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RESEARCH ARTICLE

Increased expression of plasminogen activator inhibitor-1 (PAI-1) is associated with depression and depressive phenotype in C57Bl/6J mice

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Abstract

Plasminogen activator inhibitor 1 (PAI-1), which is elevated in numerous disease states, has been implicated as a stress-related protein involved in the pathogenesis of depression. We measured PAI-1 in the plasma of healthy and depressed individuals and assessed plasminogen activator (PA) expression and regulation by PAI-1 in cultured normal human astrocytes (NHA). Elevated plasma PAI-1 levels were found in depressed patients. Brain tissues from depressed individuals also showed stronger expression of hippocampal PAI-1 by confocal imaging in comparison to healthy individuals. Using a lipopolysaccharide-induced inflammatory model of depression in mice, we measured PAI-1 in murine plasma and brain, by ELISA and immunohistochemistry, respectively. Similar elevations were seen in plasma but not in brain homogenates of mice exposed to LPS. We further correlated the findings with depressive behavior. Ex vivo experiments with NHA treated with proinflammatory cytokines implicated in the pathogenesis of depression showed increased PAI-1 expression. Furthermore, these studies suggest that urokinase-type plasminogen activator may serve as an astrocyte PA reservoir, able to promote cleavage of brain-derived neurotrophic factor (BDNF) during stress or inflammation. In summary, our findings confirm that derangements of PAI-1 variably occur in the brain in association with the depressive phenotype. These derangements may impede the availability of active, mature (m)BDNF and thereby promote a depressive phenotype.

Keywords Depression · Fibrinolysis · Hippocampus · Inflammation · LPS · PAI-1

Introduction

Major depressive disorder (MDD) is a common, debilitating, and recurrent disorder with a lifetime prevalence of roughly 20% in the United States (Global Burden of Disease Study 2013 Collaborators 2015). MDD is associated with low education, marital disruption, unstable employment, and increased risks of early mortality due to physical disorders or suicide (Kessler and Bromet 2013). Inflammatory

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biomarkers are increased in about a third of individuals with MDD (Miller and Raison 2016). Depression and inflammation may coexist with and predispose to worse outcomes after common adverse medical conditions, including cardiovascular disease, obesity, and type 2 diabetes (Agarwal et al. 2016; Mendenhall et al. 2017; Nemeroff and Goldschmidt-Clermont 2012). The brain-derived neurotropic factor (proBDNF) appears to be primarily involved in mood regulation through its cleavage by plasmin to the mature form (mBDNF) (Björkholm and Monteggia 2016). Patients with MDD generally demonstrate elevated levels of proBDNF and decreased levels of mBDNF (Idell et al. 2017).

Plasminogen activator inhibitor-1 (PAI-1) is a potent attenuator of plasmin activation via inhibition of tissue-type and urokinase plasminogen activators (tPA and uPA), all of which are expressed in astrocytes, microglia, and neurons in the brain (Hébert et al. 2015; Idell et al. 2017). Increased PAI-1 expression has been observed in a number of disease states, wherein acute inflammation and tissue remodeling occur. In empyema for example, high-grade inflammation is associated with local extravascular fibrin deposition in the pleural space and occurs with elevated levels of active PAI-1 in pleural fluids (Komissarov et al. 2016). In obese individuals with coronary artery disease, serum levels of PAI-1 and tumor necrosis factor alpha (TNF-α), a proinflammatory cytokine, are likewise significantly elevated compared to obese individuals without coronary artery disease (Bilgic Gazioglu et al. 2015). During sepsis, higher plasma PAI-1 concentrations are associated with mortality (Lorente et al. 2015). Interestingly, depression commonly occurs in individuals affected by all these conditions, including survivors of acute illness (Marra et al. 2018; Qin et al. 2007; Valero et al. 2014; Zaghloul et al. 2017).

By limiting the ability of uPA and/or tPA to convert plasminogen to plasmin, PAI-1 can downregulate conversion of pro-to mature BDNF; thereby predisposing to depression (Haastrup et al. 2014; Ventorp et al. 2015). PAI-1 may also limit tPA-mediated synaptic plasticity through interaction with the *N*-methyl-D-aspartate (NMDA) receptor (Hébert et al. 2015). In recent reports, PAI-1 was proposed as a key determinant of depression, as elevated levels of PAI-1 in cerebrospinal fluid or plasma were associated with stress response in rats and with human depression (Couch et al. 2016; Idell et al. 2017; Cassé et al. 2012; Cunningham et al. 2009; Hébert et al. 2015; Jiang et al. 2016; Tsai 2017). Collectively, the literature suggests that derangements in PAI-1 could downregulate uPA and tPA-induced fibrinolysis within the brain.

Lipopolysaccharide (LPS) is a major component of the outer protein coat of Gram-negative bacteria. LPS is also known to induce systemic inflammation, increase PAI-1 levels, and promote a depressive phenotype along with chronic neuroinflammation (Couch et al. 2016; Qin et al. 2007). These considerations led us to infer that derangements of PAI-1 contribute to the pathogenesis of depressive behavior. We examined the possibility that similar, aberrant expression of PAI-1 occurs in brain tissues harvested from patients with depression. We further sought to determine if LPS-induced depressive phenotype in mice is associated with elevations of PAI-1 in the hippocampus, the region of the brain involved in mood regulation. Finally, we attempted to characterize how LPS and other inflammatory mediators implicated in the pathogenesis of depression, such as TGF-β and TNF-α (Idell et al. 2017), interact to concurrently regulate the expression and activity of PAI-1, tPA, and uPA in cultured human astrocytes.

Methods

Animal utilization

The Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler approved the animal protocol; IACUC approval #589. C57Bl/6J mice (10–11 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). A total of 109 mice were used in this study. To induce depression-like behavior, C57Bl/6J mice were given an intraperitoneal (IP) injection of LPS (Sigma, St. Louis, MO, USA) at a dose of 0.83 mg/kg in 0.01 mL/g, as previously reported with minor modifications (O'Connor et al. 2009). Control mice were given saline by IP (vehicle). The mice were acclimated in cages for 24 h to mitigate stress prior to LPS challenge and behavioral testing.

Human brain tissues

Ten human brain tissue blocks, containing the hippocampal region, were obtained from the Brain Endowment Bank of the Miller School of Medicine at the University of Miami. The appropriate consent was given prior to the collection of tissues. These samples included five patients clinically diagnosed with major depressive disorder (MDD) and five non-depressed controls.

Tail suspension test (TST)

Mice were acclimated for 30 min prior to testing, in which a 25 cm stand was used to suspend the mice. Imaging was done with a camera (Logitech). The activity of the suspended mice was recorded for 6 min, as previously reported with minor modifications (Can et al. 2012). Scoring was done using EthoVision$^{®}$ XT software (Noldus Information Technology). The depressive phenotype is associated with reduced mobility.

Porsolt/forced swim test (FST)

At 23 h after LPS or vehicle treatment, mice were acclimated for 30 min before testing. A white backboard designed to mitigate observer-related behavioral disturbance surrounded a 16 cm × 23 cm immersion cylinder filled with tap water (22–25 °C). A camera (Logitech, Newark, CA, USA) was used to image the field. Each mouse was positioned in the water and swimming was monitored for 6 min. After swim testing, the mouse was towel-dried and warmed for 6 min. Scoring of mobility/immobility was accomplished using EthoVision® XT (Noldus Information Technology, Leesburg, VA, USA). Independent scoring by five observers was used to train/validate software scoring. Depressive phenotype was defined as increased immobility versus vehicle-treated control animals. The first 2 min of swimming were not scored as the animals equilibrated to the immersion conditions as previously described by others (Can et al. 2012).

Sucrose preference test

Mice were single housed at least 3 days prior to LPS treatment. Mice were then placed into cages with two bottles from which they could choose to drink along with free access to food, as previously reported (Eagle et al. 2016). One bottle was filled with autoclaved water and the other with 1% sucrose solution prepared in autoclaved water. The positions of the bottles were switched every 24 h. The daily intake of water was measured every 24 h until completion of the time course. Bottles were weighed and consumption calculated, assuming that 1 g ≅ 1 mL. The percent of the sucrose preference was determined as follows:

$$\frac{(\text{Day number } (N) \text{ Sucrose weight - Day } N+1 \text{ Sucrose weight})}{((\text{Day } N \text{ Sucrose weight - Day } N+1 \text{ Sucrose weight}) + (\text{Day } N \text{ Water weight - Day } N+1 \text{ Water weight}))} \times 100.$$

The data obtained were averaged per treatment group and percentages calculated. A decrease in sucrose-fluid consumption represents a depressive phenotype.

Locomotor activity

Mice were single housed in a room with red light (8 lx), as previously reported (York et al. 2013). Water, food, and bedding were removed, and a plexiglass cover with ventilation ports was placed over each cage and the mice were then acclimated for 1 h. Movement was then monitored for 30 min, with data uploaded for EthoVision® XT (Noldus Information Technology) analysis. The distance moved (in cm) was then processed using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Cardiac perfusion and harvesting of brain tissues

Mice were anesthetized with pentobarbital at a dose of 9 units/20 g via IP injection. Once unresponsive to toe pinch, blood was collected from the heart and immediately citrated. After blood collection, the left ventricle was cannulated and ice-cold citrated saline (1:9 dilution) was infused at a rate of 90 mL/h using a Genie Touch™ syringe pump (Kent Scientific, Torrington, CT, USA). A total of 10 mL of citrated saline were infused in mice prior to brain tissue collection. Tissues were subsequently used for homogenates or histologic/immunohistochemical (IHC) analyses. For IHC, citrate infusion was followed by an infusion of 6 mL of cold 10% buffered formalin. The mice were then decapitated, and the brain excised. The cerebellum and olfactory bulbs were removed, and the remaining brain was cut in a mid-sagittal plane. Each hemisphere was placed into 10% buffered formalin for further histopathological analysis. For protein or RNA analysis, brain tissue was immediately frozen in liquid nitrogen and stored at −80 °C for later use.

Paraffin embedding of brain tissue and slide preparation for histology

Brain tissue was fixed in solutions with gentle agitation in increasing concentrations of ethanol, xylene, and paraffin, as we previously reported (Tucker et al. 2010). Tissue blocks were stored at room temperature. Tissue sections were generated using a microtome (Leica, Wetzlar, Germany). Slices were 5 µm thick, placed in a water bath at 37 °C, and allowed to expand before collection on Histobond+® slides (StatLab, McKinney, TX, USA).

Immunohistochemistry (mouse)

Fixed mouse brain was blocked using horse serum (2.5%) from the ImmPRESS™ kit (Vector Labs, Burlingame, CA, USA) for 1 h. The primary antibody for PAI-1 (ab28207, Abcam, Cambridge, UK) or IgG isotypic control was incubated overnight. Tissue washes and incubations with secondary antibody were performed per the kit manufacturer's instructions. Coverslips were applied to the sections using Permount™ (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the slides were imaged by bright-field microscopy and scored. The staining intensity was determined by the reciprocal intensity method as presented by Nguyen et al. (2013).

Immunofluorescence (human)

Immunofluorescent labeling of human brain postmortem (< 24 h) tissue blocks was performed on 5 µm-thick sections, as previously described with some modification (Tucker et al. 2010). Antigen retrieval was performed using citrate buffer for heat-induced epitope unmasking (Thermo Scientific, MI, USA) at 95 °C for 20 min. Tissues were permeabilized with PBS containing 0.01% tween 20 for 10 min. Sections were then blocked with 2.5% horse serum for 1 h. Incubation with primary antibodies diluted in 2.5% horse serum was overnight at 4 °C (rabbit polyclonal to PAI-1, Abcam-66705; chicken polyclonal to GFAP, Abcam-4674,). The appropriate Alexa Fluor secondary antibodies diluted in horse serum were then applied for 1 h at room temperature (Invitrogen-Alexa Flour 488; Invitrogen-Alexa Flour 647). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAP, Molecular Probes, Hoechst 33342). Sections were mounted using Fluoro Gel with DABCO™ (Electron Microscopy Sciences, Hatfield, PA, USA). Fluorescence was visualized and imaged at 60 × magnification using a BioTek Cytation 5 multimode reader (Winooski, VT, USA).

Preparation of murine brain homogenates

Brain tissues were homogenized in T-PER™ Tissue Protein Extraction Reagent (1 g tissue/20 mL reagent) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease inhibitor (Halt™ protease inhibitor, Thermo Fisher Scientific, Rockford, IL, USA), and then centrifuged at 10,000*g* for 5 min with further homogenization through a 25-gauge needle. Samples were then centrifuged at 10,000*g* for 15 min and the supernatant was stored at − 80 °C.

PAI-1 ELISA

A mouse PAI-1 total antigen ELISA kit (Molecular Innovations, Inc., Novi, MI, USA) was used to determine plasma PAI-1 antigen levels. Samples were diluted 1:100 in saline for determinations of PAI-1 in the plasma of LPS-treated mice and 1:20 for plasma of control vehicle-treated mice.

Normal human astrocytes

Normal human astrocytes (NHA) isolated from fetal brain tissue (Lonza, Basel, Switzerland) were used for PAI-1 expression analyses. Astrocytes were first serum-starved for 12 h in serum-free RPMI-1640 media supplemented with Glutamax (SFM). Cells were then treated with PBS, LPS (5 µg/mL), TGF-β (5 ng/mL), or TNF-α (20 ng/mL) in SFM for up to 24 h. Conditioned media were collected, resolved by SDS-PAGE, and transferred to PVDF. Enzymography was performed as previously described (Karandashova et al. 2013). Western blot for PAI-1 was performed on the conditioned media of treated cells (PAI3C311, Molecular Innovations). To visualize intracellular PAI-1, serum-starved NHA cells were treated with PBS, TGF-β, TNF-α, and LPS for 6 h. Cells were then formalin fixed and permeabilized with 0.1% Triton X-100 in PBS. PAI-1 (PAI3C311) was then visualized by confocal microscopy as previously described (Boren et al. 2017). The detector settings were identical for all images.

Human subjects

This protocol was approved and monitored by the UTHSCT Institutional Review Board (#1011). A total of 26 MDD patients undergoing a major depressive episode were recruited during year 2017–2018 from the Center for Clinical Research, University of Texas Health Science Center at Tyler Texas (UTHSCT). A detailed clinical history was done to assess co-morbidities, and weight, height, and Body Mass Index (BMI; kg/m^2) were assessed. Median age was 33 years (13–56) with 19 females (67.9%). All the patients met the (DSM-IV) diagnostic criteria for MDD and the severity of depressive symptoms was assessed with the Quick Inventory of Depressive Symptomatology (QIDS-SR16) scores (Brown et al. 2008). Diabetes was present in three (11.1%) patients, COPD in one (3.7%), asthma in five (18.5%), and heart disease in one. No patients were pregnant. Five (18.5%) patients were on oral steroids, six (22.2%) on anti-inflammatory medications. The median weight was 93.4 (53.0–146.8) kg. BMI in seven patients was more than 25. Exclusion criteria included known pregnancy, age (12–65 y.o.), anemia, HIV infection, QID-S score less than 10, and immunosuppressive therapy aside from steroids. Three individuals were excluded from this study due to the stated exclusion criteria. There were 16 healthy controls (4 males and 12 females). None of the controls had a history of alcohol or drug abuse and dependence or a history of DSM-IV disorders.

Blood collection and human PAI-1 ELISA

Blood was collected by venipuncture in citrated tubes between 8:0 and 10:00 a.m. Collected blood was then centrifuged at 1600 rpm at 4 °C for 10 min and plasma stored at − 80 °C. Plasma PAI-1 antigen was determined using ELISA (HPAIKT-TOT; Molecular Innovations). All samples were analyzed in duplicate.

Statistical analyses

Statistical analyses were performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). For human studies, we expressed the data as the mean ± SEM and analyzed the data with SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7 software. Differences in PAI-1 levels between depressed patients and controls were analyzed by Welch's unequal variances *t* test. For analyses comparing saline and LPS-treated mice, Mann–Whitney test was performed. Age, gender, depression score, and body mass index were compared by student *t* test. Relationships between plasma PAI-1 and body weight or other selected factors were examined using Pearson's *r* correlation. The statistical threshold was set to $p < 0.05$.

Results

Increased plasma concentrations of PAI-1 occur in patients with clinically diagnosed depression

Patients that screened positive for depression (defined by a QIDS score ≥ 11) had significantly higher levels of PAI-1 antigen (19.3 ± 2.4 ng/mL versus 13.2 ± 1.2 ng/mL, $p = 0.02$) versus non-depressed patients/healthy people in a random selection from the population at screening (Fig. 1). A threshold of 15 indicates moderate depression on the QIDS. As such, patients were stratified by the severity of depression by QIDS scoring; < 15 (moderate group) and > 15 (severe group). Although there was a trend towards significantly increased PAI-1 levels in patients with severe disease (Table 1), there was no significant difference in patients with severe versus moderate depression. We did not find significant changes in the level of PAI-1 in depressed male and female patients by subgroup analysis (data not shown). Significant PAI-1 increments were found in patients in the > 18 age group (22.4 ± 3.1 ng/mL versus 12.2 ± 1.8 ng/mL, $p = 0.009$) as compared to < 18 age group. In Fig. 2, plasma PAI-1 levels were shown to positively correlate with weight ($r = 0.43$, $p = 0.026$). Although there was a general trend, PAI-1 levels did not significantly correlate with age or depression score (Supplementary Figure 1). Other parameters including asthma, COPD, coronary artery disease,

Table 1 Plasma PAI-1 antigen (ng/mL) differences between subjects, age, gender, depression score, and BMI

Subjects	Mean ± SEM	*p* value
Control ($n = 16$)	13.2 ± 1.23	**0.02**
Patients ($n = 26$)	19.3 ± 2.39	
Age (years)		
Age < 18 ($n = 8$)	12.2 ± 1.85	**0.009**
Age > 18 ($n = 18$)	22.4 ± 3.10	
Gender		
Male ($n = 8$)	22.1 ± 5.01	*0.488*
Female ($n = 18$)	18.0 ± 2.69	
Depression Score		
Moderate group		*0.135*
Score < 15, ($n = 10$)	15.0 ± 3.06	
Severe group		
Score > 15, ($n = 16$)	22.0 ± 3.27	
Body mass index (BMI)		
BMI < 25 ($n = 4$)	13.9 ± 2.21	*0.344*
BMI > 25 ($n = 22$)	20.3 ± 2.75	

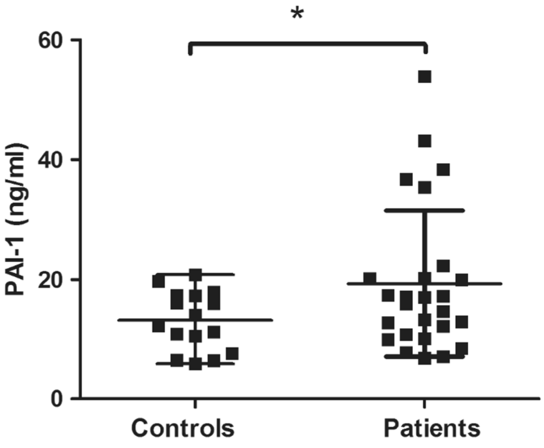

Fig. 1 Total PAI-1 antigen concentrations are increased in the plasma of patients with clinically diagnosed depression versus non-depressed population. PAI-1 antigen was measured in the plasma of normal and MDD patients. PAI-1 is significantly increased in the plasma of the depressed patient group (*$p = 0.02$). PAI-1 levels in non-depressed ($n = 16$) and depressed patients ($n = 26$) are represented using a dot box format in which the medians and outer quartile ranges are shown

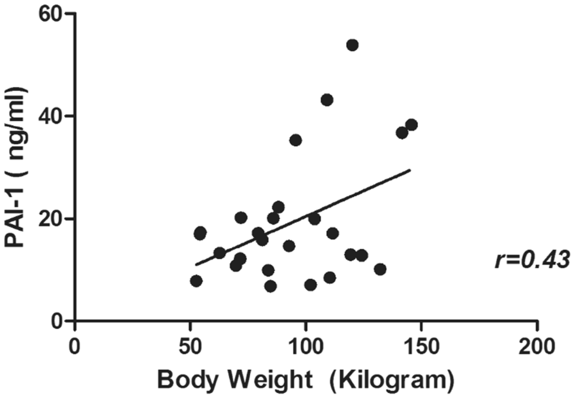

Fig. 2 PAI-1 expression correlates with increased body weight. Total PAI-1 antigen correlates with weight of the patients ($r = 0.43$, $p = 0.026$)

diabetes, anti-inflammatory medication, or BMI, likewise did not correlate with PAI-1 (data not shown).

PAI-1 is increased in the brain tissues of MDD patients

Brain tissue sections from normal and MDD patients were immunostained for PAI-1 and glial fibrillary acidic protein (GFAP, an astrocyte intermediate filament protein). PAI-1 expression was enhanced in the astrocytes within hippocampus of MDD patients ($n=5$) compared to control subjects ($n=5$, Fig. 3). While control subjects showed relatively low PAI-1 in the hippocampal astrocytes, MDD patients showed significantly more PAI-1 staining that colocalized with astrocytes ($p=0.01$, Fig. 3).

Increased plasma PAI-1 concentrations and behavioral changes with LPS challenge in C57Bl/6J mice

Behavioral tests were performed on mice treated with LPS and saline. Experimental design is illustrated in Fig. 4a. Total PAI-1 levels in the plasma were also determined after behavior testing. Total PAI-1 was significantly increased in the plasma of mice challenged with LPS ($n=12$) versus control saline ($n=14$) ($p<0.0001$). Specifically, PAI-1 increased by up to 120-fold: 16.5–77.1 ng/mL in LPS animals versus 0.6–4.1 ng/mL in saline animals (Fig. 4b). Baseline sucrose consumption was established during the 3 days prior to the LPS challenge. No differences between the groups were detected (not shown). LPS-treated mice ($n=15$) demonstrated a significant decrease (20%, $p<0.0001$, Fig. 4c) in sucrose consumption compared to saline mice ($n=15$), 24 h after LPS injection. Locomotion was likewise significantly decreased ($p=0.007$) in LPS mice ($n=15$) versus saline ($n=15$) (Fig. 4d).

Increased expression of PAI-1 in the hippocampal region of the brains of mice treated with LPS

Tissue analyses for PAI-1 were determined after behavioral testing. Immunohistochemical analyses of mouse brain sections showed increased PAI-1 expression in LPS-treated mice (Fig. 5). LPS-treated mice showed increased PAI-1 staining within the hippocampal neurons (Fig. 5a). Conversely, the brains of saline-treated mice showed

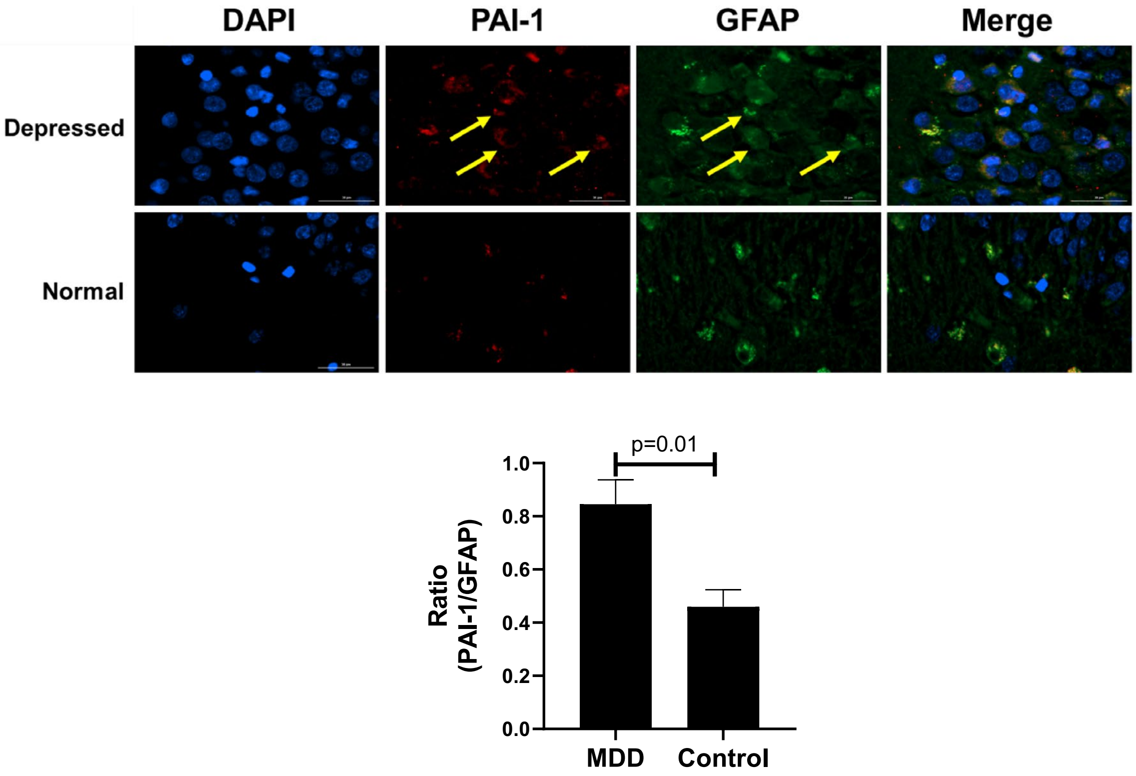

Fig. 3 PAI-1 is increased in the hippocampal astrocytes of MDD patients. **a** Brain tissue sections from normal and MDD patients were immunofluorescently stained for PAI-1 (red) and astrocyte intermediate filament protein GFAP (green) in human hippocampus of MDD subjects reveals moderate intensity of PAI-1 with GFAP in hippocampus. DAPI-positive nuclei are shown in blue. **b** In control subjects, the intensity of PAI-1 colocalization with GFAP is less in hippocampal region. Solid arrows indicate the areas of PAI-1 colocalization with the GFAP astrocytes (orange). Scale bar 30 μm. 10 fields/slide; $n=4$–5/condition

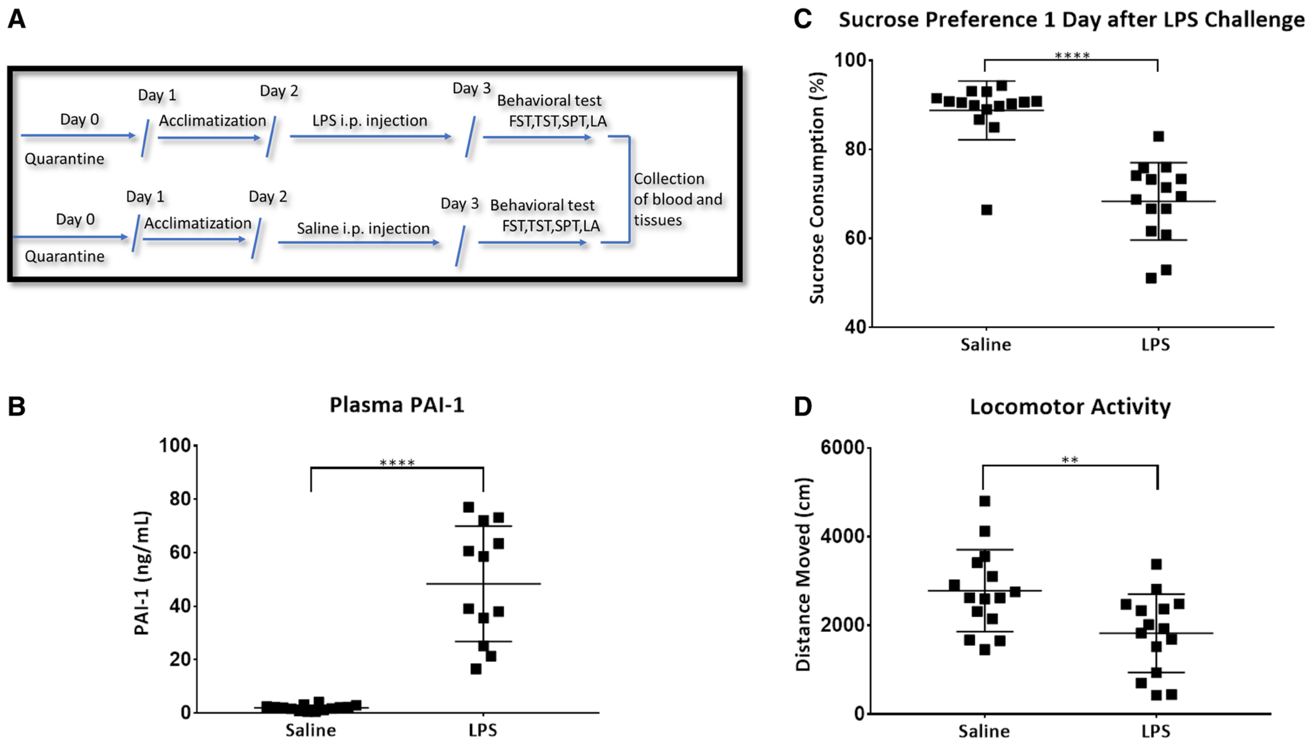

Fig. 4 LPS challenge induces PAI-1 and a depressive phenotype in C57Bl/6J mice. **a** Schematic for LPS administration, assessment of depressive phenotype and subsequent collection of fluids and tissues for analysis. Mice were then evaluated for depressive phenotypes. C57Bl/6J mice were treated with saline or LPS. **b** Total PAI-1 in the plasma of mice administered LPS ($n = 12$) or normal saline ($n = 14$), by intraperitoneal (IP) injection, are shown with the medians and ranges. PAI-1 plasma levels increased from 0.6 to 4.1 ng/mL in saline animals to 16.5–77.1 ng/mL in LPS animals ($p < 0.0001$). **c** Sucrose preference test of LPS-challenged mice ($n = 15$) versus saline controls ($n = 15$) show decreased sucrose consumption in mL throughout the experiment and saline controls. Sucrose consumption was significantly reduced ($p < 0.0001$) in mice treated with LPS at 24 h compared to controls. **d** In locomotion studies, total distance traveled over 24 h in LPS mice ($n = 15$) was decreased versus saline and control mice ($n = 15$, $p = 0.007$). Statistical differences between the groups are indicated by asterisks (**$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

significantly reduced PAI-1 staining ($p = 0.001$, $n = 10$) compared to LPS-treated mice. Staining for PAI-1 within the cortex and white matter within both groups was minimal (Fig. 5b). Hippocampal PAI-1 expression in saline and LPS-treated mice were next graphed using the reciprocal intensity method as described by Nguyen et al. (2013). LPS-treated mice demonstrated significantly higher PAI-1 levels ($p < 0.0001$) than the saline-treated controls. PAI-1 levels did not differ in brain homogenates of LPS versus control mice (data not shown).

LPS and mediators implicated in the pathogenesis of depression induce PAI-1 in human astrocytes in vitro

Given the suggestion of glial PAI-1 expression by murine and human brain by IHC, we next sought to determine if cleavage of proBDNF occurred in vitro using cultured NHA. Neither plasminogen nor BDNF was detectable in the conditioned media of serum-starved NHA by western blotting (data not shown). Plasminogen supplementation of SFM was necessary for cleavage of proBDNF by these cells (Fig. 6a). As plasminogen is not an active protease until conversion to plasmin by PAs, these studies strongly suggest that tPA and/or uPA are elaborated by these cells. NHA treated with LPS, TNF-α, and TGF-β showed enhanced PAI-1 expression compared to PBS-treated controls over the 24 h time course (Fig. 6b, c). These results are consistent with those of fibrin enzymography, where increased PAI-1 complexed with uPA or tPA were detected after treatment with TNF-α (Fig. 6d) compared to PBS-treated cells. Some induction of free tPA in the 21 h, TNF-α-treated samples was better appreciated over time using fibrin enzymography, likely attributable to the increased sensitivity of the technique. In NHA culture medium and analyzed by fibrin enzymography, TNF-α treatment increased early (5 h) expression of uPA and formation of tPA/PAI-1 complexes resulted in accumulation of a reservoir of PA that was characterized mainly as single chain (sc)-uPA (Fig. 6d) in antibody neutralization studies (not shown).

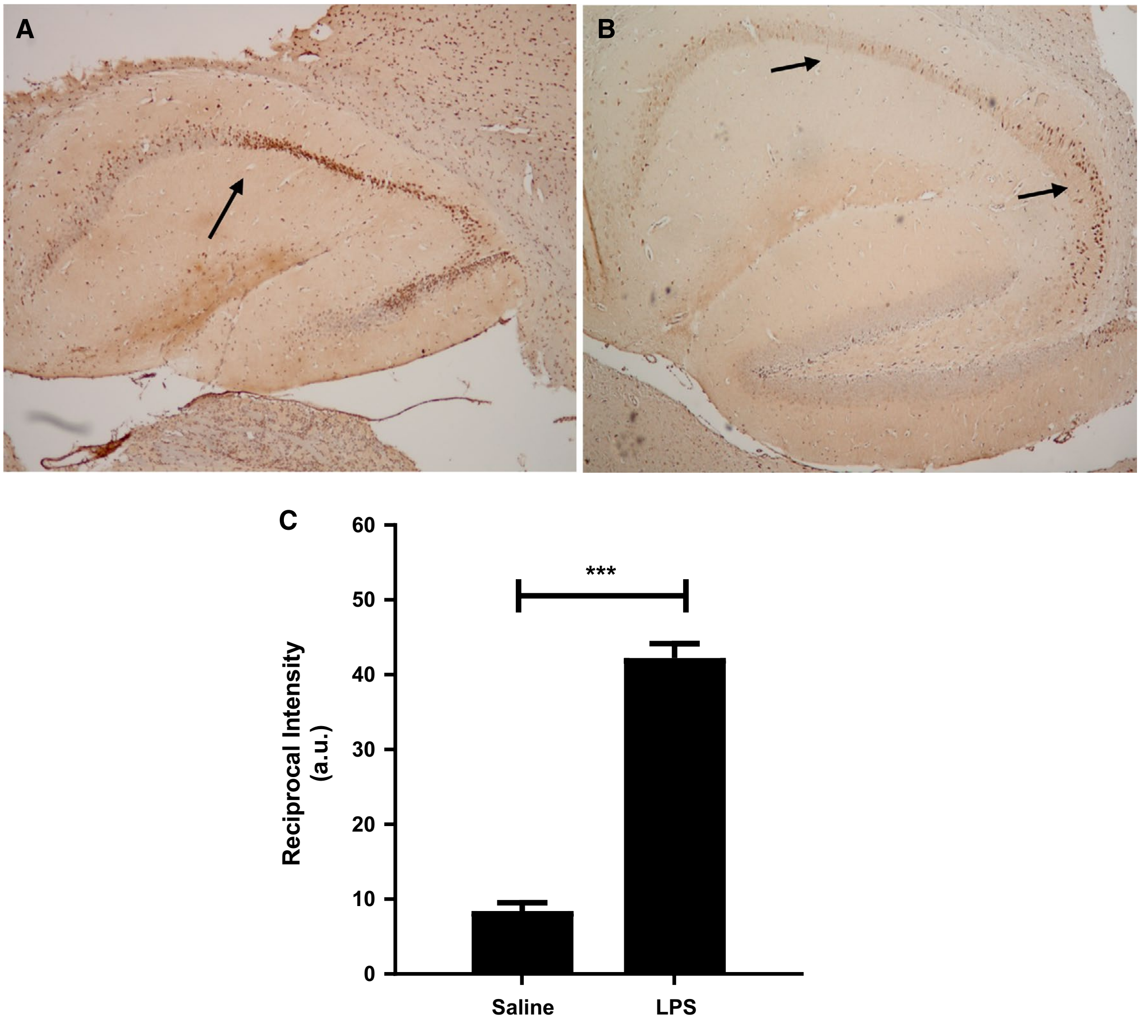

Fig. 5 PAI-1 expression is enhanced in the brains of LPS-treated mice. Brain tissue sections from saline and LPS-treated mice were immunohistochemically stained for PAI-1. **a** High neuronal PAI-1 expression was observed in the hippocampal region with relatively lower signal in the surrounding glial tissue in LPS-treated mice. Increased levels of PAI-1 are shown in the hippocampal region (solid arrows) and appear darker brown than the surrounding tissue. Image is representative of $n = 10$ LPS-challenged mice. **b** Low-to-moderate PAI-1 expression was detected in the hippocampal region of PBS-treated mice. Image is representative of $n = 9$ saline-treated mice. Images were taken at 4× optical zoom. **c** Hippocampal PAI-1 expression in saline and LPS mice were graphed using the reciprocal intensity method. Data are expressed as ± SEM comparing saline and LPS-treated mice. $^{***}p < 0.001$. $n = 9$–10 mice/group

Discussion

Our data show that a systemic injection of LPS increases PAI-1 in the plasma and variably in the hippocampus of C57Bl/6J mice, which could impair fibrinolysis, is critical to maintaining healthy synaptic plasticity and euthymia. Half of the mice treated with LPS demonstrated elevation in hippocampal PAI-1, while plasma PAI-1 was more consistently elevated (up to 120-fold) in these mice when compared to control. Furthermore, mice exposed to LPS showed a depressive behavioral phenotype as evidenced by decreased sucrose preference and decreased locomotor activity as measured 24 h after LPS injection.

Previous studies have demonstrated an increase in immobility in the tail suspension test (TST) and forced swim test (FST) in C57B6 mice injected 24 h after administering 0.83 mg/kg LPS (O'Connor et al. 2009; Walker et al. 2013). However, we were not able to reproduce these findings, possibly because of colony or microbiome differences or because C57Bl/6J animals may not respond as consistently as other murine species (Jung et al. 2014). Some have argued that immobility in the FST is an adaptive rather than a depressive behavior (Molendijk and de Kloet 2015). Optogenetic studies suggest that stress history and cortisol-mediated communication between the limbic system and frontal cortex may mediate this shift to adaptive immobility during the FST (Kloet and Molendijk 2016), or similarly

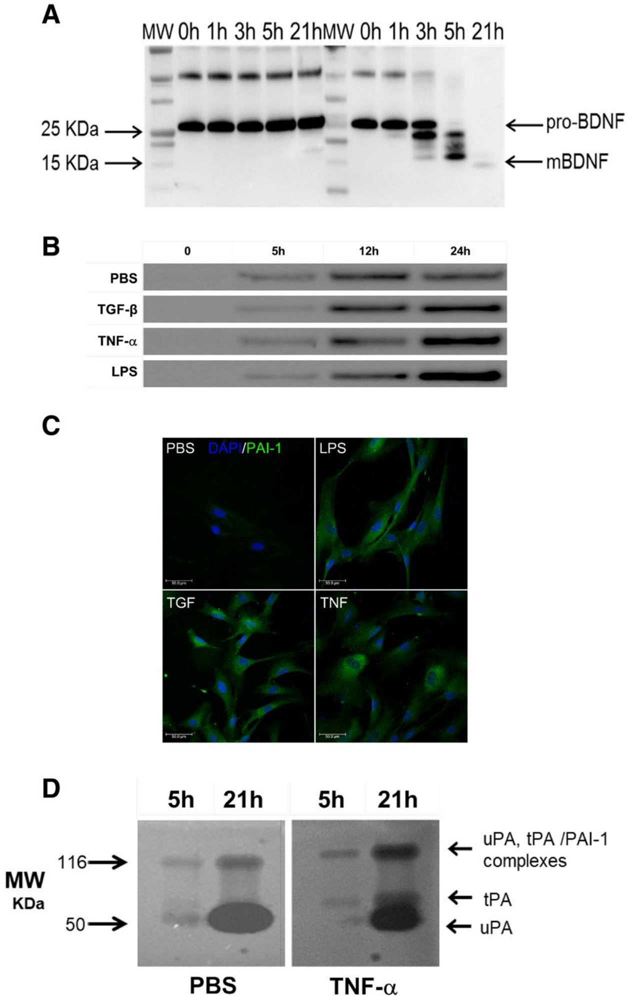

Fig. 6 Expression and regulation of PAs by PAI-1 in cultured human astrocytes. Serum-starved NHA cells were treated with proBDNF (1 µg/mL) in the presence and absence of plasminogen (20 µg/mL). **a** ProBDNF (28 kDa) was not cleaved in the conditioned media of serum-starved cells (left panel). Cleavage of proBDNF (28 kDa) to the mBDNF (14 kDa) was readily detectable by WB in the media of cells supplemented with both proBDNF and plasminogen (right panel). Serum-starved NHA cells were treated with PBS, TGF-β (5 ng/mL), TNF-α (10 ng/mL), and LPS (5 µg/mL). Conditioned media were then collected at 0, 5, 12, and 24 h. **b** Western blot analyses showed an increased accumulation of PAI-1 in the TGF-β, TNF-α and LPS-treated NHA compared to PBS-treated controls. Serum-starved NHA cells were treated with PBS, TGF-β (5 ng/mL), TNF-α (10 ng/mL), and LPS (5 µg/mL) for 6 h. **c** Confocal imaging of TGF-β, TNF-α, and LPS-treated cells showed enhanced expression of PAI-1 (green) in serum-starved NHA compared to PBS-treated control cells. Cell nuclei (blue). Serum-starved NHA were treated with PBS and TNF-α for 5 and 21 h. **d** Plasmin enzymography demonstrates an early (5 h) increment of free scuPA accompanied with the formation of uPA/PAI-1 or tPA/PAI-1 complexes in PBS-treated NHAs. A tPA-related band appeared with TNF-α treatment. Furthermore, TNF-α-treated cells also demonstrated an increase in tPA and the tPA/PAI-1 complexes, suggesting increased PAI-1 expression. The presence of uPA, tPA, and PAI-1 activity was independently confirmed by antibody-based neutralization studies (not shown). Data are representative of three independent experiments

during the TST. LPS-treated mice in our study demonstrated a decrease in mobility in the locomotor activity test, but failed to do so in the TST and FST. Mobility was actually increased in the FST. Chronic alternative models of depression such as the chronic unpredictable mild stress model have been reported to exhibit FST and TST immobility (Krishnan and Nestler 2011). Idiosyncratic differences in mouse handling and transport may influence mouse coping skills and immobility. These variations may also have contributed to the FST and TST responses observed. While not all tests which we performed showed a depressive phenotype, the locomotor and sucrose preference decrements observed were temporally related to the subacute phase at 24 h after LPS challenge in mice with indices modeling aspects of a depressive phenotype.

LPS has been used to induce a depressive behavior in numerous studies (Couch et al. 2016; Qin et al. 2007; Valero et al. 2014) and acute administration of LPS has been shown to engender chronic neuroinflammation (Qin et al. 2007). Depression is a complex heterogenous disorder that develops through complex interplay between genetics, psychosocial stress exposure, and, in certain cases, prolonged inflammation (Miller and Raison 2016). Given its ease of administration, and induction of systemic stress/inflammation, it remains a valuable tool to model depression as used by a number of groups (Andersson et al. 1992; Couch et al. 2016; Dinel et al. 2014; O'Connor et al. 2009). Interestingly, the inconsistent increase of hippocampal PAI-1 in LPS mice (approximately 50%) demonstrates that response occurs independent of genetics. The findings are consistent with those of epidemiologic twin studies, which limit heritability of depression to about 37% (Kendler et al. 2006). Early life stress-induced biochemical changes may lead to deleterious changes at the level of the neurons, glia, or blood–brain barrier that may account for decreased resiliency to stress (O'Mahony et al. 2009; Najjar et al. 2013).

Our data suggest that hippocampal PAI-1 elevations in the brains of LPS-challenged mice may be involved in the development of depressive phenotype. LPS increases systemic levels of TNF-α, which is known to suppress fibrinolysis by induction of PAI-1 and suppression of tPA in endothelial cells (Medcalf 2007). TGF-β, another mediator that induces PAI-1, has been found to be elevated in the serum of depressed individuals (Davami et al. 2016). This response is inconsistent as another study has shown decreased serum TGF-β levels (Musil et al. 2011). PAI-1 overexpression can powerfully inhibit the activity of both tPA and uPA, eliminating their ability to convert plasminogen to plasmin. Decreased plasmin generating capacity in the brain may, consequently, decrease the conversion of proBDNF to mBDNF, which can result in the accumulation of proBDNF and interfere with synaptic plasticity involved in maintaining euthymia (Idell et al. 2017).

Additionally, we analyzed the blood of depressed individuals age 12–65 from East Texas, seeking treatment for depression at the UT Health East Texas behavioral health clinic. There was a trend suggesting elevated plasma PAI-1 levels in the moderate-to-severely depressed cohort. Although we included a list of broad exclusionary conditions for this study that may contribute to increased PAI-1 (e.g., pregnancy, anemia, and HIV status), it was selective. Other factors such as age (Yamamoto et al. 2005) were not considered, as regression analyses did not show significance. This may be due to the relatively small size of this study. Altogether, the data suggest that PAI-1 overexpression may be involved in the pathogenesis of MDD in at least a proportion of predisposed subjects.

The human brain tissue of individuals with depression showed moderate to prominent PAI-1 staining in hippocampal astrocytes. Depressed patients showed PAI-1 double immunostaining with astrocytes of the hippocampus, a key structure involved in mood regulation. Stronger expression of PAI-1 in limbic structures involved in mood regulation suggests that it could be involved in decreasing neurogenesis and increasing depression during chronic stress, congruent with the murine data reported.

We cultured NHA to better understand how proinflammatory mediators linked to the pathogenesis of depression regulate PAI-1 and other components of the fibrinolytic system. These experiments show that human astrocytes are capable of converting proBDNF to mBDNF in the presence of supplemental plasminogen and proBDNF. These data suggest that the cells express plasminogen activators, which was confirmed by western blotting. In these experiments, tPA was found complexed with PAI-1, while most of uPA appeared to be in a free single-chain form. In NHA treated with TGF-β and analyzed by fibrin enzymography, increased expression of uPA and PAI-1 and formation of tPA/PAI-1 complexes result in early and continued accumulation of a reservoir of scuPA. These data allow us to infer that TGF-β may increase availability of uPA in situ to activate plasminogen during inflammation associated with depression. If similar reactions occur in vivo, this may represent a newly recognized and potentially targetable candidate pathway by which mBDNF could potentially be generated to alleviate depression.

Due to methodological limitations and inability to find significant differences in FST and TST, we switched to sucrose preference and locomotor activity where we were able to test enough mice to establish correlations in brain and plasma PAI-1 with a depressive phenotype. Of note, behavioral test conditions such as single housing during the sucrose preference test may cause distress to mice. However, we believe that the use of single-housed saline-treated mice should control for this potential confounder. We used a subacute mouse model of depression which presented another limitation, because MDD in humans is known to primarily be a stress-mediated condition that may be more durably triggered by chronic inflammation. Given our findings of dramatic elevation of PAI-1 in the peripheral circulation, with relatively inconsistent, modest increases in PAI-1 in the brain, future studies should attempt to validate our findings using models that may more selectively increase brain PAI-1 without increasing PAI-1 systemically. A study using the CUMS model demonstrated elevations in PAI-1 in the medial prefrontal cortex and hippocampus of phenotypically depressed rats reversed by escitalopram (Jiang et al. 2016). The same study demonstrated elevated serum PAI-1 in depressed humans. Based on our findings and those of others (Jiang et al. 2017; Tang et al. 2015; Tsai 2017), we posit that further study of the fibrinolytic system may yield new anti-depressant targets that may effectively improve synaptic plasticity and reverse the depressive state.

Our data in a murine model of depressive phenotype in patients with clinically diagnosed depression, and postmortem tissue samples from patients with depression, demonstrate that overexpression of PAI-1 occurs in association with depressive behavior. IHC analyses suggest that hippocampal overexpression of PAI-1 is associated with, and may contribute to, a depressive phenotype. PAI-1 was likewise increased in the plasma of patients with clinical depression. In vitro data show that PA expressed by NHA activate plasminogen and convert proBDNF to mBDNF, thereby promoting euthymia. Conversely, TGF-β-treated NHA showed that tPA/PAI-1 complexes form, promoting formation of a scuPA reservoir during cellular stress. In conclusion, hippocampal PAI-1 overexpression variably occurs in association with depressive phenotype and the derangements of PAI-1 in a proportion of depressed subjects may be targetable to raise the possibility of next generation therapeutics targeting PAI-1 to be salutary in predisposed patients.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest to report.

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