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Review Article

Characterisation of serpin polymers *in vitro* and *in vivo*

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ABSTRACT

Neuroserpin is a member of the serine protease inhibitor or serpin superfamily of proteins. It is secreted by neurones and plays an important role in the regulation of tissue plasminogen activator at the synapse. Point mutations in the neuroserpin gene cause the autosomal dominant dementia familial encephalopathy with neuroserpin inclusion bodies or FENIB. This is one of a group of disorders caused by mutations in the serpins that are collectively known as the serpinopathies. Others include α_1-antitrypsin deficiency and deficiency of C1 inhibitor, antithrombin and α_1-antichymotrypsin. The serpinopathies are characterised by delays in protein folding and the retention of ordered polymers of the mutant serpin within the cell of synthesis. The clinical phenotype results from either a toxic gain of function from the inclusions or a loss of function, as there is insufficient protease inhibitor to regulate important proteolytic cascades. We describe here the methods required to characterise the polymerisation of neuroserpin and draw parallels with the polymerisation of α_1-antitrypsin. It is important to recognise that the conditions in which experiments are performed will have a major effect on the findings. For example, incubation of monomeric serpins with guanidine or urea will produce polymers that are not found *in vivo*. The characterisation of the pathological polymers requires heating of the folded protein or alternatively the assessment of ordered polymers from cell and animal models of disease or from the tissues of humans who carry the mutation.

1. Introduction

The past two decades have seen the emergence of a new class of disorder that are collectively termed the conformational diseases [1,2]. These are characterised by the aggregation and tissue deposition of aberrant conformations of protein. It is the predominant pathology in a number of neurological conditions including Alzheimer's and Huntington's disease, the spongiform encephalopathies and the serpinopathies [3–5]. The serpinopathies result from point mutations in members of the serine protease inhibitor or serpin superfamily. They are characterised by the formation of ordered polymers that are retained within the cell of synthesis. This causes disease by a 'toxic gain of function' from the accumulated protein and a 'loss of function' as a result of the deficiency of inhibitors that control important proteolytic cascades. The serpinopathies are exemplified by the Z mutation of α_1-antitrypsin (Glu342Lys) that results in the retention of polymers within the endoplasmic reticulum of hepatocytes [6]. These polymers form the periodic acid Schiff (PAS) positive, diastase resistant inclusions that are associated with neonatal hepatitis, cirrhosis and hepatocellular carcinoma [7]. A second example results from mutations in neuroserpin. This leads to the formation of ordered polymers that are retained within sub-cortical neurones to form PAS positive, diastase resistant inclusions that are termed Collins' bodies. These inclusions underlie the autosomal dominant dementia familial encephalopathy with neuroserpin inclusion bodies or FENIB [8].

Five mutations have been described that result in the dementia FENIB (Table 1) [9]. Perhaps the most striking are Ser49Pro and His338Arg, that are homologous to Ser53Phe (the Siiyama mutation) and His334Asp in α_1-antitrypsin that cause liver disease [10,11]. This strongly indicates a common molecular mechanism of polymer formation. This was confirmed by the finding that entangled polymers of neuroserpin formed in the neuronal inclusion bodies of FENIB with identical morphology to those present in hepatocytes from a child with α_1-antitrypsin deficiency related cirrhosis [8].

There is a direct relationship between the rate of polymerisation, the magnitude of the intracellular accumulation of a serpin

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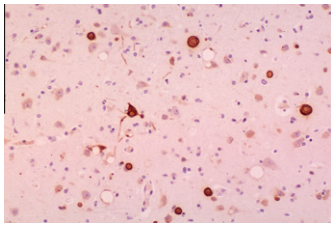
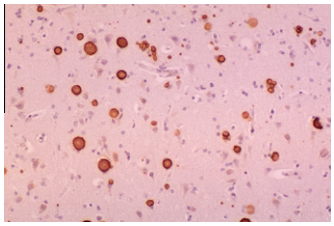
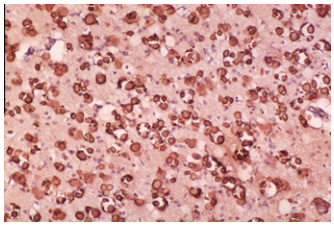
Table 1
The increasing propensity for the mutants of neuroserpin to polymerise is associated with a decreased age of onset and increasing severity of the disease. The magnitude of intracellular neuroserpin accumulation is evident in the cross sections of cerebral cortex stained for inclusions. The least-destabilizing mutation (Ser49Pro) has scattered small neuronal inclusions, with slowly developing disease, associated with an average age of onset of dementia of 48 years [8]. As instability increases, the number of cells with inclusions increases, and thus neuronal cell toxicity. This is strikingly evident for the most destabilizing mutants (Gly392Glu and Gly392Arg), which are associated with the onset of dementia at 13 and 8 years, respectively [12,13,16]. Abbreviation: N/A, not available. The + signs indicate the relative rates of polymerization. From [9].

Mutation	Rate of polymerisation	Age of onset of symptoms	Clinical manifestations	Histology of inclusions at post-mortem
Ser49Pro	+	48	Dementia, tremor, seizures in terminal stages	
Ser52Arg	++	24	Myoclonus, status epilepticus, dementia	
His338Arg	+++	15	Myoclonic seizures, dementia, tremor, dysarthria	N/A
Gly392Glu	++++	13	Myoclonus, status, epilepticus, dementia, chorea	
Gly392Arg	++++	8	Dementia, epilepticus of slow- wave sleep	N/A

and the severity of disease [12,13]. This is best illustrated for individuals with the dementia FENIB. In the original family with Ser49-Pro neuroserpin (neuroserpin Syracuse) the affected family members had diffuse small intraneuronal inclusions of neuroserpin with an onset of dementia between the ages of 45 and 60 years [8,14,15]. However, in a second family, with a conformationally more severe mutation (neuroserpin Portland; Ser52Arg) and larger inclusions, the onset of dementia was in early adulthood; and in a third family, with yet another mutation (His338Arg), there were more inclusions and the onset of dementia in adolescence. The most striking example was the family with the most 'polymerogenic' mutation of neuroserpin, Gly392Glu (Table 1). This replacement of a conserved residue in the shutter region resulted in large multiple inclusions in every neurone, with affected family members dying by age 20 years [12,13]. More recently a fifth mutation has been described (Gly392Arg) which caused a profound intellectual decline in an 8 year old girl, seizures and electrical brain activity in keeping with 'epilepticus of slow-wave sleep (ESES)' [16]. Thus FENIB can cause a spectrum of disease from dementia to epilepsy with variable electrical status.

The characterisation of conformers of neuroserpin or α_1-antitrypsin associated with disease requires an understanding of the folding pathway [17–19], transitions to the polymeric and latent conformers [20] and the mechanisms by which these intermediates and folded conformers are handled by cells. There is strong evidence that mutants of both neuroserpin and α_1-antitrypsin are associated with a significant delay in folding [18,19]. This folding defect can be studied by treating the folded protein with urea or guanidine. However in the case of α_1-antitrypsin the polymers that form as a consequence of treatment with these agents are not recognised by the 2C1 monoclonal antibody that recognises the pathological polymers that form *in vivo* [21]. This implies that the long-lived folding intermediate is efficiently degraded by the proteasome via the pathway of endoplasmic reticulum-associated degradation (ERAD) [22–25]. Moreover experiments performed *in vitro* with guanidine or urea produce conformers that, whilst interesting [26] are unlikely to be relevant to disease. The quality control pathway is able to fold some of the material to a near-native conformation. Some of this will continue to fold to a native conformation, traffic through the Golgi apparatus and then be secreted. However a proportion will form the latent species [20] and some will form polymers in which the reactive centre loop of one molecule is inserted into β-sheet A of another (Fig. 1) [21,27]. Pathologically relevant conformational transitions may be recapitulated by heating the folded monomeric protein [11]. The resulting polymers of α_1-antitrypsin are recognised by the 2C1 antibody (Fig. 1).

Studies of cell biology and animal models allow investigation of the disposal of misfolded protein by the proteasome, the trafficking of the fully folded protein through the secretory pathway and the formation of the latent and polymeric conformers. There is growing recognition that autophagy plays a role in clearing the intracellular polymers of α_1-antitrypsin [28,29]. Our own studies show that autophagy plays a background role in clearing both wildtype and mutant of neuroserpin but it is not increased in cells expressing the mutant protein [30]. It is the proteasome that is mainly responsible for degradation with more severe mutations being associated with more degradation in cell models of disease.

The detection of specific serpin conformations by biochemical methods has been described previously [31]. We review here the recent advances in the characterisation of conformers of neuroserpin. Much of this work is based on previous studies characterising

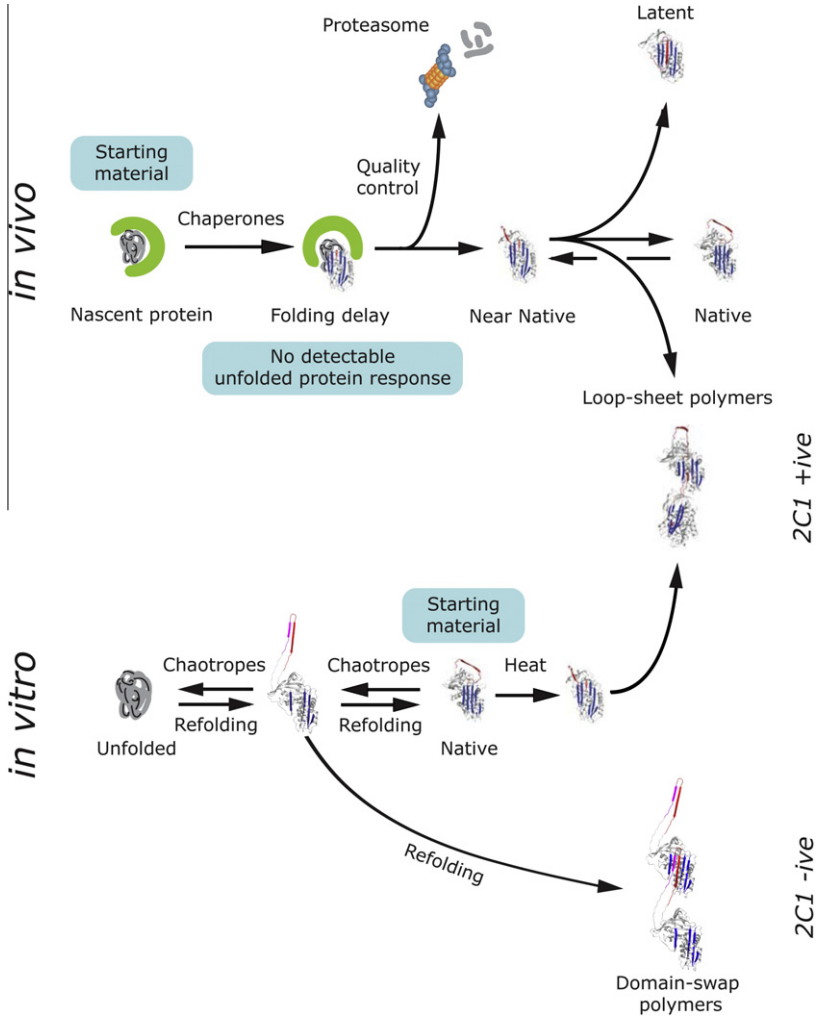


Fig. 1. The 'Unified Fold Pathway', a unifying hypothesis to explain the cellular handling of mutants of neuroserpin and α_1-antitrypsin. The nascent protein binds to chaperones whilst in the folding pathway. The mutants cause a significant delay to folding [18,19] causing much of the newly synthesised protein to be 'timed out' [72] and targeted for endoplasmic reticulum-associated degradation (ERAD) via the proteasome [23,30]. Efficient chaperone function and ERAD prevent accumulation of incompletely folded protein and a failure of ER homeostasis, thus no unfolded protein response is detected [22]. A proportion of the protein is folded by the chaperone machinery to a near-native conformation. Some of this will fold fully and be trafficked through the Golgi apparatus for secretion. However the near native structure can form either the monomeric latent conformer or loop-sheet polymers that are linked by the reactive loop of one molecule inserting into β-sheet A of another. These polymers are retained within the endoplasmic reticulum and, when formed by mutants of α_1-antitrypsin, can be stained with the 2C1 monoclonal antibody [11]. The *in vivo* pathway can be recapitulated by *in vitro* experiments. Monomeric proteins can be unfolded in urea and guanidine and then refolding monitored over time [19]. The folding delay is followed by the formation of polymers that are not recognised by the 2C1 antibody [21]. Thus these polymers are likely to be folding artefacts that are not found *in vivo*. Heating the monomeric protein produces polymers that bind the 2C1 antibody and so recapitulate the epitope associated with disease.

conformers of α_1-antitrypsin and so we have also included these within this chapter where appropriate.

2. Protocols

2.1. Biochemical characterisation of neuroserpin conformations

A previous publication in Methods has described the different means of characterising the conformations of serpins [31]. We describe here how to form the conformations of neuroserpin and briefly recapitulate on how to differentiate between these conformers, *i.e.* monomers, oligomers, reactive loop cleaved, complexed with protease and the latent form of neuroserpin.

2.1.1. Materials

Preparation of conformers: PBS, Protease inhibitor cocktail EDTA free (Roche Diagnostics, 11873580001), Hepes, Dodecyl maltoside, NaCl, tPA single-chain (Calbiochem, Merck Chemicals, 612200), tPA inhibitor 1,5 dansyl-Glu-Gly-Arg CMK (Calbiochem, Merck Chemicals, 251700). SDS–PAGE: Novex NuPAGE 10% w/v Bis–Tris gel (Invitrogen, NP0302). Non-denaturing PAGE: 3–12% w/v Bis–Tris gel (Invitrogen, BN2011). TUG–PAGE: 30% w/v Acrylamide–Bis acrylamide 37.5:1 (Severn Biotech, 20-2100-10), 8 M urea, 1.5 M Tris–HCl pH 8.8, 0.75 M Tris–HCl pH 6.8, TEMED, 10% w/v ammonium persulfate.

2.1.2. From monomers to polymers

The most common method to form polymers from a monomeric serpin is by heating. This approach has been used to prepare polymers of α_1-antitrypsin and neuroserpin, amongst others [6,21,27,32,33]. Polymers of neuroserpin are prepared by heating a 0.4 mg/ml solution of monomeric neuroserpin in PBS at 45 °C for different times. In practice, 110 µl of a 0.4 mg/ml solution in PBS is prepared in a PCR tube and heated at 45 °C in a thermal cycler. Ten microliter aliquots are taken over time for further analysis (Fig. 2A). It is important to use an appropriate concentration of

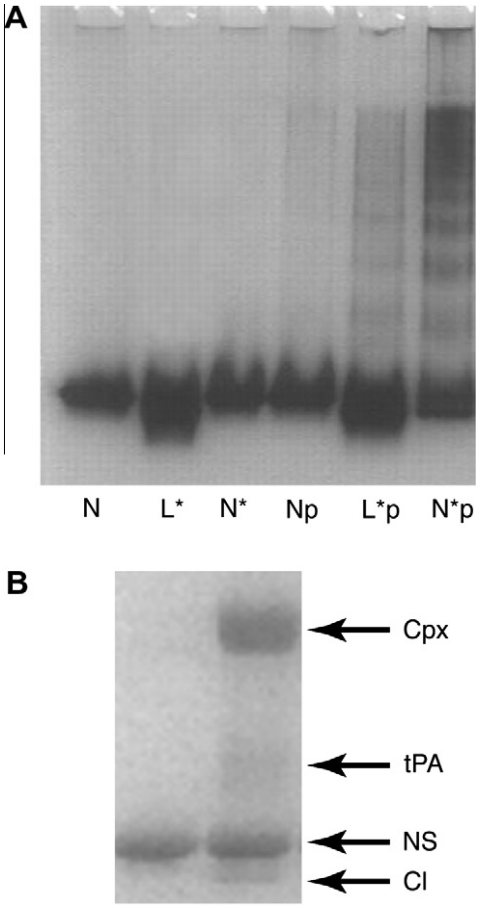


Fig. 2. (A) Examples of different conformers of wildtype and Ser49Pro neuroserpin. Native wildtype neuroserpin (N), latent Ser49Pro neuroserpin (L*), and native Ser49Pro neuroserpin (N*) were incubated at 0.25 mg/ml and 37 °C for 2 h and then analyzed by non-denaturing PAGE. The proteins were visualized by silver staining. Np, native wildtype neuroserpin polymers; L*p, latent Ser49Pro neuroserpin polymers; N*p, native Ser49Pro neuroserpin polymers. From [20]. (B) Inhibition of tissue plasminogen activator (tPA) by neuroserpin visualised by SDS–PAGE. Native wildtype neuroserpin (NS) was incubated with tPA at a 2:1 ratio for 5 min and then analysed by SDS–PAGE. Cpx, SDS-stable complex of neuroserpin and tPA; Cl, reactive loop cleaved neuroserpin. From [27].

protein, in the case of neuroserpin between 0.4 and 1 mg/ml, to avoid the formation of aggregates or the latent species (see Section 2.1.3). It should also be noted that the pH of the buffer is critical for the polymerisation of neuroserpin and α_1-antitrypsin [33,34].

2.1.3. Formation of latent neuroserpin

Low concentrations of neuroserpin favour the formation of the monomeric latent species rather than polymers (Figs. 1 and 2A). These two processes are competitive and specific conditions are required in order to exclusively obtain the latent form. Neuroserpin is first dialysed against 20 mM Na-Phosphate buffer pH 7.4. A 0.01 mg/ml solution is heated at 45 °C for 12 h and then 55 °C for 20 h. The samples are concentrated by ultracentrifugation (Amicon Ultra, 10 kD MW cut-off) to a concentration of 1 mg/ml [20]. The latent species is formed by the intra-molecular incorporation of the reactive loop (Fig. 1). It forms spontaneously in association with Ser49Pro neuroserpin and can be isolated from the neuronal inclusions of individuals with FENIB [20].

2.1.4. Formation of complexed and reactive loop cleaved neuroserpin

Serpins form SDS-stable complexes with their target enzymes [35]. Neuroserpin forms complexes with tissue plasminogen activator (tPA), but these complexes are unstable [27,36]. Complexes are formed by incubating neuroserpin and tPA at a ratio of 2:1 in 50 mM Hepes, 150 mM NaCl, 0.1% w/v dodecyl maltoside. A 10 µl sample is prepared with neuroserpin at 2 µM and tPA at 1 µM and incubated for 5 min at room temperature. The reaction is stopped by the addition of 1 mM final 1,5 dansyl-Glu-Gly-Arg-Chloromethyl Ketone for 1 min and the samples snap-frozen in liquid nitrogen. The samples are analysed by SDS–PAGE (10% w/v acrylamide gel). Both complexed and cleaved forms can be observed (Fig. 2B).

2.1.5. Limited proteolysis of serpin polymers

Limited proteolysis can be used to explore the availability of structural domains within the serpin monomer and polymer. This method has been used to assess the loop-sheet and β-hairpin models of α_1-antitrypsin polymers formed *in vitro* and in material isolated from the liver *of* homozygotes for the severe Z deficiency mutant (Glu342Lys) [21,26]. Heat-induced Z α_1-antitrypsin polymers are formed by incubating monomeric protein purified from the plasma of a Z α_1-antitrypsin homozygote at 0.2 mg/ml and 41 °C in 40 mM Tris, pH 8.0 for 6 days. In practice the sample solution is prepared in a PCR tube and heated at 41 °C in a thermal cycler. As the polymerisation is concentration dependent we find 0.2 mg/ml to be an appropriate concentration for the reproducible formation of a ladder of α_1-antitrypsin polymers. Polymers are separated from residual monomer by gel filtration on a Superdex 200 gel filtration column (GE Healthcare) and confirmed by 7.5% w/v acrylamide non-denaturing PAGE.

The endopeptidases Lys-C (cleaves peptide bonds C-terminal to lysine residues) and Asp-N (cleaves peptide bonds N-terminal to aspartic acid residues) are used for the enzymatic digestion of separated Z α_1-antitrypsin polymers. Polymers are incubated overnight with Lys-C or Asp-N in 40 mM Tris, pH 8.0 at a range of molar ratios at room temperature. Digestion fragments are then separated on 10% w/v acrylamide SDS–PAGE before transfer onto a PVDF membrane (Immobilon-P^{SQ}, Millipore) (see Section 2.3.5). N-terminal sequencing of the first 5 amino acids of each digestion fragment is used to identify endopeptidase cleavage sites on the α_1-antitrypsin structure.

Digestion fragments can also be separated by HPLC reverse phase chromatography to guarantee the recovery of a complete α_1-antitrypsin fragment map. A silica based C18 reverse phase column is cleaned with 10-column volumes of 95% v/v acetonitrile with 0.1% v/v trifluoroacetic acid (TFA) at a flow rate of 1–2 ml/min. The digested polymer sample is loaded onto the column at 0.5 ml/min with 0.1% v/v TFA. Bound peptides are eluted with an increasing concentration of organic solvent (10–65% v/v gradient of acetonitrile in 0.1% v/v TFA) at 0.5 ml/min and peptide detection at 214 nm. Samples corresponding to elution peaks are frozen in liquid nitrogen, lyophilised and N-terminal sequenced.

Comment: recombinant serpin polymers may need incubation with endopeptidase for only a few hours in contrast to polymers formed from plasma-purified protein. The gel filtration separation of monomer from polymer may be avoided by forming polymers at a higher temperature but this may favour the formation of insoluble aggregates as opposed to soluble polymers.

2.1.6. Refolding of neuroserpin

Recently the refolding of neuroserpin and the effect of folding on polymerisation had been studied [19]. The protein was first denatured in 5 M guanidine-HCL and then refolded by 30-fold dilution in a buffer without guanidine-HCL. Different folding intermediates can be characterised by biochemical or biophysical methods as described in Takehara et al. [19]. In this study, an initial refolding intermediate I_{IN} was reported for wildtype, Ser49Pro and the severe His338Arg mutant of neuroserpin, followed by a late intermediate I_R. This intermediate can then form either the native protein or polymers.

2.2. Biophysical characterisation of serpin polymerisation

2.2.1. Materials

Intrinsic tryptophan fluorescence: a spectrofluorimeter capable of excitation and emission wavelengths of light in the 280–360 nm range, such as a Perkin Elmer LS 50B, with a Peltier-type or water-jacketed sample holder connected to a thermostat-regulated water bath and pump; fluorescence cuvette (Hellma 108002F-10-40).

Circular dichroism spectroscopy: a spectropolarimeter with a temperature-controlled cuvette holder, such as a Jasco J-810; a narrow path length cuvette (Hellma 100-1-40); phosphate buffer; sodium fluoride.

2.2.2. Comments

There is no one biophysical method that is directly applicable to all serpin polymerisation; for instance, intrinsic tryptophan fluorescence can be used to monitor polymerisation of α_1-antitrypsin [34] but not α_1-antichymotrypsin or neuroserpin [27,37]. In addition, the mechanisms of serpin polymerization, and their biophysical hallmarks, remain contentious [21,26]. Thus, the data resulting from indirect approaches should be interpreted in conjunction with other evidence. For example, where a serpin is subjected to heat, denaturants and/or extended incubation, representative aliquots should be removed at regular intervals for analysis using physical approaches such as non-denaturing polyacrylamide gel electrophoresis, transverse urea gradient gel electrophoresis or size exclusion chromatography.

Typically, a serpin polymerisation experiment will be undertaken at close to neutral pH, with the application of heat. Sodium or potassium phosphate is therefore a recommended buffer, as it provides a pH that is insensitive to temperature (unlike Tris–HCl) and that is readily achieved by mixing the acid and base components (thereby avoiding introduction of chloride ions from adjustment with hydrochloric acid which would interfere with circular dichroism measurements).

2.2.3. Intrinsic tryptophan fluorescence

This technique utilises the fluorescent properties of the protein itself. Tryptophan residues have an excitation maximum around 280 nm and, depending on their proximity to solvent and protonated acidic side chains, an emission peak around 340 nm. Whilst several factors confound direct structural inference from intrinsic fluorescence readings, relative changes of emission peak character can be used to monitor global changes in a protein such as unfolding or polymerisation.

Sample cuvette: we used a 0.5 ml fluorescence cuvette with four transparent sides, a 1 cm (excitation) by 0.2 cm (emission) path length, and a lid to prevent evaporation. If a larger cuvette is used, then the volume of sample should be increased proportionally and a stirrer used to maintain sample and thermal homogeneity.

Instrument set up: the excitation slit width, determining both the wavelength range and the intensity of light used to excite the sample, is set at 2.5 nm. If there is sufficient material, brief runs are conducted to ensure that the emission slit width gives optimal signal-to-noise ratio, starting with 2.5 nm and increasing to 5, 10 and 15 nm. If necessary, the photomultiplier tube gain is adjusted at this stage. The excitation wavelength is set at 295 nm, and the emission range at 300–400 nm, with a scan rate of 50 nm/min.

Buffer blank: the 0.5 ml cuvette, containing 360 μl of phosphate buffer (20 mM sodium phosphate, 100 mM NaCl, 0.1 mM EDTA, and 0.1% w/v polyethylene glycol 8000, pH 7.4), is allowed to equilibrate to the temperature of the holder. Ninety microliters of a buffer matching that of the protein sample is added to the cuvette, and after a 30 s delay spectra are collected for the different slit widths.

Sample check: the 0.5 ml cuvette, containing a fresh 360 μl aliquot of phosphate buffer, is allowed to equilibrate and 90 μl of a 0.5 mg/ml stock of α_1-antitrypsin is added to the cuvette. After a 30 s delay, spectra are collected using the same parameters as the blanks. The parameters that yield a tryptophan peak value as close to the middle of the linear range of the instrument as possible are then selected for the assay. Once the measurements are complete, the absorbance at 295 nm of the sample is measured using a spectrophotometer: this should not exceed 0.1 to minimise internal filter effects.

Time course assay: the instrument is instructed to read the fluorescence at 340 nm, with 0.5–5 s integration time, approximately once every 1–5 min for 24–48 h. The cuvette holder, cuvette and 360 microlitres of phosphate buffer are pre-warmed to 45 °C, then 90 μl of a 0.5 mg/ml stock of α_1-antitrypsin is added to the cuvette, and data collection started immediately. Three microlitres of stock are snap frozen at −80 °C (time 0 s), and at four regular time points thereafter 15 μl is withdrawn from the sample cuvette and snap frozen. Once the assay is concluded a final 15 μl is set aside. These samples are analysed using an 8% w/v non-denaturing polyacrylamide gel and correlated with the fluorescence data. Typically, the fluorescence data are fitted using a single exponential function to determine the resulting rate of polymerisation [38].

2.2.4. Circular dichroism spectroscopy

This form of spectroscopy is sensitive to buffer components, particularly in the far-UV region. Care should be taken in sample preparation, which will have a higher concentration of protein in general than for tryptophan fluorescence. Samples should have an absorption below 1 at the wavelengths and path length that are tested and be free of chloride ions (an increased ionic strength can be obtained by replacing NaCl with NaF), citrate, DTT and β-mercaptoethanol. We tend to use 50 mM sodium phosphate pH 7.4. Samples should be centrifuged or filtered before use.

Sample cuvette: this should have a narrow path length, around 0.1–0.5 mm, and be specifically designed for circular dichroism applications.

Buffer and protein check: an absorption spectrum of the buffer alone should be collected to ensure that it does not have a composition that will interfere with the circular dichroism assay. The protein sample should have absorbance values below 1.0 at the cuvette path length used.

Instrument set up: do not forget to check that the nitrogen flow is sufficient for your intended use of the spectropolarimeter. The progress of polymerisation can be monitored by collecting spectra between 195 and 260 nm at 5–10 min intervals over a 24 h period at 45 °C.

Time course assay: non-denaturing polyacrylamide gel electrophoresis should be used on a pre-incubation and post-incubation sample to check for sample loss and polymerisation. After high-speed centrifugation of the heated sample, the absorption at 280 nm of pre- and post-incubation samples should be compared to check for precipitation of material.

Analysis of results: while there are methods available to deconvolute circular dichroism spectra into fractional secondary structure content, these results are at best qualitative in nature. The greatest utility of this method in the context of polymerisation was achieved by selecting a single wavelength, for example 216 or 222 nm, and fitting the change in circular dichroism signal to a single exponential equation to derive a rate for polymerisation [27].

2.2.5. Characterisation of serpin polymerisation by ion mobility-mass spectrometry

Ion mobility-mass spectrometry (IM-MS) provides a multidimensional separation of gas phase protein ions based on both their

relative mobilities in a gas filled drift tube and their mass to charge ratio. The drift times of protein ions in the drift tube can be converted into collision cross-sections [39–41]. We used this method successfully to assess different species of α_1-antitrypsin formed during polymerisation [21]. Polymers used for ion mobility-mass spectrometry are formed by heating M or Z α_1-antitrypsin at 60 °C in 200 mM ammonium acetate pH 6.5. For time course polymerisation experiments, aliquots are snap frozen in liquid nitrogen and stored at −80 °C until required for electrospray ionisation.

IM-MS experiments are performed on a Synapt HDMS quadrupole-ion trap-IM-MS instrument (Waters, Manchester, UK). Nanoflow electrospray capillaries are prepared with a nanoESI source as previously described [42]. Mass spectra parameters are optimised for the transmission of non-covalent complexes. Typical values are: capillary voltage 1.1 kV, cone voltage 30 V, cone gas 80 l/h, extractor 0.3 V, ion transfer stage pressure 3.0 mbar, transfer voltage 10 V, bias 20 V, trap voltage 20 V, IMS wave velocity 250 m/s, IMS wave height either ramped or varied between 10 and 14 V, transfer wave velocity 100 m/s, transfer wave height 5 V, trap and transfer pressure 5.1×10^{-2} mbar, IMS pressure 5.0×10^{-1} mbar, and time-of-flight analyser pressure 1.2×10^{-6} mbar. The collision cross-sections acquired from fixed wave height data are calibrated externally as described previously [43]. Spectra are analysed using MassLynx V4.1 and DriftScope V2.1 (Waters).

2.2.6. Other methods

Other biophysical techniques can be used to assess the different conformers of a serpin and the pathways by which they are formed. Two colour coincidence detection (TCCD), a single molecule fluorescence method, has been used to study the early stages of neuroserpin polymerisation [44]. In this method two distinct fluorophores are excited with two lasers, which permit the detection and identification of associated molecules and oligomers. These data show that neuroserpin polymerisation proceeds first by the unimolecular formation of an active monomer, followed by competing processes of polymerisation and formation of a latent monomer from the activated species.

X-ray crystallography allows the direct identification of conformers of neuroserpin and α_1-antitrypsin. The structure of both cleaved [45] and native [46,47] neuroserpin have been described by this method. Numerous crystal structures of α_1-antitrypsin have been published, including native [48,49], in complex with a protease [50,51] and a cleaved polymer [52,53].

2.3. Detection of polymers in cell models of disease

Cells that over express serpins have been used to analyse pathways of polymerisation. Two of the best studied are neuroserpin and α_1-antitrypsin. This has required the refinement of western blots of non-denaturing gels and the development of conformation specific ELISA assays.

2.3.1. Materials

Transient transfection of COS-7 cells: poly-L-lysine (Sigma P1524), Lipofectamine 2000 (Invitrogen 11668027), Opti-MEM I (Invitrogen 31985047), DMEM (Sigma 6429), fetal bovine serum (FBS, Sigma).

Antibody preparation: Freund's Complete Adjuvant (FCA, Sigma F5881), Freund's Incomplete Adjuvant (FIA, Sigma, F5506), mouse myeloma cells (cell line P3-X63-Ag8-653), DMEM (Dulbecco's modified Eagle's medium containing glucose and L-glutamine, Sigma D5796), complete DMEM (DMEM plus sodium piruvate (Sigma P3662), Hepes (Sigma H0887)), foetal bovine serum (Sigma F2442), DMEM-20 (complete DMEM plus 20% v/v foetal bovine serum), cloning supplement (PAA Ltd, F05-009; this substitutes for feeder cells), polyethyleneglycol (Sigma, P7181), HAT Media Supplement (Sigma H0262), HT Media Supplement (Sigma H0137), serum free-hybridoma culture medium (Sigma 14610C).

ELISA assays: 96 well EIA/RIA plate (Costar, Corning Incorpotated, 3590, flat bottom, without lid, high binding surface), substrate solution (Sigma T0440), rabbit anti-mouse HRP-labelled antibody (Sigma A9044).

Western blot: Nonidet P40 (Roche Diagnostics), PMSF (Roche Diagnostics), protease inhibitor cocktail EDTA free (Roche Diagnostics, 11873580001), Immobilon-P PVDF membrane (Millipore, IPVH00010), BSA (Fisher Scientific), Bio-Rad protein assay (Bio-Rad, 500-0006), goat anti-mouse HRP (Thermo Scientific, 32430), Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34095), Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, 34077).

Confocal microscopy: poly-L-lysine (Sigma P1524), paraformaldehyde, mounting medium (FluroSave, Calbiochem 345789) plus 2% w/v DABCO (1,4-diazabicyclo-[2.2.2] octane, Sigma D2522) and 3 μg/ml DAPI (4,6-diamidino-2-phenylindole, Sigma D9542, for DNA staining in the blue channel). Primary antibodies: anti-calreticulin (Santa Cruz Biotechnology sc-7431), anti-ERGIC-53/p58 (Sigma E1031), anti-GM130 (BD Biosciences Pharmigen), secondary antibodies: Abcam anti-rabbit TRITC (ab7081), anti-mouse FITC (ab7057), anti-goat FITC (ab7121).

2.3.2. Transient transfection of COS-7 cells

Transient transfection of COS-7 cells has been used to study serpin polymerisation in a cellular context. The pcDNA 3.1 plasmid encoding the wildtype or the mutant variants of α_1-antitrypsin and neuroserpin are introduced into cells using Lipofectamine 2000, following the manufacturer's protocol. COS-7 cells are plated on six well plates previously coated with poly-L-lysine by incubating the wells with 0.5 ml of poly-L-lysine solution (0.1 mg/ml in water) for 5 min, rinsing with sterile water and leaving to dry for 2 h in a sterile air flow cabinet. Typically, 200,000 cells are plated in 2 ml of culture medium (DMEM plus 20% v/v FBS) per well. The next day, 4 μg of DNA and 10 μl of Lipofectamine are added to two separate tubes with 250 μl of Opti-MEM I each, then mixed together and left to react for 20 min at room temperature. The mix is then added to the cells, on top of the normal culture medium, and cells are incubated for 4–6 h before changing the medium to 2 ml of Opti-MEM I per well. Transfected cells are cultured for 1, 2 or 3 days before collection of the conditioned culture medium and cells for analysis.

Comments: COS-7 cells are able to grow in serum free Opti-MEM I for up to 3 days, which allows the analysis of the culture medium supernatant of transfected cells without the interference of serum proteins. In order to assess the efficiency of transfection in each well, a fraction of the total DNA (4 μg per well) can be substituted with a plasmid expressing a control protein (for example luciferase) that can be later detected by western blot or any other appropriate technique.

2.3.3. Production of antibodies against serpin polymers

We have produced several types of antibodies, including rabbit polyclonal antibodies against monomeric native serpins (wildtype neuroserpin and Z α_1-antitrypsin) and monoclonal antibodies (mAb) against polymerised serpins (Ser49Pro neuroserpin and Z α_1-antitrypsin) [11,54]. As with any antibody production process, the type and quality of the antigen is critical for the immunisation and screening steps, in order to maximise the chances to obtain the desired antibodies [55]. In our studies we have used the following antigens: (i) monomeric wildtype neuroserpin, expressed in *E. coli*, purified and refolded as described in [27]; (ii) monomeric Z α_1-antitrypsin, purified from plasma from homozygous patients using the protocol described in [56]; (iii) polymers of Ser49Pro neuroserpin, made by heating purified protein obtained from

E. coli [20,27]; iv) polymers of Z α_1-antitrypsin made from plasma-purified protein by heating at 0.2 mg/ml and 60 °C for 1 h in PBS buffer.

We immunised rabbits and mice following standard procedures as described below. Before starting the immunisation a sample of pre-immune serum from each animal should be obtained.

2.3.3.1. Rabbit polyclonal antibodies. Briefly, on day 1, each rabbit is injected subcutaneously at multiple points with the antigen (125, 250 or 500 μg of purified serpin conformer) emulsified with FCA. After the first immunisation, the rabbits receive a booster dose every 3 weeks (on days 21, 42 and 63) with the same dose of antigen but emulsified with FIA. Blood samples are collected 1 week after each booster dose for antibody titration by ELISA. The final bleed is performed 1 week after the third booster dose.

2.3.3.2. Mouse monoclonal antibodies. We use 7–10 week old female Balb/c mice. Mice are first injected intraperitoneal with 10 μg of antigen emulsified with FCA, followed by four booster doses of 10 μg each (emulsified in FIA) at 3 weeks intervals through the same route. Serum samples are collected from the tail veins 5 days after each injection for antibody titration by ELISA. The pre-fusion boosts are given according to the following plan: 3 days before spleen collection, mice are injected intravenously (morning) and then intraperitoneal (evening) with 10 μg of antigen in PBS; the intraperitoneal injection is repeated two days before culling the mice for spleen extraction. On that day, mice are sacrificed by cervical dislocation, bled for the terminal serum sample and the spleen cells collected aseptically in serum free-DMEM medium. They are then frozen in freezing medium (8% v/v DMSO in DMEM-20) and kept in liquid nitrogen until needed for fusion.

On fusion day, the spleen cells are thawed in DMEM-20 and washed once with DMEM before mixing with the myeloma cells at a ratio of 4 spleen cells/1 myeloma cell (about 100×10^6 spleen cells and 25×10^6 myeloma cells). The cells are fused by adding 1 ml of 50% w/v polyethyleneglycol to the cell pellet drop-wise at 37 °C with gentle stirring. The fusion products are diluted in HAT-20 plus 5% v/v cloning supplement and distributed in ten 96-well plates at different densities: 2 plates at 120 μl/well, 2 plates at 60 μl/well and 6 plates at 30 μl/well, in a final volume of approximately 200 μl/well. Two columns of myeloma cells are included to control for the selectivity of the medium and two columns of spleen cells to control for background IgG from non-fused spleen cells.

Selection: after 4–5 days in HAT-20 most myeloma cells have died and the first hybridoma colonies are visible. On day 5 after fusion 50 μl/well of fresh medium are added. Afterwards, the cells are fed every other day by replacing half of the volume with fresh medium.

Screening: the primary screening is aimed at identifying wells containing hybrids producing antibodies specific to the antigen. This can be done approximately 8–10 days after fusion, if most of the colonies are large enough. The primary screening is performed by antigen mediated ELISA, using the same antigen as the immunogen (see the ELISA section below). The positive wells are expanded into 24-well plates, grown for 1–2 days until 25–50% confluent and subjected to secondary screening.

Secondary screening: the aim of the secondary screen is to select among the positive wells, those displaying specificity for a particular conformation of the antigen. Antigen mediated ELISA is used to confirm the positivity of the expanded wells, then a sandwich ELISA is used with different serpin conformers as the antigen (native monomer, polymer, serpin-protease complex, cleaved serpin, latent monomer). At this point, the hybridoma culture medium supernatant can also be analysed by other techniques of interest, such as immunocytochemistry or western blot, according to the use intended for the antibodies. Wells that maintain the positivity and display an interesting specificity are then cloned by limiting dilution, and cells from the 24-well stage are frozen as a backup.

Cloning: this is done in HT-20 medium containing 10% v/v of cloning supplement. We use the "diagonal cloning" method, where 25–250 μl (depending on cell density) of cells from a well of the 24-well plate are added to well 1A of a 96 well plate, then serially diluted 1:2 down the first column, and then all the wells from the first column are serially diluted 1:2 along the rows, all across the plate. The cloning plates are screened after approximately 10 days by ELISA as in the primary screening. Three positive wells are selected from the lowest possible density area and expanded into 24-well plates in HT-20 medium with cloning supplement, and subsequently into 6-well plates, where cells are transferred from HT-20 to HT/DMEM-20 and then DMEM-20. Throughout this expansion process, the positivity and specificity of the hybridoma clones must be assessed by ELISA.

Expansion: The selected clones are expanded into cell lines by stepwise culture into larger vessels and transferring gradually to DMEM-10, low serum medium (down to 1% v/v) and serum-free medium. Frozen aliquots from these cultures are prepared and stored in liquid nitrogen.

Comments: the use of cloning supplement avoids the need for feeder cells to support the growth of the hybridoma cells at critical steps such as fusion and cloning. Gradually removing the cloning supplement (or the feeder cells) in the expansion steps should be fine for most hybridoma clones, but for some the supplement becomes a necessary additive, especially after thawing and during low density growth.

2.3.4. ELISA assays

We use two types of assay: antigen mediated and sandwich ELISA. In both cases, the volume is 50 μl/well in all the steps (except blocking and developing) and all the incubations are undertaken in a moist chamber or by wrapping the plate with cling film to avoid evaporation.

Coating. For antigen mediated ELISA, the plate is coated with the antigen diluted in PBS at 1–2 μg/ml. For sandwich ELISA, the plate is coated with antigen-purified rabbit polyclonal antibody (capture antibody), diluted at 2 μg/ml in PBS. In both cases, the plates are incubated overnight at room temperature. The protocol is then as follows:

- wash the plates three times for 5 min with washing solution (0.9% w/v NaCl, 0.05% v/v Tween20), filling the wells to the rim with a washing bottle. If you have access to good quality distilled water, you can wash two times with water and the last time with washing solution.
- block the plates by adding 300 μl of blocking buffer per well (PBS, 0.25% w/v BSA, 0.05% v/v Tween20). Block for at least 1 h at room temperature. The plates can be left in blocking buffer (with added 0.1% w/v sodium azide) at 4 °C for several days. We do not recommend freezing down the saturated plates, since we have observed random signals and higher background.
- empty the wells by inverting the plate and dry it by flicking the wrist several times.

For the sandwich ELISA, incubate with the antigen. For the standard curve, add 100 μl of the purified antigen solution in the first well of row A, then perform 1:2 serial dilutions in blocking buffer from well 1 to 11, by transferring 50 μl from each well to the next and mixing five times. In well 11 discard the excess 50 μl, and leave well 12 blank for the background control. We usually start the standard curve at approximately 500 ng/ml. For the test samples, we use the blocking buffer to do the dilutions, starting from

at least 1:2 to reduce problems with background. When analysing samples from cell cultures, the cell lysates are prepared in the same way described for western blot analysis, while culture media supernatants can be analysed directly as they are. The final well volume should always be 50 μl per well. Incubate for 2 h at room temperature. Wash the plates as described above.

Empty and drain the plate and add the binding antibody diluted in blocking buffer. Antigen mediated ELISA is usually performed by starting at a high concentration (1:100 for immune serum, 1:1 or 1:2 for culture medium supernatant from hybridoma cells, 10 μg/ml for purified antibodies) and performing serial dilutions along the row or column, in order to obtain the affinity curve of the antibody for the antigen used in the coating.

For the sandwich ELISA, the binding antibody is generally used at a fixed concentration, although affinity curves can also be performed in this format by incubating with a fixed concentration of antigen. In our serpin polymer assays, the binding antibody is an anti-polymer monoclonal antibody used at 1 μg/ml or at an appropriate dilution of the hybridoma culture supernatant. When detecting total amounts of neuroserpin, we use a cocktail of three monoclonal antibodies that detect all conformers of neuroserpin (1A10, 10B8 and 10G12, at 333 ng/ml each), while for detection of total amounts of α_1-antitrypsin we use monoclonal antibody 9C5 (culture medium supernatant at 1:250 dilution). Incubate for 2 h at room temperature and wash the plates as described above. Then

- add 50 μl /well of the secondary rabbit anti-mouse HRP-labelled antibody, diluted 1:20,000 in blocking buffer without sodium azide (which inhibits HRP activity). Incubate for 1 h at room temperature. We use a rabbit anti-mouse antibody in order to prevent cross-reactivity with the capture antibody. Wash the plates as described above.
- add 50 μl/well of substrate solution and let it develop in the dark at room temperature for approximately 10 min. Stop the reaction by adding 50 μl/well of 1 M H_2SO_4 (final volume: 100 μl). Read the absorbance at 450 nm in a plate reader. It is convenient to bring the developing solution to room temperature for a few minutes before use.

Comments: the combined use of polyclonal and monoclonal antibodies allows the establishment of a sandwich ELISA. This is a powerful technique for the detection and quantification of any specific antigen in solution. To this end, we have used this type of ELISA as our main strategy for secondary screening. This has also allowed the development of sandwich ELISAs for the quantification of total and polymerised neuroserpin [54] which have been used for the detection of wildtype human neuroserpin in cerebrospinal fluid [57] and neuroserpin secreted from monocytes [58]. We have also developed a sandwich ELISA to quantify polymerised α_1-antitrypsin in cell culture samples and human serum [11]. When using these techniques, it is important to keep in mind that the concentration values obtained for the samples are relative, since they are determined from the concentration calculated for the antigen used as the standard (Fig. 3A). Note also that the standard curve is highly sensitive to the conformation of the antigen; the same monoclonal antibody used as the detection antibody tends to give higher signals in a polymer standard than in a monomer one for the same concentration of protein. This is probably due to the higher concentration of epitopes in the polymerised serpin.

2.3.5. Detection of α_1-antitrypsin polymers by western blot analysis

We will focus on α_1-antitrypsin as an example but the same protocols apply for neuroserpin. The cells are collected by trypsinisation and pelleted at 700 g for 10 min at room temperature. The pellet is lysed in 100 μl of lysis buffer (10 mM Tris, 150 mM NaCl, pH 7.4, 1% v/v Nonidet P40, 1 mM PMSF, protease inhibitor cocktail) by pipeting, vortexing gently and incubating on ice for 20 min. The lysate is cleared by spinning at top speed in a bench centrifuge at 4 °C for 15 min. The protein concentration is measured by a Bradford assay (Bio-Rad) and between 25 and 50 μg of protein are loaded in each lane for non-denaturing PAGE. For supernatant analysis, the sample is centrifuged at 700*g* for 10 min (this removes floating cells and cell debris). The supernatant is then carefully collected and concentrated 10 times using a Vivaspin 2 column (Sartorius, Germany).

7.5% w/v non-denaturing gels are prepared and run on a Mini Protean III gel system (Bio-Rad) in a discontinuous buffer [31]. A standard loading buffer without SDS or reducing agent is used (it is important not to boil the samples). The gels are run at 90 V at room temperature until the front reaches the bottom of the gel. The anode buffer is 100 mM Tris–HCl, pH 7.8 and the cathode buffer 50 mM Tris–HCl and 70 mM Glycine at pH 8.9. The proteins are then transferred to a PVDF membrane (Immobilon P, Millipore) in 20 mM Tris and 140 mM Glycine, without ethanol/methanol or SDS. The transfer is performed at room temperature at 200 mA for 2 h, with an ice pack in the transfer tank (Mini Protean III wet transfer system, Bio-Rad). The membrane is washed in PBT (PBS plus 0.1% v/v Tween 20) for 5 min and blocked in PBT–BSA (3% w/v BSA) or PBT-milk (5% w/v milk) overnight at 4 °C or 2 h at room temperature. The membrane is then incubated with the primary antibody. A monoclonal antibody against human α_1-antitrypsin from Abcam (Cambridge, UK) is used at 1:10000 (diluted in PBS plus 3% w/v BSA and 0.1% w/v sodium azide), incubating overnight at 4 °C or 2 h at room temperature.

The membrane is washed six times for 5 min with PBT and incubated with a goat anti-mouse HRP secondary antibody (Pierce) at 1:2000 in PBT-milk (no sodium azide, since this inhibits HRP activity) for 1.5 h at room temperature. The membrane is again washed six times for 5 min with PBT and an extra three times for 5 min with PBS. Two different chemoluminescence kits are mixed to develop the membrane: Supersignal West Femto Maximum Sensitivity Substrate and Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific). As a general rule the membrane is incubated in 2 + 2 ml of the Pico kit plus 5–10% v/v of the Femto kit (100 + 100 to 200 + 200 μl of each Femto reagent). The exact amount of Femto reagents will depend on the amount of protein in the gel and more than one mix might have to be tested. Always start with the minimal percentage of Femto reagents.

Comments: when detecting different conformers of the serpins by western blot of non-denaturing gels, it is important to note that the immunoreactivity of the polymers is higher than that of the monomeric forms; hence polymers tend to give stronger signals even if the same amount of protein is loaded per lane.

2.3.6. Confocal microscopy

Cells are grown in 13 mm diameter glass coverslips at the bottom of wells of a 24 well plate. Before plating the cells, the coverslips are made adherent by treatment with poly-L-lysine: 500 μl of poly-L-lysine solution (0.1 mg/ml in water) are added, left for 5–10 min, aspirated, rinsed with sterile water and left to dry for 2 h in a sterile air flow cabinet. Once dry, they are kept in sterile conditions at room temperature for a few days. The coverslips are then prepared by following the protocol below:

– aspirate the culture medium and wash the cells with PBS. The number of washes depends on how well your cells adhere to the coverslips, but three is recommended and at least one should be done.
– add 300 μl of ice-cold 4% v/v PFA per well and fix for 30–45 min at room temperature.
– wash the cells three times for 5 min with PBS.

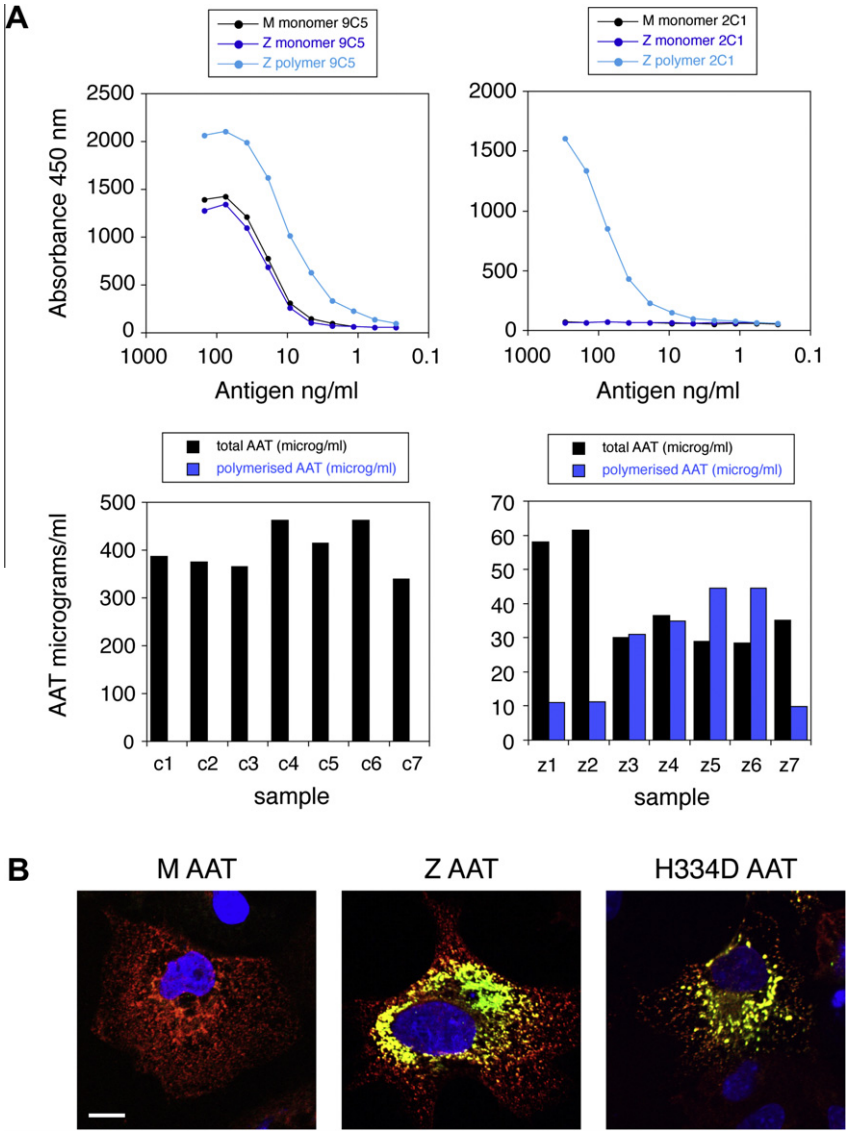


Fig. 3. (A) Serum samples from PiZZ individuals (z) and control subjects (c) were analysed by sandwich ELISA, using the monoclonal antibody 9C5 as the detection antibody to quantify all conformers and the 2C1 monoclonal antibody to quantify only polymers. The reactivity of these antibodies is shown in the standard curves (top graphs, see graph legends). The serum from control subjects showed no signal with the polymer specific 2C1 antibody, while serum from PiZZ patients showed reduced levels of α_1-antitrypsin with the 9C5 antibody and detectable levels of α_1-antitrypsin polymers with the 2C1 antibody. (B) COS-7 cells were transiently transfected with M, Z or His334Asp α_1-antitrypsin (AAT), fixed and immunostained with mAb 2C1 (green) and with a polyclonal antibody that recognizes all forms of α_1-antitrypsin (red). Only the merged panels are shown, with overlapping signals in yellow. The DNA is stained blue (DAPI). The polyclonal antibody recognised all conformers of α_1-antitrypsin in cells expressing the M, Z and His334Asp variants, while the polymer specific monoclonal antibody 2C1 only reacted with Z and His334Asp α_1-antitrypsin contained in perinuclear inclusions (green and yellow staining in middle and right panels). Scale bar: 10 μm. From [11].

- add 300 μl of blocking buffer (PBS, 1% w/v BSA, 0.1% v/v Triton X, 0.1% w/v sodium azide) and incubate at room temperature for 45 min. This is an optional blocking step, it can be avoided if there is no background in the staining or the cells detached easily.
- remove the blocking buffer and incubate with 300 μl of the primary antibody (diluted in blocking buffer) at an appropriate dilution for 1–2 h at room temperature or overnight at 4 °C. If the antibody is expensive or in short supply, incubate the coverslip up side down over a 50 μl drop of the antibody solution, by placing the drop on a flat piece of Parafilm inside a humidified box to avoid evaporation. After the antibody step, place the coverslip back into the well for the washes, paying attention to put it right side (cells side) up.
- wash the cells three times for 5 min with PBS.
- incubate in 300 μl of labelled secondary antibody diluted in blocking buffer, at an appropriate antibody dilution for 1–2 h in the dark at room temperature. We use secondary antibodies that are all developed in donkey to avoid cross-reactions, diluted 1:750.
- wash the cells three times for 5 min with PBS.
- put a drop of mounting media in a microscope slide (you can mount up to three coverslips per slide), pick up the coverslip with sharp tweezers, drain the excess of PBS in a piece of tissue and place the coverslip with the cells facing down on top of the drop. Tap on the top of the coverslip gently to set it down.

The coverslips are then left to dry in the dark, at room temperature or overnight at 4 °C, before analysis in the microscope. Samples must be kept at 4 °C in the dark for the fluorescence to last for a few days.

Comments: we use a variety of primary antibodies, including our home made polyclonal and monoclonal antibodies against neuroserpin and α_1-antitrypsin. Two of these, 7C6 and the 2C1, are specific to polymers of neuroserpin and α_1-antitrypsin respectively (Fig. 3B) [11,54]. We have also used a panel of antibodies against resident proteins of different compartments of the secretory pathway for our co-localisation studies, which are described in the reagents section above. When applying two primary antibodies in the same preparation, they can be mixed and incubated at the same time, provided that they are from different species. In the next step, the two corresponding secondary antibodies can also be incubated simultaneously, which greatly reduces the length of the whole procedure. If there is a possibility that the primary antibodies will compete for binding to the same antigen, we apply the strategy of incubating with the weakest antibody first, followed by the corresponding secondary antibody and a fixation step (4% v/v PFA for 15 min at room temperature), then proceed with the second primary antibody and its secondary antibody. We have used this strategy when staining to compare the distribution of total (with a rabbit polyclonal antibody) vs. polymerised (with the 7C6 monoclonal antibody) neuroserpin [54]. As in any antibody application, it is very important to know the specificity of your antibody and its limitations in immunostaining. For example, our anti-neuroserpin polymers monoclonal antibody 7C6 has a much greater affinity for polymers than for monomers of neuroserpin, as seen by sandwich ELISA, but it detects polymers only in immunocytochemistry if used at the right concentration [54]. When imaging serpin polymers by immunofluorescence, special attention has to be given to adjust the gain for the channel where polymers are detected, since they produce a very intense signal.

2.4. *Drosophila melanogaster* as an *in vivo* model for serpin polymer disease

Drosophila melanogaster is an ideal model organism in which to translate *in vitro* experiments. Indeed, its short lifespan and distinguishable adult stage allow rapid analysis of *in vivo* data; its large population number allows statistical analysis of results, and its extensively studied genetics allow easy experimental design.

2.4.1. Expression of neuroserpin in *Drosophila melanogaster*

The *UAS-GAL4* expression system has been widely used in *Drosophila* to model neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases [59,60]. Two lines are designed, one driver line with a tissue specific promoter controlling the expression of the GAL4 protein, and one responder line with an upstream activation sequence (UAS) controlling the expression of the transgene of interest, here neuroserpin. Following mating of these two *Drosophila* lines, the progeny carries both constructs. The GAL4 protein binds to the UAS and activates the expression of the transgene in a tissue specific manner. This expression system has been used to express toxic proteins such as α-synuclein [61], amyloid β [62] and neuroserpin [54] in the brain of *Drosophila*. The response to these toxic proteins can be studied in different cell types, such as photoreceptors. Therefore the effect of drugs and other proteins on aggregation can be studied *in vivo* in *Drosophila*. Finally, with the use of inducible drivers, it is possible to express toxic proteins transiently to study accumulation and clearance processes and their link with aging [63].

2.4.1.1. Expression of neuroserpin in neurons. The pan-neuronal *elav-GAL4* driver allows expression of transgenes in all neurons [64]. *Drosophila* has a relatively short lifespan permitting the study of the impact of various proteins on longevity [65]. To perform such experiments, 100 flies of a given genotype are collected on the day of eclosion (hatching), placed in tubes in groups of 10 and counted on day 1, 3 and 5 of a 7-day cycle. Expression of wild type neuroserpin with the elav driver is toxic to flies and so we engineered Pro–Pro residues at the P1–P1' positions in order to remove inhibitory activity. Expression of the resulting inactive neuroserpin, and indeed the mutants that form polymers did not significantly reduce the median lifespan of flies [54].

Locomotor ability can also be measured. This experiment is undertaken by simply putting 10 flies in a 25 ml plastic pipette. The flies are tapped down to the bottom of the pipette, and left to climb for 15 seconds. The number of flies above the 25 ml line and under the 2 ml line is recorded. A performance index (PI) is calculated as PI = 0.5 × [(total flies + flies at top − flies at bottom)/total flies]. This index was plotted against levels of polymers measured by ELISA in five whole flies and showed that the accumulation of polymers reduced climbing ability in *Drosophila* [54]. Recently, recording methods have been improved, relying on a video camera associated with software that calculates the coordinates of each fly in a group of 10 placed in a tube. The data obtained allows the assessment of velocity and distance covered by the flies.

The availability of specific antibodies for neuroserpin combined with the small size of the *Drosophila* head allows microscopy imaging experiments in order to locate neuroserpin accumulation. Immunohistochemistry is performed on paraffin sections (Fig. 4). Heads are dissected from female flies 25 days after eclosion. The heads are fixed in 4% v/v PFA in PBS overnight and then gradually dehydrated in increasing concentrations of ethanol and finally 100% v/v butanol. The heads are then fixed in paraffin (Mass Histology, MA, USA). Paraffin sections are deparaffinated, rehydrated and treated for 10 min in the dark with 10% v/v methanol, 3% v/v H_2O_2 in PBS to inactivate endogenous peroxidase activity. Sections are then incubated with 1A10 monoclonal antibody at 25 μg/ml in blocking reagent (PBS, 10% w/v BSA, 0.1% v/v Triton X-100, 0.1% w/v Na azide) overnight at room temperature. After three washes in PBS, sections are incubated with anti-mouse IgG at 80 μg/ml for 45 min and mouse PAP complex at 1:200 for 30 min (in Na azide-free blocking reagent), with three washes in PBS after each incubation step. HRP activity is developed with SIGMAFAST-DAB tablets (Sigma) and sections are subsequently dehydrated and mounted with DePex (VWR International). Pictures are obtained with a Zeiss AxioSkope2 microscope using AxioVision software. Heads of flies expressing wild type, Ser49Pro, Ser52Arg, His338Arg and Gly392Glu neuroserpin have been assessed. This showed the accumulation of mutant neuroserpin within cortical neuronal cell bodies (Fig. 4 and [54]). The small size of the *Drosophila* brain also allows whole mount brains to be treated with antibodies and observed by confocal microscopy [66]. 3D images can then be acquired. Moreover, fluorescent proteins driven through the *UAS-GAL4* expression system can also be used to locate neurons or other cell types.

2.4.1.2. Expression of neuroserpin in photoreceptors. The *UAS-GAL4* system also allows the specific expression of neuroserpin in *Drosophila* photoreceptors. The ommatidia that compose *Drosophila* eyes are organised in a regular pattern. As a consequence, ectopic expression of toxic proteins may cause developmental disruption leading to a lack of regularity in the organisation of the eye - a 'rough eye' phenotype. This can be observed with a dissecting microscope or by scanning electron microscopy to obtain a detailed image of the surface of the *Drosophila* eye. Expression of neuroserpin mutants in the *Drosophila* eye leads to small disruption of the ommatidia as shown by the presence of ectopic bristles and fused ommatidia.

Toxicity of proteins can also be assessed in the eye by the pseudopupil technique. The fly head is cut away from the body and light is directed through the eye. Microscopy allows the

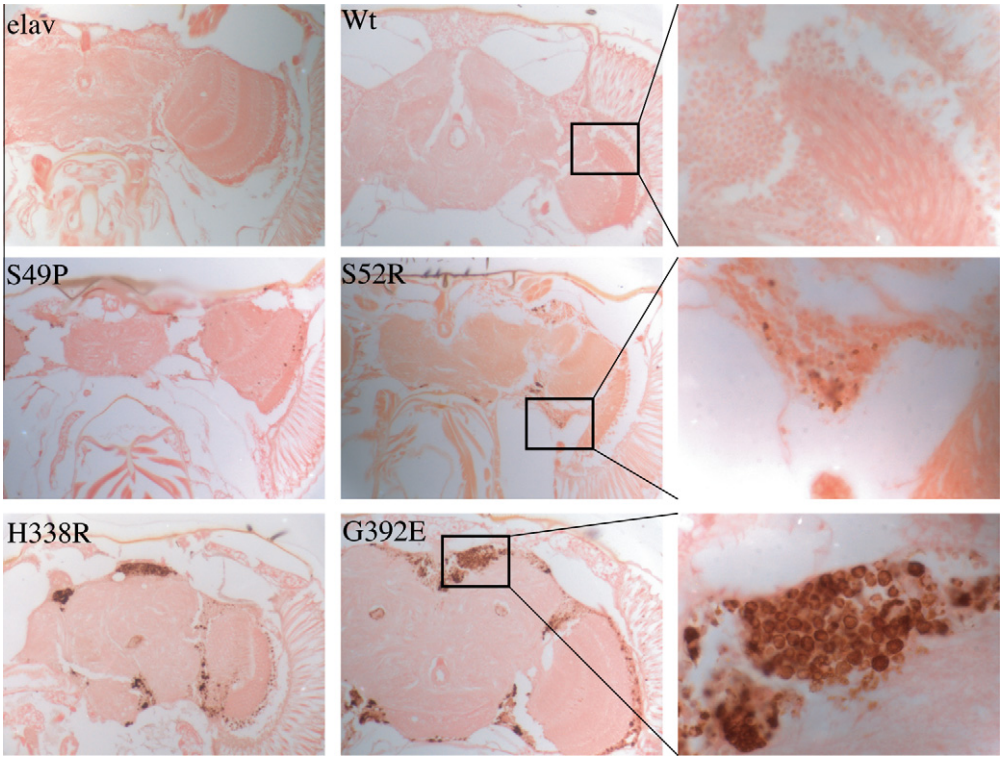


Fig. 4. Accumulation of mutants of neuroserpin in *Drosophila* neurones. Intracellular accumulation of mutant neuroserpin (brown staining) was located within cortical neuronal cell bodies, adjacent to the mushroom bodies and lobula. Left and middle panels were taken with a 20x objective; enlarged details in right panels were obtained with a 100x oil immersion objective. Sections of 25 days old flies cultured at 29 °C were stained with mouse monoclonal 1A10 antibody that detects total neuroserpin. elav, transgenic control flies with only the elav driver; Wt, transgenic flies expressing wildtype neuroserpin; S49P, S52R, H338R, G392E, transgenic flies expressing the different neuroserpin mutants. From [54].

identification of 7 of the 8 rhabdomeres that form each ommatidia. Neurodegeneration can be measured as the number of degenerated rhabdomeres that do not allow light to pass, as shown in a *Drosophila* model of Huntington's disease [67].

2.4.2. Quantification of different conformers

The small size of *Drosophila* also allows quantification of protein in the head or whole body. Three aliquots of 5 whole flies are homogenised in 100–300 μl of 150 mM NaCl, 50 mM Tris pH 7.5, 1% v/v Nonidet p40, 5 mM EDTA with EDTA-free protease inhibitor cocktail tablets (Roche). The samples are homogenised using either a hand held motorised pestle (Kimble Kontes, Vineland, NJ, USA) or tungsten balls and a mixer mill (Retsch, Haan, Germany) in 96 well 1.2 ml storage plates (Abgene, Epsom, UK). The samples are centrifuged and 70 μl of supernatant retrieved. This is then analysed by ELISA as described in Section 2.3.4. Flies expressing wild type neuroserpin do not form polymers whereas flies expressing Ser49Pro, Ser52Arg, His338Arg and Gly392Glu neuroserpin accumulate polymers as measured by ELISA [54]. Such experiments, associated with the use of inducible drivers, allow the quantification of aggregation and clearance kinetics of mutant neuroserpin *in vivo* [30].

2.5. Mouse models of FENIB

Transgenic mice have been used to assess the neuroprotective effect of wildtype neuroserpin in the central nervous system [68]. These mice show resistance to injury induced by focal ischemia. Transgenic models have also been used to study the neurodegenerative disease progressive motor neuropathy [69]. The overexpression of neuroserpin in these mice increased myelination of motor axons, improved motor behaviour and increased longevity. Mice have also been used to model FENIB by expressing either the mild Ser49Pro mutant, or the more severe Ser52Arg mutant, of human neuroserpin in the central nervous system [70]. These mice recapitulate the morphological, biochemical and clinical features of the human disease. Mutant neuroserpin accumulates as intraneuronal inclusions within the brain long before the development of clinical symptoms. There were larger neuroserpin deposits within the endoplasmic reticulum, and more severe clinical features, in mice that expressed Ser52Arg neuroserpin than in those that expressed Ser49Pro neuroserpin. These data confirm the association between the structural instability of neuroserpin and the severity of disease. A more striking phenotype was seen in transgenic mice that express the very severe Gly392Glu mutant of neuroserpin [71]. These mice accumulated very large aggregates of neuroserpin in the endoplasmic reticulum and occasionally in lysosomes. The aggregates increased with age and the mice were more susceptible to seizures in keeping with the clinical phenotype. These mice represent a valuable tool for the study of neuroserpin polymerisation *in vivo*.

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