Plasminogen activator system homeostasis and its dysregulation by ethanol in astrocyte cultures and the developing brain

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Abstract

In utero alcohol exposure can cause fetal alcohol spectrum disorders (FASD), characterized by structural brain abnormalities and long-lasting behavioral and cognitive dysfunction.

Neuronal plasticity is affected by *in utero* alcohol exposure and can be modulated by extracellular proteolysis. Plasmin is a major extracellular serine-protease whose activation is tightly regulated by the plasminogen activator (PA) system. In the present study we explored the effect of ethanol on the expression of the main components of the brain PA system in sex-specific cortical astrocyte primary cultures *in vitro* and in the cortex and hippocampus of post-natal day (PD) 9 male and female rats.

We find that ethanol alters the PA system in astrocytes and in the developing brain. In particular, the expression of tissue-type PA (tPA), encoded by the gene *Plat*, is consistently upregulated by ethanol in astrocytes *in vitro* and in the cortex and hippocampus *in vivo*. Astrocytes exhibit endogenous plasmin activity that is increased by ethanol and recombinant tPA and inhibited by tPA silencing. We also find that tPA is expressed by astrocytes of the developing cortex and hippocampus *in vivo*. All components of the PA system investigated, with the exception of Neuroserpin/*Serpini1*, are expressed at higher levels in astrocyte cultures than in the developing brain, suggesting that astrocytes are major producers of these proteins in the brain. In conclusion, astrocyte PA system may play a major role in the modulation of neuronal plasticity; ethanol-induced upregulation of tPA levels and plasmin activity may be responsible for altered neuronal plasticity in FASD.

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1. Introduction

In utero alcohol exposure can cause fetal alcohol spectrum disorders (FASD), characterized by structural brain abnormalities and behavioral and cognitive dysfunctions that affect FASD individuals throughout life (Hellemans et al., 2010; Riley et al., 2011). A significant body of research has identified strong co-morbidity between FASD and several mental disorders. More than 90% of individuals with FASD develop mental health problems including attention deficit/hyperactivity disorders, depression, anxiety, and alcohol and drug abuse and dependence; also common among individuals exposed to alcohol during gestation are learning and intellectual disabilities (Burd et al., 2003; Popova et al., 2016; Streissguth and O'Malley, 2000; Weyrauch et al., 2017). Because of this evidence, the category "neurodevelopmental disorder associated with prenatal alcohol exposure" was included as a condition requiring further study in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (Association, 2013).

Clinical and preclinical studies indicate that neuronal plasticity and connectivity are affected by *in utero* alcohol exposure; these alterations may be responsible for some of the cognitive and behavioral abnormalities present in individuals with FASD (Lebel et al., 2012; Medina, 2011; Wozniak et al., 2013). Extracellular proteolytic activity can affect neuronal plasticity and excitability by regulating the availability of Brain-Derived Neurotrophic Factor (BDNF), by modulating the activity of NMDA receptors, and by degrading the extracellular matrix (ECM) proteins (Tsilibary et al., 2014; Wojtowicz et al., 2015). Plasmin is a major extracellular serine-protease whose proteolytic activation from its zymogen plasminogen is tightly regulated by the plasminogen activator (PA) system. Main components of the brain PA system include two PAs, tissue-type (tPA) and urokinase-type (uPA), encoded by the genes *Plat* and *Plau* respectively, two plasminogen activator inhibitors, plasminogen activator inhibitor 1 (PAI-1) and neuroserpin, encoded by the genes *Serpine1* and *Serpini1* respectively, and the zymogen plasminogen, encoded by *Plg* (Mehra et al., 2016). Fig. 1 is a schematic representation of these components of the PA system and their function.

The ECM in the developing brain is involved in neuronal guidance, neurite outgrowth, and synaptogenesis (Song and Dityatev, 2017). Astrocytes release a plethora of ECM proteins and modulators that can promote or inhibit neuronal development in the developing brain (Asher et al., 2000; Hamel et al., 2005; Moore et al., 2009; Tom et al., 2004). We have previously shown that carbachol-treated astrocytes upregulate the expression and release of PAI-1 which increases the extracellular levels of the ECM proteins laminin and fibronectin in the same cells and neurite outgrowth in co-cultured hippocampal pyramidal neurons (Guizzetti et al., 2008).

We also reported that ethanol-treated astrocytes inhibit hippocampal neuron neurite outgrowth through the inhibition of carbachol-induced PAI-1 expression and release, which

decreases the extracellular levels of neuritogenic laminin and fibronectin (Guizzetti et al., 2010). Furthermore, ethanol increases expression and release of tPA in astrocytes; this effect is mediated by reduced DNA methylation in the promoter region of the *Plat* gene leading to increased *Plat*/tPA expression (Zhang et al., 2014b).

Exposure of neonatal mice to ethanol (an animal model for FASD mimicking ethanol exposure during the third trimester of human gestation) increases tPA activity and induces neurodegeneration, which is attenuated in tPA−/− mice, indicating that ethanol-induced neurodegeneration is mediated, at least in part, by increased tPA activity (Noel et al., 2011). In adult mice chronic ethanol exposure and ethanol withdrawal increase the activity of tPA, which, through the activation of plasminogen to plasmin, proteolytically degrades laminin γ −1 and induces neurodegeneration (Pawlak et al., 2005; Skrzypiec et al., 2009). This observation is in agreement with the observation that chronic ethanol exposure and ethanol withdrawal increase tPA expression in adult mice (Smith et al., 2016; Hashimoto and Guizzetti, unpublished). The effects of alcohol on the ECM and ECM protease systems, including the PA system, have been recently described in a review paper (Lasek, 2016).

Published evidence indicates that the majority of the components of the PA system investigated in this study are developmentally regulated in the brain. In rodents, *Plat*/tPA expression in the brain reaches maximal levels during the first two postnatal weeks in rodents (corresponding to the third trimester of human gestation) (Friedman and Seeds, 1995; Yu et al., 2014). *Plau*/uPA is highly expressed in neurons, microglia, and astrocytes of the developing brain; while in the adult brain its expression is lower and restricted to astrocytes and a few neuronal populations (Mehra et al., 2016; Merino et al., 2017). *Serpine1*/PAI-1, an irreversible inhibitor of tPA and uPA, is expressed at relatively low levels in the brain compared to other tissues and mostly by astrocytes and endothelial cells (Docagne et al., 1999; Mehra et al., 2016). On the other hand the brain expresses high levels of another PA inhibitor, *Serpini1*/neuroserpin, a brain-specific transient inhibitor of tPA and, to a lesser extent, uPA (Gravanis and Tsirka, 2005; Lee et al., 2015; Mehra et al., 2016). Neuroserpin expression is higher in neurons, though it is also found in astrocytes (Docagne et al., 1999). Neuroserpin expression starts increasing between embryonic day (E)14 and E17, reaches maximal levels perinatally, and declines in the adult (Krueger et al., 1997). *Plg*/ plasminogen is expressed at low levels everywhere in the adult and developing brain and is not developmentally regulated (Basham and Seeds, 2001; Taniguchi et al., 2011). Also in the human brain, PLAT, SERPINE1, and PLAU show developmentally regulated expression with high levels of expression at 37 weeks post-conception in the hippocampus, cortex, and other brain regions compared to other time-points. SERPINI1 reaches a peak in expression after birth and shows high levels of expression throughout neonatal development. Similar to what is reported for rodents, in humans PLG is not developmentally regulated and shows low levels of expression throughout the brain with little variation between brain regions (Miller et al., 2014).

The role tPA plays in brain physiology and pathology has been characterized mostly in the adult rodent brain and in *in vitro* cultures of brain cells (Chevilley et al., 2015; Fredriksson et al., 2017; Hebert et al., 2016; Mehra et al., 2016). tPA has been shown to be involved in neuronal plasticity (Seeds et al., 2003), axonal regeneration (Minor et al., 2009; Seeds et al.,

1997), excitotoxicity (Nicole et al., 2001; Tsirka et al., 1995, 1996), neuroprotection (Echeverry et al., 2010), microglial activation and neuroinflammation (Rogove and Tsirka, 1998; Tsirka et al., 1997), and increased blood-brain barrier permeability (Niego et al., 2012; Su et al., 2008; Yepes et al., 2003).

The role of tPA in the developing brain is not as well explored and most of the developmental studies focus on the cerebellum, where tPA is involved in granule cell and interneuron migration (Friedman and Seeds, 1995; Raoult et al., 2014; Seeds et al., 1999), while tPA overexpression inhibits dendritic arborization and induces cell death in developing Purkinje cells (Li et al., 2013).

tPA is expressed by astrocytes *in vitro* and after brain injury *in vivo* (Adhami et al., 2008; Docagne et al., 1999; Kim et al., 2011; Zhang et al., 2014b), but was not detected in astrocytes of the healthy adult rodent brain (Louessard et al., 2016). The expression of tPA in astrocytes of the developing brain has not been investigated.

In this study we show that ethanol alters the expression of several genes of the PA system and, in particular, upregulates *Plat*/tPA expression *in vitro* and in the developing hippocampus and cortex *in vivo*. Astrocytes exhibit endogenous plasmin activity that is increased by ethanol and recombinant tPA and inhibited by tPA silencing. In addition, we show that tPA is expressed in astrocytes of the developing cortex and hippocampus and that most of the components of the PA system investigated (with the exception of Neuroserpin/ *Serpini1*) are expressed at higher levels in astrocyte cultures than in the developing brain, indicating that astrocytes may be the major regulators of plasmin activity in the developing brain parenchyma.

2. Materials and methods

2.1. Animals and experimental design

This study employed two experimental models: *an in vitro* model, *i.e.* rat primary astrocyte cultures, and an *in vivo* model, *i.e.* rat pups. For both models, timed-pregnant gestational day (GD) 15 Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) and housed at the VA Portland Health Care System vivarium. All the employed 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.

2.1.1. In vitro experiments—We examined the effects of ethanol on gene expression of 5 main components of the PA system, *Plat, Plau, Serpine1, Serpini1,* and *Plg* (Fig. 1), in sex-specific cortical astrocyte primary cultures by qPCR. We then evaluated ethanol-induced changes in expression of the two proteins encoded by genes showing ethanol regulation, tPA and uPA. In addition, because Serpine1 was upregulated in the hippocampus and in the cortex of post-natal day (PD) 9 pups neonatally exposed to ethanol (Fig. 8C and 9C), we also tested the effect of ethanol on the *Serpine1* gene product PAI-1. We hypothesized that changes in gene and protein expression of components of the PA system pathway may lead to altered plasmin activity in astrocytes (Fig. 1). For this reason, we also investigated the

effect of ethanol on plasmin activity. Because the regulation of endogenous plasmin activity in astrocyte cultures has not been characterized, we tested the effects of exogenous tPA and tPA silencing on endogenous plasmin activity. Because we found that the effects of ethanol were similar in male and female cultures, we carried out protein level and plasmin activity experiments in mixed astrocyte cultures, which were prepared from equal numbers of male and female fetuses.

In the *in vitro* studies we incubated astrocytes with 75 mM ethanol (corresponding to 0.35 g/dl) for 24 h; this ethanol concentration is clinically relevant, as it can be found in the blood of individuals after high ethanol intake (Adachi et al., 1991) and is within the range of concentrations recommended for *in vitro* studies (Deitrich and Harris, 1996). The actual average concentrations of ethanol at the beginning and the end of the *in vitro* incubations were measured by gas chromatography and are reported in Fig. 2A.

The number of replicates (n) was 6 for most of the *in vitro* experiments, with the exception of experiments described in Fig. 6A (n = 4). Outliers were identified as described in the statistical analysis section and removed from analysis. One outlier was removed from each of the following groups: male astrocyte control group in Fig. 3A; male astrocyte control and male astrocyte ethanol groups in Fig. 3B; astrocyte control group in Fig. 4A; for these groups the n is 5.

2.1.2.   In vivo experiments—While tPA is expressed by astrocytes *in vitro* and after brain injury *in vivo* (Docagne et al., 1999; Zhang et al., 2014b), a recent study suggested that tPA is not expressed by astrocytes in the adult rat and mouse brain (Louessard et al., 2016). However, to date, no studies have investigated the expression of tPA in astrocytes of the developing brain. We therefore assessed the co-localization of astrocytic markers with tPA in the cortex and hippocampus of PD9 rats by double-labeling fluorescent immunohistochemistry. We next investigated whether the ethanol effects on gene and protein expression of components of the PA system observed in astrocytes *in vitro* were recapitulated in a well-established *in vivo* model of FASD, which mimics ethanol exposure during the third trimester of human gestation (Tran et al., 2007; Zhang et al., 2014a). In these *in vivo* studies we used a postnatal alcohol exposure paradigm in which ethanol is administered by intragastric intubation from PD4 to PD9. The reason for choosing this paradigm of ethanol exposure lies in the fact that the first 9 postnatal days in rats correspond approximately to the third trimester of gestation in humans; this is a developmental stage characterized by rapid brain growth (so-called brain growth spurt) during which several important events occur in the hippocampus and other brain regions, including astrocyte proliferation, dendritic arborization and synaptogenesis (Rice and Barone, 2000) leading to accelerated and synchronized functional maturation of these brain regions. Exposure to ethanol during this period has been associated with behavioral dysfunctions (Thomas et al., 2004, 2007). Using a split-litter design, rat pups were administered 5.0 g/Kg/day ethanol in milk formula or were sham intubated from PD 4 through PD 9 as described in details in Section 2.5. Alcohol concentrations were measured by gas chromatography in the blood of male and female animals sacrificed 2 h after the end of the last ethanol administration; no differences between male and female pups were found (Fig. 2B).

All the *in vivo* data were generated from 6 litters. 3 litters were used in the collection of all data points (i.e. cortical and hippocampal mRNA and protein data); due to an unpredicted loss of samples, 3 of the litters used to generate cortex gene expression data were different from 3 of the litters used to generate hippocampus gene expression and hippocampus and cortex protein expression data. In all experiments, the left cortex and hippocampus were used for mRNA studies, the right cortex and hippocampus for protein studies. The number of replicates (ranging between 4 and 9) were not always the same in all the groups and end-point measured in the *in vivo* studies for the following reasons: 1) different number of pups and different male/female distribution across litters; 2) accidental death of animals during the intubation process (<than 1 animal per litter); 3) removal of outliers. Additionally, in some cases we carried out protein or gene expression determinations in 2 pups per treatment per sex per litter, while in other cases we analyzed 1 pup per sex per treatment per litter. One outlier was removed from each of the following groups: female ethanol group, male control group in Fig. 8A; female ethanol group in Fig. 8B; female control group, male control group, male ethanol group in Fig. 8C, E; female control group, male ethanol group in Fig. 9A; female control group in Fig. 9B; female ethanol group, male ethanol group in Fig. 9C, female control group, male control group in Fig. 9E; female control group in Fig. 10C.

2.2. Sex-specific and sex-balanced cortical rat astrocyte cultures

Sex-specific and sex-balanced cortical astrocyte cultures were prepared from E21 Sprague-Dawley fetuses as we described previously (Guizzetti et al., 1996, 2008) with the exception that fetuses were sexed and grouped by sex (in the case of sex-specific cultures) or an equal number of male and female fetuses were used (for sex-balanced cultures). Astrocyte cultures were grown in 75 cm^2 flasks in Dulbecco's modified Eagle medium (DMEM; ThermoFisher, Waltham, MA) containing 10% fetal bovine serum, 100units/mL penicillin, and 100 μg/mL streptomycin. After 9–10 days in culture, astrocytes were sub-cultured in 100 mm dishes (2.5×10^6 cells/dish), 6-well plates (4×10^5 cells/well), or 24 well plates (1×10^5 cells/well), and maintained for an additional 4 days in complete medium. Sex determination of fetuses was carried out by observation of the ano-genital distance (McCarthy, 2015) and confirmed by *Sry* genotyping.

2.3. Ethanol exposure via intubation and brain isolation

Neonatal rats between PD4 and PD9 were intragastrically administered 5.0 g/kg of ethanol per day in milk formula (Similac Advance Early Shield with iron) as described previously (Zhang et al., 2014a). The ethanol administration was divided into two feedings 2 h apart. Ethanol-treated animals were also given two subsequent milk formula only feedings at 2 h intervals following ethanol treatment to compensate for the lack of nursing in intoxicated pups, as previously described (Tran et al., 2007; Zhang et al., 2014a). Intubation volumes were adjusted based on animal weight at a rate of 0.0278 ml/g. The control groups were sham-intubated at the same intervals as the ethanol-treated group. Animals were euthanized 2 h after the last intubation with 50 μl of rat cocktail (500 mg/mL ketamine and 10 mg/mL acepromazine in 0.9% saline). Hippocampi and cortices were rapidly dissected, snap frozen in liquid nitrogen, and stored at −80 °C.

2.4. Sry genotyping

Genomic DNA extraction and amplification was carried out as recommended by the manufacturer using the SYBR Green Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO). Briefly, 1–2 mm pieces of tails were collected in 200 μL PCR tubes and digested at 25 °C for 10 min in 100 μL extraction/tissue preparation solution followed by heat denaturation for 3 min at 95 °C immediately followed by the addition of 80 μl neutralization solution. Quantitative PCR was then carried out on a Bio-Rad CFX96 Real-Time System using genomic primers for *Sry* (ratSryF – CATC-GAAGGGTTAAAGTGCCA; ratSryR-ATAGTGTGTAGGTTGTTGTCC) (An et al., 1997), and *Gapdh* (ratGapdhPromoterF –ACCATGCTTCACTGACATTCTGA; ratGapdhPromoterR-GGTCTGCCTCCCTGCTAACC) (Cha-Molstad et al., 2009). Samples with *Sry* Cq – *Gapdh* Cq < 0 are males.

2.5. Blood and medium alcohol concentration analyses

Alcohol levels in the cell culture medium were determined at the beginning and at the end of the 24-h incubation with ethanol. For blood alcohol measurement, trunk blood from ethanol-treated pups was collected after euthanasia. Blood and culture medium samples (20 μl) were diluted into 500 μL of a matrix of 4 mM *n*-propanol in deionized water. Ethanol concentrations were determined by head-space gas chromatography as previously described (Finn et al., 2007).

2.6. RNA isolation and qPCR

RNA was isolated using the Trizol reagent (ThermoFisher Scientific, Inc. Waltham, MA) and Direct-zol RNA MiniPrep Plus kit (Zymo Research, Orange, CA), including the DNase treatment for removal of residual genomic DNA, following the manufacturer's recommendations. RNA concentration and purity were determined by UV absorption at 260 nm, with 260/280 ratios between 1.9 and 2.1, and RNA integrity was assessed using a 1% agarose gel stained with SYBR Gold (ThermoFisher Scientific, Inc. Wlatham, MA). Quantitative RT-PCR was carried out using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA) with 10 ng of RNA per well using a CFX96 Touch thermocycler (Bio-Rad Laboratories, Hercules, CA). Relative expression was determined using the Ct method after normalizing expression to total RNA measured with the Quant-iT RiboGreen kit (ThermoFisher Scientific, Inc. Waltham, MA) as previously described (Hashimoto et al., 2004) using the primers listed in Table 1. Primers for *Plau, Plg, Serpine1*, and *Serpini1* qRT-PCR were designed using NCBI Primer-BLAST software. All used primers had efficiencies between 90 and 110% and a single peak in melt-curve analysis; *Plat* primers were based on published primer sequences (Zhang et al., 2014b) with confirmation of specific, efficient amplification in our laboratory. All the gene expression data were expressed as fold over female control, to allow for ANOVA analysis of sex as well as treatment differences (*2.12 Statistical Analysis*).

2.7. tPA and PAI-1 ELISAs

Lysates were prepared on ice using lysis buffer (Cell Signaling Technology; Danvers, MA) supplemented with the cOmplete™ mini protease inhibitor cocktail (Roche Diagnostics,

Basel, Switzerland). Samples were homogenized with a hand-held pellet pestle homogenizer, followed by five sonication pulses of 5 s each at 30%. Samples were then incubated at 4 °C for 40 min on a rotary shaker and then centrifuged at $20,000 \times g$ for 10 min at 4 °C; the pellets were discarded. Levels of tPA were determined in lysates from rat cortices, hippocampi, and mixed-sex astrocyte cultures using a tPA enzyme-linked immunosorbent assay (ELISA) kit (Innovative Research, Novi, MI) as described in the manufacturer's protocol. Levels of PAI-1 were determined in lysates from rat cortices and mixed-sex astrocyte cultures using a PAI-1 ELISA kit (Innovative Research, Novi, MI) as described in the manufacturer's protocol. tPA and PAI-1 concentrations in the samples were interpolated from their respective standard curves and normalized to total protein content.

2.8. Western blot

Cells were lysed in 200 μl cell lysis buffer (Cell Signaling Technology; Danvers, MA) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland); protein concentrations were determined with the BCA protein assay (Thermo-Fisher, Waltham, MA). After electrophoresis on 4–12% SDS-PAGE gels, proteins were transferred to a PVDF membrane and blocked for 1 h in Tris-buffered saline with 5% nonfat milk at room temperature, similarly to what was previously described (Guizzetti et al., 2007). The PVDF membranes were incubated with an anti-uPA antibody (Abcam, Cambridge, UK; dilution 1:1000) for 2 h at room temperature followed by an anti-rabbit HRP-conjugated secondary antibody (BD Biosciences, San Jose, CA; dilution 1:5000) for 1 h at room temperature. After detection of uPA immunoreactivity, the membranes were stripped and re-probed with a β-actin antibody (Sigma, St. Louis, MO; 1:10,000 dilution) for 30 min followed by an anti-mouse HRP-conjugated secondary antibody (BD Biosciences, San Jose, CA; dilution 1:3000) for 30 min at room temperature. Densitometric analyses of uPA and β-actin immunoreactivity bands were carried out using the software ImageJ.

2.9. Plasmin activity

For ethanol-treatment experiments mixed-sex primary astrocyte cultures were grown in 100 mm dishes. At the end of the treatments, cells were washed twice, scraped in PBS, and centrifuge at $200 \times g$. Cell pellets were re-suspended in deionized water and sonicated 5 times at 30% for 5 s each on ice. Cell homogenates were then centrifuged for 10 min at $250 \times g$ and the supernatant used for plasmin activity determinations using the Plasmin Activity Assay kit (AnaSpec, EGT Group, Fremont, CA) as described by the manufacturer.

For siRNA- and recombinant tPA-treatment experiments, primary astrocytes were grown in 24-well plates. At the end of each treatment, wells were washed 2x with PBS and 150 μl of 1× assay buffer was added to each well. An additional 150 μl of 1× assay buffer containing 150 μM plasmin substrate was then added to each well. Plates were incubated for 1 h at 37 °C in a tissue-culture incubator under an atmosphere of 5% CO_2/95% air after which fluorescence intensity was read (Ex/Em = 380 nm/500 nm) using a SpectraMax Gemini EM (Molecular Devices, Sunnyvale, CA, USA). The relative fluorescence units (RFU) measured in each sample was normalized to the protein added to each well as determined by the BCA method.

2.10. Ethanol treatments and siRNA transfections in astrocyte primary cultures

Twenty-four hours before the beginning of ethanol treatments, astrocytes were switched to a serum-free medium consisting of DMEM supplemented with 0.1% bovine serum albumin (BSA), 100units/mL penicillin, and 100 μg/mL streptomycin. Ethanol treatments (75 mM) were carried out in serum-free medium for 24 h. We previously reported that astrocyte exposure to up to 100 mM ethanol did not cause cytotoxicity in astrocytes (Guizzetti and Costa, 1996). To reduce ethanol evaporation, cultures were placed in sealed chambers with a reservoir tray containing water supplemented with ethanol at the same concentration used in the culture medium and gassed with a 5% CO_2/95% air gas mixture, as previously described (Chen et al., 2013; Guizzetti et al., 2007; Zhang et al., 2014b). Astrocyte transfections were carried out using lipofectamine RNAi-MAX Transfection Reagent in Opti-MEM I medium (Invitrogen; Carlsbad, CA) and "Stealth" *Plat* siRNA and non-target siRNA (Qiagen, Valencia, CA). On the day of transfection, primary rat astrocytes were switched to antibiotic-free and serum-free medium (DMEM with 0.1%BSA) and supplemented with 50 nM *Plat* siRNA or 50 nM non-target control, lipofectamine RNAiMAX Transfection Reagent, and Opti-MEM I according to the manufacturer's instructions for 24 h; the transfection medium was then replaced with fresh DMEM/BSA medium for an additional 24 h (for total of 48 h since the beginning of transfection). The specific silencing of *Plat* was confirmed by qRT-PCR.

2.11. Immunohistochemistry and fluorescence microscopy

Animals were anesthetized with rat cocktail and perfused *via* the left ventricle with ice cold 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffered solution (PBS, pH 7.3). Brains were removed and stored in 4% paraformaldehyde overnight at 4 °C. Fixed brains were paraffin embedded and cut into 6 μm coronal sections. Four adjacent brain sections collected in series were stained following the standard procedures for double labeling. The slides were deparaffinized and re-hydrated in xylene and a graded series of ethanol, followed by two 5 min washes in water. Slides were placed into boiling citrate buffer (0.01M; pH 6.0) for antigen retrieval and were then allowed to cool at room temperature for 2 h. The tissue was blocked with 5% normal serum diluted in 0.5% Triton X-100 in PBS for 1 h and incubated overnight with the primary antibodies at 4 C. Double staining for tPA (Abcam, Cambridge, UK; 1:250 dilution) and glial fibrillary acidic protein (GFAP; Millipore, Darmstadt, Germany; 1:500 dilution) and tPA and S100β (Abcam, Cambridge, UK; 1:50 dilution) was carried out to label tPA-expressing astrocytes in the hippocampus and cortex respectively. Secondary detection of tPA used Alexafluor 488 conjugated goat anti-rabbit antiserum (Invitrogen, Carlsbad, CA; 1:250 dilution). GFAP and S100β were detected using Alexafluor 555 conjugated goat anti-mouse antiserum (Invitrogen, Carlsbad, CA; 1:250 dilution). Counterstaining was then performed with 4′,6-diamidino-2-phenylindole (DAPI). Some slices were incubated with secondary antibodies only, to verify the specificity of the immunostaining. Staining was observed and imaged by indirect fluorescence microscopy using a Leica DM5000B microscope equipped with a DFC365 FX camera.

2.12. Statistical analysis

In vivo and *in vitro* studies carried out in male and female pups or in sex-specific astrocyte cultures were analyzed by two-way Analysis of Variance (ANOVA) with sex and ethanol treatment as the two factors; *post-hoc t*-test analysis was carried out only when ethanol treatment and sex (Fig. 3A and B) or ethanol treatment and sex × treatment interactions (Fig. 8A) were statistically significant in two-way ANOVA. Studies carried out in mixed cultures were analyzed by one-way ANOVA followed by the Fisher's LSD post-hoc test or by the Student's t-tests. Data are shown as mean ± standard error of the mean (SEM). Outliers in the qPCR data were identified as values 1.5 * inter quartile range (IQR = Q3-Q1) less than the 1st quartile or 1.5 * IQR greater than the 3rd quartile; outliers in the protein levels and activity data were identified by the Grubb's test analysis; outliers were removed before data analysis in an unbiased manner. Data analysis was carried out using Prism v6.04 (Graphpad Software, Inc., La Jolla, CA) or R software.

3. Results

3.1. Effect of alcohol on the expression of Plat, Plau, Serpine 1, Serpini 1, and Plg in sex-specific astrocyte cultures

Two-way ANOVA of sex by ethanol treatment revealed a significant main effect of ethanol [$F(1, 19) = 18.84$, $p = 0.0003$], with increased *Plat* gene expression in sex-specific astrocytes cultures (Fig. 3A), similar to what we reported in mixed cultures (Zhang et al., 2014b). Furthermore, we found that the expression of *Plau* was down-regulated by ethanol [main effect of ethanol treatment; $F(1, 18) = 55.84$, $p < 0.0001$; Fig. 3B]. Ethanol did not alter the expression of *Serpine1, Serpini1*, or *Plg* (Fig. 3C, D, E). A small, but significant sex difference was identified in *Plat* [$F(1,19) = 6.04$, $p = 0.0238$] and *Plau* [$F(1,18) = 5.109$, $p = 0.0364$] gene expression with female astrocytes expressing slightly lower levels of *Plat* and slightly higher levels of *Plau*.

3.2. Effect of alcohol on the expression of tPA, uPA, and PAI-1 in sex-balanced astrocyte cultures

Ethanol increased the levels of tPA, the protein encoded by the *Plat* gene (Fig. 4A), in agreement with increased *Plat* gene expression (Fig. 3A). On the other hand, despite decreased expression of the *Plau* gene (Fig. 3B), the levels of the encoded protein uPA were not affected by ethanol (Fig. 4C and D); finally, because *Serpine1* was upregulated in the hippocampus and in the cortex of PD9 pups neonatally exposed to ethanol (Fig. 7C and 8C), we also tested the effect of ethanol on the levels of *Serpine1* gene product PAI-1, which were decreased by ethanol (Fig. 4B).

3.3. Effect of alcohol, recombinant tPA, and tPA silencing on plasmin activity in sex-balanced astrocyte cultures

Together, an increase in tPA expression and a decrease in PAI-1 expression suggest a shift of the PA system equilibrium toward the formation of more active plasmin (Fig. 1). We therefore tested whether ethanol increased astrocyte endogenous plasmin activity. We found that plasmin activity was increased after *in vitro* ethanol exposure (Fig. 5A). To test whether

the increased plasmin activity was due to a direct effect of ethanol on plasmin, we measured the activity of recombinant plasmin in the presence and absence of 75 mM ethanol and observed that ethanol did not directly enhance plasmin activity (Fig. 5B).

To our knowledge, this is the first time that endogenous plasmin activity was measured in primary astrocyte cultures; therefore, in order to verify that the enzymatic activity we measured was specific plasmin activity, we treated astrocytes with the plasmin inhibitor D-Val-Phe-Lys Chloromethyl Ketone Dihydrochloride and found a robust inhibition in plasmin activity (Fig. 6A). Furthermore, exogenous recombinant tPA increased (Fig. 6B) while tPA silencing (using a specific *Plat* SiRNA) decreased (Fig. 6C) endogenous plasmin activity in astrocytes. We also verified that *Plat* siRNA strongly reduced *Plat* expression (Fig. 6D) but did not trigger a compensatory upregulation of *Plau* expression (Fig. 6E). These data suggest that ethanol increases plasmin activity by upregulating the expression of *Plat*/tPA and, possibly, decreasing the expression of PAI-1.

3.4. Co-localization of tPA and astrocytic markers in the cortex and hippocampus of PD9 rats

Because a recent study suggested that tPA is not expressed by astrocytes in the mature rodent brain (Louessard et al., 2016), we investigated the expression of tPA in the developing brain and showed that tPA is expressed in astrocytes of the cingulate cortex and of the CA1 region of the hippocampus in the developing rat brain (PD9), assessed by double-staining immunohistochemistry of tPA and S100β in the cortex (Fig. 7A) and tPA and GFAP in the hippocampus (Fig. 7B). tPA was localized in the astrocyte cell body as well as in *puncta* that appeared to be vesicular packing for extracellular release.

3.5. Effect of alcohol on the expression of Plat, Plau, Serpine 1, Serpini 1, and Plg in the cortex and hippocampus of female and male PD9 pups neonatally exposed to alcohol between PD4 and PD9

Gene expression results from the cortex of PD9 rats neonatally exposed to alcohol for the most part recapitulated the results obtained in cortical astrocyte cultures, as we found that ethanol increased *Plat* and decreased *Plau* gene expression (Fig. 8A and B). Two-way ANOVA of sex and ethanol treatment revealed a significant main effect of ethanol for both genes [$F(1, 26) = 10.77$, $p = 0.0029$ for *Plat* and $F(1, 28) = 5.38$, $p = 0.0278$ for *Plau*]. Interestingly, we identified a significant sex × ethanol treatment interaction in *Plat* regulation in the cortex [$F(1, 26) = 4.809$, $p = 0.0375$]; *post-hoc* *t*-test confirmed that Plat expression was significantly upregulated in the cortex of female, but not male, pups (Fig. 8A). Also in agreement with what was found in primary cortical astrocyte cultures, we did not find significant changes in the expression of *Serpini1* and *Plg* in the cortex of neonatal rats exposed to ethanol (Fig. 8D and E). On the other hand, while Serpine1 expression was not changed in ethanol-treated astrocytes, it was upregulated by ethanol in the cortex [$F(1, 26) = 12.01$, $p = 0.0019$; Fig. 8C].

The hippocampus of neonatal rats exposed to ethanol displayed significantly altered expression of nearly all genes involved in the PA system. Two-way ANOVA of sex and ethanol treatment revealed a significant main effect of ethanol on *Plat* expression in the

hippocampus [$F(1, 18) = 19.61$, $p = 0.0003$; 9A]. The effect of ethanol on hippocampal *Plau* expression was not significant by two-way ANOVA, although a trend toward an increase in expression was observed [$F(1, 19) = 3.335$, $p = 0.084$; 9B]. Serpine1 expression was upregulated by ethanol in in the hippocampus [$F(1, 19) = 3.43$, $p = 0.0794$; Fig. 9C], similar to what we observed in the cortex. Finally, *Serpini1* expression was downregulated [$F(1,20) = 4.613$, $p = 0.0442$; Fig. 9D] and *Plg* expression was upregulated [$F(1, 16) = 10.39$, $p = 0.0053$; Fig. 9E] by ethanol in the hippocampus.

3.6. Effect of alcohol on tPA and PAI-1 protein expression in the cortex and hippocampus of female and male PD9 pups neonatally exposed to alcohol between PD4 and PD9

In agreement with the upregulation in *Plat* gene expression, tPA protein levels were upregulated in both the cortex and the hippocampus of neonatal male and female rats exposed to ethanol [$F(1, 27) = 13.46$, $p = 0.0011$ for the cortex and $F(1,= 11.88$, $p = 0.0029$ for the hippocampus; Fig. 10A, C]. PAI-1 levels were found to be upregulated by ethanol in the cortex [$F(1, 27) = 6.02$, $p = 0.0209$; Fig. 10B]. Hippocampal PAI-1 levels were not measured as the amount of proteins extracted from the hippocampus was not sufficient to allow this determination.

3.7. Relative expression of Plat, Plau, Serpine 1, Serpini 1, and Plg in cortical rat astrocyte cultures and in the cortex and hippocampus of PD9 rats

Table 2 reports the relative expression of the investigated genes in the developing (PD9) cortex and hippocampus and in astrocyte cultures normalized to total RNA content by the Quant-iT Ribo-Green method, as previously described (Hashimoto et al., 2004). Four of the investigated genes, *Plat, Plau, Serpine1*, and *Plg*, were enriched in astrocyte cultures compared to brain tissue, while *Serpini1* was expressed at higher levels in the hippocampus and cortex. *Plg* was by far the least expressed of the five genes, with similar expression in the cortex and hippocampus; similarly expressed genes in these two brain regions were also *Serpine1* and *Serpini1*, while the genes encoding for the two PAs, *Plat* and *Plau* were expressed at higher levels in the hippocampus than in the cortex. *Plat* was the most highly expressed gene in astrocyte cultures, while *Serpini1* was the most highly expressed in both the cortex and hippocampus (Table 2). In agreement with the gene expression data relative to *Plat*, we found that the highest tPA protein levels under control conditions were found in astrocyte cultures (536.55 ± 141.38 ng/mg protein) and that tPA levels in the developing hippocampus (98.56 ± 3.03 ng/mg protein) were higher than in the developing cortex (73.86 ± 3.25 ng/mg). Also in agreement with gene expression data, we found that PAI-1 expression was lower than tPA in both astrocytes and the cortex and was higher in astrocytes than in the cortex (153.02 ± 35.24 ng/mg in astrocytes and 0.75 ± 0.04 ng/mg in the cortex).

4. Discussion

The PA system was initially described in the vasculature, where, through the activation of the zymogen plasminogen to the serine protease plasmin, it regulates the degradation of fibrin clots. During the last twenty years, this system has been described to be present and highly active in the brain parenchyma where it is implicated in several brain functions and dysfunctions (Chevilley et al., 2015). In this study we compared the effect of ethanol on the

expression of genes encoding five major players in the PA system of the brain, namely tPA, encoded by *Plat*, uPA, encoded by *Plau*, PAI-1, encoded by *Serpine1*, neuroserpin, encoded by *Serpini1*, and plasminogen, encoded by *Plg* (Fig. 1) in cortical astrocyte cultures and the brain (cortex and hippocampus) of PD 9 rats neonatally exposed to ethanol, which represent *in vitro* and *in vivo* models of FASD, respectively.

Changes in gene expression following ethanol exposure observed in cortical astrocytes (Fig. 3) and in the cortex (Fig. 8) extensively overlapped, showing an increase in *Plat*, a decrease in *Plau*, and no changes in *Serpini1* and *Plg* expression. The only discrepancy observed between these two systems, *i.e.* the upregulation of *Serpine1* in the cortex (Fig. 8C) but not in cultured astrocytes (Fig. 3C), may be due to ethanol induction of *Serpine1* expression not in astrocytes, but in endothelial cells, which indeed express high levels of *Serpine1*/PAI-1 (Simpson et al., 1991). In the hippocampus (Fig. 9) the expression of all five genes investigated was somehow altered by ethanol. Similar to what we observed in astrocyte cultures and the cortex, *Plat* expression was upregulated and similar to what observed in the cortex, *Serpine1* expression was upregulated by ethanol. In contrast to what we observed in astrocytes and in the cortex, *Plau* expression was upregulated in the hippocampus of female animals, and *Serpini1* was downregulated while *Plg* was upregulated in hippocampal samples from ethanol-treated males and females.

These results indicate that the effects of alcohol on cortical astrocytes are remarkably recapitulated in the PD9 cortex. On the other hand, the hippocampus differs from both cortical astrocyte cultures and PD9 cortices, suggesting regional differences in the response to ethanol that may be due in part to the different developmental stages of these two regions at PD9 (Bayer et al., 1993). Our studies focused on the neocortex and hippocampus, because these two regions are highly affected by *in utero* alcohol exposure during the third trimester-equivalent of human gestation in animal models (Berman and Hannigan, 2000; Ikonomidou et al., 2000) and human imaging studies revealed alterations in these regions caused by fetal alcohol exposure associated with functional impairments (Archibald et al., 2001; Lebel et al., 2011; Riikonen et al., 1999; Riley and McGee, 2005).

Of the five genes investigated, only *Plat* displayed a consistent upregulation by ethanol in cultured cortical astrocytes and in the two brain regions examined, cortex and hippocampus (Figs. 3A, 8A and 9A), which was corroborated by the upregulation of tPA protein levels (Figs. 4A; 10A, C).

Our studies also show an enrichment of four components of the PA system (*Plat, Plau, Serpine1*, and *Plg*) in primary astrocyte cultures in comparison to cortex and hippocampus, while *Serpini1* was more highly expressed in the cortex and hippocampus than in astrocytes *in vitro* (Table 2), in agreement with a published study that found higher expression of *Serpini1*/neuroserpin in neurons compared to astrocytes (Docagne et al., 1999). Genes that show higher expression in enriched astrocyte cultures are likely to be more highly expressed by astrocytes than by other cell types in the brain, while genes that are expressed at higher levels in brain homogenates than in enriched astrocyte cultures are likely to be more expressed in cell types other than astrocytes. A limitation of this interpretation is that astrocytes may behave differently *in vitro* and *in vivo*.

Published studies reported tPA expression in astrocytes in culture (Docagne et al., 1999; Zhang et al., 2014b) or after brain injury (Adhami et al., 2008; Kim et al., 2011), while another study did not find tPA expression in astrocytes of the healthy adult mouse or rat brain (Louessard et al., 2016). Here we show that, in the developing rat brain, tPA is expressed by astrocytes (Fig. 7). We hypothesize that astrocytes of the developing brain express high levels of *Plat*/tPA as well as other components of the PA system. The regulation of plasmin proteolysis through the PA system may represent an important mechanism by which astrocytes modulate plasticity in the developing brain. We did not attempt to quantify the number of astrocytes vs other cell types expressing tPA because astrocytic markers do not label the whole cell, therefore making this determination inaccurate. Indeed, it was shown that GFAP staining detects only astrocyte major processes (accounting for approximately 15% of the total volume of an astrocyte), but does not penetrate their spongiform ramifications that are apparent when astrocytes are individually injected with a dye (Bushong et al., 2002). On the other hand, the *ex vivo* isolation of neonatal astrocyte mRNA revealed that *Plat* is enriched by about twofold in astrocytes compared to the whole brain supporting our hypothesis that astrocytes express high levels of tPA in the developing brain (Hashimoto and Guizzetti, unpublished).

The role of astrocytes in modulating the PA system is not fully elucidated. Recently, astrocytes have been shown to serve as a surface for the activation of exogenous plasminogen by exogenous tPA and to be involved in the uptake and lysosomal degradation of plasmin and plasminogen (Briens et al., 2017). However, to our knowledge, the regulation of endogenous plasmin activity by endogenous (and exogenous) tPA has never been shown before in these cells. Here we show that cortical astrocytes *in vitro* have specific endogenous plasmin activity (Fig. 6A) that can be increased by exogenous tPA (Fig. 6B) and can be decreased by tPA silencing (Fig. 6C). Furthermore, we show that ethanol treatments upregulate plasmin activity (Fig. 5A); an effect that is not due to a direct interaction between plasmin and ethanol, as when recombinant plasmin is incubated with ethanol, its activity is not altered (Fig. 5B). Rather, the ethanol-induced increase in plasmin activity is likely mediated by the upregulation of *Plat*/tPA (Figs. 3A and 4A), and, possibly, by the downregulation of PAI-1 (Fig. 4B) in astrocytes. Together these data are consistent with the hypothesis that astrocytes play a major role in modulating plasmin activity in the developing brain by regulating the expression of endogenous components of the PA system.

Plasmin has a broad spectrum of targets; the PA system, and in particular tPA, by modulating plasmin activity, play a major role in the regulation of the ECM through two mechanisms: 1) by regulating plasmin-mediated activation of the pro-forms of matrix metalloproteinases to their active form, which are able to degrade ECM components, and 2) by modulating plasmin-mediated direct degradation of ECM components, such as fibronectin, laminin, and proteoglycans (Bukhari et al., 2011; Guizzetti et al., 2008, 2010; Mehra et al., 2016). While several cell types in the brain express components of the PA system (Chevilley et al., 2015; Fredriksson et al., 2017; Hebert et al., 2016; Mehra et al., 2016), we hypothesize that the astrocyte PA system in the developing brain plays a major role in the modulation of ECM proteins and ECM-mediated neuronal plasticity, as astrocytes are also major producers of ECM proteins (Moore et al., 2009).

Given the broad range of plasmin substrates, this proteolytic enzyme has profound effects on brain physiology and pathology, as detailed above. Therefore, it is not surprising that multiple mechanisms exist to regulate plasmin activity. In this study we investigated the effects of ethanol on factors that modulate the activation of plasmin (Fig. 1). However an additional level of regulation of plasmin activity occurs down-stream to its activation and is mediated by the extracellular plasmin inhibitors α2-antiplasmin and α2-macroglobulin (Al-Horani and Desai, 2014). The relatively small, but highly reproducible and statistically significant changes in plasmin activity induced by ethanol in astrocytes are therefore likely due to a balance between the upregulation of tPA, down-regulation of PAI-1, and the effect of plasmin extracellular inhibitors, which prevent sharp increases in plasmin activity. In support of this hypothesis, our proteomic study of the astrocyte secretome identified α2-macroglobulin as the most abundant protein released by astrocytes in culture (Moore et al., 2009). Additional evidence of the presence of abundant levels extracellular plasmin inhibitors in astrocytes derives from our observation that the activity of recombinant plasmin is highly reduced when this enzyme is added to astrocyte cultures, compared to when is tested in a cell-free system (Wilhelm and Guizzetti, unpublished observation).

In addition to its role in activating plasmin, tPA can also modulate neuronal plasticity through plasmin-independent mechanisms, as it interacts with and upregulates the NRD2 subunit of the NMDA receptor, therefore enhancing NMDA receptor activity (Norris and Strickland, 2007; Pawlak et al., 2005). In addition, tPA binds to low-density lipoprotein (LDL) receptor-related protein (LPR) and modulates the late phase of long-term potentiation in hippocampal neurons (Zhuo et al., 2000).

Despite public health warnings against the use of alcohol during pregnancy, heavy drinking during gestation is still of concern particularly in some communities (May et al., 2014, 2018; May and Gossage, 2011). A recent study of maternal alcohol use and nutrition in South Africa found that a group of pregnant women consumed an average of 8.3 drinks per occasion across pregnancy (Carter et al., 2017). Drinking at these levels can translate to BACs of 45–65 mM, according to an online BAC calculator (http://www.clevelandclinic.org/health/interactive/alcohol_calculator.asp). Therefore, BACs in the range of the ones used in this study, though high, can be reached in pregnant women.

Based on the presented data and the published literature, we hypothesize that ethanol increases *Plat*/tPA expression in astrocytes, which leads to increased plasmin activity and increased degradation of the neuritogenic ECM, resulting in altered neuronal development. We have previously shown that astrocytes *in vitro* release many ECM proteins and ECM modulators, including members of the PA systems (Moore et al., 2009). We have also shown that changes in PAI-1 in astrocyte cultures regulate levels of the neuritogenic ECM proteins fibronectin and laminin released by these cells as well as neurite outgrowth in neurons co-cultured with astrocytes (Guizzetti et al., 2008, 2010).

In conclusion, we find that astrocyte cultures express *Plat, Plau, Serpine1, Serpini1,* and *Plg*; all of these genes with the exception of *Serpini1* are expressed at higher levels in astrocyte cultures than in the cortex and hippocampus of the developing rat brain. Astrocytes also exhibit endogenous plasmin activity that can be modulated by changing tPA levels through

pharmacological (recombinant tPA), genetic (*Plat* siRNA) and toxicological (ethanol) manipulations. Furthermore, we find that tPA is expressed *in vivo* in astrocytes of the developing cortex and hippocampus. We also show that ethanol alters the PA system in astrocyte cultures and in the developing brain. In particular, *Plat*/tPA expression is consistently upregulated in astrocytes *in vitro* and in the cortex and hippocampus of neonatal animal exposed *in vivo*. Changes in plasmin extracellular proteolysis in astrocytes due to changes in the expression of components of the PA system may be responsible for altered plasticity after developmental ethanol exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations:

ECM	extracellular matrix
FASD	fetal alcohol spectrum disorders
PA	plasminogen activator
Plat	plasminogen activator tissue-type (gene)
Plau	plasminogen activator urokinase-type (gene)
PAI-1	plasminogen activator inhibitor 1
Plg	Plasminogen (gene)
Serpine1	serine proteinase inhibitor E1 (gene)
Serpini1	serine proteinase inhibitor I1 (gene)
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator

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PLASMINOGEN ACTIVATOR SYSTEM

PAI-1 (*Serpine1*)
Neuroserpin (*Serpini1*)

uPA (*Plau*)　　tPA (*Plat*)

Plasminogen (*Plg*) ⟶ Plasmin

ECM (fibronectin, laminin, etc..) proteolysis

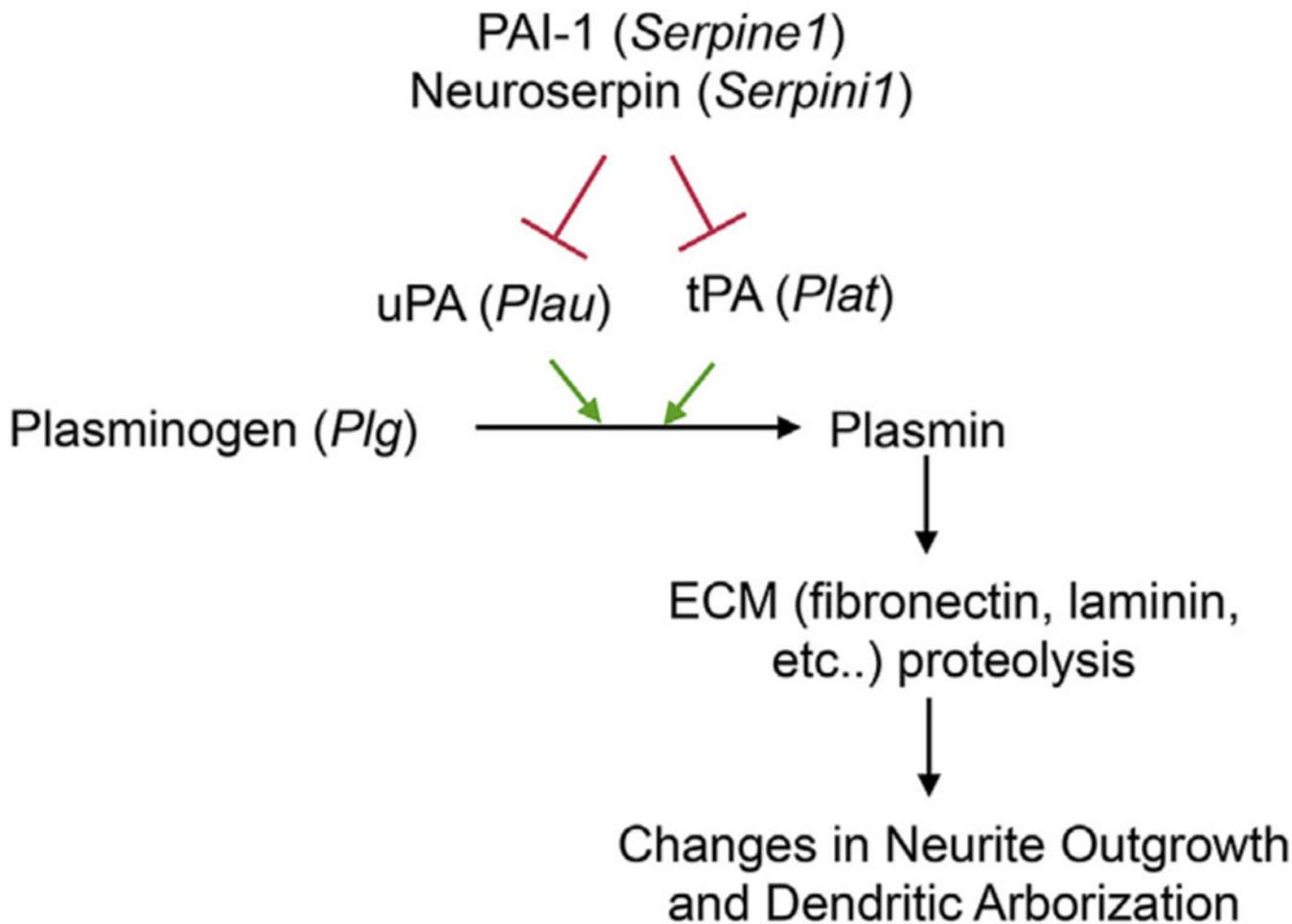Changes in Neurite Outgrowth and Dendritic Arborization

Fig. 1. Plasminogen activator system.
Schematic representation of the components of the plasminogen activator (PA) proteolytic cascade investigated in this study. The PA system regulates the formation of the proteolytic enzyme plasmin from its zymogen plasminogen. There are two mammalian PAs: tissue-type (tPA) and urokinase-type (uPA). PAs are serine proteases that cleave plasminogen to generate the active protease plasmin. Plasmin is a potent extracellular protease, which degrades ECM proteins. This protease cascade is tightly regulated by serine protease inhibitors of PAs; the two main PA inhibitors in the brain are PA inhibitor-1 (PAI-1), and neuroserpin. The gene name of each component is given in parenthesis.
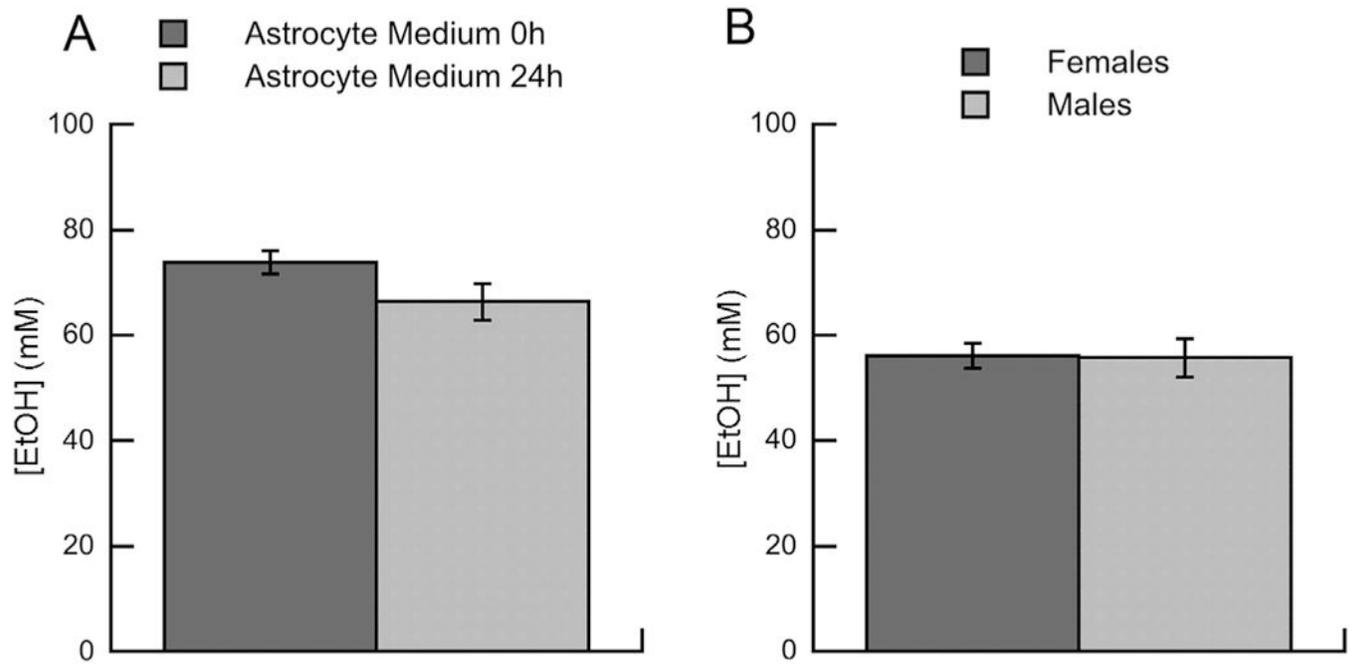
Fig. 2. *In vitro* **and** *in vivo* **alcohol levels.**
Alcohol levels were quantified by gas chromatography as described in "Methods". The media of astrocyte cultures both at the beginning (0 h) and the end (24 h) of exposures were tested to insure that the alcohol concentration remained relatively constant (**A**). Blood alcohol levels were assayed in male and female PD9 rat pups 2 h after the final ethanol administration (**B**).
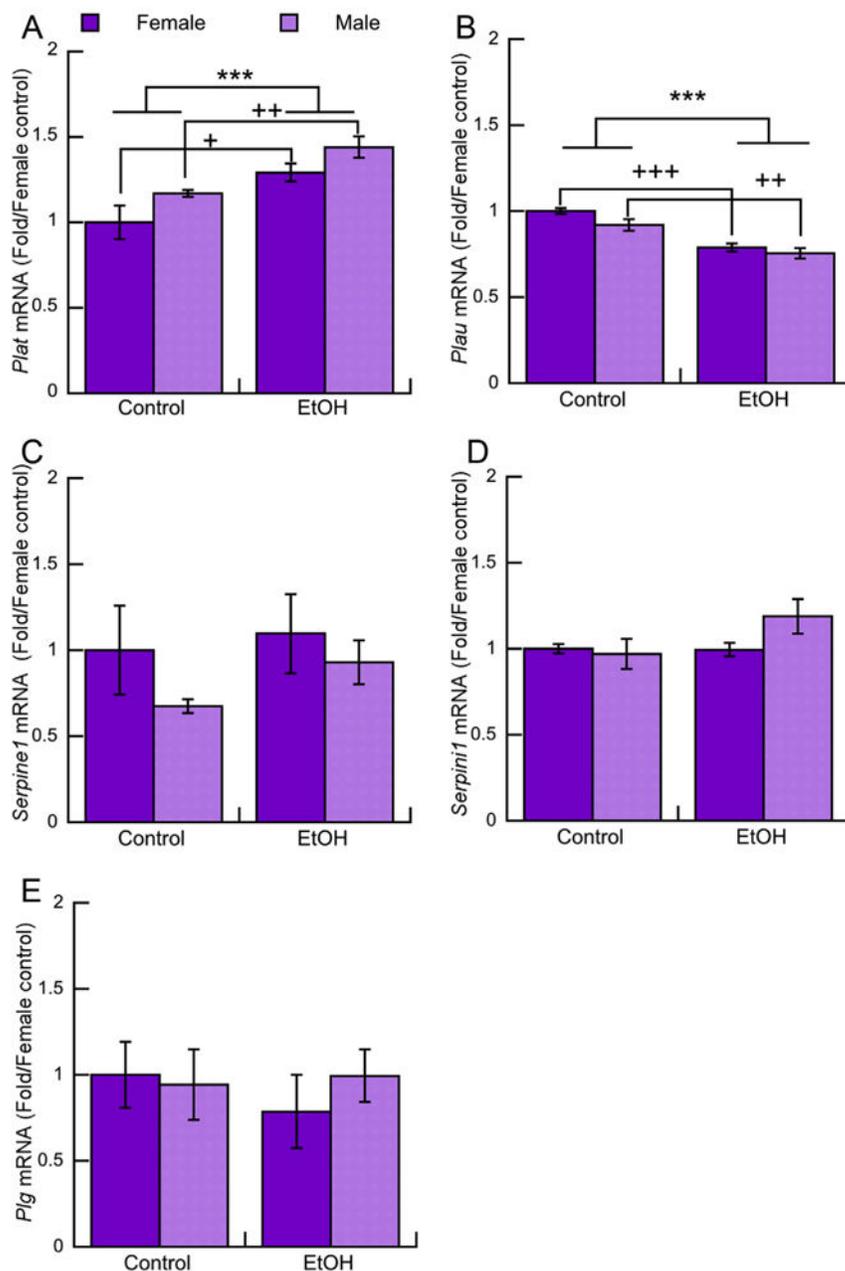
Astrocyte Cultures

Fig. 3. Effect of ethanol on *Plat, Plau, Serpine1, Serpini1*, and *Plg* expression in primary astrocyte cultures.

Sex-specific primary rat cortical astrocytes were incubated for 24 h in the presence or absence of 75 mM ethanol. RNA was extracted and *Plat* (**A**) (n = 5–6), *Plau* (**B**) (n = 5–6), *Serpine1* (**C**) (n = 6), *Serpini1* (**D**) (n = 6), and *Plg* (**E**) (n = 6) mRNA levels were quantified by qPCR, normalized to total RNA and expressed as fold over female control. ***$p < 0.001$; indicates main effect of ethanol by two-way ANOVA. +$p = 0.05$; ++$p = 0.01$; +++$p = 0.001$ Student's t-test between the two groups delimited by the conjoined lines.

Astrocyte Cultures
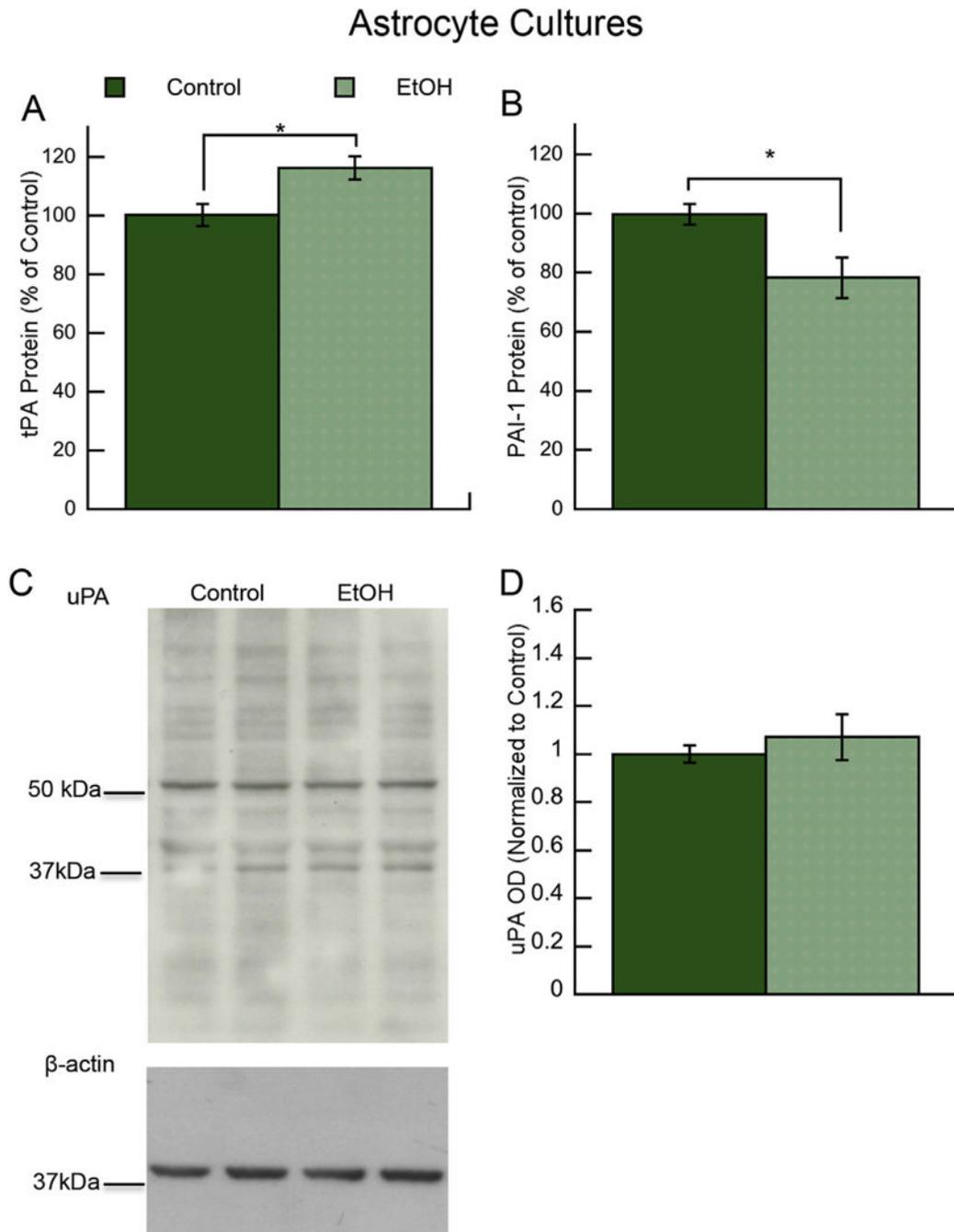
Fig. 4. Effect of ethanol on tPA, uPA, and PAI-1 protein levels in primary astrocyte cultures.
Mixed-sex primary rat cortical astrocytes were incubated for 24 h in the presence or absence of 75 mM ethanol. tPA (**A**) (n = 5e6) and PAI-1 (**B**) (n = 6) protein levels were quantified in cell lysates by ELISA; uPA protein levels were quantified in the cell lysate by Western blot normalized to β-actin and expressed as fold over control (**D**) (n = 7). Panel **C** shows representative immunoblots of uPA (upper) and β-actin (lower) in astrocyte cell lysate. *p < 0.05 compared to control by Student's *t*-test.
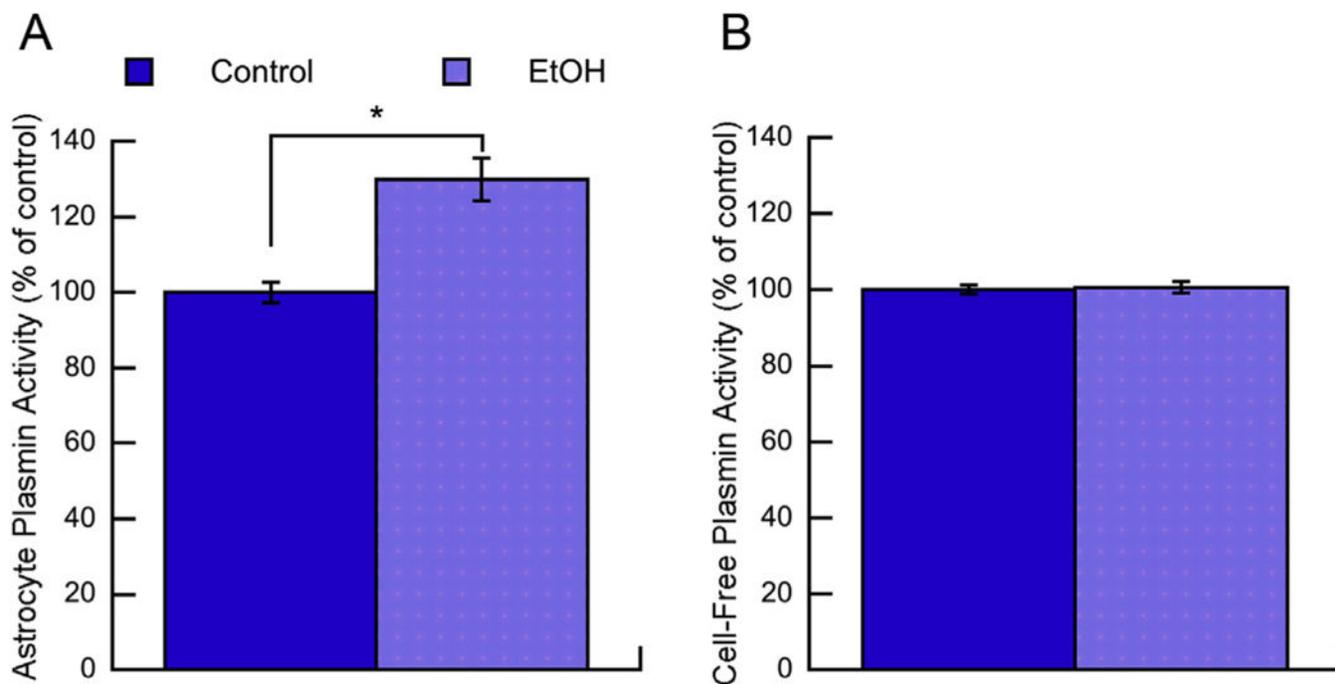
Fig. 5. Effect of ethanol on plasmin activity in primary astrocyte cultures and in a cell-free system.

Mixed-sex primary rat cortical astrocytes were incubated for 24 h in the presence or absence of 75 mM ethanol. Plasmin activity was quantified in astrocyte homogenates, normalized to total protein content, and expressed as percent of control (**A**) (n = 6). Solutions containing human recombinant plasmin (250 ng/ml) were incubated in the presence or absence of 75 mM ethanol; plasmin activity was determined and expressed as percent of control (**B**) (n = 6). *p < 0.05 compared to control by the Student's t-test.
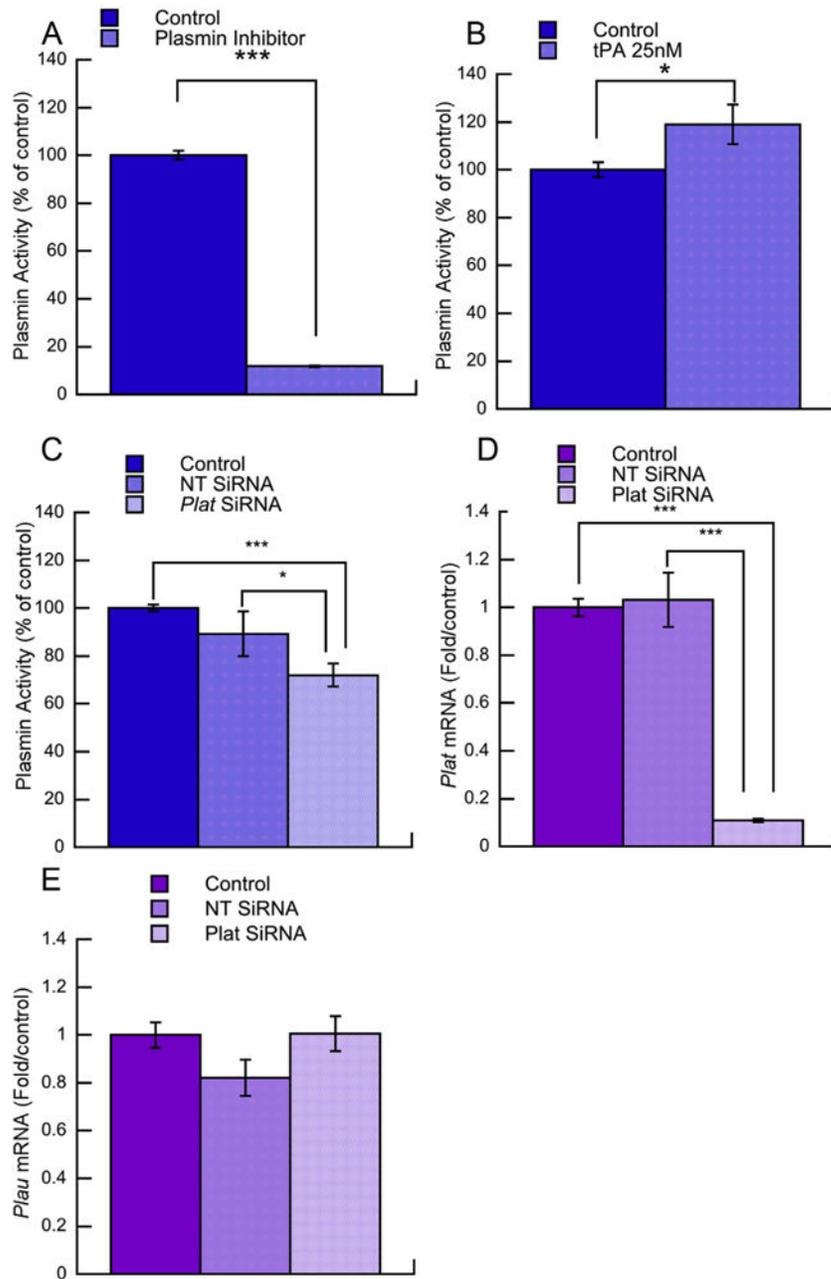
Astrocyte Cultures

Fig. 6. Effect of plasmin inhibition, recombinant tPA and *Plat* silencing on endogenous plasmin activity in primary astrocyte cultures.

Mixed-sex primary rat cortical astrocytes were incubated with or without 10 μM plasmin inhibitor (**A**) (n = 4); 25 nM recombinant tPA for 4 h (**B**) (n = 6); or transfected with a non-target (NT siRNA) or a *Plat* specific siRNA (*Plat* siRNA) using lipofectamine RNAiMAX for 48 h (**C**) (n = 6). Plasmin activity was quantified in astrocyte homogenates as described in "Methods". *Plat* (**D**) (n = 6) and *Plau* (**E**) (n = 6) mRNA were quantified in NT siRNA-

and *Plat* siRNA-transfected cells, normalized to total mRNA, and expressed as fold over control. *: $p < 0.05$; ***$p < 0.001$ compared to control by the Student's t-test.
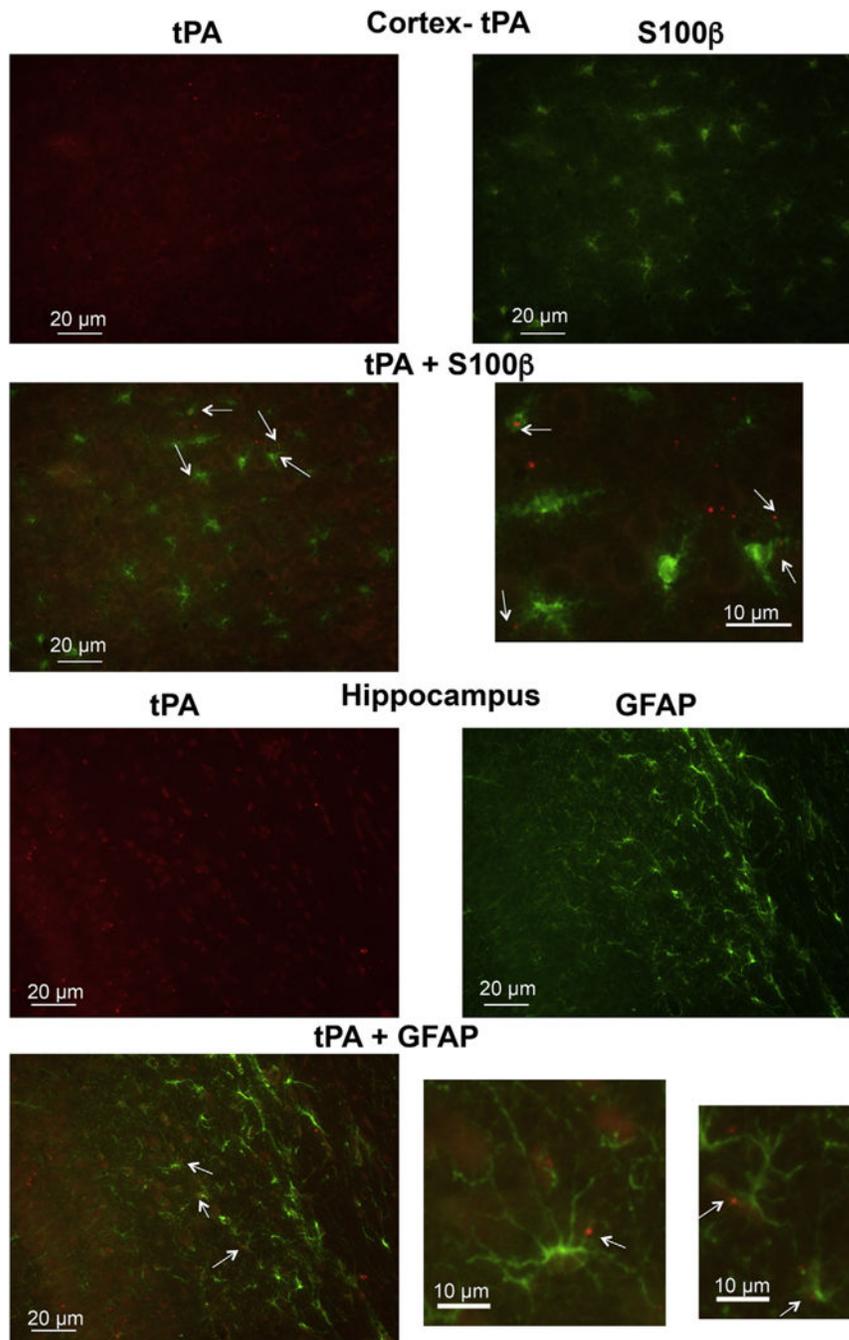
Fig. 7. Fluorescence immunohistochemistry of tPA localization in astrocytes and neurons of the developing cortex and hippocampus.

Brains from PD9 rats were fixed, paraffin-embedded, and double-immunolabeled with tPA and S100β (**A**) or tPA and GFAP (**B**) antibodies to determine tPA expression in cortical (**A**) and hippocampal (**B**) astrocytes respectively, as described in "Methods". **A:** Shown are tPA staining alone (in red, upper left image); S100β alone (in green, top right image); combined tPA and S100β staining (lower left image); a detail of the same image is shown in the lower right. **B:** Shown are tPA staining alone (in red, upper left image); GFAP alone (in green, top

right image); combined tPA and GFAP staining (lower left image); details of the same image are shown in the two images in the center and lower right. Arrows indicate some of the places where tPA puncta co-localize with S100β (**A**) or GFAP (**B**) staining.
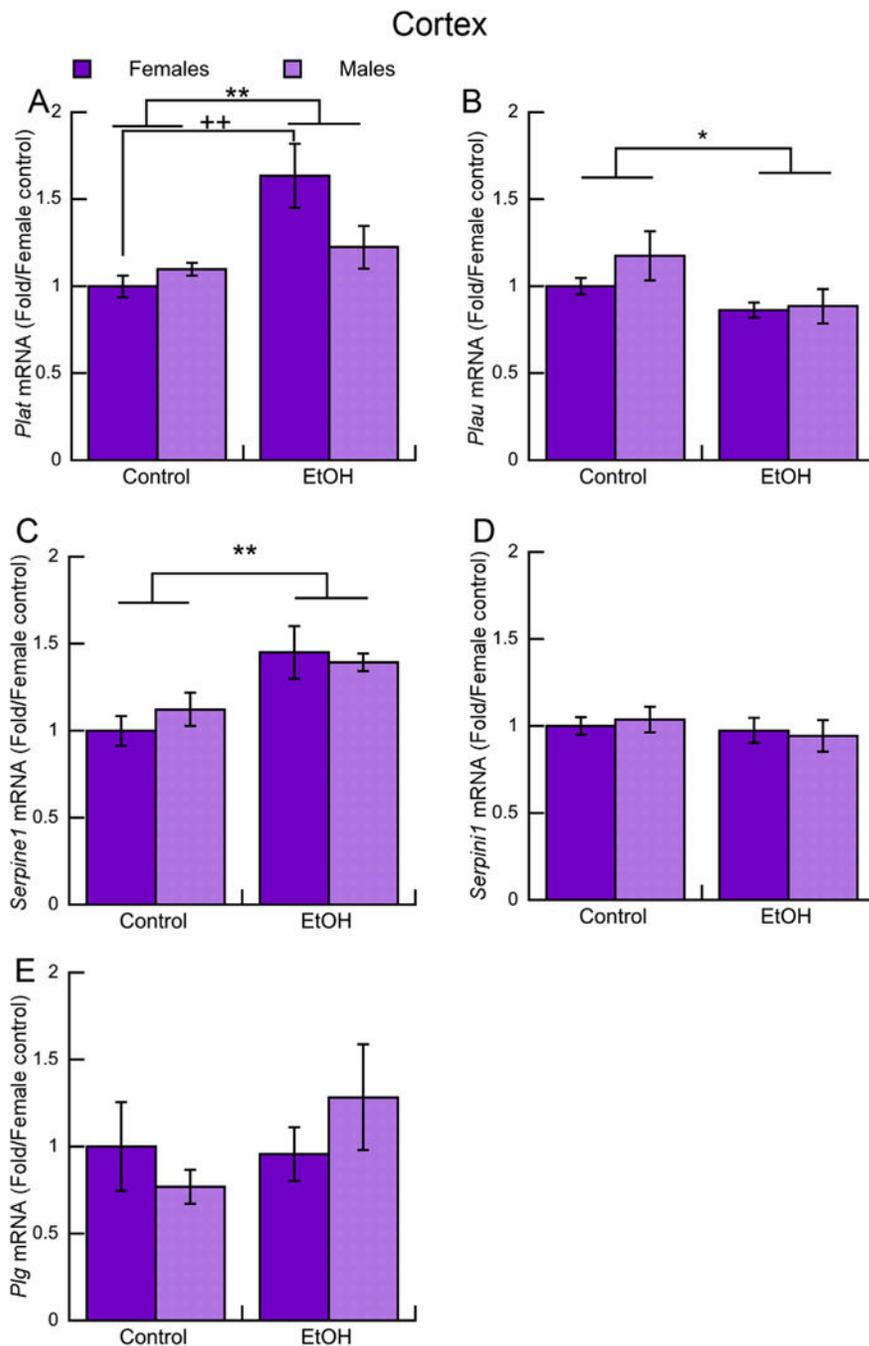
Fig. 8. Effect of *in vivo* ethanol exposure on *Plat, Plau, Serpine1, Serpini1*, and *Plg* expression in the developing cortex.

Female and male rat pups were intubated with 5 g/kg/day ethanol or were sham (control) intubated from PD4 to PD9. RNA was extracted from the dissected cortices and *Plat* (**A**) (n = 7–9), *Plau* (**B**) (n = 7–9), *Serpine1* (**C**) (n = 7–8), *Serpini1* (**D**) (n = 4–6), and *Plg* (**E**) (n = 6–8) mRNA levels were quantified by qPCR, normalized to total RNA and expressed as fold over female control. *: $p < 0.05$; **$p < 0.01$; indicates main effect of ethanol by two-way

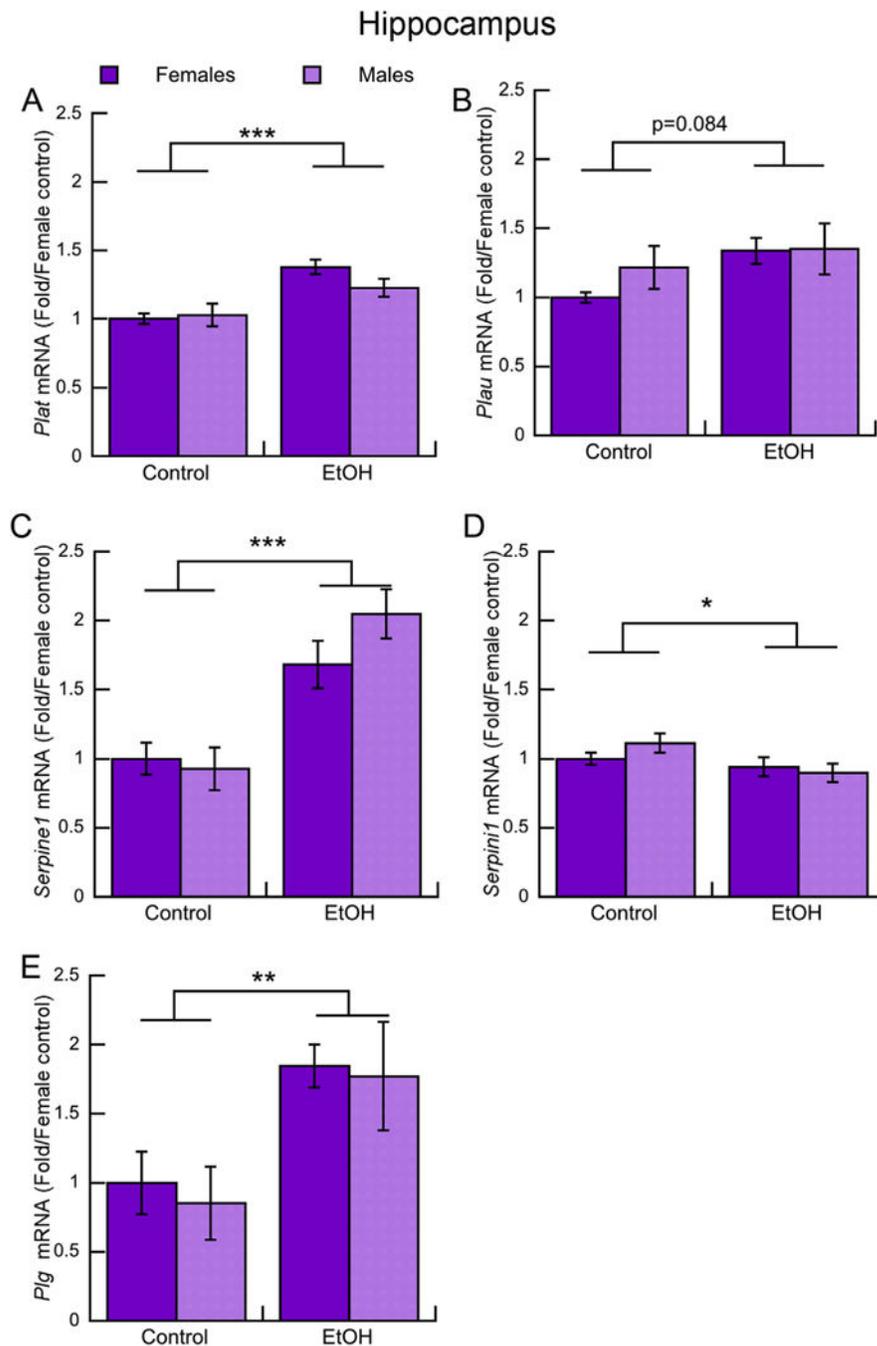ANOVA. $++p = 0.01$ Student's t-test between the two groups delimited by the conjoined lines.

Fig. 9. Effect of *in vivo* ethanol exposure on *Plat, Plau, Serpine1, Serpini1*, and *Plg* expression in the developing hippocampus.

Female and male rat pups were intubated with 5 g/kg/day ethanol or were sham (control) intubated from PD4 to PD9. RNA was extracted from the dissected hippocampi and *Plat* (**A**) (n = 4–7), *Plau* (**B**) (n = 5–7), *Serpine1* (**C**) (n = 4–6), *Serpini1* (**D**) (n = 5–7), and *Plg* (**E**) (n = 4–6) mRNA levels were quantified by qPCR, normalized to total RNA and expressed as fold over female control. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ indicate main effect of ethanol by two-way ANOVA.
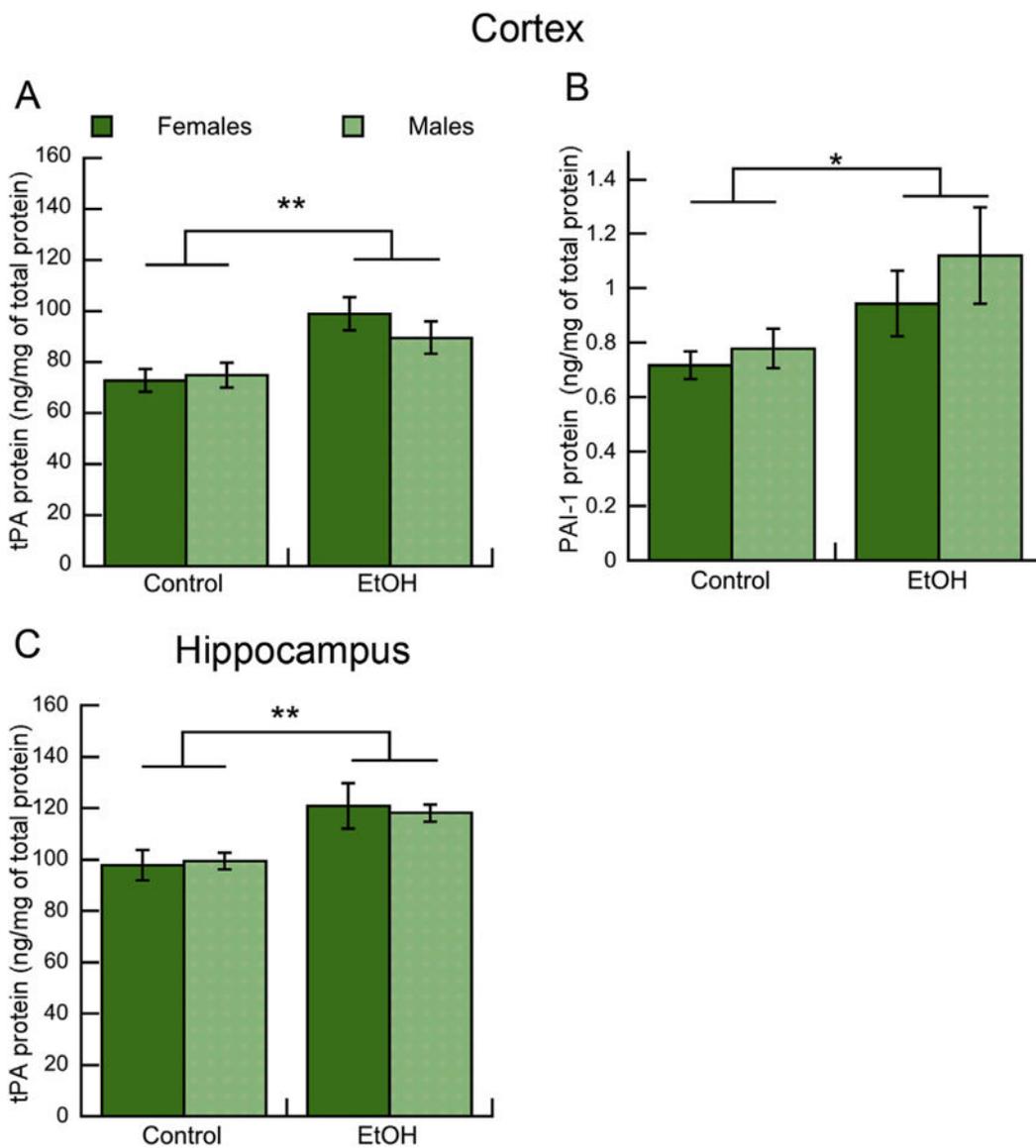
Cortex

Fig. 10. Effect of *in vivo* ethanol exposure on tPA and PAI-1 protein expression in the developing cortex and hippocampus.

Female and male rat pups were intubated with 5 g/kg/day ethanol or were sham (control) intubated from PD4 to PD9. Proteins were extracted from the dissected brain areas and cortical tPA (**A**) (n = 7–8) and PAI-1 (**B**) (n = 7–8) and hippocampal tPA (**C**) (n = 5–6) protein levels were quantified by ELISA, normalized to total protein levels and expressed as ng/mg of total protein. *$p < 0.05$; **$p < 0.01$ indicate main effect of ethanol by two-way ANOVA.

Table 1

Sequences of the primers used to measure gene expression.

Primer	Sequence 5′–3′
Gapdh-F	ACCATGCTTCACTGACATTCTGA (Cha-Molstad et al., 2009)
Gapdh-R	GGTCTGCCTCCCTGCTAACC (Cha-Molstad et al., 2009)
Plat-F	AGCAAGGCACGGGACACGGA (Zhang et al., 2014b)
Plat-R	GGTCAGGCAACGTGAACGCC (Zhang et al., 2014b)
Plau-F	TACCAGCACGGGCCAATGCG
Plau-R	TGGCACTCTCTTGTCCGAAGCCA
Plg-F	ACTTCCCAGATGCTGGCTTG
Plg-R	ACCGCTTCAGGTTGCAGTAT
Serpine1-F	CGTCTTCCTCCACAGCCATT
Serpine1-R	GTTGGATTGTGCCGAACCAC
Serpini1-F	AGGTGTGAGGGAGACTTGAAAC
Serpini1-R	CTGACCACTCGGCTATGGTT
SrY-F	CATCGAAGGGTTAAAGTGCCA (An et al., 1997)
SrY-R	ATAGTGTGTAGGTTGTTGTCC (An et al., 1997)

Primers used in the studies described in the manuscript. *Plau, Plg, Serpine1*, and *Serpini1* primers were designed during the course of this study using NCBI Primer-BLAST software. Publications from which *Plat, SrY*, and *Gapdh* primers were derived are referenced. All primer efficiencies were tested and were between 90 and 110% and had a single peak in melt-curve analysis.

Table 2

Comparative expression in astrocyte cultures and hippocampus and cortex of PD9 pups of *Plat, Plau, Plg, Serpine1*, and *Serpini1*.

Gene symbol	Tissue/Cell	Normalized Cq	Expression relative to Cortical *Plg*
Plat	Astrocytes	17.09	10,131
Plat	Cortex	22	342
Plat	Hippocampus	19.03	2570
Plau	Astrocytes	18.84	2970
Plau	Cortex	25.97	21
Plau	Hippocampus	23.72	100
Plg	Astrocytes	27.57	7.79
Plg	Cortex	30.57	1.00
Plg	Hippocampus	30.22	1.25
Serpine1	Astrocytes	22.02	354
Serpine1	Cortex	25.48	30
Serpine1	Hippocampus	25.26	39
Serpini1	Astrocytes	21.60	437
Serpini1	Cortex	16.67	13,234
Serpini1	Hippocampus	17.02	10,281

Relative expression of the indicated genes was measured by qPCR in primary astrocyte cultures (Astrocytes) and cortical or hippocampal tissue from PD9 rats. The called cycle threshold (Cq) for expression was normalized to RNA weight as determined by the Ribo Green assay (Normalized Cq). Expression levels are presented relative to the gene with the lowest overall expression (cortical Plg).