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Astrocyte tissue plasminogen activator expression during brain development and its role in pyramidal neuron neurite outgrowth

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Abstract

The serine protease tissue plasminogen activator (tPA), encoded by the gene *Plat,* exerts a wide range of proteolysis-dependent and proteolysis-independent functions. In the developing brain, tPA is involved in neuronal development *via* the modulation of the proteolytic degradation of the extracellular matrix (ECM). Both lack of and excessive tPA are associated with neurodevelopmental disorders and with brain pathology. Astrocytes play a major role in neurite outgrowth of developing neurons as they are major producers of ECM proteins and ECM proteases. In this study we investigated the expression of *Plat* in developing and mature hippocampal and cortical astrocytes of Aldh1l1-EGFP-Rpl10a mice *in vivo* following Translating Ribosome Affinity Purification (TRAP) and the role of tPA in modulating astrocyte-mediated neurite outgrowth in an *in vitro* astrocyte-neuron co-culture system. We show that *Plat* is highly enriched in astrocytes in the developing, but not in the mature, hippocampus and cortex. Both the silencing of tPA expression in astrocytes and astrocyte exposure to recombinant tPA reduce neuritogenesis in co-cultured hippocampal neurons. These results suggest that astrocyte tPA is involved in modulating neuronal development and that tight control of astrocyte tPA expression is important for normal neuronal development, with both experimentally elevated and reduced levels of this proteolytic enzyme impairing neurite outgrowth. These results are consistent with the hypothesis that the ECM, by serving as adhesive substrate, enables neurite outgrowth, but that controlled proteolysis of the ECM is needed for growth cone advancement.

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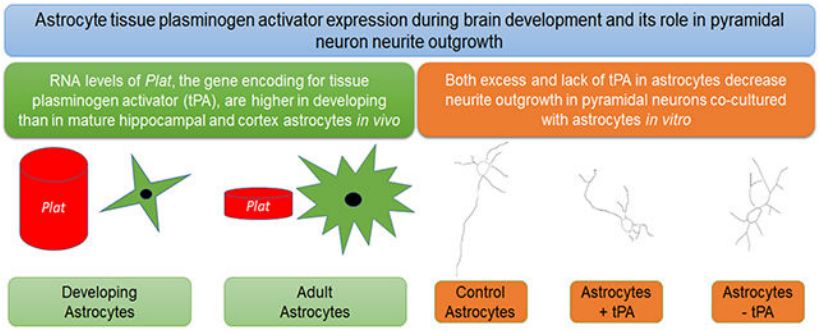
Declaration of Competing Interest

None.

CRediT authorship contribution statement

Calla M. Goeke: Investigation, Data curation, Formal analysis, Writing - original draft. **Xiaolu Zhang:** Investigation, Data curation, Formal analysis, Writing - original draft, Writing-review & editing. **Joel G. Hashimoto:** Investigation, Data curation, Formal analysis, Writing-review & editing. **Marina Guizzetti:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

Graphical Abstract



Keywords

Astrocytes; Tissue plasminogen activator; Pyramidal neurons; Neurite outgrowth; Translating Ribosome Affinity Purification; Brain Development

1. Introduction

Tissue Plasminogen Activator (tPA), encoded by the *Plat* gene, is a serine protease that was initially identified in the blood and studied for its profibrinolytic role in the vasculature [1, 2]. The best-characterized function of tPA is the proteolytic activation of the zymogen plasminogen to the extracellular proteolytic enzyme plasmin [3, 4]. In the brain tPA is produced by neurons, astrocytes, microglia, and oligodendrocytes [3] and plays a role in neuronal plasticity, axonal regeneration, excitotoxicity, neuroprotection, microglial activation, neuroinflammation, and increased blood-brain barrier permeability [3-5].

Developing astrocytes are involved in dendrite and axon growth, synaptogenesis, and synapse elimination [6, 7]. The extracellular space, which accounts for approximately 40% of the neonatal brain volume, contains the highly dynamic ECM, which modulates cell proliferation, cell migration, the growth of dendrites and axons, and the formation of synapses [8, 9]. Extracellular proteases, including tPA, are responsible for the remodeling of the ECM [10, 11], and the dysregulation of ECM and extracellular proteases is involved in several brain pathologies [10, 12]. Astrocytes express and release numerous ECM proteins and extracellular proteases [13].

In this study we investigated the effects of the modulation of astrocyte tPA on neuronal development using an *in vitro* astrocyte-neuron co-culture system that allows us to understand the effects of treatments carried out in astrocytes on neurite outgrowth of pyramidal neurons [14, 15]. To test the relative expression of *Plat* in developing and mature astrocytes *in vivo,* we employed a transgenic mouse model (the Aldh1l1-EGFP-Rpl10a mouse model) and the Translating Ribosome Affinity Purification (TRAP) method to isolate astrocyte-specific translating RNA [16-18].

2. Materials and methods

2.1. Animals

Timed-pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA). Adult hemizygous Aldh1l1-EGFP-Rpl10a transgenic mice [B6; FVB-Tg (Aldh1l1/EGFP/Rpl10a) JD130Htz/J] [17] (the Jackson Laboratory; Stock N0: 030247) were bred with C57BL/6 J mice (the Jackson Laboratory) to obtain hemizygous offspring. Postnatal day (PD)7 mice were genotyped by tail biopsy using a rapid DNA isolation protocol [19] followed by qPCR with primers targeting eGFP for wild-type/transgenic identification [20], and *Gapdh* as a positive control. All animals were housed at the VA Portland Health Care System Veterinary Medical Unit, on a 12hr light/dark cycle at 22 ± 1°C. All the animal procedures were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the VA Portland Health Care System's Institutional Animal Care and Use Committee.

2.2. Isolation of astrocyte RNA from Aldh1l1-EGFP-Rpl10a mice by TRAP

The cortex and hippocampus of PD7, PD14, and PD93 Aldh1l1-EGFP-Rpl10a TRAP mice were dissected, snap frozen in liquid nitrogen, and stored at −80° C until processing. The TRAP procedure to isolate astrocyte-enriched RNA was carried out as previously described [17, 18] using anti-GFP antibodies (Memorial-Sloan Monoclonal Antibody Facility). Following the final wash of the RNA-Antibody-Bead complex, RNA was isolated from input and pull-down samples.

2.3. qRT-PCR

RNA was extracted using TRIzol (Thermo-Fisher Scientific, Waltham, MA) and Direct-zol RNA MiniPrep Plus Kit (Zymo Research, Orange, CA). RNA concentration and purity were determined by UV absorption at 260 nm, with 260/280 ratios between 1.9 and 2.1. Quantitative RT-PCR (qPCR) was carried out using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA) with 10ng of RNA per reaction using a CFX96 Touch thermocycler (Bio-Rad Laboratories, Hercules, CA). Mouse primers for *Plat, Plau, Plg, Serpine1,* and *Serpini1* and rat primers for *Plat* and *Plau* are described in "Supplemental Methods". mRNA levels were normalized using RiboGreen [21].

2.4. Primary astrocyte cultures

Cortical astrocyte cultures were prepared from gestational day 21 fetuses, as previously described [15, 22]. Astrocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Thermo-Fisher Scientific, #11885092), supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Flowery Branch, GA, #S12450), 1,000 units/mL penicillin-streptomycin (pen-strep, Thermo-Fisher Scientific, #15140122) in a humidified incubator at 37°C under a 5% CO_2/95% air atmosphere until confluent (10-16 days). Astrocytes were then sub-cultured on glass coverslips placed into 24-well plates at the density of $0.1x10^6$ cells/well for astrocyte/neuron co-culture experiments or in 6-well plates at the density of $0.25\ x10^6$ cells/well for mRNA level and enzymatic activity experiments.

2.5. Astrocyte treatment with recombinant tPA (rtPA)

Three days after sub-culturing astrocytes in the appropriate vessels, medium was changed to DMEM containing 0.1% Bovine Serum Albumin (BSA; Sigma Aldrich, St. Louis, MO) and pen/strep (DMEM/0.1% BSA/pen-strep) and replaced 24 hr later with fresh DMEM/0.1% BSA/pen-strep medium containing 50 nM of active recombinant rat tPA (rtPA, Molecular Innovations, Novi, MI, catalog # 25692) for 4hr or 16hr. Each time-point had its own control, consisting of astrocytes incubated for the same length of time in the same medium without rtPA.

2.6. Astrocyte transfection with siPlat

Two days after sub-culture, astrocytes were transfected in DMEM/0.1% BSA without antibiotics supplemented with 10μl/well (24-well plates) or 0.5 ml/well (6-well plates) Lipofectamine RNAiMAX (Thermo-Fisher Scientific, #13778030) diluted with Opti-MEM I Reduced Serum Media (Opti-MEM; 1:50 dilutions; Thermo-Fisher Scientific, #31985062) containing 1nM Stealth siRNA targeting the rat *Plat* (Invitrogen/Thermo-Fisher Scientific, Carlsbad, MA; catalog # 1330001) or 1nM Stealth siRNA non-target control, medium guanosine and cytosine (GC) content (non-target siRNA; Invitrogen/Thermo-Fisher Scientific, #462001) for 24hr. Transfection medium was then replaced with DMEM/0.1% BSA/pen-strep for additional 24hr.

2.7. Astrocyte-neuron co-cultures, immunocytochemistry, and microscopy

Astrocytes sub-cultured on glass cover slips placed into 24-well plates were treated with rtPA or transfected with Plat-siRNA or non-target siRNA control as described above. At the end of these treatments, the medium was removed and freshly isolated hippocampal neurons prepared from fetuses at GD21 as previously described [15, 22] and suspended in DMEM/0.1% BSA/pen-strep medium were plated on top of the pre-treated or control astrocytes ($1x10^4$ neurons/cover slip) for an additional 16hr. At the end of the incubation, astrocyte-neuron co-cultures were fixed in 4% paraformaldehyde and immunolabeled with a neuronal-specific anti-β-III tubulin antibody (1:150 dilution; Millipore, Burlington, MA, #MAB1637), followed by a goat anti-mouse Alexa Fluor 488 secondary antibody (1:300 dilution; Thermo-Fisher Scientific, #A11001), as previously described [15]. The glass coverslips were then mounted on microscope slides. β-III tubulin-labeled neurons were imaged on a Leica DM5000b microscope attached to a DFC36 FX camera. Neurons were selected as measurable if they 1) had three or more neurites that were all longer than the cell body, 2) did not overlap with other neurons, 3) were fully on top of the astrocyte monolayer, 4) were pyramidal neurons and 5) had no breakage in the cell bodies or neurites. All neurons that met these requirements were measured. Pictures of neurons obtained with the 40x objective were traced using Neurolucida and analyzed with Neurolucida Explorer (Version 11, MBF Bioscience, Williston, VT, USA) by a researcher blind to the experimental treatments. Three parameters were analyzed from each cell: the length of the longest neurite, the average length of the minor neurites, and the number of neurites.

2.8. tPA activity assay

At the end of the treatments, astrocytes plated in 6-well plates were scraped in cold phosphate buffered saline and centrifuged at 200g for 10 minutes. Cell pellets were re-suspended in deionized water and sonicated 5 times at 18% power for 5 seconds each on ice. The cell lysates were then centrifuged again at 250g for 10 minutes and the supernatants were collected for analysis. tPA activity was measured by the Sensolyte AMC tPA activity kit (AnaspeC, Inc., Fremont, CA, #AS-72160) according to the manufacturer's protocol. Relative Fluorescent Units were measured using a plate reader (BMG Labtech Fluorescent) at 354 nm excitation and 442 nm emission and normalized to protein content in the same sample. The cellular protein content was quantified by the Bradford assay (Thermo-Fisher Scientific, #23200) [14].

2.9. Statistics

Neurite measurement data were analyzed with the statistical program R [23] and the package lme4 [24]. We used a nested multilevel analysis [25] to account for the measurement of multiple neurons on the same coverslip. Data-points in Figs. 2 and 4 represent the average measurements from neurons on the same coverslip. Benjamini-Hochberg tests were run to correct for multiple comparisons and to control for the false discovery rate (FDR), as three different parameters were analyzed in the same neurons. All other data were analyzed with Student's t-test or one-way ANOVA followed by Tukey's post-hoc test as appropriate using Prism v8.0.2 (GraphPad Software, Inc., La Jolla, CA). Data are shown as mean ± standard error of the mean; p or q values <0.05 were considered significant. Outliers were identified by the Grubb's test analysis and were removed before data analysis in an unbiased manner.

3. Results

3.1. Plat expression in developing and adult astrocytes in vivo

We examined *Plat* relative expression in hippocampal and cortical astrocytes and in the whole hippocampus and cortex of PD7, PD14, and adult (PD93) Aldh1l1-EGFP-Rpl10a mice. At PD7 *Plat* was highly enriched in astrocytes from both areas compared to the respective inputs (whole hippocampus or cortex). At PD14 Plat was still enriched in astrocytes; while in adult animals, *Plat* was equally expressed in astrocytes and in the whole tissue (Fig. 1A, B, Tables 1, 2). Besides tPA, encoded by the gene *Plat,* the main components of the brain plasminogen activating system include urokinase-type PA (uPA), encoded by the gene *Plau,* two PA inhibitors, plasminogen activator inhibitor 1 (PAI-1) and neuroserpin, encoded by the genes *Serpine1* and *Serpini1* respectively, and the zymogen plasminogen, encoded by the gene *Plg* [4]. The relative expression of these genes in astrocytes and the whole hippocampus across developmental stages normalized to *Plat* expression in the input fraction at PD7 are shown in Table 1 with some of these genes also analyzed in the cortex (Table 2). *Plat* expression peaked in adulthood in the whole hippocampus and at PD14 in the whole cortex. *Plat* was the second mostly highly expressed gene in the input samples at all developmental stages and in both brain areas. *Plau* was expressed at much lower levels than *Plat* at all developmental stages (suggesting that tPA is the major PA in the brain) and was significantly enriched in hippocampal astrocytes at PD7 and PD14 and significantly de-enriched in adult hippocampal astrocytes and in the PD14 and adult cortical astrocytes.

Serpini1 was the most highly expressed gene in the whole hippocampus and cortex, its expression peaked in adulthood, and it was greatly de-enriched in astrocytes both during development and in adulthood, in agreement with a published report indicating *Serpini1*/neuroserpin is primarily expressed by neurons [26]. *Serpine1* was expressed at low levels in both brain areas, and it was de-enriched in astrocytes at all time-points. This suggests that *Serpini1*/neuroserpine is the major PA inhibitor in the brain and that astrocytes, especially during brain development, are highly involved in the activation of the plasminogen activating system, but less involved with its inhibition. *Plg* was by far the least expressed of the four genes, with similar expression in astrocytes and the whole hippocampus and it was not developmentally regulated. There were no sex differences in the expression of these genes and data were collapsed across sexes.

3.2. Effect of astrocyte exposure to recombinant tPA on pyramidal neuron neurite outgrowth

To test the effect of increased tPA activity in astrocytes on neurite outgrowth in co-cultured neurons, we incubated astrocytes without (control) or with 50 nM rtPA for either 4hr or 16hr; the treatment was then removed, and freshly isolated hippocampal neurons were plated on top of the astrocyte monolayer for an additional 16hr. Nested multilevel analysis with corrections for multiple comparisons showed a main effect of astrocyte exposure to rtPA on the longest neurite lengths ($\chi^2_{(1,42)}$=15.93, q<0.0001) and on the average minor neurite length ($\chi^2_{(1,42)}$=10.97, q<0.0001). Astrocyte treatment with rtPA decreased the length of the longest neurite in neurons co-cultured for 4hr ($\chi^2_{(1,42)}$=13.68, q<0.0001, 12.84% decrease) and 16hr ($\chi^2_{(1,42)}$=9.55, p=0.0020, 11.72% decrease) (Fig. 2B) as well as the minor neurite length in neurons co-cultured for 4hr ($\chi^2_{(1,42)}$=5.72, q=0.0168, 11.10% decrease) and 16hr ($\chi^2_{(1,42)}$=8.75, q=0.0031, 12.55% decrease) (Fig. 2C). There was no effect of rtPA exposure on the number of neurites (Fig. 2D). The incubation of astrocytes with rtPA resulted in increased tPA activity in the astrocyte monolayer (p<0.0001, 55.82% increase, Fig. 2A), compared to the control group, in agreement with the observation that tPA from the extracellular space adheres to the astrocyte surface, where it exerts its proteolytic effect on plasminogen [27]. The inhibitory effect of rtPA-treated astrocytes on neurite outgrowth was not due to a cytotoxic effect of rtPA on astrocytes, as assessed by the MTT assay (Supplementary Fig. S1A).

3.3. Effect of Plat silencing in astrocytes on pyramidal neuron neurite outgrowth

To test the effect of tPA/*Plat* knockdown in astrocytes on neurite outgrowth we co-cultured neurons with astrocytes transfected with si*Plat* or a non-target siRNA (non-target control) or with astrocytes treated only with transfection reagents (naïve control), si*Plat*, but not the non-target control, strongly and selectively reduced *Plat* expression by over 80% in astrocytes 48h after transfection without affecting the expression of *Plan* (Fig. 3).

Nested multilevel analysis with corrections for multiple comparisons showed a main effect of *siPlat* on the longest neurite length ($\chi^2_{(2,38)}$=6.97, q=0.0307) and on the average minor neurite length ($\chi^2_{(2.38)}$=10.56, q=0.0051). Post-hoc analysis revealed a significant decrease in the longest neurite length (p=0.0055, 18.57% decrease, Fig. 4A) and in the minor neurite length (p=0.0051, 13.87% decrease, Fig. 4B) in the si*Plat* group compared to the

non-target control group. There were no changes associated with any treatment on the number of neurites (Fig. 4C). The inhibitory effect of si*Plat*-transfected astrocytes on neurite outgrowth was not due to cytotoxicity of si*Plat* on astrocytes, as assessed by the MTT assay (Supplementary Fig. S1B).

4. Discussion

Astrocytes contribute to neuronal development, as they are involved in axon and dendrite outgrowth and elaboration and in synaptogenesis [7, 28], effects in part modulated by the numerous ECM proteins and ECM proteases released by astrocytes [13]. tPA is expressed by several cell types in the brain, including astrocytes [3]. However, there is remarkably little information about the contribution of tPA derived from astrocytes to brain functions. Previous research has shown that astrocytes serve as an attachment surface for tPA and plasminogen and allow the conversion of plasminogen to plasmin by the proteolytic action of tPA [27].

tPA is also involved in neurodevelopmental disorders. Elevated levels of tPA were recently reported in the serum of male children with autism spectrum disorder [29, 30]. Post-mortem studies demonstrated the involvement of neuroinflammation in autism [31]. As tPA is upregulated during neuroinflammation, modulates neuroinflammatory processes in the brain, and affects the integrity of the blood brain barrier [4], increased plasma tPA levels may be the result of brain tPA leaked into the blood stream as a consequence of ongoing neuroinflammation. Furthermore, tPA upregulation is involved in the developmental effects of ethanol as seen in Fetal Alcohol Spectrum Disorders [32, 33]. Developmental ethanol exposure increases tPA activity and induces neurodegeneration, which is attenuated in $tPA^{-/-}$ mice [34]. We previously reported that tPA upregulation by ethanol occurs in *in vitro* astrocytes [14, 35].

The first novel finding of this study is that *Plat* is enriched in astrocytes of the developing (PD7 and PD14) hippocampus and cortex compared to the whole hippocampus and cortex (input) but is not enriched in astrocytes in the adult brain (Fig. 1; Table 1), suggesting that astrocyte-derived tPA plays a role in the events occurring during this stage of brain development characterized by axonal and dendritic arborization and synaptogenesis [6].

The second novel finding of this study is that both the exposure of astrocytes to excessive tPA and the inhibition of tPA expression in astrocytes result in inhibition of neurite outgrowth (Figs. 2 and 4). The inhibition of neurite outgrowth by tPA silencing in astrocytes was significant only when compared to astrocytes treated with non-target siRNA control, which represents the most appropriate control for this experiment, but not when compared to naïve astrocytes. This is the first time that modulation of tPA levels in astrocytes has been shown to alter neurite outgrowth in neurons. Published reports provide support for our finding that both abnormal increase and abnormal decrease in tPA lead to the inhibition of neurite outgrowth and dendritic arborization [38, 39].

The observation that both decreased and excess tPA result in inhibition of neurite outgrowth is consistent with the role played by the ECM in neurite outgrowth. Indeed, while the

neuritogenic ECM serves as substrate that provides neurons with the adhesion necessary for neurite extension, controlled proteolysis of the ECM at the growth cone and controlled detachment of the growth cone from the substrate are also necessary for growth cone advancement [36]. The effect of astrocyte tPA on neurite outgrowth is likely due to tPA-induced proteolytic activation of plasmin and the subsequent degradation of the ECM by plasmin. Indeed, we have previously reported that increased PAI-1 in astrocytes induces neurite outgrowth in co-cultured neurons [37], that astrocyte exposure to function-blocking antibodies against fibronectin and laminin inhibit neurite outgrowth in co-cultured neurons [22], and that exposure to rtPA increases plasmin activity in astrocytes [14].

Limitations of the study include the fact that we co-cultured cortical astrocytes with hippocampal neurons, an approach that minimized the use of animals needed for these studies. To in part attenuate this limitation, we characterized the expression of *Plat in vivo* by TRAP in both the cortex and the hippocampus. Another limitation is the fact that the mechanistic studies were carried out *in vitro.* Future studies will examine the effects of *Plat* knock-down and overexpression in *in vivo* astrocytes on neuronal dendritic arborization.

In conclusion, this is the first study to explore the levels of *Plat* in developing and adult astrocytes and to identify astrocytes as the main producers of *Plat* during development but not in the adult brain. The developing brain is characterized by heightened plasticity that can be modulated by astrocyte tPA; for this reason, tPA levels need to be tightly regulated as both excess and decreased tPA in astrocytes alter neuronal development, likely by affecting the optimal levels of astrocyte-released ECM required by neurons for their normal development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BSA	Bovine Serum Albumin
DMEM	Dulbecco’s Modified Eagle’s Medium
ECM	extracellular matrix
PAI-1	plasminogen activator inhibitor-1
PD	postnatal day
rtPA	recombinant tissue plasminogen activator

siRNA	small interfering ribonucleic acid
tPA	tissue plasminogen activator
TRAP	translating ribosome affinity purification

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Highlights

- *Plat* expression is enriched in astrocytes in the developing, but not in the mature, hippocampus and cortex
- Both the silencing of tPA expression and the addition of recombinant active tPA in astrocytes inhibit neurite outgrowth in co-cultured hippocampal neurons
- The modulation of astrocyte tPA levels plays an important role in neuronal development

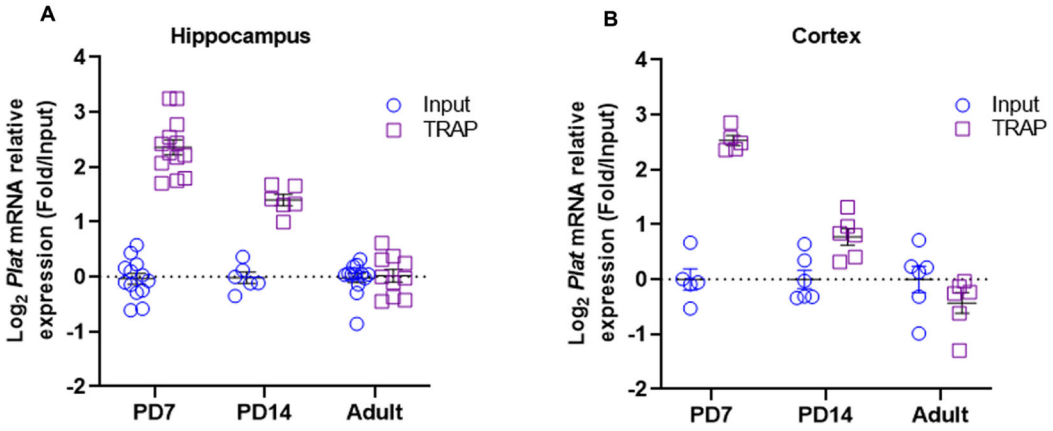

Fig. 1.

Plat expression in astrocytes of developing and adult Aldh1l1EGFP-Rpl10a mice. The relative expression of *Plat* in astrocytes from the hippocampus (A) and cortex (B) of PD7, PD14, and PD93 Aldh1l1-EGFP-Rpl10a mice was measured by qPCR after TRAP and compared to the expression in the whole hippocampus or cortex (input), respectively. Data are shown as $\log_2$ fold/input (mean ± SEM) and are normalized to total RNA. n=6-13 male and female mice.

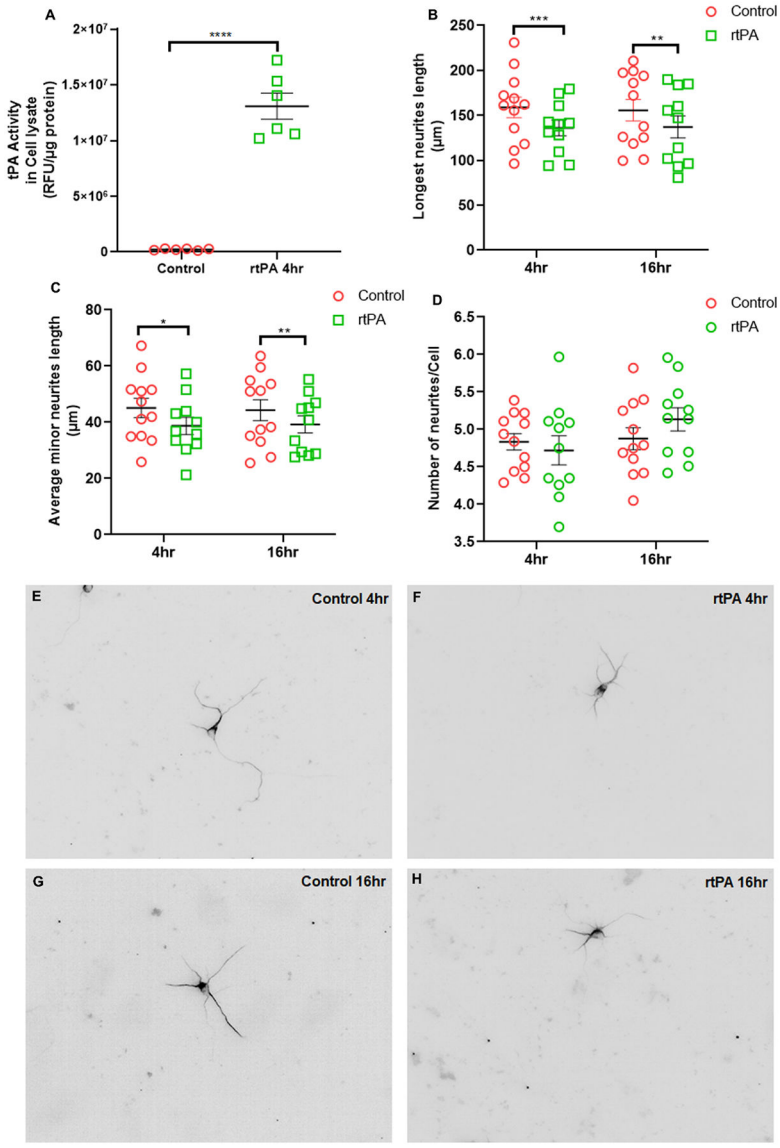

Fig. 2.

Effect of rtPA-treated astrocytes on pyramidal neuron neurite outgrowth. (A) Primary rat astrocytes were incubated for 4hr in the presence or absence of 50nM rtPA; tPA activity was measured in cell lysates. ****, $p<0.0001$ tPA *vs.* control by Student's t-test (n=6). (B-H) Astrocytes were incubated with 50nM rtPA for 4hr or 16hr followed by treatment wash-out. Hippocampal neurons were plated on top of treated astrocyte for an additional 16hr. Cultures were then fixed and stained with neuron-specific βIII-tubulin antibody and a fluorescent secondary antibody. Neurites were traced and analyzed using Neurolucida and Neurolucida Explorer. (B) Longest neurite length was decreased by rtPA pre-treatment of co-cultured astrocytes. (C) Average length of minor neurites was decreased by rtPA pre-treatment of co-cultured astrocytes. (D) Number of neurites/cell was not altered by rtPA. (E-H) Representative images of pyramidal neurons in (E) Naïve control 4hr, (F) rtPA 4hr, (G) Naïve control 16hr, and (H) rtPA 16hr. Data were analyzed using a nested multilevel

analysis with Benjamini-Hochberg to correct for multiple comparisons. *q<0.05, **q<0.01 (n=11-12).

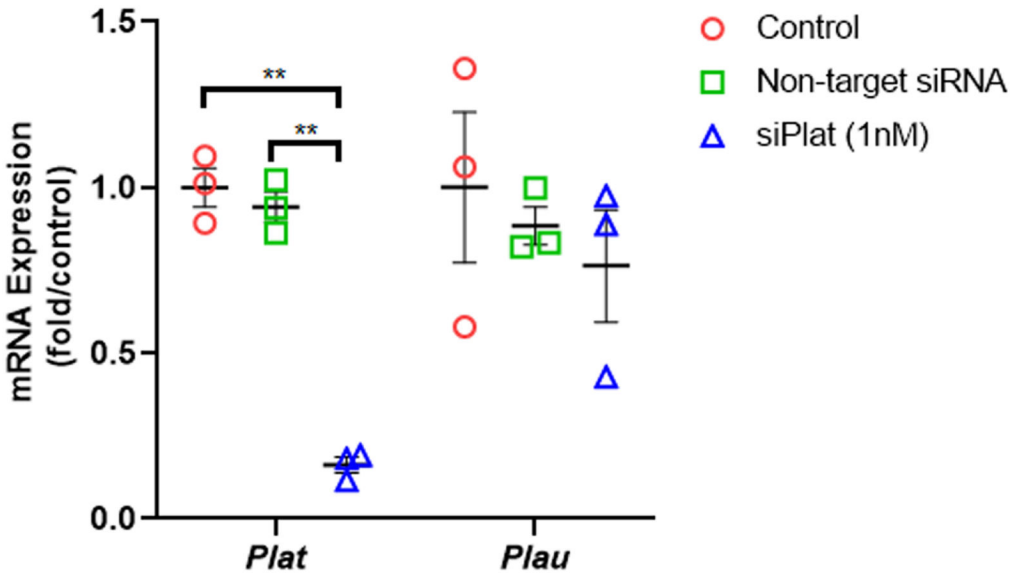

Fig. 3.

Effect of *Plat* silencing on *Plat* and *Plan* mRNA expression in astrocytes. Primary rat astrocytes were treated with transfection reagents (control), or transfected with a non-target siRNA or with a *Plat* specific siRNA (*siPlat*). 48hr after transfection, *Plat* mRNA levels were quantified in astrocytes cell lysates using qPCR. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{**}p<0.01$ (n=3).

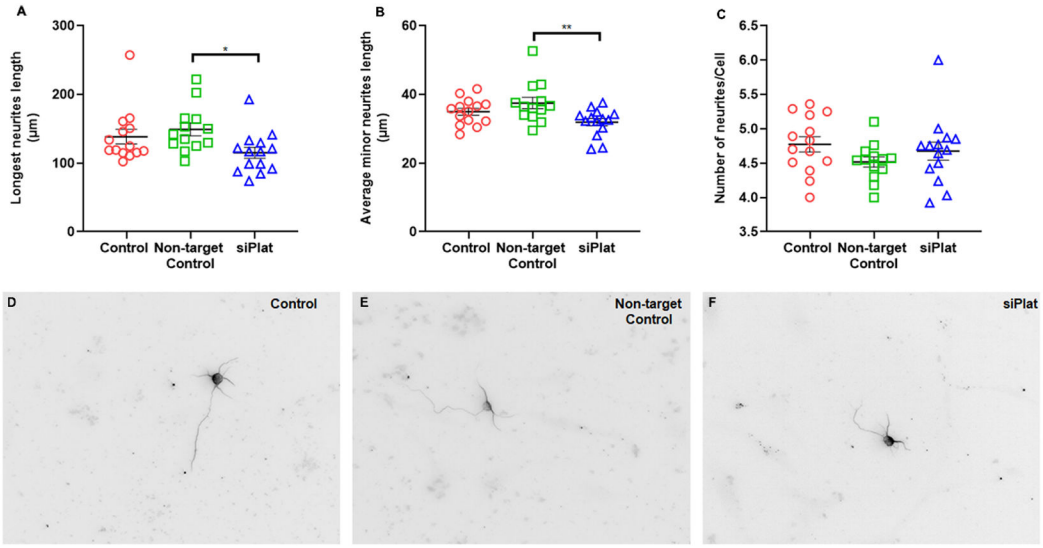

Fig. 4.

Effect of *Plat* silencing in astrocytes on pyramidal neuron neurite outgrowth. Primary rat astrocytes were treated with transfection reagents (control), transfected with a non-target siRNA (non-target control), or transfected with a *Plat* specific siRNA (*siPlat*). 48hr after transfection, hippocampal neurons were plated on top of the astrocyte monolayer for an additional 16hr. Cultures were then fixed and stained with neuron-specific βIII-tubulin antibody and a fluorescent secondary antibody. Neurites were traced and analyzed using Neurolucida and Neurolucida Explorer. (A) Longest neurite length was decreased by *Plat* silencing in co-cultured astrocytes. (B) Average minor neurite length was decreased by *Plat* silencing in co-cultured astrocytes. (C) Number of neurites/cell was unchanged by *Plat* silencing. (D-F) Representative images of pyramidal neurons in (D) Naïve control, (E) Non-target control, and (F) *siPlat.* Data were analyzed using a nested multilevel analysis with Benjamini-Hochberg to correct for multiple comparisons. *$q<0.05$, **$p<0.01$ (n=13-14).

Table 1.

Plasminogen Activating System gene expression in hippocampal astrocytes of *Alh1l1*-EGFP-Rpl10a mice

Target	Age	Input (whole hippocampus)	TRAP
Plat	PD7	1.0000±0.0679	5.4356±0.5738***
Plau	PD7	0.0096±0.0005	0.0162±0.0008***
Serpine1	PD7	0.0089±0.0011	0.0025±0.0005***
Plat	PD14	1.2038±0.0845	3.2117±0.2244***
Plau	PD14	0.0171±0.0010	0.0256±0.0011***
Plg	PD14	0.0009±0.0001	0.0013±0.0002
Serpine1	PD14	0.0068±0.0010	0.0011±0.0001***
Serpini1	PD14	6.5853±0.2960	0.7011±0.0281***
Plat	Adult	2.2707±0.1085	2.3642±0.1911
Plau	Adult	0.1054±0.0064	0.0441±0.0040***
Plg	Adult	0.0016±0.0003	0.0020±0.0003
Serpine1	Adult	0.0224±0.0019	0.0144±0.0018**
Serpini1	Adult	29.5804±1.7205	5.5950±0.3996***

Relative Expression calculated as fold PD7 *Plat* Input (whole hippocampus) ± SE.

** $p < 0.01$

*** p<0.001 indicates the comparison of gene expressions in input and TRAP mice by the student's t test.

Table 2.

Plasminogen Activating System gene expression in cortex astrocytes of *Alh111*-EGFP-Rpl10a mice

Target	Age	Input (whole cortex)	TRAP
Plat	PD7	1.7788±0.1341	9.9632±0.8275***
Plat	PD14	5.9539±0.7403	10.1091±1.0614**
Plau	PD14	0.3687±0.0466	0.1827±0.0195**
Serpine1	PD14	0.0678±0.0077	0.0160±0.0022***
Serpini1	PD14	38.5537±2.8563	6.9219±0.4865***
Plat	Adult	4.0127±0.5899	2.9125±0.3219
Plau	Adult	0.4054±0.0624	0.1225±0.0113*
Serpine1	Adult	0.0550±0.0103	0.0134±0.0021**
Serpini1	Adult	61.1063±8.4417	10.6520±0.9958***

Relative Expression calculated as fold PD7 *Plat* Input (whole hippocampus) ± SE.

* $p < 0.05$

** $p < 0.01$

*** $p<0.001$ indicates the comparison of gene expressions in input and TRAP mice by the student's t test.