dr. Cynthia Martin using the same data set. This corroborated the result obtained via Sholl analysis as fractal analysis also showed a statistically significant increase in neuronal complexity following 24-hour treatment of 10 nM PAI-1. This further suggests that PAI-1 increased the dendritic arborization of neurons.

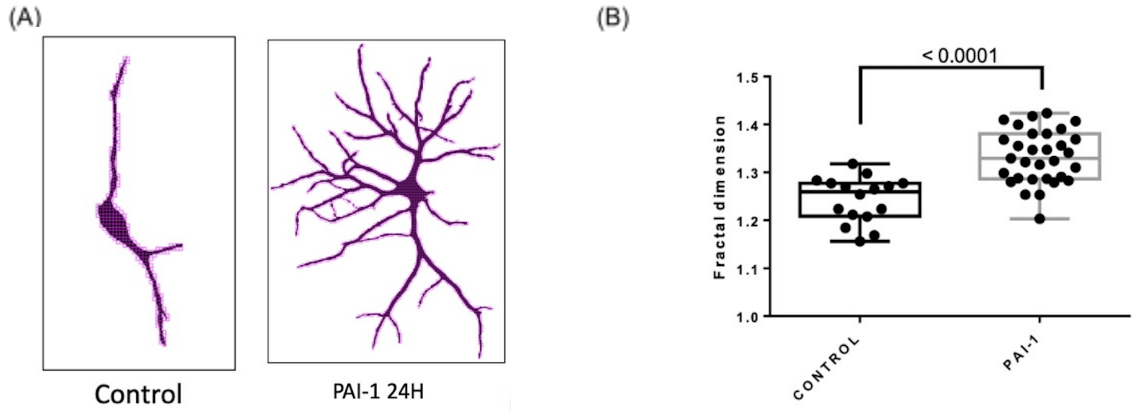
Work presented in this paper shows the effect of uPA and tPA on astrocytes (wound healing potential of uPA on astrocytic growth and the modulation of the NVU with the increase of AQP4 in tPA) and the effect of PAI-1 on neuronal complexity. In order to have a greater understanding of the components of the PAS on the NVU as a whole, it would be beneficial to study the effect of complexes, as clinical data has shown differential pharmacokinetics of complexes.[37] Although investigating the complexes of activators and inhibitors may sound counterintuitive, it further highlights the role of the PAS in the CNS in hopes to shed light on

mechanisms to hijack the possible therapeutic potential for neurovascular and neurodegenerative diseases.

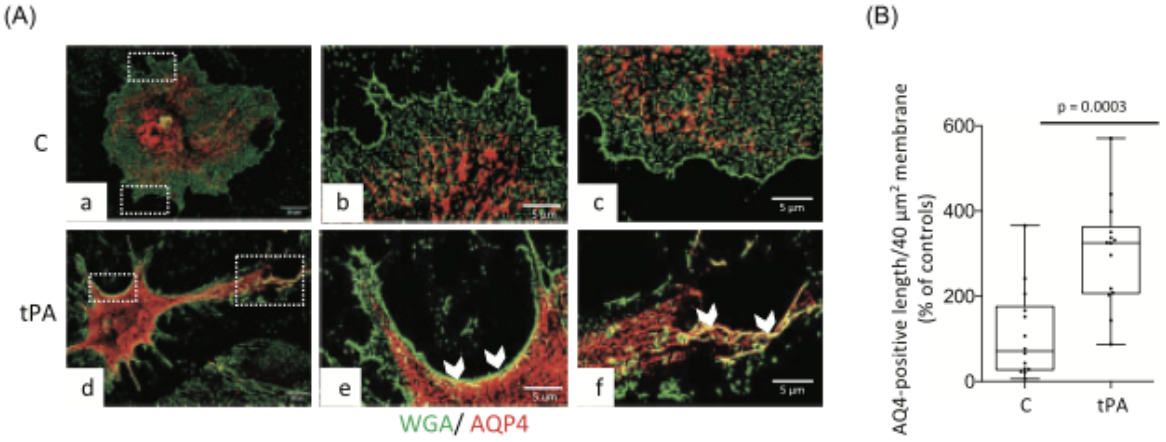
Conclusions

The work presented here supports the growing body of literature of the role of the PAS in the CNS. uPA is shown to induce astrocytic wound healing via a suggested activation of the β-catenin/WNT pathway. tPA is shown to increase AQP4 expression in the astrocytic endfeet, serving as the basis of proposing the TNF-α mediated modulation of the BBB, further linking the role of cerebrovasculature and astrocytic activation. PAI-1 was shown to increase dendritic neuronal complexity. By studying the PAS in both neuronal and astrocytic components of the NVU, the PAS shows potential for druggable targets for neurovascular and neurodegenerative diseases.

Supplemental Information



Supplemental Figure 1: Fractal Analysis for Neuronal Complexity by Dr. Cynthia Martin-Jimenez. (A) Representative images of fractal analysis for both control and PAI-1 24 hour treatment. (B) Fractal dimension criterion for both control and PAI-1 treated neurons.



Supplemental Figure 2: Increased cellular localization of AQP4 on astrocytic end feet by Dr. Ariel Diaz. (A) Representative images of confocal immunohistochemistry (B) Quantification of AQP-4 in astrocytic end-feet for control and compared to 5 nm tPA treatment. n=14 cells from three different animal groups for both control and tPA treatment. Adapted from Diaz et al., 2021.[21]

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