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Activators and inhibitors of the plasminogen system in Alzheimer's disease

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Abstract

Accumulation and deposition of Aβ is one of the main neuropathological hallmarks of Alzheimer's disease (AD) and impaired Aβ degradation may be one mechanism of accumulation. Plasmin is the key protease of the plasminogen system and can cleave Aβ. Plasmin is activated from plasminogen by tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). The activators are regulated by inhibitors which include plasminogen activator inhibitor-1 (PAI-1) and neuroserpin. Plasmin is also regulated by inhibitors including α2-antiplasmin and α2-macroglobulin. Here, we investigate the mRNA levels of the activators and inhibitors of the plasminogen system and the protein levels of tPA, neuroserpin and α2-antiplasmin in post-mortem AD and control brain tissue. Distribution of the activators and inhibitors in human brain sections was assessed by immunoperoxidase staining. mRNA measurements were made in 20 AD and 20 control brains by real-time PCR. In an expanded cohort of 38 AD and 38 control brains tPA, neuroserpin and α2-antiplasmin protein levels were measured by ELISA. The activators and inhibitors were present mainly in neurons and α2-antiplasmin was also associated with Aβ plaques in AD brain tissue. tPA, uPA, PAI-1 and α2-antiplasmin mRNA were all significantly increased in AD compared to controls, as were tPA and α2-antiplasmin protein, whereas neuroserpin mRNA and protein were significantly reduced. α2-macroglobulin mRNA was not significantly altered in AD. The increases in tPA, uPA, PAI-1 and α2-antiplasmin may counteract each other so that plasmin activity is not significantly altered in AD, but increased tPA may also affect synaptic plasticity, excitotoxic neuronal death and apoptosis.

Keywords: plasminogen system • Alzheimer's disease • amyloid β

Introduction

The abnormal accumulation and deposition of amyloid β (Aβ) peptide is one of the key neuropathological hallmarks of Alzheimer's disease (AD) and is thought to initiate a series of processes that cause synaptic dysfunction and neuronal death [1,2]. In recent years, reduced activity of enzymes capable of degrading Aβ has been suggested as a potential contributor to AD pathogenesis [3–5]. These enzymes include angiotensin-converting enzyme [6,7], neprilysin [8], endothelin-converting enzymes [9], insulin-degrading enzyme [10] and plasmin [11]. Plasmin is the key protease of the plasminogen system, the primary function of which is fibrinolysis, but has also been shown to be important in cell matrix degradation and cell migration [12,13]. Plasmin is activated from its inactive precursor plasminogen by two plasminogen activators, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) which are, in turn, regulated by inhibitors that include plasminogen activator inhibitor-1 (PAI-1) and neuroserpin. Plasmin itself is inhibited by α2-antiplasmin and α2-macroglobulin [14–16].

Aβ activates the plasminogen activators both *in vitro* and *in vivo* [11,17–19]. This has the potential to be a protective mechanism to limit the accumulation of Aβ. Plasmin cleaves Aβ at multiple sites, is capable of degrading Aβ fibrils and reduces Aβ deposition [11,20]. Plasmin protects cultured neurons from Aβ-induced cell death [11,21,22] and enhances clearance of Aβ *in vivo* [23].

We previously found that plasmin protein and activity were not significantly altered in the human AD brain compared to controls [24]. This suggests that the activating influence of Aβ on the plasminogen system may be counterbalanced by changes affecting other activators and inhibitors of the system. Apart from plasmin

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activation, these activators and inhibitors mediate a range of additional effects that may also be of relevance to the development of AD. Neuroserpin, for example, was shown to interact directly with Aβ and reduce Aβ fibril formation and toxicity to cultured neurons [25]. tPA was shown in several studies to influence synaptic plasticity, a process essential in learning and memory [26–30], but is also a mediator of excitotoxic neuronal death [31–33] and apoptosis [34]. The activators and inhibitors of the plasminogen system have not been much studied in human brain tissue and in the few published reports, the findings are somewhat contradictory. One immunohistochemistry-based study showed increased tPA in AD, with highest levels in amyloid-rich regions of the brain [35]; a further study found no significant alteration in tPA or uPA proteins, but decreased tPA activity in AD, possibly resulting from an increased neuroserpin level [36]; yet another study reported no significant alteration of tPA activity in AD but did show a negative correlation between tPA activity and the level of Aβ [37].

Our aim in this study was to investigate tPA, uPA, neuroserpin, PAI-1, α2-antiplasmin and α2-macroglobulin in AD; to look at their distribution in regions of human brain tissue relevant to AD pathology; to compare the expression of the genes encoding them in AD and control brain tissue; and to measure tPA, neuroserpin and α2-antiplasmin proteins, in order to identify differences in AD that may contribute to the disease.

Materials and methods

Brain tissue

This study had local Research Ethics Committee approval. The tissue was obtained from the Human Tissue Authority-licensed South West Dementia Brain Bank, University of Bristol. The tissue was dissected from brains that had been removed from patients within 72 hrs of death. The left cerebral hemisphere had been sliced and frozen at −80°C. The right cerebral hemisphere had been fixed in 10% formalin for approximately 3 weeks before tissue was taken, processed and paraffin sections cut for neuropathological assessment and diagnosis. The area fractions of cerebral cortex immunopositive for phospho-τ (τ load) and Aβ after excluding Aβ-laden blood vessels (Aβ plaque load) had been measured as previously described [38,39] and *APOE* genotype characterized. These studies involved immunoperoxidase staining and measurement of gene expression and proteins for which different cohorts were used, as indicated later. The AD cases were selected on the basis of a diagnosis according to CERAD [40] of ‘definite AD’ and a Braak tangle stage of IV–VI.

For the immunoperoxidase studies, a cohort of five AD (ages 78–90, mean 84, S.D. 5.79; post-mortem delays of 4–49.5 hrs, mean 24.5, S.D. 19.42) and five controls (ages 59–83 years, mean 73.2, S.D. 11.69; post-mortem delays of 3–72 hrs, mean 22, S.D. 29.21) was chosen to demonstrate the distribution of tPA, uPA, PAI-1, neuroserpin, α2-antiplasmin and α2-macroglobulin in the human brain and to show any obvious differences in expression between AD and control.

For the gene expression studies, we selected a larger cohort of 20 AD (ages 54–90 years, mean 76.4, S.D. 10.4; post-mortem delays of 4–43.5 hrs, mean 17.4, S.D. 10.8) and 20 matched controls (ages 58–93 years, mean 77, S.D. 9.8; post-mortem delays of 3–24 hrs, mean 15.7, S.D. 7.9). For the tPA, neuroserpin and α2-antiplasmin protein measurements, we expanded this cohort to 38 AD (ages 54–98 years, mean 78.5, S.D. 9.6; post-mortem delays of 4–48 hrs, mean 23.6, S.D. 11.9) and 38 matched controls (ages 58–95 years, mean 78.8, S.D. 9.2; post-mortem delays of 3–48 hrs, mean 25.7, S.D. 14.2). mRNA and protein measurements were made on frontal and temporal cortex (BA6 and BA22) with the exception of the tPA protein measurements which, because of the high cost of the assay, were made on the temporal cortex only.

Western blots

The specificities of the antibodies used for the immunoperoxidase studies and for protein measurements by ELISA were confirmed by Western blot. Brain tissue from the frontal cortex (BA6) was homogenized in 1 ml 1% SDS lysis buffer in a Precellys homogenizer (Stretton Scientific, Derbyshire, UK). Total protein levels were quantified using Total Protein Kit (Sigma-Aldrich, Dorset, UK) according to the manufacturer’s instructions. Recombinant protein standards were loaded (as summarized in Table 1), along with 30 μg (total protein) homogenate per sample onto a 4–20% Tris-HCL pre-cast gel (Bio-Rad, Hercules, CA, USA) and electrophoresed at 150 V for 1 hr. The proteins were transferred to nitrocellulose membrane overnight at 4°C. Non-specific binding was blocked by incubation of the membrane with 10% non-fat milk in 0.05% tris-buffered saline-Tween-20 (TBST) for 1 hr at room temperature, with agitation. The membrane was incubated with the appropriate primary antibody, diluted (as summarized in Table 1) in 5% milk/TBST, for 1 hr at room temperature with agitation. After three washes in TBST, the membrane was incubated for 1 hr at room temperature with the appropriate peroxidase-conjugated secondary antibody, diluted in 5% milk/TBST. The membrane was washed three times in TBST then ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK) were applied for 1 min. before exposure to photographic film in the dark for 7 min. then development.

Immunoperoxidase staining

We performed immunoperoxidase staining to show the distribution of tPA, uPA, PAI-1, neuroserpin, α2-antiplasmin and α2-macroglobulin in brain tissue. Antibody specificity was assessed by Western blot as described earlier. We used 3-amino-propyl-triethoxy silane-coated slides to collect 7-μm-thick paraffin sections of temporal lobe (including Brodmann areas BA20-22). Sections were incubated overnight at 60°C, dewaxed, dehydrated and incubated in 3% hydrogen peroxide in methanol for 30 min. to block endogenous peroxidase. Antigen-retrieval step were performed as outlined in Table 2, then the sections were washed in cold running water, and non-specific antibody binding was blocked by incubation with blocking serum (Vecatastain Universal Elite Kit; Vector Labs, Burlingame, CA, USA) for 20 min. at room temperature. Sections were washed twice in PBS and the primary antibodies were applied as outlined in Table 2, overnight at room temperature. Sections were again washed twice in PBS, and the biotinylated Universal Antibody (Vector Labs) applied for 20 min. at room temperature. After two washes in PBS, VectaElite ABC complex (ABC), made up previously and incubated at room temperature for 30 min., was applied for 20 min. After 2 washes in PBS 3,3’-diaminobenizidine was applied for 7 min., the sections were washed in running water, then immersed in copper sulfate for 4 min. and counterstained in Harris’ haematoxylin for 15 sec. The sections were rinsed, dehydrated, cleared and mounted. Negative controls comprised sections treated as above apart from omission of the primary antibody.

Table 1 Summary of the recombinant protein, primary and secondary antibody dilutions used in Western blots to demonstrate antibody specificity

Antibody	Antibody supplier and product code	Application in this study	Recombinant protein supplier and product code	Recombinant protein dilutions	Primary antibody dilution	Secondary antibody (Vector Labs) and dilution
tPA	Santa Cruz sc-5241	Immunoperoxidase, luminescent sandwich ELISA	ProSpec ENZ-263	1:10	1:100	Anti-goat 1:10000
tPA	Abcam ab51518	Luminescent sandwich ELISA	N/A	N/A	1:100	N/A*
uPA	Abcam ab24121	Immunoperoxidase	ProSpec ENZ-264	1:100	1:200	Anti-rabbit 1:5000
PAI-1	Abcam ab12499	Immunoperoxidase	ProSpec ENZ-356	1:10	1:100	Antimouse 1:5000
Neuroserpin	Abcam ab55587	Immunoperoxidase, direct ELISA	N/A	N/A	1:400	Antimouse 1:5000
α2-Antiplasmin	Abcam ab30994	Immunoperoxidase	Haematologic Technologies HA2AP-0230	1:10	1:500	Antimouse 1:5000
α2-Antiplasmin	Abcam ab62770	Sandwich ELISA	Haematologic Technologies HA2AP-0230	1:100	1:500	Anti-rabbit 1:5000
α2-Antiplasmin	Santa Cruz sc-67510	Sandwich ELISA	Haematologic Technologies HA2AP-0230	1:1000	1:300	Anti-goat 1:10000
α2-Macroglobulin	Abcam ab36995	Immunoperoxidase	N/A	N/A	1:100	Antimouse 1:5000

*****tPA ab51518 was biotinylated, so HRP-conjugated streptavidin (R&D Systems), diluted 1:500 in TBST, was used in place of a secondary antibody.

Table 2 Summary of antigen retrieval steps and antibody dilutions used for immunoperoxidase studies

Target antigen	Antibody supplier and product code	Antigen retrieval	Antibody dilution
tPA	Santa Cruz sc-5241	Boiled in EDTA for 10 min., left to cool for 10 min.	1:50
uPA	Abcam ab24121	Boiled in EDTA for 10 min., left to cool for 10 min.	1:400
PAI-1	Abcam ab12499	Boiled in trisodium citrate for 5 min., rested for 5 min., boiled again for 5 min., left to cool for 15 min.	1:20
Neuroserpin	Abcam ab55587	Boiled in EDTA for 10 min., left to cool for 10 min.	1:400
α2-Antiplasmin	Abcam ab30994	Boiled in EDTA for 10 min., left to cool for 10 min.	1:100
α2-Macroglobulin	Abcam ab36995	Boiled in EDTA for 10 min., left to cool for 10 min.	1:200

RNA isolation, cDNA production and Real-time PCR (RT-PCR)

We homogenized frontal and temporal neocortex in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in a Precellys homogenizer. Chloroform was added to the homogenates for 3 min., after which they were centrifuged at 13,000 × *g* for 15 min. at 4°C then the aqueous phase removed and mixed with an equal volume of isopropyl alcohol and 30 μg of glycogen. After a 10-min. incubation at room temperature, the samples were centrifuged at 13,000 × *g* for 10 min. at 4°C to precipitate the RNA, which was washed then twice in 75% ethanol and re-suspended in water (Sigma-Aldrich, Gillingham, UK). The RNA was treated with DNaseI (Roche Diagnostics Ltd., West Sussex, UK) to remove any DNA and the RNA concentration determined using the RiboGreen quantification kit (Invitrogen).

High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) was used, as per the manufacturer's instructions, to produce cDNA. Quantification of the cDNA was performed using the PicoGreen quantification kit (Invitrogen).

Real-time PCR was carried out using an ABI 7000 sequencing detection system (ABI Prism; Applied Biosystems). Assay on Demand Gene Expression Products for tPA, uPA, neuroserpin, PAI-1, α2-antiplasmin and α2-macroglobulin, and for calibrating genes glyceraldehyde 3-phosphate (GAPDH) and neuron-specific enolase (NSE) (Taqman probes; Applied Biosystems) were incubated with SensiMix dT (Quantace, London, UK) and 10 ng cDNA (total volume of 20 μl) at 50°C for 2 min., 95°C for 10 min. followed by 40 cycles of 95°C for 15 min. and 60°C for 1 min. Samples were analysed in triplicate and gene expression relative to mean control tissue expression was calculated using the $2^{-\Delta\Delta Ct}$ method [41]. We used two calibrator genes for the assays: NSE (because in the brain all of the plasminogen system components are expressed predominantly in neurons) and GAPDH (expressed by all cell types). As $2^{-\Delta\Delta Ct}$ is an exponential function (the fold-increase or decrease in mRNA), the data for each cohort were expressed as the geometric mean and 95% confidence interval [42].

Measurement of neuroserpin protein by direct ELISA

A seven-point homogenate standard curve (total protein range 50–500 μg), brain homogenates (100 μg total protein per sample) diluted in PBS and blanks of PBS were incubated (in duplicate) in a clear 96-well microplate (Fisher Scientific, Loughborough, UK) for 2 hrs at room temperature with agitation. The plate was then washed five times with 0.05% PBS-Tween20 (PBST), tapped dry on the final wash and incubated for 2 hrs at room temperature with the mouse monoclonal anti-neuroserpin antibody (Abcam, Cambridge, UK) (the specificity of which was confirmed by Western blot) diluted 1:400 in PBS. After five washes in PBST, the plate was incubated with the peroxidase-conjugated anti-mouse secondary antibody (Vector Labs) diluted 1:100 in PBS for 30 min. at room temperature, in the dark. The plate was washed five times with PBST, tapped dry on the final wash, then 100 μl of peroxidase substrate (R&D Systems, Minneapolis, MN, USA) was added to all wells for 3 min., followed by 50 μl Stop solution, then absorbance measured at 450 nm using a multidetection microplate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Relative neuroserpin levels were interpolated from the standard curve.

Measurement of tPA protein by luminescent sandwich ELISA

Black 96-well microplates (Fisher Scientific) were coated with capture goat polyclonal anti-tPA (Santa Cruz Biotechnology, CA, USA) antibody diluted 1:100 in coating buffer (10 mM sodium carbonate, 30 mM sodium bicarbonate, pH 9.6) at 4°C overnight. After five washes, the plate was tapped dry and incubated with 1% BSA/PBS for 90 min. at room temperature with agitation. The plate was washed five times, tapped dry and the recombinant tPA protein (ProSpec-Tany TechnoGene Ltd., Ness-Ziona, Israel) standard curve of 5 five-fold dilutions (concentration range 0.08–50 ng/ml), homogenate samples (100 μg total protein per sample), blanks and controls (where either capture antibody, sample of detection antibody were omitted) were diluted in PBS, loaded in duplicate and incubated for 2 hrs at room temperature with agitation. The plate was again washed five times, tapped dry and incubated in the dark for 2 hrs at room temperature with biotinylated sheep polyclonal anti-tPA antibody (Abcam) diluted 1:100 in 1% BSA. Following five further washes, the plate was again tapped dry and incubated with peroxidase-conjugated streptavidin (R&D Systems) diluted 1:500 in 1% BSA (Sigma-Aldrich) for 30 min. in the dark at room temperature. The plate was again washed five times and 100 μl luminescent peroxidase substrate (R&D Systems) added for 5 min., after which luminescence was measured using a multidetection microplate reader (BMG Labtech). Absolute protein levels were interpolated from the standard curve.

Measurement of α2-antiplasmin by sandwich ELISA

Clear, 96-well microplates (Fisher Scientific) were coated with capture goat polyclonal anti-α2-antiplasmin antibody (Santa Cruz) diluted 1:1000 in coating buffer at 4°C overnight. After five washes in PBST, the plate was tapped dry and incubated with 1% BSA (Sigma-Aldrich) for 90 min. at room temperature with agitation. The plate was washed five times, tapped dry and the samples incubated for 2 hrs at room temperature with agitation. The samples that made up the standard curve consisted of 6 five-fold serial dilutions of full length human purified α2-antiplasmin protein (Haemotologic Technologies, VT, USA) diluted in PBS (protein range 0.003–1.7 μg/ml). For the homogenate samples, 100 μg total protein diluted in 50 μl PBS was loaded and for the blanks, 50 μl PBS. Additional controls where either capture antibody, sample or detection antibody were omitted were also included. All samples were loaded in duplicate. After 5 washes, the plate was tapped dry and incubated with the detection rabbit polyclonal anti-α2-antiplasmin antibody (Abcam) diluted 1:1000 in 1% BSA (Sigma-Aldrich) for 2 hrs at room temperature with agitation. The plate was again washed five times, tapped dry and peroxidase-conjugated secondary antibody (Vector Labs) was applied for 30 min. in the dark at room temperature. The plate was again washed five times and 100 μl peroxidase substrate (R&D Systems) added for 5 min. after which 50 μl Stop solution was added and the absorbance measured using a multidetection microplate reader (BMG Labtech) at 450 nm. Absolute protein levels were interpolated from the standard curve.

Measurement of post-mortem stability of tPA, neuroserpin and α2-antiplasmin

Tissue from the frontal cortex of an AD brain removed 4 hrs after death and a control brain removed 10 hrs after death was dissected and divided into 10 aliquots. These were incubated for 0, 6, 12, 18, 24, 48 or 72 hrs at room temperature or 24, 48 or 72 hrs at 4°C. The aliquots were then homogenized in 1 ml 1% SDS lysis buffer and tPA, neuroserpin and α2-antiplasmin protein measurements made as described earlier.

Statistical analysis

Differences in mRNA expression and protein level between the AD and control groups were analysed by Mann–Whitney *U*-test. The relationships of *APOE* genotype and Braak stage to mRNA and protein level were assessed by Kruskall–Wallis test, followed by Dunn's post-tests if appropriate. Relationships between individual mRNAs and the corresponding proteins, proteins and Aβ load, and between age or post-mortem delay and the level of individual activator and inhibitor mRNAs and proteins were assessed by Spearman's correlation. *P*-values < 0.05 were considered significant.

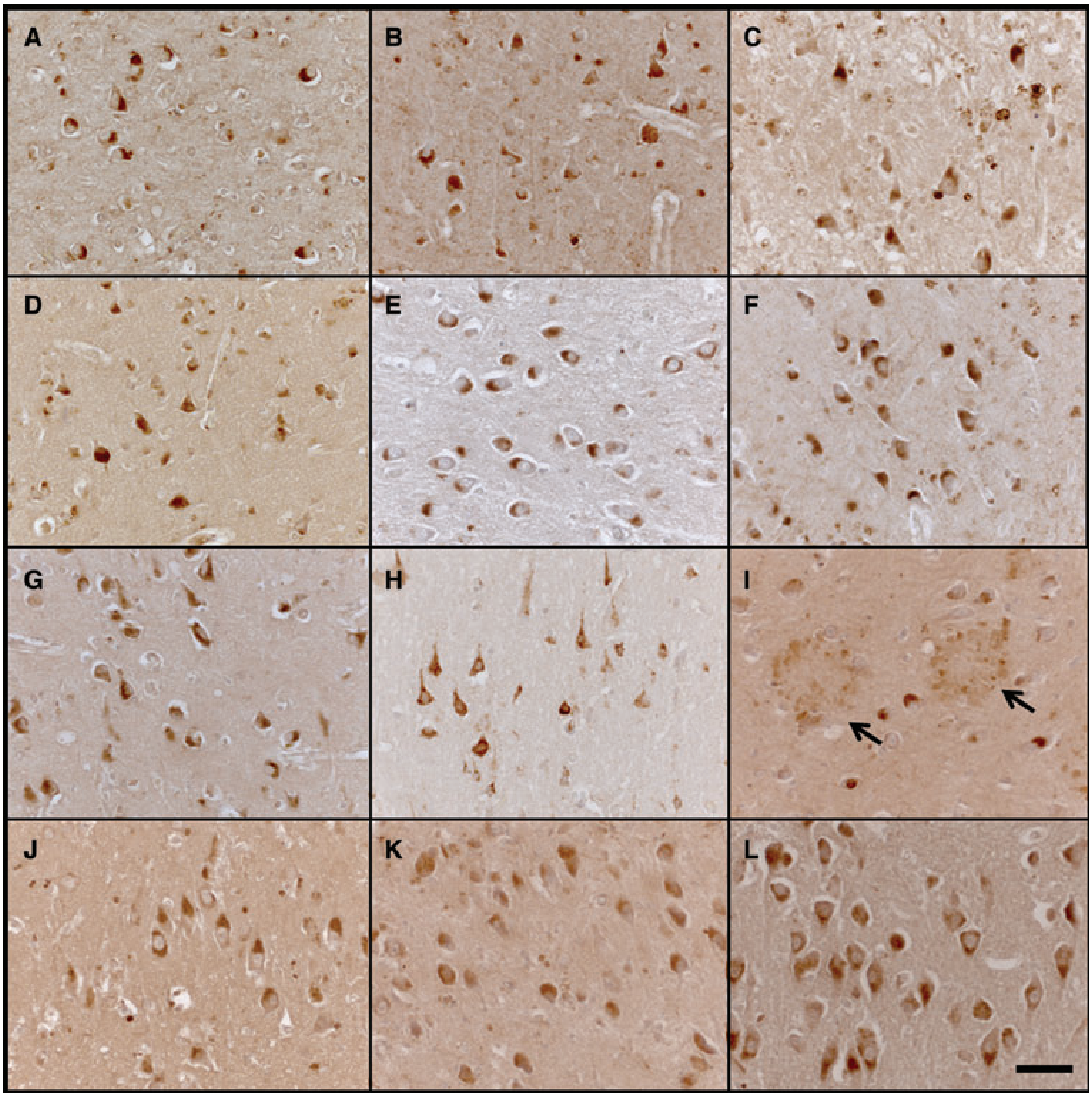

Fig. 1 Distribution of tPA (**A**, **B**), uPA (**C**, **D**), PAI-1 (**E**, **F**), neuroserpin (**G**, **H**), α2-antiplasmin (**I**, **J**) and α2-macroglobulin (**K**, **L**) in AD and control brain tissue. All proteins were present predominantly in the neurons. α2-antiplasmin was also associated with swollen processes in the peripheral part of some Aβ plaques in AD brain tissue (arrows in **I**). No labelling was present in negative control sections. Scale bar = 50 μm for all sections.

Results

Localization of the plasminogen system activators and inhibitors in the human brain

The distribution of tPA, uPA, PAI-1, neuroserpin, α2-antiplasmin and α2-macroglobulin in sections from human brain is shown in Figure 1. The images are representative of the findings across the cohorts. The specificities of all the antibodies used were confirmed by Western blot (Fig. S1) and no labelling was present in the negative control sections included in each experiment. tPA and uPA were predominantly in neurons but occasional small clusters of immunopositive granular material were present in microglia or astrocytes (Fig. 1A–D). PAI-1 and neuroserpin were both present in neurons (Fig. 1E–H). α2-Antiplasmin was present predominantly

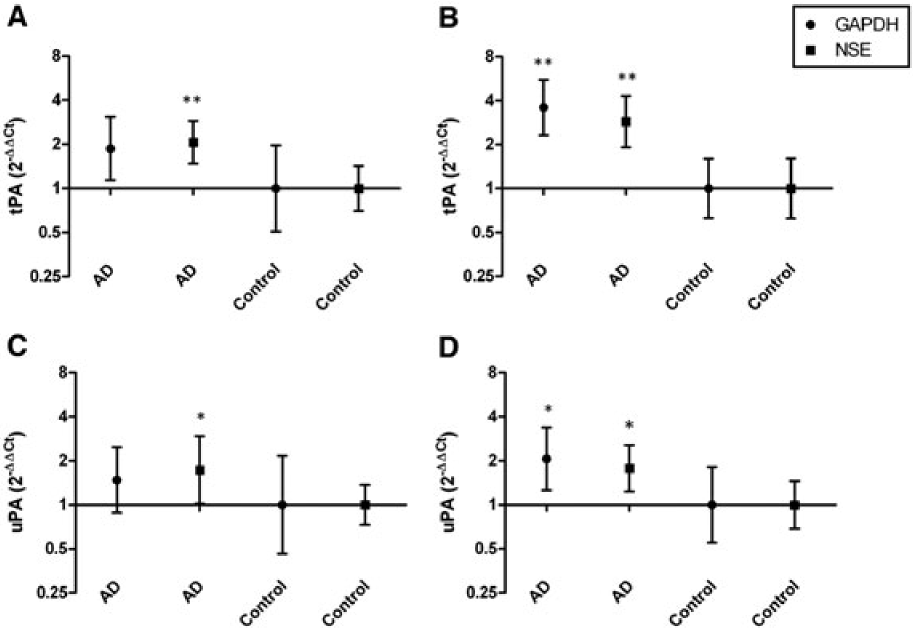

Fig. 2 The fold change in tPA mRNA expression in the frontal (**A**) and temporal cortex (**B**) and in uPA mRNA expression in the frontal (**C**) and temporal (**D**) cortex in AD and control brains calibrated against endogenous GAPDH and NSE. The geometric mean and 95% confidence intervals are shown for each group on a logarithmic scale to the base 2. tPA mRNA expression was significantly increased in AD compared to control in the temporal cortex when calibrated against GAPDH ($P = 0.001$) and NSE ($P = 0.002$) and in the frontal cortex relative to NSE ($P = 0.007$). uPA mRNA expression was significantly increased in AD compared to control in the temporal cortex relative GAPDH ($P = 0.03$) and NSE ($P = 0.032$) and in the frontal cortex relative to NSE ($P = 0.017$).

in neurons but was additionally found in swollen cell processes at the periphery of some Aβ plaques (Fig. 1I–J). α2-Macroglobulin was predominantly neuronal (Fig. 1K and L). Our results did not replicate previous findings of co-localization of tPA and PAI-1 with Aβ plaques [36,43]. None of the proteins was associated with neurofibrillary tangles and, with the exception of co-localization of α2-antiplasmin with Aβ plaques in AD, there were no obvious differences in the distribution of any of the proteins in AD and control brains.

mRNA expression

The mRNA expression of the genes encoding the plasminogen activators tPA and uPA is shown in Figure 2 and all the data for the mRNA expression studies are summarized in Table 3. tPA expression relative to both GAPDH and NSE was higher in AD than controls; this reached statistical significance in the temporal cortex ($P = 0.001$ tPA relative to GAPDH, $P = 0.002$ tPA relative to NSE) and in the frontal cortex when tPA was calibrated against NSE ($P = 0.007$; Fig. 2A and B). uPA expression was also increased in AD compared to control in the temporal cortex ($P = 0.03$ uPA relative to GAPDH, $P = 0.032$ uPA relative to NSE) and in the frontal cortex when uPA was calibrated against NSE ($P = 0.017$; Fig. 2C–D). tPA and uPA mRNA expression did not vary significantly with *APOE* genotype, or Aβ plaque load in the AD cohort. tPA mRNA relative to GAPDH varied significantly with Braak stage ($P = 0.028$). Post-testing revealed the expression to be higher in temporal cortex from brains of Braak tangle stages V–VI than Braak stages 0–II ($P < 0.05$). uPA mRNA showed no significant correlation with Braak stage.

Table 3 Summary of changes in tPA, uPA, PAI-1, neuroserpin, α2-antiplasmin and α2-macroglobulin mRNA relative to GAPDH and NSE mRNAs in AD compared to controls

Protein	Frontal		Temporal	
	GAPDH	NSE	GAPDH	NSE
tPA	ns	** ↑	** ↑	** ↑
uPA	ns	* ↑	* ↑	* ↑
PAI-1	ns	ns	* ↑	* ↑
Neuroserpin	**↓	ns	**↓	ns
α2-Antiplasmin	ns	** ↑	ns	*** ↑
α2-Macroglobulin	ns	ns	ns	ns

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (↑: significantly increased; ↓: significantly decreased); ns: not significant.

The mRNA expression of the inhibitors of the plasminogen system is shown in Figure 3. PAI-1 expression was increased in AD compared to controls in both the frontal and temporal lobes but this was only statistically significant in the temporal cortex ($P = 0.026$ PAI-1 relative to GAPDH, $P = 0.029$ PAI-1 relative to NSE; Fig. 3A and B). Neuroserpin expression in relation to GAPDH was significantly decreased in AD compared to controls in both brain regions ($P = 0.001$ in the temporal cortex, $P = 0.007$ in the frontal cortex) but there was very little alteration in the expression of neuroserpin in relation to NSE in either brain region (Fig. 3C and D). α2-Antiplasmin mRNA in relation to NSE was significantly higher in AD than controls in both regions ($P = 0.001$

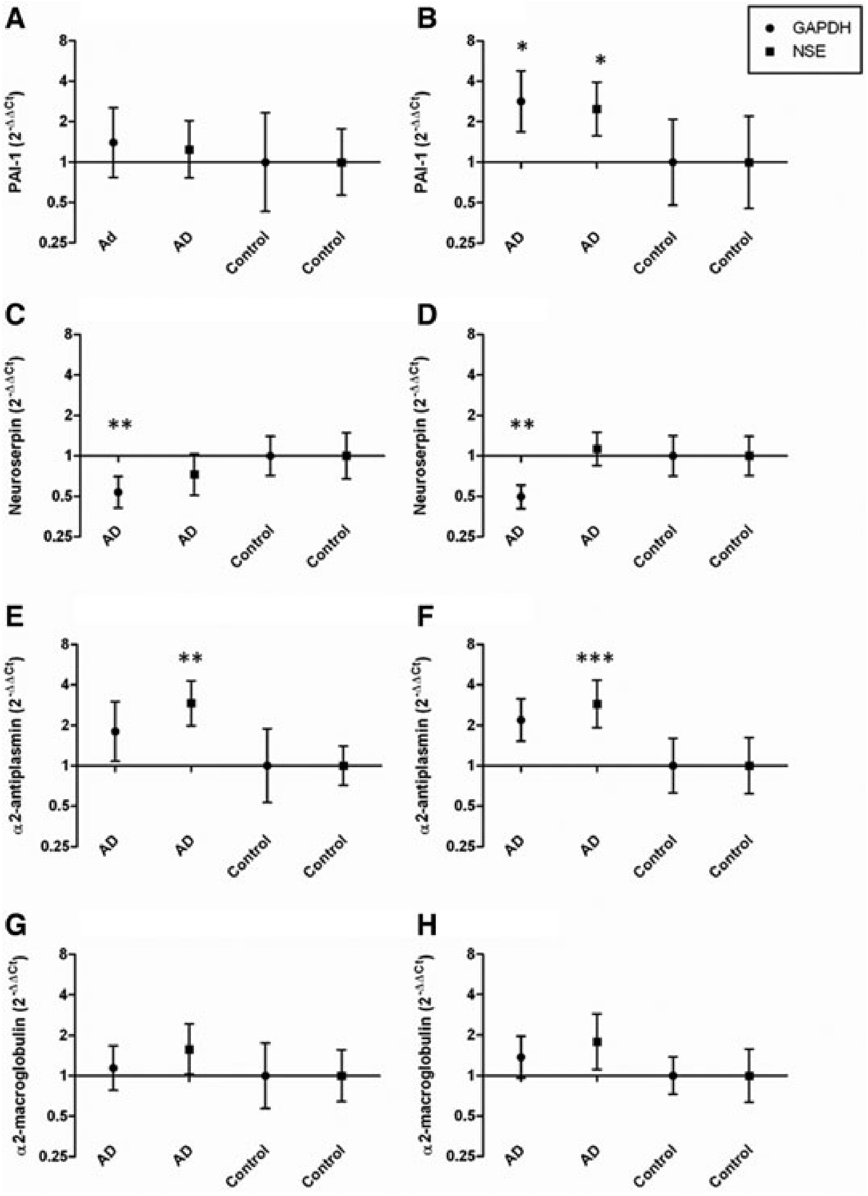

Fig. 3 The fold change in PAI-1 mRNA expression in the frontal (**A**) and temporal cortex (**B**), in neuroserpin mRNA expression in the frontal (**C**) and temporal (**D**) cortex, in α2-antiplasmin mRNA in the frontal (**E**) and temporal (**F**) cortex and in α2-macroglobulin mRNA in frontal (**G**) and temporal (**H**) cortex in AD and control brains calibrated against endogenous GAPDH and NSE. The geometric mean and 95% confidence intervals are shown for each group on a logarithmic scale to the base 2. PAI-1 mRNA expression was significantly increased in AD compared to control in the temporal cortex with GAPDH calibration ($P = 0.026$) and with NSE calibration ($P = 0.029$). The increase in PAI-1 in the frontal region did not reach statistical significance. Neuroserpin mRNA expression was reduced in AD compared to control when calibrated against GAPDH ($P = 0.001$ in the temporal cortex, $P = 0.007$ in the frontal cortex). α2-Antiplasmin mRNA was increased in both brain regions in AD compared to control; this was significant when calibrated against NSE ($P = 0.001$ in the temporal cortex and $P = 0.0004$ in the frontal cortex) and against GAPDH in the temporal cortex ($P = 0.012$). α2-Macroglobulin mRNA was not significantly altered in AD compared to control.

in the temporal cortex, $P = 0.0004$ in the frontal cortex); in relation to GAPDH, the increase was significant in the temporal cortex only ($P = 0.012$; Fig. 3E and F). α2-Macroglobulin mRNA showed a non-significant increase in AD compared to controls in both brain regions (Fig. 3G and H).

PAI-1, neuroserpin and α-macroglobulin mRNA all showed no significant relationship to *APOE* genotype or Aβ load in the AD cohort, or with Braak stage. α2-antiplasmin mRNA did not vary significantly with *APOE* genotype or Aβ load but, in the temporal cortex, was significantly altered with Braak stage (α2-antiplasmin mRNA in relation to GAPDH $P = 0.003$, in relation to NSE $P = 0.018$) and post-tests showed increased expression in Braak stages V–VI compared to 0–II (α2-antiplasmin mRNA in relation to GAPDH $P < 0.01$, in relation to NSE $P < 0.05$).

tPA, neuroserpin and α2-antiplasmin proteins

Data from the protein measurements are summarized in Table 4. tPA protein was measured in the temporal cortex only. tPA was increased in the AD group compared to controls but this did not reach statistical significance (Fig. 4A). The mean tPA concentrations ± S.E.M. were: AD 0.431 ng/ml ± 0.112, controls 0.277 ng/ml ± 0.0573. Neuroserpin was significantly reduced in

Table 4 Summary of changes in tPA, neuroserpin and α2-antiplasmin proteins in AD compared to controls

Protein	Frontal	Temporal
tPA	NA	ns
Neuroserpin	**↓	*↓
α2-Antiplasmin	ns	* ↑

*$P < 0.05$; **$P < 0.01$ (↑: significantly increased; ↓: significantly decreased); ns: not significant; NA: not assessed.

both the frontal and temporal cortex in AD compared to controls ($P = 0.003$) in the frontal cortex and $P = 0.035$ in the temporal cortex; Fig. 4B and C). The mean relative neuroserpin levels ± S.E.M. were: in the frontal region, AD 1.647 relative units ± 0.091, controls 2.099 relative units ± 0.107; in the temporal region AD 0.616 relative units ± 0.048, controls 0.771 relative units ± 0.052. α2-antiplasmin was increased in AD compared to controls but this only reached significance in the temporal region ($P = 0.018$; Fig. 4D and E). The mean α2-antiplasmin concentrations ± S.E.M. were: in the frontal region, AD 0.151 μg/ml ± 0.057, controls 0.074 μg/ml ± 0.017; in the temporal region, AD 0.245 μg/ml ± 0.069, controls 0.063 μg/ml ± 0.012.

The protein concentrations all correlated positively with the levels of the corresponding mRNAs; the correlation was significant for temporal neuroserpin (neuroserpin mRNA in relation to GAPDH, $P = 0.004$) and for α2-antiplasmin in both brain regions (α2-antiplasmin mRNA in relation to GAPDH: frontal cortex $P = 0.019$, temporal cortex $P = 0.048$).

tPA and α2-antiplasmin protein concentrations increased with increasing number of *APOE* ε4 alleles and with Braak stage but these correlations did not reach statistical significance.

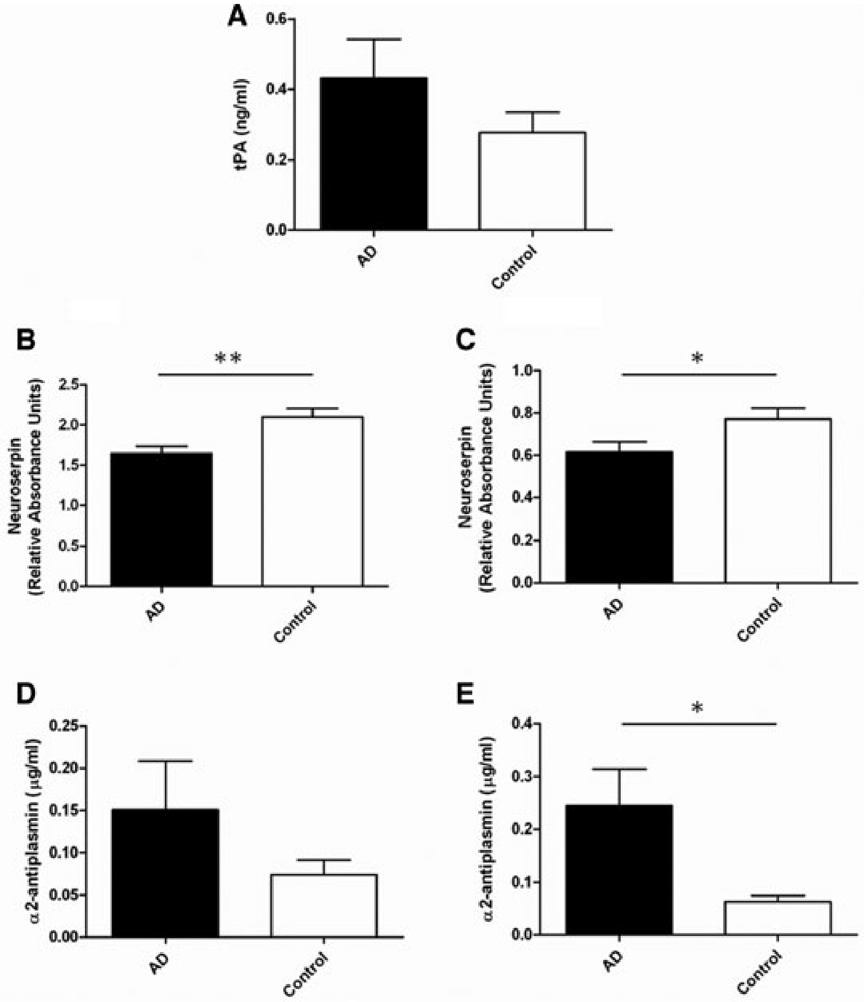

Fig. 4 tPA protein in temporal lobe AD and control brain (**A**), neuroserpin protein in the frontal (**B**) and temporal (**C**) cortex and α2-antiplasmin protein in the frontal (**D**) and temporal (**E**) cortex. The bars indicate the mean values and standard errors. tPA protein was increased in AD compared to control but this did not reach statistical significance. Neuroserpin was significantly reduced in both brain regions in AD compared to control: in the frontal cortex $P = 0.003$, in the temporal cortex $P = 0.035$. α2-Antiplasmin protein was increased in AD compared to control; this reached statistical significance in the temporal cortex ($P = 0.018$).

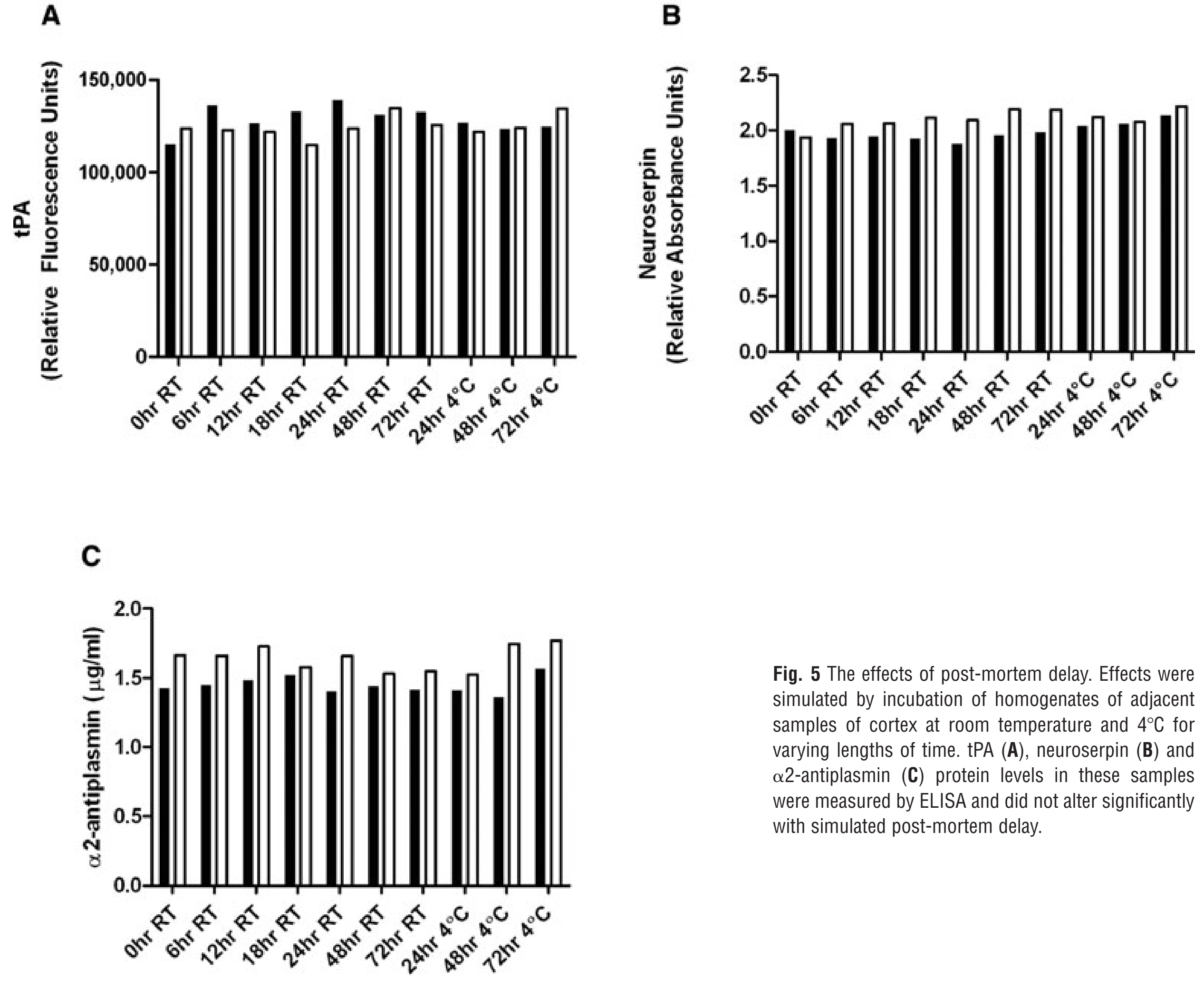

Fig. 5 The effects of post-mortem delay. Effects were simulated by incubation of homogenates of adjacent samples of cortex at room temperature and 4°C for varying lengths of time. tPA (**A**), neuroserpin (**B**) and α2-antiplasmin (**C**) protein levels in these samples were measured by ELISA and did not alter significantly with simulated post-mortem delay.

Neuroserpin protein decreased with increasing Braak stage, a relationship that was significant in the frontal region ($P = 0.028$); *post hoc* testing showed the difference to be significant between Braak stages V–VI and 0–II ($P < 0.05$). Neuroserpin did not vary significantly with *APOE* genotype. None of the proteins correlated with Aβ plaque load in the AD samples.

Age, gender and post-mortem delay

The protein and mRNA measurements showed no relationship to age or post-mortem delay and did not vary significantly with gender, either in the individual cohorts or when the cohorts were combined. tPA, neuroserpin and α2-antiplasmin protein concentrations did not alter significantly when unfixed brain tissue was incubated for up to 72 hrs at room temperature or 4°C (Fig. 5). The protein measurements are therefore unlikely to have been affected by variations in post-mortem delay between the different cases or phenotypic groups.

Discussion

In recent years, there has been mounting interest in the contributions of enzymes capable of degrading Aβ to the clearance of this peptide from the brain. Plasmin is one such enzyme but the plasminogen system has not been much studied in the human brain. We recently found no significant alterations in protein concentration or enzyme activity of plasmin in AD and concluded, therefore, that alterations in plasmin are not generally responsible for Aβ accumulation, although they may contribute in some cases [24]. We have now shown that there are marked changes in the expression of the activators and inhibitors of the plasminogen system in AD: significant increases in tPA, uPA, PAI-1 and α2-antiplasmin mRNA and increased tPA and α2-antiplasmin protein, and significant reduction in neuroserpin mRNA and protein. Although the net effect may be little or no change in plasmin activity, altered expression of plasminogen activators and inhibitors could have other consequences in AD; this is particularly so for tPA.

The stimulus responsible for the increase in tPA mRNA and protein in AD is unclear. Although the previous immunohistochemical study of Medina *et al.* [35] suggested that plaque-associated Aβ may induce tPA synthesis, we did not find tPA to co-localize with plaques, and tPA mRNA and protein levels did not correlate with Aβ plaque load in the AD samples. There was a significant increase in tPA mRNA in Braak stages V–VI compared to stages 0–II, which would suggest that tPA synthesis increases with progression of the disease; however, this relationship did not follow through to the protein expression. One possible explanation is the significant increase in PAI-1 mRNA expression in AD which, if also translated to the protein level, would result in increased inhibition and removal of tPA. Alternatively, tPA expression may be under translational control, as hypothesized by Salles and Strickland [44], such that the protein is synthesized only under certain conditions [45].

The activation of uPA by Aβ has been somewhat controversial, some studies reporting activation [11] and others no activation [17,19]. We found an increase in uPA mRNA in AD but this did not correlate with Aβ plaque load. α2-Antiplasmin has not previously been shown to interact directly with Aβ. Our immunohistochemical demonstration of α2-antiplasmin around Aβ plaques raises the possibility that there is indeed interaction between the two but again, in the AD samples there was no correlation between α2-antiplasmin mRNA or protein level and Aβ plaque load. α2-Antiplasmin mRNA, like tPA mRNA, increased with Braak stage and was significantly higher in stages V–VI than 0–II; again, this increase was not translated to the protein level.

The balanced increases in the expression of the plasminogen activators tPA and uPA, and the inhibitors PAI-1 and α2-antiplasmin in AD may account for the lack of overall alteration in plasmin activity in AD, which we showed previously. However, these proteins have other actions that may be relevant in AD. On the positive side, tPA enhances synaptic plasticity in mice and restores cognition and improves performance in memory tasks [26]. However tPA is a mediator of excitotoxic neuronal death [31] and apoptosis [34]. uPA and PAI-1 have also been shown to be anti-apoptotic [46–52] so the observed increased expression of these proteins may be neuroprotective. The consequence of increased α2-antiplasmin expression may be limited to a reduction in plasmin activity as, to our knowledge, it has not been shown to have any other effects to date.

Our finding of reduced neuroserpin mRNA and protein differs from that in a previous study which showed an increase in neuroserpin protein in AD compared to controls [36]. The reasons for this disparity are not entirely clear but some of our other observations may suggest a partial explanation. We found neuroserpin gene expression to be reduced relative to GAPDH (constitutively expressed in all cells) but not relative to NSE (expressed solely by neurons). This suggests that the reduction in neuroserpin in our AD cohort was secondary to neuron loss, which may have been more pronounced than in the study by Fabbro and Seeds [36]. In the frontal region neuroserpin protein level was significantly lower in Braak stages V–VI than 0–II, in keeping with the interpretation that neuroserpin level falls late in the disease, after most neuronal damage has occurred. It is not clear at what stage of disease were the AD patients studied by Fabbro and Seeds or, indeed, what criteria were used to make the diagnosis. Other differences that may be relevant relate to cohort size (12 AD and 12 control samples in the previous study and 38 AD and 38 controls in this study), and the method for measuring neuroserpin (densitometry of Western blots calibrated with a single standard sample on each gel, rather than direct ELISAs with each plate calibrated against a seven-point standard). A decrease in neuroserpin could help to limit the continued accumulation of Aβ, as tPA activity, and hence plasmin activity, will be prolonged. However, the findings of Kinghorn *et al.* [25] indicate that neuroserpin is also capable of reducing $A\beta_{1-42}$ toxicity, both in rat pheochromocytoma cells and in *Drosophila*.

One limitation of this study is that the enzymatic activities of tPA and uPA have not been assessed. A second limitation of this study is that the expression of the activator and inhibitor proteins has not been measured in corresponding plasma samples. The levels of these proteins are higher in the plasma than the brain parenchyma. Unfortunately, matched plasma and brain tissue samples are difficult to obtain, and in any case post-mortem measurements of plasmin-related proteins in blood samples are likely to be affected by coagulation of blood after death. The aim of this study was therefore limited to investigation of the plasminogen system in the brain, where its potential role in the clearance of Aβ deposition may be of relevance in AD.

Our finding of several significant alterations in the plasminogen system highlights the need for further investigation of this system in the CNS.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Supporting information

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

Fig. S1 The specificities of the antibodies used for the immunoperoxidase and protein studies were assessed by Western blot.

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