Cell. Mol. Life Sci.

DOI 10.1007/s00018-005-5077-4
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Cellular and Molecular Life Sciences

Review

Neuroserpin: a serpin to think about

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Received 25 February 2005; received after revision 16 November 2005; accepted 28 November 2005

Abstract. Proteinases and their inhibitors play important roles in neural development, homeostasis and disease. Neuroserpin is a member of the serine proteinase inhibitor (serpin) superfamily that is secreted from the growth cones of neurons and inhibits the enzyme tissue-type plasminogen activator (tPA). The temporal and spatial pattern of neuroserpin expression suggests a role in synaptogenesis and is most prominent in areas of the brain that participate in learning, memory and behaviour. Neuroserpin also provides neuronal protection in pathologies such as cerebral ischaemia and epilepsy by preventing excessive activity of tPA. Point mutations in neuroserpin cause aberrant conformational transitions and the formation of loop-sheet polymers that are retained within the endoplasmic reticulum of neurons, forming inclusion bodies that underlie an autosomal dominant dementia that we have called familial encephalopathy with neuroserpin inclusion bodies or FENIB. We review here the role of neuroserpin and other proteinase inhibitors in brain development, function and disease.

Key words. Serpin; tissue-type plasminogen activator (tPA); proteinases; proteinase inhibitors; nervous system; polymers; FENIB; serpinopathies; conformational disease.

The discovery and initial characterisation of neuroserpin

In 1989 Peter Sonderegger and colleagues identified several proteins that were secreted from chicken neuronal axons in culture, including one that they called axonin-2 [1, 2]. Further characterization demonstrated that axonin-2 was secreted from axons of both the central and peripheral nervous system, and sequence analysis showed it to be a new member of the serine proteinase inhibitor or serpin superfamily. This was subsequently termed neuroserpin or, more recently, proteinase inhibitor 12 (PI12, gene symbol SERPINI1) [3–5].

The chicken neuroserpin gene contained an open reading frame of 1230 nucleotides encoding a secreted protein of 394 amino acids (45 kDa). The sequence predicted two N-glycosylation sites that explained the higher molecular mass of the mature secreted protein (55 kDa) that was formed following cleavage of the secretory signal peptide [3]. Cloning of the human and mouse neuroserpin genes showed the sequences to be highly conserved, with significant homology (between 33 and 42%) to other members of the serpin superfamily [3, 6, 7]. The human neuroserpin gene was localised to chromosome 3q26 [6].

Human neuroserpin, like its chicken homologue, is expressed in neurons of the central and peripheral nervous system [8]. It is most widely expressed during the late stages of development, when neurons migrate and synapses are formed. A more restricted pattern is found in the adult brain that is stronger in the cortex, hippocampus and amygdala, areas that retain synaptic plasticity during adulthood [3, 6, 7]. Neuroserpin expression is not solely localized to the brain, and messenger RNA (mRNA) and/or protein have also been detected in the

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pancreas, heart, kidney and testis [6–8]. More recently, neuroserpin has also been identified in the pituitary and adrenal glands, where it is contained within regulated secretory granules [9].

Neuroserpin *in vitro*: structure and inhibitory activity

The first clues to the structure and function of neuroserpin came from its sequence homology to other members of the serpin superfamily [3]. This family contains inhibitory and non-inhibitory members and is characterised by a very well known and conserved mechanism of action (fig. 1A). The serpin molecules present an exposed peptide, the reactive centre loop, as a pseudosubstrate for the target enzyme. After binding, the proteinase cleaves the reactive centre loop, releasing it and initiating a conformational transition in which the bound proteinase is translocated from the top to the bottom of the serpin molecule. At the same time the reactive centre loop inserts into the β-sheet A of the serpin, and the proteinase is irreversibly inactivated by molecular rearrangement [10]. The structure of native neuroserpin is likely to be very similar to that of the archetypal serpin, α_1-antitrypsin. However only the reactive loop cleaved form of neuroserpin has been crystallised to date [11]. The crystal structure of cleaved neuroserpin is almost identical to that of cleaved α_1-antitrypsin and shows that the reactive centre loop completely inserts into β-sheet A [11]. This is in keeping with the enzyme complexed form of a serpin shown in fig. 1A.

Sequence alignment suggested that neuroserpin inhibited trypsin-type proteinases, but unlike other serpins (such as antithrombin) there was no heparin-binding domain [3, 6, 8]. The amino acid sequence in the reactive centre loop was identical between chicken and mouse neuroserpin and had only one conservative substitution in man [6, 7]. The inhibitory activity of recombinant purified neuroserpin has been assessed *in vitro* by the formation of SDS-stable complexes with putative target proteinases. Mouse, chicken and human neuroserpin inhibit tissue-type plasminogen activator (tPA) and to a lesser extent urokinase-type plasminogen activator (uPA) and plasmin, but they have no inhibitory activity against thrombin [7, 8, 12]. Complex formation between a serpin and its cognate enzyme results in formation of a covalent bond between the serine at the active site of the proteinase and the P1 residue of the serpin [13]. In most cases, the complex is very stable and results in an irreversible reaction that permanently inactivates the proteinase [10]. This stable complex presents new epitopes that are recognised by membrane receptors (typically the LDL receptor-related protein, LRP) that mediate its internalisation and intracellular degradation [14–16]. However the covalent complex formed between neuroserpin and tPA is short-lived *in vitro* [17, 18]. This leads to cleavage of the neuroserpin reactive centre loop and the release of cleaved neuroserpin (40 kDa) and active tPA. In this scenario, neuroserpin is a transient inhibitor of tPA, and this may be the physiological role of neuroserpin *in vivo*. Despite its short life *in vitro*, the internalisation of the tPA-neuroserpin complex via LRP has been reported in mouse cortical cells and embryonic fibroblasts in culture, suggesting a more stable interaction *in vivo* or a temporal/spatial coupling between inactivation and uptake [16]. Moreover, a high molecular mass band that could correspond to neuroserpin-tPA complex has been identified in murine brain lysates and conditioned culture media from cells expressing both neuroserpin and tPA [8, 9]. Thus the complex seems to be sufficiently stable to be detected and have a physiological role *in vivo*.

Within the serpin superfamily, neuroserpin is most closely related to plasminogen activator inhibitor type 1 (PAI-1) and proteinase nexin 1 (PN-1) in terms of both structure and function. However, these proteins display different inhibitory profiles and patterns of expression within the brain. The main targets for PN-1 in the brain are thrombin, uPA and tPA, whilst PAI-1 inhibits both tPA and uPA [19, 20]. PAI-1 is expressed in many tissues but is poorly detected in the nervous system, whereas both neuroserpin and PN-1 are expressed at moderate levels in the adult brain [3, 6, 7, 21, 22,]. Finally, whilst neuroserpin is synthesised in neurons, PN-1 is found both in neurons and glial cells [8, 22].

Neuroserpin *in vivo*: physiological roles

The main activity of neuroserpin is the extracellular inhibition of tPA in the nervous system. It is becoming clear that neuroserpin provides a natural counter balance to excessive tPA, both in health and disease. The functions of tPA in the central nervous system and its control by neuroserpin have been recently reviewed by Yepes and Lawerence [23, 24], so we will describe them here only briefly. The main substrate for tPA is plasminogen, which upon cleavage by tPA becomes plasmin, a proteinase with a broad specificity whose role in the central nervous system is not yet completely defined. Although most of the effects of tPA seem to be mediated by plasmin, there is evidence that some of the roles of tPA in the central nervous system are independent of plasmin, which suggests that tPA has other substrates [25, 26].

During development, neuroserpin and plasminogen activators are widely expressed in nervous tissues, with high expression levels at the stages of axonogenesis and synaptic connexion [3, 7, 27, 28]. They are secreted from the growth cones at the tip of neuronal axons [3, 29]. Moreover, the expression of neuroserpin in both pitu-

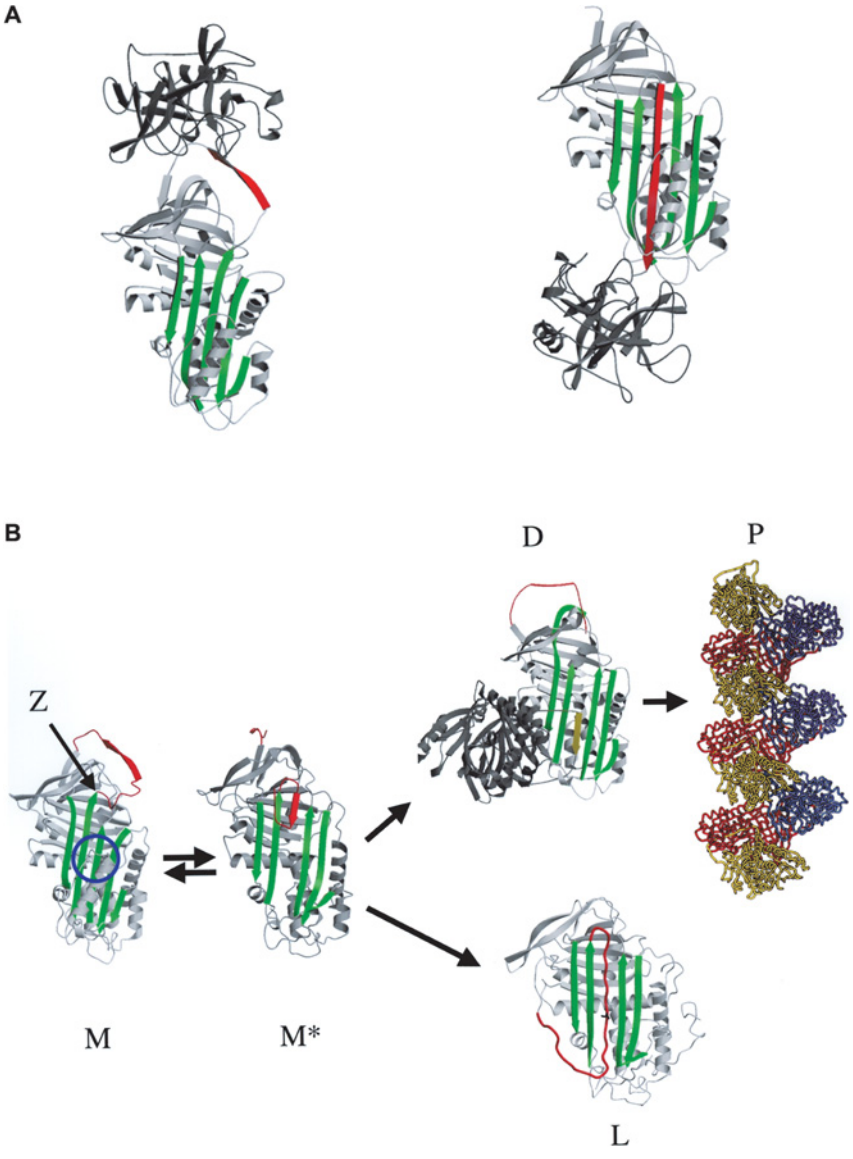


Figure 1. (*A*) Mechanism of inhibition of proteinases by α_1-antitrypsin (and other serpins) [10, 165]. After docking (left) to the reactive centre loop of α_1-antitrypsin (red) the target proteinase (in this case neutrophil elastase, dark grey) is inactivated by translocation from the upper to the lower pole of the serpin (right). At the same time, the reactive loop inserts as an extra strand into the β-sheet A of α_1-antitrypsin (green). Reproduced from [166] with permission. (*B*) Mechanism of polymerisation of mutants of α_1-antitrypsin. Mutations in the shutter domain of the serpin (blue circle) or the Z mutation (Glu342Lys at P17, black arrow) open β-sheet A, allowing the partial insertion of the loop (step 1) and the formation of an unstable intermediate (M*) [140, 141]. The gap in the β-sheet A can accept the loop of another molecule (step 2) to form a dimer (*D*) that then extends into polymers (P) [142, 144, 165]. Alternatively, the gap in the M* intermediate can accept its own loop (step 3) and adopt a latent conformation (L) [155, 167, 168]. In the polymer, the individual α_1-antitrypsin molecules are coloured in yellow, red and blue for clarity. Reproduced from [140] with permission.

itary AtT-20 and rat pheocromocytoma (PC12) cells regulates the extension of processes similar to neurites [9, 30]. The tPA/plasmin system has also been shown to affect neuritogenesis in PC12 cells [31]. Taken together, these data support a role for neuroserpin in neurite outgrowth and synaptogenesis during development of the nervous system.

In the adult brain, neuroserpin and tPA expression overlap in areas that retain synaptic plasticity, i.e. the hippocampus, hypothalamus, cerebellum, amygdala and sympathetic nerves [3, 6, 7, 32]. The transcription of neuroserpin is enhanced by neuronal depolarisation, which implies that neuroserpin is activity-regulated, a characteristic of proteins involved in synaptic plasticity [33]. There is cumulative evidence that tPA is involved in learning and memory [23, 24, 34–36,], and its co-expresssion and functional interaction with neuroserpin suggest a role for neuroserpin in modifying these processes. Neuroserpin is

also involved in other complex brain functions, such as behaviour and emotion. The overexpression or knockout of neuroserpin in mice causes a reduction in locomotor activity in new environments and a neophobic response to new objects [37]. Moreover, knocking out neuroserpin does not affect the levels and distribution of tPA proteolytic activity, suggesting that neuroserpin has tPA-independent functions [37].

A new role in the nervous system has been recently proposed for the tPA/neuroserpin axis. It has been suggested that they regulate the permeability between the vascular and nervous system compartments during normal brain function and in disease [23, 24]. Under normal conditions, the effect of tPA on neurovascular permeability is mediated through its interaction with LRP [38]. However, in pathological conditions such as ischaemia an increase in tPA activity leads to increased vascular permeability, which can be prevented by injection of neuroserpin into the cisterna magna [26]. The finding that mice deficient in tPA were resistant to excitotoxic cell death induced by brain ischaemia initiated a series of investigations assessing the possible neuroprotective role of neuroserpin after cerebral ischaemia induced by middle cerebral artery occlusion [39]. The first report showed that the expression of PAI-1 was upregulated after ischaemia, whereas the expression levels of tPA itself and of its two other inhibitors, neuroserpin and PN-1, were unchanged [40]. In contrast Yepes and colleagues found that tPA expression was transiently upregulated and neuroserpin activity was increased for several days after ischaemia [41]. Moreover, they showed that exogenous neuroserpin administrated intracerebrally after stroke was neuroprotective, decreasing the volume of the stroke, the proteolysis of the basement membrane and the number of apoptotic cells. This led to the hypothesis that following ischaemia upregulation of neuroserpin balances the activity of the overexpressed tPA, thereby limiting brain injury [41]. Similar results were obtained in mice overexpressing neuroserpin, where the volume of the lesion after ischaemia was 30% smaller than in normal mice [42]. In this case, the neuroprotection provided by neuroserpin was twofold: first by inhibiting tPA activity after the stroke and second by reducing the number of activated microglial cells that in turn produce both tPA and uPA in the injured area [42]. Neuroserpin has also been shown to be neuroprotective by blocking the tPA-dependent propagation of seizures and preventing seizure-induced neuronal cell death [25].

Evidence is now emerging on the physiological control of neuroserpin levels. As mentioned above, the transcription of neuroserpin is enhanced by neuronal depolarisation [33]. The RNA-binding protein HuD stabilises neuroserpin mRNA binding to its 3′-end, allowing a higher degree of translation [43]. This post-transcriptional regulation is under the influence of thyroid hormones, as hypothyroidism leads to decreased levels of neuroserpin in several areas of the brain [44]. The expression of neuroserpin is also upregulated during adaptation to dark background in *Xenopus* melanotrope cells [45]. After neuroserpin has exerted its inhibitory function, its complex with tPA is recognised and internalised by the LRP receptor in neural cells. Active neuroserpin is also internalised by the same pathway, providing an additional mechanism of regulating neuroserpin levels *in vivo* [16].

Neuroserpin in context: extracellular serpins and serine proteinases in the nervous system

Although neuroserpin and its target enzyme tPA are the focus of this review article, there is growing evidence that extracellular serine proteinases and their counterbalancing serpin partners play fundamental roles in the development, normal function and pathology of the nervous system (some of them summarised in fig. 2). In this section, we assess the roles of these other enzymes and inhibitors, in order to provide a framework to understand the function of neuroserpin.

The plasminogen activators, tPA and uPA, and their serpin inhibitors, neuroserpin, PAI-1 and PN-1, play several roles in the nervous system. In 1981, Krystosek and Seeds described the release of a plaminogen activator from neuroblastoma growth cones *in vitro* [29, 46]. The secretion of uPA was also identified in primary neuronal cultures [47]. *In situ* hybridisation studies in the rat demonstrated strong, widespread expression of uPA in neurons of the central and peripheral nervous system. This was apparent at the onset of axonogenesis and was maintained in adult neurons. However, only transient expression was reported in oligodendrocytes during myelination [27, 28]. In contrast, tPA was predominantly detected in the floor plate and ventricular ependymal cells *in utero* and during postnatal development [27, 28, 48]. In the adult, tPA was widely expressed throughout the brain, in particular in the Purkinje and granule cells in the cerebellum, meninges and ventricular ependyma, with high concentrations in the hippocampus and hypothalamus [32, 49, 50]. Expression of the substrate for uPA and tPA, plasminogen, has been found in similar areas, confirming their functional interaction in the brain [51]. PAI-1 is found in low levels in the adult brain [21], but its expression can be upregulated in astrocytes by transforming growth factor β1 (TGFβ1) and plasminogen [52, 53]. PN-1, previously known as glia-derived neurite promoting factor and glia-derived nexin, has also been detected in the brain and shown to be expressed by astrocytes and neurons [22, 54–57, 58].

The demonstration of tPA receptors and a peak of tPA expression in cerebellar granule cells at the time of migration prompted the hypothesis that migrating cells need to exert

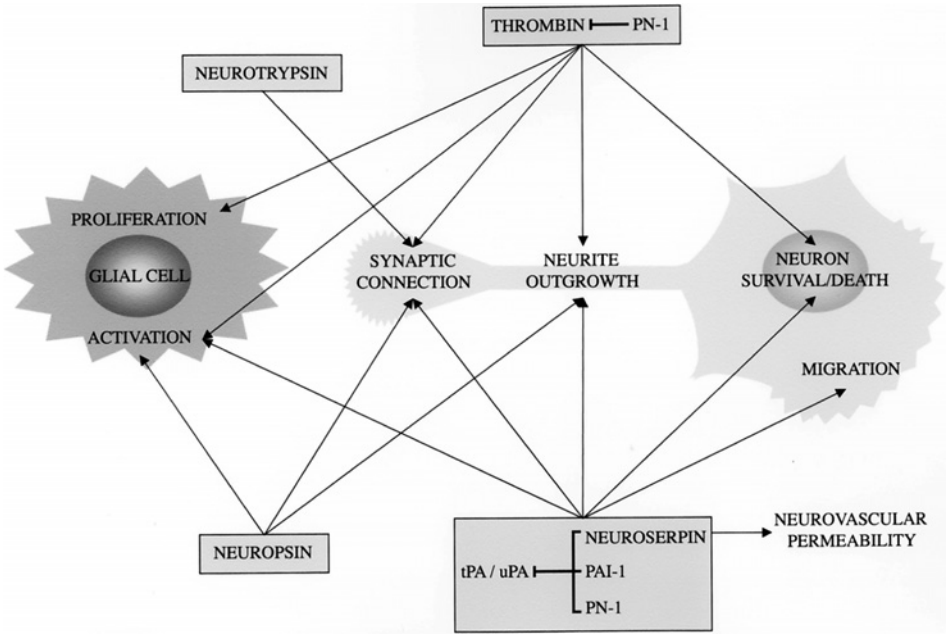


Figure 2. Diagram summarising the main activities of extracellular serine proteinases and serpins in the nervous system. PN-1, proteinase nexin 1; PAI-1, plasminogen activator inhibitor type 1; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

proteolytic activity in the extracellular matrix [59, 60]. However, inhibiting tPA failed to block neuronal cell migration *in vitro* [61], and an excess of tPA resulted in migration defects and cell death in the weaver mutant mouse model [62]. Binding sites for tPA and uPA have also been found in other neuronal cell types where inhibition of both plasminogen activator activities leads to an increase in neurite outgrowth [63]. Conversely, the expression of plasminogen activators in neurons has been associated with fibrinogen degradation during axonal outgrowth [64], and plasminogen activator activity has been found to increase the migration and neurite extension of PC12 cells in three-dimensional gels [65]. These discrepancies may be partly explained by the finding that agents that inhibit plasmin or plasminogen activation cause a decrease in neurite outgrowth within three-dimensional matrices but promotes it in two-dimensional substrates [66].

tPA and uPA also participate in synaptic plasticity as evidenced by their temporal expression during muscle synapse elimination [67], induction of tPA after seizure, kindling and long-term potentiation in the brain [68], impairment in learning following overexpression of uPA in mice [34] and reduction of long-term potentiation in tPA knockout mice [35]. It has also been proposed that tPA acts as a cytokine in the central nervous system, as it can activate microglial cells *in vitro* independent of its proteinase activity [69]. The plasminogen activators and their inhibitors also play important roles in pathological events in the nervous system, as indicated by the involvement of tPA in excitotoxic cell death and ischaemia (discussed above in relation to neuroserpin), their participation in inflammation and demyelination [70] and an increase in PAI-1 levels in the cerebrospinal fluid in correlation with neurological diseases such as encephalitis and multiple sclerosis [71].

The interactions between thrombin, its receptors (proteinase activated receptors, PAR, 1–4) and its serpin inhibitor PN-1 are also recognised to play fundamental roles in the development, maintenance and pathology of the nervous system [72, 73]. The precursor of thrombin, prothrombin, is produced throughout the developing and adult nervous system [74, 75], all four PARs are widely expressed in neurons and glia [76], and PN-1 is also present in the brain, as discussed above [22, 54–57, 58]. PN-1 and prothrombin have also been found in normal cerebrospinal fluid [77, 78]. Thrombin exerts a plethora of activities in the nervous system, many of them mediated by PAR-1 and modulated by PN-1. Thrombin causes neurite retraction through interaction with PAR-1, whereas PN-1 inhibition of thrombin enhances neurite outhgrowth [79–82]. The balance between thrombin and PN-1 is also critical for synapse dynamics [83], and for the modulation of astrocyte morphology, a process that involves PAR-1 signalling through protein kinase C [84, 85]. Low levels of thrombin stimulate the proliferation of astrocytes in culture, an activity that can be blocked by PN-1 [84, 86], suggesting that thrombin can be neuroprotective after injury or following insults such as hypoglycemia and oxidative stress, by increasing the number of cells able to provide neurotrophic factors. Thrombin and PN-1 levels are sequentially upregulated after nerve injury, suggesting their involvement in nerve damage repair [87]. Conversely, in-

creased signalling of thrombin through PAR-1 causes apoptotic neuronal cell death, a toxicity that can be prevented by PN-1 [88–90]. Thrombin and PN-1 have also been implicated in the pathology of Alzheimer's disease. Studies have shown a reduction in PN-1 levels in the brain of patients with Alzheimer's disease [91], the presence of thrombin in Aβ plaques and neurofibrillary tangles [92] and modulation of Aβ toxicity by both thrombin and PN-1 [93, 94]. However, their exact roles in the pathogenesis of Alzheimer's disease have yet to be elucidated.

In the late eighties, the serpin α_1-antichymotrypsin (ACT) was detected in the human adult brain but not in the foetal central nervous system [95]. Since then, expression of ACT has been confirmed and extensively studied in astrocytes. In these cells, ACT production is stimulated by oncostatin M, the interleukin-6/soluble interleukin-6 receptor complex [96], interleukin-1 and tumor necrosis factor-alpha (TNFα), the last through the activation of nuclear factor kappa B (NFκB) [97]. This regulation of expression suggests that ACT could be part of the inflammatory response in the brain. The finding of ACT in amyloid plaques both in the brain of elderly individuals and those with Alzheimer's disease [98, 99] has focused research on the relationship between ACT and neurodegeneration. Amyloid plaques are largely composed of the toxic amyloid $\beta_{1–42}$ peptide (A$\beta_{1–42}$). This is the product of proteolytic processing of the amyloid precursor protein (APP) and is central to the pathology of Alzheimer's disease. However, there is considerable controversy as to the identity of the toxic A$\beta_{1–42}$ species (early oligomers vs. mature fibrils) [100]. The concentration of ACT is higher in the cerebrospinal fluid of patients with Alzheimer's disease than age-matched controls [101]. This serpin has been found to promote fibrilisation of Aβ [102] and to inhibit Aβ degradation [103], as well as to increase the plaque burden in transgenic mouse models of Alzheimer's disease [104, 105]. Outside the central nervous system the main targets for ACT are cathepsin-G and chymotrypsin [106]. The identity of its target proteinase in the brain is not yet clear, despite the reports of a cathepsin-G-like proteinase in astrocytes [107], and a chymotrypsin-like proteinase (clipsin) in rat brain that is inhibited by ACT [108]. Recently, it has been reported that activated human microglial cells secrete proteins that are toxic to neuroblastoma cells. These can be inhibited with some chymotrypsin inhibitors but not by ACT. This suggests that chymotrypsin-like proteases can participate in inflammatory processes in the brain [109].

The serpin C1-inhibitor has also been found in normal brain tissue [110], and its expression has been confirmed in human microglia and neuroblastoma cell lines [110, 111]. Interestingly, it has also been identified in Aβ plaques and abnormal neuronal processes in the brain of patients with Alzheimer's disease. However, its role in the disease progression is unknown [110].

The kallikrein (KLK for genes, K for proteins) family of serine proteinases includes several members that can be found in the nervous system [112]. One of these, KLK8 (K8/neuropsin), has extracellular serine protease activity and is important in the physiology as well as disease of the nervous system. Neuropsin was first identified in the brains of mice, where it is mainly expressed in the neurons of the adult limbic system. Its expression levels change after electrical stimulation, suggesting a role in hippocampal plasticity related to learning, memory and cognition [113, 114]. The cloning of human neuropsin has shown the existence of two splicing isoforms, type 1 and type 2 [115, 116]. Type 2 neuropsin is preferentially expressed in the adult human brain with a similar pattern to expression in the mouse [116]. Subsequent studies on neuropsin showed that it has proteolytic activity toward the extracellular matrix protein fibronectin [117] and the presynaptic adhesion molecule L1 [118], and that it may regulate long-term potentiation [119], synaptic connectivity [120], and neurite outgrowth and fasciculation [121] in the hippocampus. Neuropsin levels increase after brain kindling [122], and blocking of neuropsin may inhibit the progression of kindling [123]. In contradiction to these reports, targeted disruption of the neuropsin gene in mouse showed no effect in physiological synaptic function but an increased susceptibility to seizures [124]. Neuropsin expression is also induced in oligodentrocytes after injury of the central nervous system, suggesting it could be involved in myelin processing [125, 126]. Furthermore, neuropsin mRNA is upregulated in the hippocampus of brains of individuals with Alzheimer's disease [127]. Two inhibitors have been identified in the brain of adult mouse that regulate the activity of neuropsin: the serpin SPI3, which is proposed to act as intracellular inhibitor, and murinoglobulin I, which belongs to the α_2-macroglobulin family and functions as an extracellular inhibitor [128].

Neurotrypsin is a novel neuronal serine proteinase that was first identified in the mouse and then in the human nervous system [129, 130]. Its structure consists of a multidomain mosaic protein with a catalytic domain that displays high sequence homology with the trypsin-like subfamily of serine proteinases [129]. In an exhaustive study of the expression pattern of neurotrypsin in mouse, Wolfer and collaborators showed significant expression of this serine proteinase in the developing nervous system, with a distribution indicative of a role for neurotrypsin in neuronal plasticity, both during development and in adult life [131]. Recently, a truncating mutation of neurotrypsin has been linked with nonsyndromic autosomal recessive mental retardation [132]. Together with the expression of neurotrypsin in the presynaptic membrane of cortical synapses, this suggests that neurotrypsin is a regulator of adaptive synaptic functions involved in cognition.

In a recent study, the phosphatidylethanolamine-binding protein (PEBP) has been proposed as the prototype of a

novel family of serine proteinase inhibitors, as it has no sequence homology with the serpins but it is able to inhibit thrombin, chymotrypsin and neuropsin *in vitro* [133]. PEBP is present and highly conserved in organisms from plants [134] and yeast [135] to humans [136]. In rat, it has a broad tissue distribution, with increased expression in the brain, adrenal gland, heart, testis, liver and kidney [137]. Despite its conservation and ubiquitous presence, the role of PEBP *in vivo* remains largely unknown. It is regarded as the precursor of the hippocampal neurostimulating peptide, which is involved in neuronal differentiation [138]. It has also been described as being an inhibitor of Raf-1 kinase and therefore able to modulate intracellular signalling [139]. This activity is compatible with its cytoplasmic localisation, which correlates with the absence of a secretion signal peptide in the mammalian PEBP gene [136]. Despite this, recombinant PEBP has been found in the culture medium of transfected cells, supporting its possible role as an extracellular inhibitor of serine proteinases [133].

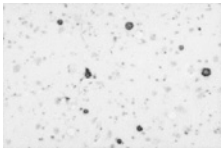
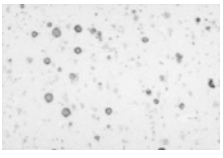
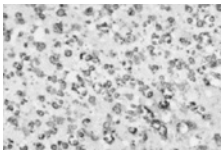
Neuroserpin and disease: mutations in neuroserpin cause the dementia FENIB

The refined 'mousetrap-like' mechanism of inhibition that characterises the serpins (fig. 1A) is also their weak point. Point mutations may destabilise and 'open' β-sheet A [140, 141]. This can then be readily filled by the insertion of the reactive centre loop of a second serpin molecule. The propagation of this linkage is the basis for a mechanism of polymerisation that has been described for mutants of the serpin α_1-antitrypsin (fig. 1B) [142–144]. These mutants are retained within hepatocytes as periodic acid Schiff (PAS)-positive inclusions that are associated with neonatal hepatitis, juvenile cirrhosis and adult hepatocellular carcinoma. The resulting lack of circulating α_1-antitrypsin reduces the protein available to protect the tissues of the lung, which in turn leads to an imbalance with neutrophil elastase and early onset emphysema [145, 146]. Polymerisation also underlies several diseases associated with mutants of other serpins (antithrombin, C1-inhibitor, α_1-antichymotrypsin), and consequently these diseases have been grouped together as the serpinopathies [147].

The same aberrant protein linkage in mutants of neuroserpin causes intracerebral inclusions and dementia. Four different mutations of neuroserpin (Ser49Pro, Ser52Arg, His338Arg and Gly392Glu) have been described in humans [148–151]. All four mutations cause the spontaneous formation of neuroserpin polymers that are retained as inclusion (or Collins) bodies within neurons in the deeper layers of the cortex and the substantia nigra (table 1) [148, 151]. We have called this autosomal dominant dementia familial encephalopathy with neuroserpin inclusion bodies, or FENIB [148, 149]. FENIB has several clinical manifestations (dementia, tremor, seizures, progressive myoclonus epilepsy, dysarthria) that present in different degrees depending on the severity of the mutation (table 1) [151].

The striking phenotype-genotype correlation between mutations in neuroserpin and disease severity is more ap-

Table 1. The effects of neuroserpin mutations on the severity of FENIB [151]. Reproduced from [145] with permission. N/A, not available.

Mutation	Post-morten brain histology	Predicted instability	Age at onset of symptoms	Clinical manifestations
Ser49Pro		+	48	dementia, tremor, seizures in terminal stages
Ser52Arg		++	24	myoclonus, status epilepticus, dementia
His338Arg	N/A	+++	15	myoclonus, seizures, dementia, tremor, dysarthria
Gly392Glu		++++	13	myoclonus, status epilepticus, dementia, chorea

parent than with many other genetic disorders. The first disease-related mutations found in neuroserpin were Ser49Pro and Ser52Arg [148, 149]. The Ser49Pro mutation, also known as the Syracuse mutant, is homologous to the Siiyama mutant of α_1-antitrypsin (Ser53Phe) that promotes polymerisation, hepatic inclusions and plasma deficiency [152, 153]. It was described in members of a family in Syracuse (New York) who presented with unusual signs of dementia in their fifties [148, 149]. Post-mortem examination of brain tissue revealed the presence of novel inclusion bodies in the deep layers the cortex, that when purified and analysed by SDS-polyacrylamide gel electrophoresis (PAGE) revealed a single protein band that was subsequently identified as neuroserpin [148, 149]. Genetic analysis of the neuroserpin gene from an affected member of the family showed that it contained a point mutation with the substitution of the serine for a proline at position 49 [148, 149]. All members of the family with dementia were heterozygous for the mutation [148, 149]. The second family to be described had the Ser52Pro mutation (neuroserpin Portland) and was characterised by an earlier age of onset, more severe clinical symptoms and a greater number of inclusions in the brain [148, 149]. Two more severe disease-related mutations have now been reported in the neuroserpin gene, His338Arg and Gly392Glu [151]. Both were associated with an even earlier age of onset, more severe clinical manifestations and more abundant inclusions within the brain (table 1) [151]. The two least severe mutants of neuroserpin that cause FENIB, Ser49Pro and Ser52Arg, have been characterised *in vitro* and in cell culture [17, 154–156]. Recombinant human Ser49Pro neuroserpin has a reduced inhibitory activity (0.3×10^4 M^{-1} s^{-1} versus 1.2×10^4 M^{-1} s^{-1} for the wild-type form at 25 °C), while Ser52Arg is inactive as an inhibitor of tPA [17, 154]. When incubated at 37 °C, wild-type neuroserpin does not polymerise, whereas both mutants polymerise readily, with Ser52Arg polymerising 15 times faster than Ser49Pro neuroserpin [17, 154]. These rates of polymerisation are in keeping with the number of inclusions and the severity of the dementia associated with each mutation.

The trafficking of wild-type, Ser49Pro and Ser52Arg neuroserpin has been characterised by transient transfection in COS-7 cells [156]. In this cell system, both mutants showed a high degree of accumulation within the endoplasmic reticulum (ER) (fig. 3A–D), and when assessed by non-denaturing PAGE and immunoblotting, these accumulations were shown to be formed of polymers (fig. 3E). Polymers were seen both when mutant neuroserpin was expressed alone or when co-expressed with the wild-type molecule as occurs in heterozygous patients. The rate of secretion of wild-type, Ser49Pro and Ser52Arg neuroserpin was evaluated by pulse-chase assays in the same cell model of FENIB [156]. These showed that the wild-type protein was readily secreted

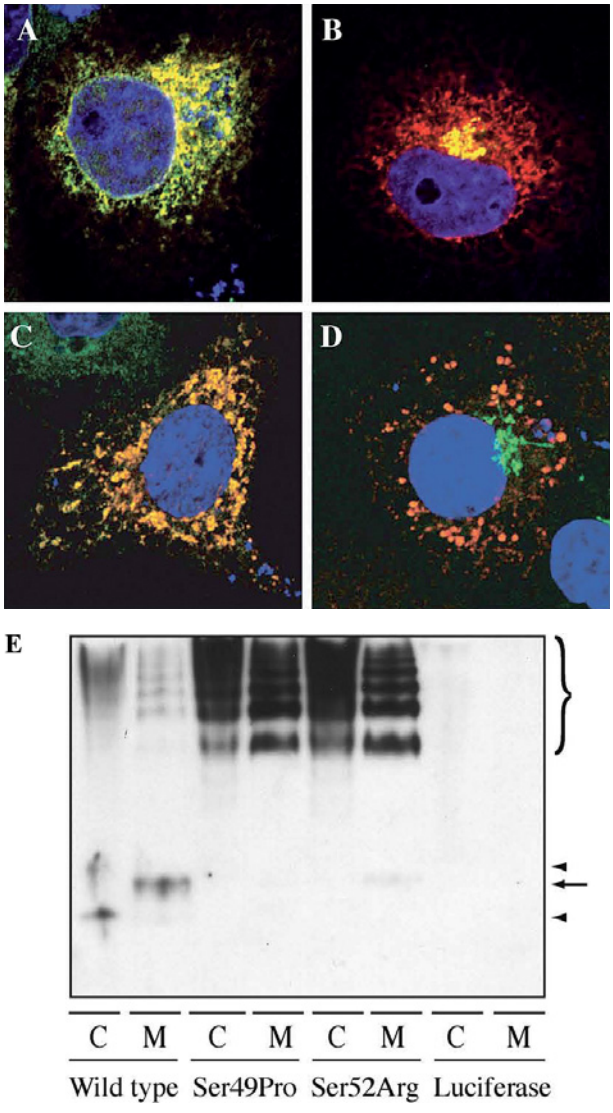


Figure 3. (*A–D*) Confocal microscopy analysis of the trafficking of wild-type and mutant neuroserpin in transiently transfected COS-7 cells. After 24 h of expression, neuroserpin (Texas Red labelling) and the ER marker calreticulin or the Golgi marker GM130 (green fluorescein labelling) were detected by immunostaining. Co-localisation results in yellow staining. In *A*, wild-type neuroserpin co-localises with the ER marker in a reticular pattern of staining. In *B*, wild-type neuroserpin co-localises with the Golgi marker (yellow) showing active secretion. In *C*, mutant Ser49Pro neuroserpin co-localises with the ER marker (yellow) as punctuate accumulations within the ER. In *D*, mutant Ser49Pro neuroserpin shows the same punctuate pattern of accumulation and no co-localisation with the Golgi marker, suggesting that the trafficking from the ER to the Golgi is a limiting step in the secretion of the mutant. (*E*) Native polyacrylamide gel electrophoresis and Western Blot analysis of COS-7 cells 3 days after transfection with wild-type or mutant neuroserpin or with a control plasmid expressing luciferase. The cell lysates and culture media from cells expressing either Ser49Pro or Ser52Arg show the ladder typical of polymerised serpins (curly bracket), while the lanes corresponding to wild-type neuroserpin show the intracellular (arrowheads, two different glycosylation forms) and extracellular (arrow) monomeric forms of neuroserpin. C, cell lysates; M, culture medium. Reproduced from [156] with permission.

(half time for secretion, 1 h) while both mutants showed significant delay in their secretion, more so in the case of Ser52Arg neuroserpin (half time for secretion, 11 h versus 5 h for Ser49Pro neuroserpin); again, this is in keeping with the severity of disease caused by the mutants. When compared with Z α_1-antitrypsin, both neuroserpin mutants are more readily secreted [157], but the retained neuroserpin is more resistant to intracellular degradation. This results in a greater accumulation of protein and the more ready detection of polymers [157, 156].

It is possible that the clinical features of FENIB are caused by the lack of active neuroserpin in the extracellular environment or by toxicity of the retained mutant polymers within the ER of neurons or a combination of the two. In the case of α_1-antitrypsin, which is synthesised in the hepatocytes and inhibits neutrophil elastase in the lungs, it is easier to distinguish between the consequences of the lack of α_1-antitrypsin activity (emphysema) and the gain of toxicity due to polymerisation of the mutant protein (liver disease). For neuroserpin it is more difficult to differentiate between the effects of loss and gain of function, as there is no physiological separation of the site of synthesis and the site of action of this serpin. In the central nervous system, an excess of tPA activity has been linked to excitotoxin-mediated cell death, so neuroserpin activity could be important for neuron survival [158, 159]. On the other hand, it is likely that the accumulation of neuroserpin polymers within neurons has similar deleterious effects upon cerebral function as α_1-antitrypsin polymers have within the liver.

The cellular pathways involved in the cytotoxicity of serpin polymers are not yet defined. In recent years it has been recognised that the ER responds to the accumulation of misfolded proteins in its lumen by activating the unfolded protein response, an ER signalling pathway that can eventually lead to apoptosis [160]. It is easy to imagine that polymerisation of serpins prevents their normal trafficking, but polymers are ordered structures and so do not elicit the unfolded protein response [161–163]. In contrast, the accumulation of polymers within the ER is sufficient to activate the ER overload response [162, 163]. This is a different ER stress pathway that can promote inflammation and cell death [164]. These studies have been performed on the Z variant of α_1-antitrypsin [161–163], but it is likely that neuroserpin polymers will be handled by the ER of neurons as α_1-antitrypsin polymers are handled by the ER of hepatocytes. A major question still remains as to how these polymers cause neuronal cell death and dementia.

Conclusions and future perspectives

The present knowledge of the neural roles of serine proteinases and their serpin inhibitors clearly shows their importance for the development and function of the nervous system as well as in neural diseases. Neuroserpin is an important inhibitor of tPA and plays a central role in neuronal development, learning, behaviour and memory. Point mutations in neuroserpin explain the novel inclusion body dementia FENIB. There is a close correlation between the molecular consequences of point mutations in neuroserpin and the severity of the associated disease that can be investigated in protein, cell and animal models of disease. Future elucidation of the cellular responses to neuroserpin polymers, combined with investigations of the mechanism of neuroserpin polymerisation *in vitro*, will provide valuable knowledge about FENIB and will help to develop strategies to prevent diseases caused by serpin polymerisation.

Acknowledgements. We are grateful to K. Römisch for critical reading of the manuscript and to all the members of the Lomas laboratory for helpful discussions. Our work is funded by the Medical Research Council (UK), the Wellcome Trust and Papworth Hospital.

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