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Neuroserpin

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1. ABSTRACT

Neuroserpin is a member of the serpin family of serine protease inhibitors. Tissue distribution analysis reveals a predominantly neuronal expression during the late stages of neurogenesis and, in the adult brain, in areas where synaptic changes are associated with learning and memory (synaptic plasticity). *In vitro* studies revealed complex formation between neuroserpin and different serine proteases, i.e. tPA, uPA, and plasmin. The neuroserpin-target complex has so far not been characterized *in vivo.* However, some investigations help to understand the functional role of this serpin. Neuroserpin was shown to be involved in the regulation of the morphology of neuroendocrine cells in culture, possibly by modulating the degradation of the extracellular matrix by proteolytic enzymes such as tPA. Moreover, a role of neuroserpin in mood regulation has been deduced from the over- and underexpression of neuroserpin in genetically modified mice, which showed increased anxiety and novelty-induced hypo-locomotion. In pathological conditions of the central nervous system (i.e. stroke and seizures), neuroserpin plays a neuroprotective role, probably by blocking the deleterious effects of tPA. A familial form of a neurodegenerative disease, termed familial encephalopathy with neuroserpin inclusion bodies, is caused by point mutations in the neuroserpin gene. This condition is characterized by the intracellular polymerization and accumulation of mutated neuroserpin, leading to neuronal death and dementia.

2. EARLY PHASE OF THE CHARACTERIZATION OF NEUROSERPIN

2.1. Isolation and initial characterization of neuroserpin

Neuroserpin was isolated in a screen for neuronal proteins involved in axonal growth and synapse formation (1). It was initially identified as a protein, 54-60 kDa in size, expressed by neurons of the central and peripheral nervous system in a compartmental cell culture system used to characterize proteins secreted from the axons of neurons. Purification of the protein from the ocular vitreous fluid of chicken embryo allowed performing partial amino acid microsequencing (2). Based on N-terminal and internal peptide sequences, degenerate oligonucleotide primers for PCR were designed and used to generate cDNA fragments, which in turn were used to isolate the cDNA from a chicken embryonic brain library. Database search of the amino acid sequence deduced from the unique long open reading frame revealed a high degree of similarity to members of the serpin family of serine protease inhibitors. The low overall identity to other human serpins (in range of 33-42%) excluded the possibility that the novel serpin was the chicken homologue of any known mammalian serpin. For this reason, and due to the specific neuronal expression, the novel protein was termed neuroserpin and received the denomination Proteinase Inhibitor 12 (PI12), or *SERPINI1* in the official terminology for serpins (3). Based on the sequence of the chicken neuroserpin cDNA, the human and mouse counterparts were isolated (4, 5). Recently, a neuroserpin-like inhibitor has been characterized in Drosophila by computational analysis of the genome (6). The gene is called Spn4 (serine protease inhibitor 4) and was reported as one of six serpin-like genes expressed in Drosophila oocytes.

2.2. The neuroserpin gene

Chromosomal localization by fluorescence *in situ* hybridization mapped the human neuroserpin gene to chromosome 3 at position 3q26 (4). The neuroserpin gene is composed of nine exons (7). The first one is non-coding, the translation initiation codon is found in exon 2 and the stop codon in exon 9. All exon-intron boundaries of neuroserpin followed the GT-AG rule. The same exon-intron organization had already been described for two other serine protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) (8) and protease nexin-1 (PN-1) (9), suggesting that the three genes belong to the same subfamily of serpin genes.

Drosophila Spn4 has four coding exons (6). Analysis of the cDNA and genomic sequences of Spn4 revealed the existence of two mRNAs, generated by alternative splicing, encoding for Spn4.1 and Spn4.2 proteins.

2.3. The neuroserpin mRNA

The open reading frame of neuroserpin is 1230 base pairs in length and is flanked by a short 5' untranslated region (UTR) and by a 3' UTR containing two polyadenylation sites, giving rise to two transcripts of different length. Only one putative polyadenylation site was identified in the human sequence; however, a discrepancy between the size of the mRNA on Northern blots and the length of the isolated cDNA clone could be explained by alternative polyadenylation. The human and chicken sequences have an overall identity of 78% on the nucleotide and of 80% on the amino acid level (4). The mouse counterpart also shows a high degree of evolutionary conservation; the identity to chicken neuroserpin is of 87% on the nucleotide and of 76% on the amino acid level (5). Drosophila Spn4, finally, shows 30% identity on amino acid level to the vertebrate neuroserpin (6).

2.4. Structural aspects of neuroserpin

Analysis of the amino acid sequence revealed the presence of a hydrophobic stretch of 16 residues at the amino terminus of the protein, which was postulated to serve as signal peptide. Moreover, two potential sites for N-linked glycosylation were identified in the chicken sequence, three in the mouse, rat, and human sequence, thus explaining the difference in molecular weight observed between the calculated mass and the size estimated by SDS-PAGE. An arginine and a methionine at position P_1 and P_1' of the reactive site loop, respectively, have been found in other serpins targeted against trypsin-like serine proteases, such as PAI-1 and alpha2-antiplasmin. On the other hand, the absence of the heparin-binding exosite (a stretch of five to seven basic residues at the N-terminus of other serpins, such as PN-1) indicates that the anti-proteolytic activity of neuroserpin is not enhanced by heparin.

Both Drosophila Spn4 proteins also have characteristics required for inhibitory serpins, but they could have different target proteases due to alternative splicing of exon 4, encoding the reactive site loop (6). Spn4.1 is probably targeted against serine proteases with basic substrate specificity. Together with vertebrate neuroserpin it shares a similar motif at the C-terminus, reminiscent of an ER-retention signal. Therefore, both neuroserpin and Spn4.1 could be intracellular regulators of serine protease activity.

Analysis of the crystal structure of a cleaved form of neuroserpin (missing 13 amino acids at the C-terminus) revealed a topology identical to that of other serpin structures, e.g. alpha1-antitrypsin, alpha1-antichymotrypsin, and antithrombin III (10).

3. EXPRESSION OF NEUROSERPIN

3.1. Regulation of neuroserpin expression

So far, a single promoter has been identified. However, the use of an alternative exon 1 during embryogenesis cannot be ruled out (7). Luciferase reporter gene assays in PC12 pheochromocytoma cells of rat adrenal medulla showed that a 200 base pairs region of the promoter near the transcription initiation site is sufficient to drive transcription (11). No TATA-like sequence could be found in the 5´ UTR of the gene (7). The initiation of transcription is probably regulated by the interplay of an Inr-binding protein and the transcription factor Sp1, as postulated for other TATA-less promoters. Indeed, binding

sites for these two proteins were localized in the neuroserpin promoter. Binding sites for neuron-specific transcription factors were also detected, e.g. for AP-2, olf-1 (found in sensory neurons), zif/268, and the thyrotroph embryonic factor TEF (expressed in the mouse hippocampus). At position –56 to -71 a CAAT-box was localized, a sequence used as binding site for several trans-activating factors involved in the regulation of the general transcription machinery.

Experiments performed in primary neuronal hippocampal cells and in PC12 cells showed that the transcription of neuroserpin mRNA is regulated by neuronal depolarization with elevated extracellular KCl (11). Whereas no change in neuroserpin expression was detected in the early phase, an increase in neuroserpin mRNA was observed eight hours after depolarization. This late increase in neuroserpin expression might be explained by the expression of zif/268, a transcription factor that was found to bind to the neuroserpin promoter and suppress its activity in PC12 cells. Interestingly, zif/268 has been reported to be induced by various forms of neuronal electrical activity as an immediate-early gene. Therefore, it could act as a silencer for the neuroserpin gene in the early response, when the proteolytic activity of tissue-type plasminogen activator (tPA) is needed, and subsequently allow the termination of the tPA activity by enhancing the expression of the serpin.

Post-transcriptionally, neuroserpin is controlled by HuD, a trans-acting factor that belongs to the embryonic lethal abnormal vision (Elav)-like family (12). The spatial and temporal expression of HuD correlates with that of neuroserpin under physiological conditions. HuD was shown to bind three AU-rich sequences in the 3´UTR of neuroserpin *in vitro* and in PC12 cells, thereby prolonging the lifetime of the mRNA and increasing the neuroserpin protein level. Another study by the same group shed further light into the process of post-transcriptional regulation of neuroserpin expression (13). This study describes the regulation of neuroserpin expression by the thyroid hormone in rat brain and PC12 cells at the level of RNA turnover by a mechanism involving HuD as well as other uncharacterized proteins.

3.2. Expression of neuroserpin during development

During embryonic development, neuroserpin expression starts when neurons become postmitotic and migrate away from the ventricular zone, suggesting a role for the serpin in late developmental processes, such as synaptogenesis and synaptic plasticity. The expression is predominant in the nervous system, with exception of a faint *in situ* hybridization signal observed in the mouse liver at very early stages (embryonic day 14 and earlier) (5) and of a Northern blot signal in fetal human liver and kidney (14).

In the central nervous system, neuroserpin is initially expressed homogeneously at low levels in most regions. Northern blot analysis shows a strong expression in chicken retina, brain, cerebellum, and spinal cord at E14 (2). Starting from embryonic day 15, the distribution is then more differentiated, with highest levels in neocortex, hippocampus, cerebellar primordium, pons and medulla (*in situ* hybridization results).

The detailed expression pattern of neuroserpin in the developing mouse dentate gyrus and cerebellum was investigated by adaptor-tagged competitive PCR starting from postnatal day 2 up to the sixth week of life (15, 16). Both studies showed a similar expression pattern for the cerebellum, with a drop in neuroserpin expression during the first week of life followed by a linear increase in the later period. The expression in the dentate gyrus did not follow any specific pattern.

In the peripheral nervous system, the presence of neuroserpin mRNA in the mouse was detected by *in situ* hybridization as early as embryonic day 10 in dorsal root ganglia of the developing spinal cord. Expression was also detected in other cells, i.e. cranial nerve ganglia, sympathetic and parasympathetic ganglia and cells of the olfactory epithelium (5).

Neuroserpin protein could be detected in the mouse brain starting from E14. The expression then increases steadily until it reaches a peak perinatally, and subsequently declines to an intermediate level in the adult (5).

Drosophila Spn4 is expressed during all developmental stages in neurons of the central nervous system and in the secretory epitracheal Inka cells (6).

3.3. Expression of neuroserpin in the adult

In the adult, a strong neuroserpin mRNA expression was detected by Northern blot in the adult brain. Lower levels of expression were found in spinal cord, heart, kidney, testis (mouse), lung (chicken), and pancreas (human) (2, 4, 5, 14). In the brain the size and morphology of the stained cells and the specific expression within the gray matter indicate that neuroserpin is expressed predominantly by neurons. *In situ* hybridization localized a very strong neuroserpin expression in the olfactory bulb, the isocortex, the hippocampus, and the amygdala (5). In other regions, such as many midbrain, pontine, and medullary areas, as well as in a subset of cells in the cerebellum, moderate expression was observed. No signal could be found in the caudate putamen and in most thalamic nuclei. In the spinal cord, positive cells were detected in the ventral horn, the intermediate zone, and the ventral laminae of the dorsal horn.

The protein distribution in the various organs of the mouse reflects the mRNA localization. Within the brain, high amounts of protein were detected by Western blot analysis in neocortex, hippocampus, and olfactory bulb, and at lower levels in striatum, thalamus, and cerebellum (5). In heart and kidney no neuroserpin protein could be isolated although the presence of mRNA was demonstrated. The absence of the protein was postulated to be due to protein turnover or to posttranscriptional processes.

Immunohistochemical analyses on mouse adult tissue sections showed positive signal in brain and spinal cord (14, 17). Positive cells were primarily neurons, however cells of microglial origin seem also to be stained. In the brain, staining was observed in Purkinje cells of the cerebellum, in most cells of the hypothalamus, in large motor neurons of medulla oblongata, in the hippocampus, and in some neurons throughout the cortex. Most neurons of dorsal and ventral horn of the spinal cord are also strongly positive.

Finally, as neurons and neuroendocrine cells share a common developmental program, the expression of neuroserpin was also investigated in neuroendocrine tissue (18). Two neuroserpin clones were isolated from a rat anterior pituitary cDNA library; they differed in the length of the 3´ UTR, reflecting the alternative use of two polyadenylation signals. *In situ* hybridization showed expression in the entire pituitary gland, and in the medullary tissue of the adrenal gland. The strongest expression was detected in melanotropes of the intermediate lobe and in most cells of the anterior pituitary. No signal was identified in the adrenal cortex.

In the same tissues, the presence of neuroserpin protein was shown by Western blot analysis. In particular, neuroserpin was detected in both the membrane and soluble fraction of the secretory granules from bovine intermediate and neural lobe of the pituitary gland and in chromaffin granules from bovine adrenal medulla and from PC12 cells (18, 19). In these cells, neuroserpin reactivity was detected by immunohistochemistry in the Golgi apparatus, in the secretory vesicles as well as in the growth cones that were generated at the neurite tips after nerve growth factor (NGF) stimulation. No neuroserpin immunoreactivity was detected in endosomes and synaptic vesicles.

3.4. Investigation of neuroserpin expression using DNA microarray analysis

Neuroserpin was shown to be differentially expressed in several screens employing DNA microarrays (20-32). Three studies are of particular interest, because they help to understand the physiological and pathological role of neuroserpin. In the first study, neuroserpin expression was reported to be upregulated by 1.52-fold in the dorsolateral prefrontal cortex of schizophrenic vs. control patients (20). In the second one, male individuals with schizophrenia showed reduction in neuroserpin expression by two fold compared to male controls, whereas neuroserpin expression in female patients did not differ (32). The divergence between the two studies was proposed to be due to the different age of the cohorts that were investigated and/or to the different arrays used (oligonucleotide vs. cDNA microarrays). However, changes in neuroserpin expression in schizophrenia, a condition often associated with emotional distress (33) is very interesting, because it is consistent with data obtained from neuroserpin overexpressing and knock-out mice, which display an anxiety-like behavior with a novelty-induced hypo-locomotion (see Chapter 4.3).

In the third study, gene expression was investigated in the brain of mice subjected to caloric restriction, a model system for the retardation of aging (21). Caloric restriction was found to induce the expression of 120 genes by 1.7-fold or more in the neocortex; one of these genes was identified to be neuroserpin. As neuroserpin is thought to promote neuronal plasticity (see Chapter 4.4), its overexpression was suggested to contribute to the improved psychomotor performance observed in the mice studied (34).

4. PHYSIOLOGICAL FUNCTION OF NEUROSERPIN

4.1. *In vitro* complex formation

Based on homology of the reactive site loop of neuroserpin and other serpins, an inhibitory activity against trypsin-like serine proteases was postulated. In order to demonstrate the anti-proteolytic activity and to determine which neuronal serine proteases were possible targets, recombinant neuroserpin protein was produced and complex formation with various proteases was assessed. SDS-stable complexes were formed with tPA, urokinase plasminogen activator (uPA), and NGF-gamma?(5, 14, 35). Plasmin and trypsin, in contrast, seemed to be involved in a substrate-like interaction with neuroserpin. Indeed, upper bands representing covalent complexes could hardly be detected in SDS-PAGE followed by Western blot analysis. Instead, a strong band representing the cleaved, inactive form of neuroserpin appeared (14, 35). No complex was detected with thrombin at any concentration.

Drosophila Spn4.1 formed SDS-stable complexes after incubation with trypsin, plasmin, and thrombin, but not with chymotrypsin or elastase (6). Moreover, due to the similarity between the reactive site sequence of Spn4.1 and the cleavage site sequence for furin and other members of the subtilisin-like proprotein convertase (SPC) family, the interaction between Spn4.1 and furin was tested. *In vitro*, soluble human furin formed a covalent complex with Spn4.1, and its amidolytic activity could be reduced up to 65% by an eightfold molar excess of recombinant Spn4.1. Interestingly, overexpression of Spn4.1 or neuroserpin in Drosophila led to molting defects in larvae similar to those reported for SPC2 as well as for peptide factor ETH mutants. It was hypothesized that Spn4.1 could inhibit the SPC that normally processes ETH or other peptides involved in the molting process.

In summary, these studies demonstrated that neuroserpin is a functional serine protease inhibitor that forms covalent acyl-enzyme complexes with proteases via the standard serpin mechanism resulting in cleavage of the predicted P_1-P_1´ bond of the reactive site loop, as showed by N-terminal sequencing of the neuroserpin-tPA complex (14).

The stability of the complexes formed *in vitro* between neuroserpin and different serine proteases is an important issue because it may provide insights into the physiological interactions of neuroserpin. In a first study, the stability of the complexes formed between neuroserpin and tPA or uPA was assessed (35). For both proteases similar results were obtained. Once formed most of the

complexes remained stable for at least five hours on ice. Incubation at 37°C lead to a slightly increased decay, as indicated by the stronger intensity of the band representing the proteolytically cleaved form of neuroserpin. However, a band representing the covalent complex was still present after five hours of incubation.

Different results were obtained from another study. The complex between neuroserpin and tPA at 37°C could be detected at early time points, up to 80 minutes for single-chain tPA, 20 minutes for two-chain tPA (36). Subsequently, the high molecular weight band was not detected anymore, and the band representing the cleaved form of neuroserpin increased in intensity. Therefore, the complex formed by neuroserpin with tPA was quite unstable, leading to a rapid hydrolysis followed by release of the cleaved form of the serpin. Moreover, kinetic experiments demonstrated that tPA cleavage of the physiological substrate plasminogen was poorly inhibited by neuroserpin, even when the serpin was present at a thousand-fold molar excess over tPA (36). On the contrary, the reaction was efficiently inhibited by PAI-1. Furthermore, when tested in a chromogenic substrate-based assay, tPA was first inhibited by neuroserpin, as shown in previous experiments (5, 35), but then with increasing time the protease recovered its activity. No recovery of activity was detected with PAI-1. In summary, these studies suggest that neuroserpin is neither a pure inhibitor, nor a pure substrate of tPA. The profiled neuroserpin inhibition of tPA suggests that it acts like a competitive substrate.

In conclusion, *in vitro* data seem to indicate that neuroserpin binds and inhibits different serine proteases. Different observations point toward tPA as the most likely physiological target. Neuroserpin interacts relatively fast with and inhibits both single-chain and two-chain forms of tPA (5, 35). Neuroserpin and tPA are co-expressed in neurons of many central nervous system regions, with the highest levels of co-expression in neocortex and hippocampus (14, 17). A 106 kDa band was observed by analyzing a mouse brain extract by Western blot with an affinity purified anti-neuroserpin antibody (14). Because the size of the band corresponded approximately to that expected for a complex with tPA, this was taken as evidence for the *in vivo* interaction between the two proteins. However, the composition of the band was not further investigated.

Although the neuroserpin-tPA complex has been shown to be less stable than the usual serpin-protease complexes *in vitro* (36), evidence for neuroserpin inhibition of tPA was demonstrated *in vivo* using a transgenic mouse model overexpressing neuroserpin under the control of the Thy1.2 promoter (37). Moreover, the deleterious effects of tPA following stroke and seizures could be inhibited by neuroserpin injection or by its transgenic overexpression (37-41).

4.2. Neuroserpin function in the neuroendocrine tissue

The predicted role of neuroserpin as a tPA inhibitor and the expression of both proteins in the pituitary and adrenal gland prompted investigators to study the role of neuroserpin in the endocrine tissue (18, 19). AtT-20 cells, a neuroendocrine cell line derived from mouse anterior pituitary gland that develop neurites in culture, synthesize low levels of neuroserpin. Two AtT-20 cell lines were generated that stably overexpress neuroserpin. These cells showed morphological changes, resulting in an increase in the extension of the neurites, with processes terminating in growth-cone-like structures. The cells had a slightly higher tPA activity in the cell lysate and a slightly lower tPA activity in the medium when compared to the parental cells. Moreover, a faint higher-molecular-weight band was observed in the medium of the parental cells at the size of the neuroserpin-tPA complex. The band was absent from the medium of the neuroserpin-overexpressing cells. Taken together, these results demonstrate a clear effect of neuroserpin overexpression on cell morphology, but only a small difference in the activity of the putative neuroserpin target, tPA. As a consequence, neuroserpin was postulated to regulate neurite outgrowth by modulating proteases other than tPA or by a mechanism independent from its function as an inhibitor.

The same group published a similar study where the effect of neuroserpin on neurite outgrowth was studied in another neuroendocrine cell line, PC12 cells (19). When treated with NGF, PC12 cells differentiate into a neuronal phenotype and extend neurites. Stably transfected PC12 cells were generated, which expressed the sense or antisense neuroserpin cDNA, leading to an overexpression or down-regulation of the serpin. Transfected and parental cells were then subjected to NGF treatment. Cells overexpressing neuroserpin extended a reduced amount of neurites and showed a reduction in total free neurite length. In contrast, reduced levels of neuroserpin led to an increase in both neurite-extending cells and in total free neurite length. The effect was concentration-dependent, indicating a strong negative linear relationship between neuroserpin level and total free neurite length. Similar results were achieved by stimulating the cells with other growth factors, namely epidermal growth factor and basic fibroblast growth factor. Again, the possible involvement of neuroserpin as a tPA inhibitor was investigated. As PC12 cells overexpressing tPA are known to produce increased neurite outgrowth (42), and as an increased neuroserpin expression seems to reduce the effect, the serpin was postulated to regulate the process by modulating the degradation of the extracellular matrix (ECM) by proteolytic enzymes such as tPA. Complex formation between the two molecules was detected *in vitro* at pH 7.0-7.5. However, neuroserpin was unable to complex tPA at the acidic pH (5.0-5.5) typically found in the secretory vesicles, demonstrating that intracellular complexation cannot occur.

These results are in contrast to those obtained with AtT-20 cells, where neuroserpin overexpression was shown to increase the extension of neurites in a tPA-independent way. However, in both cell lines neuroserpin seems to play a role in regulating changes in cell adhesion required for axon elongation. Depending on the cell line, neuroserpin could exert its function either by inhibiting tPA, or by inhibiting other proteases involved in ECM degradation, or by other mechanisms that do not involve its inhibitory function.

4.3. Role of neuroserpin in the regulation of the emotional state

Behavioral studies were conducted on neuroserpin knock-out and overexpressing mice (43). Knock-out mice were generated by insertion of a neo cassette into the second coding exon. Transgenic mice were generated by overexpressing neuroserpin in the brain under the control of the Thy1.2 promoter (37). In both cases, mutant mice were born in a mendelian ratio and developed normally. Brain weight and morphology were normal, no seizures, neurological deficits, or obvious changes in the spontaneous home cage behavior were detected. The brain tPA proteolytic activity was normal in the knock-out mice, but reduced by 70% in neuroserpin overexpressing mice (37). However, during these investigations, neuroserpin-deficient mice appeared less active. Since motor impairment could be excluded, the mice were subjected to different behavioral studies (forced and free exploration tests) to assess whether the emotional state was responsible for the reduced activity. Homozygous neuroserpin-deficient mice ($Ns^{-/-}$) exhibit a reduced locomotor activity in all tests. They showed a reduction in time and distance spent in scanning, with the effect being stronger in the aversive exploration zones, e.g. on the elevated O-maze and in the novel object test. Moreover, on the elevated O-maze, the $Ns^{-/-}$ mice reduced the visits not only in the open sector but, in contrast to wild-type mice, also in the intermediate zone. This behavior is typical for an anxiety- like response. In the novel object test, the mice showed a neophobic response, characterized by a strong avoidance and by a complete lack of approach to the object. The time spent in investigating the object was reduced, whereas the time spent in the corners of the arena increased. The change in zone preference suggests that the mice were able to perceive the object normally, excluding the altered perception as a cause for the disturbances.

The heterozygous neuroserpin-deficient mice ($Ns^{+/-}$) showed the same behavioral alterations as the $Ns^{-/-}$ mice in the forced exploration tests, but they were indistinguishable from the wild-type mice in free exploration tests. Since these mice express only reduced amounts of neuroserpin, the decreased severity of the phenotype suggests that the reduction in activity is a direct consequence of the neuroserpin deletion.

Neuroserpin overexpressing mice showed no reduction in activity, they had even a slightly increase in activity. However they showed increased avoidance in the open-field test and a neophobic response in the novelty test. In the latter, the phenotype was similar to the one detected in $Ns^{-/-}$ mouse, but less severe, at an intermediate level between $Ns^{-/-}$ and control mice.

In summary, the over- and underexpression of neuroserpin is associated with an anxiety-like behavior, in particular with a novelty-induced hypo-locomotion. Neuroserpin is the first protease inhibitor reported to play a role in mood regulation.

4.4. Role in synaptic plasticity

The spatio-temporal expression pattern of neuroserpin suggests a role of the serpin in the regulation of extracellular proteolytic events taking place in the late stages of neurogenesis when the axons have reached their target, during synaptogenesis and the subsequent remodeling processes including synapse elimination. In the adult central nervous system, neuroserpin is found in areas where synaptic changes are associated with learning and memory.

In the primary visual cortex, synaptic plasticity occurs after monocular deprivation (MD) performed during the critical period, which is defined as the period that allow experience-dependent refinements of the neuronal connections. The MD-model was used to study the influence of neuronal activity on synaptic plasticity and in particular the role of neuroserpin in this process (44). In non-deprived mice, the expression of neuroserpin in hippocampus and visual cortex was shown by RT-PCR and *in situ* hybridization. A signal was already detectable at birth; its intensity increased up to postnatal day 13 (P13) and was still detectable in six months old animals. In visually-deprived mice neuroserpin expression in the hippocampus was very similar to the one observed in non-deprived mice. In the visual cortex, however, RT-PCR showed low levels of neuroserpin at P13. On the side ipsilateral to MD the levels at P18 were normal again, whereas on the contralateral side they remained low at least until P33. Interestingly, when MD was performed after the critical period, no differences in neuroserpin expression were noted. These results suggest a regulatory role of neuroserpin in the refinement of synaptic contacts in the visual cortex after visual deprivation.

4.5. Neuroserpin catabolism

Neuroserpin is cleared from the extracellular space via its interaction with low-density lipoprotein receptor-related protein (LRP), a member of the low-density lipoprotein receptor family. LRP is a transmembrane protein of approximately 600 kDa present in the brain that was shown to bind and mediate the internalization of complexes of tPA and uPA with the serpin PAI-1 (45). *In vitro* studies with native and cleaved neuroserpin showed low binding to immobilized LRP, whereas substantial binding was observed when neuroserpin was preincubated with tPA (46). A similar situation had already been described for other serpins (47). In murine primary cortical cultures, surprisingly, not only the complex, but also active neuroserpin was internalized into endosomal compartments and degraded by the cells in an LRP-mediated mechanism. The possibility that neuroserpin was internalized in complex with other proteases could be ruled out. However, the need for an accessory molecule or for alterations in neuroserpin that would allow the internalization in the absence of a protease could not be excluded. The internalization of an active serpin has not been reported before.

5. PATHOLOGICAL FUNCTIONS OF NEUROSERPIN

5.1. Neuroprotective role of neuroserpin in stroke

Since acute ischemic stroke is often caused by a thromboembolytic event in cerebral arteries, the intravenous administration of recombinant tPA within three

hours of symptom onset was recommended in the 1980s as a thrombolytic therapy to restore the cerebral blood flow in the ischemic brain (48, 49). Patients treated with tPA achieved greater neurologic recovery and experienced fewer disabilities than patients treated with placebo (50). Beyond the three hours time window, the treatment increased the risk of serious side effects. However, some animal studies seem to contradict the conclusions drawn from human patients. tPA-deficient mice showed a decrease in stroke size and an increase in neuronal survival after focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO) when compared to wild-type mice (51). Moreover, plasminogen-deficient mice showed an increase and animals deficient in alpha2-antiplasmin a decrease in stroke volume, suggesting a plasminogen-independent function of tPA in the thrombolytic therapy (52). Despite the contradictory results, the effect of neuroserpin, a putative tPA inhibitor, was studied in a focal cerebral ischemic model produced by MCAO. In a first study, the expression of neuroserpin as well as tPA and uPA was shown to increase after stroke in rats (39). Immunohistochemical analysis revealed that neuroserpin was elevated as early as six hours after stroke, reached a peak at 48 hours and remained elevated for one week in neurons surrounding the ischemic core. Similarly, zymographic analysis showed a 50% increase in tPA synthesis six hours after stroke onset. This enhanced proteolytic activity is thought to be responsible for the degradation of the basement membrane observed as early as 10 minutes after reperfusion. Moreover, increased tPA activity was detected in the perivascular tissues of the ischemic penumbra and was shown to be necessary and sufficient for the increase in brain-blood-barrier (BBB) permeability observed after MCAO (41). Intracerebral administration of neuroserpin reduced the stroke volume by 64%, suppressed the increase of tPA synthesis, preserved the integrity of the basement membrane, reduced BBB permeability, and inhibited apoptosis in the ischemic penumbra by at least 50%. The effect is due to the inhibitory activity of neuroserpin, since administration of an inactive molecule did not reduce the stroke size. The following model was proposed: tPA, released by vascular endothelial cells in response to acute ischemia, damages the BBB, thus increasing vascular permeability and allowing its extravasation. As a consequence, the basement membrane is degraded (by tPA or by other proteases), and the neuronal cells, which depend on contacts with the matrix for survival, undergo apoptosis. Neuroserpin, by inhibiting the deleterious effects of tPA, could be a naturally occurring neuronal survival factor.

In a second study, similar results were achieved using a transgenic mouse that overexpresses neuroserpin in the brain under the control of the Thy1.2 promoter (37). In these mice, zymographic analysis confirmed a reduction in tPA activity in the brain by 70% when compared to wild-type mice. After MCAO, besides a reduction in the stroke volume, a decrease in the number of tPA/uPA-producing, activated microglia in the marginal region of the infarct could be demonstrated. This later effect is thought to be responsible for the decreased tPA and uPA activity observed in the transgenic mice after stroke when compared to wild-type mice. Furthermore, the activity of tPA was also inhibited by complex formation with neuroserpin, whereas an uPA-neuroserpin complex could not be detected. This work added further evidence to the model proposed by the first study: tPA, produced by hyperexcited neurons following ischemic stroke, promotes the activation of microglia. Activated microglial cells synthesize (1) uPA, which supports microglial migration via degradation of ECM; (2) tPA, which maintains and enhances microglial activation; and (3) neurotoxic agents (e.g. tumor necrosis factor-alpha, nitric oxide, glutamate, matrix metalloproteases) which digest the ECM and thus lead to neuronal apoptosis. The activation of microglia by tPA is independent from its proteolytic activity, since plasminogen-deficient mice have normal microglial activation. So tPA has a dual role in stroke: a plasminogen-dependent, leading to ECM degradation, as described by Yepes et. al. (41), and a plasminogen-independent, causing the activation of microglia. Neuroserpin, again, acts as a neuroprotective agent by reducing the activation of microglia and thus uPA and tPA production.

Moreover, the beneficial effect of neuroserpin was also demonstrated in rats by administrating the inhibitor three hours and tPA 4 hours after MCAO (38). The administration of tPA at such a late time point had been previously shown to augment the risk of side effects (49). However, the pre-treatment with neuroserpin led to reduction of the volume of the ischemic lesion, of the BBB leakage, and of the brain edema when compared to rats treated with tPA alone or with saline. So, neuroserpin could help in widening the therapeutic window of tPA treatment.

In a recent study, reduction of neuroserpin mRNA level after MCAO has been hypothesized to be responsible for the exacerbation of the ischemic injury observed in diabetic rats (53). Hyperglycemia has been proposed to aggravate the cerebrovascular disorder in stroke patients and animal models. The study shows that at early phase following ischemia/reperfusion, plasminogen activator (PA) activity as well as PA/PAI-1 ratio is reduced in diabetic rats compared to normal ones. At a later phase (11-23 hours after reperfusion) neuroserpin mRNA level was also reduced in the hyperglycemic rats, whereas the infarct volume increased. Therefore, a model was hypothesized where a reduction in PA activity in early phase is responsible, via reduced fibrinolysis, of an enhancement of ischemic lesion and neuronal damage. These are in turn exacerbated by reduced levels of a neuroprotective agent, neuroserpin.

5.2. Neuroprotective role of neuroserpin in seizures

Kainic acid (KA), a potent glutamate analog, can cause massive depolarization and cell death in neurons (54). When injected into the amygdala, it causes hyperstimulation and subsequent propagation of impulses to the dentate gyrus and then to the hippocampus. Using this model, it is possible to study the effects caused by seizure activity in the hippocampus without direct application of the excitotoxin. Yepes and colleagues performed this experiment in rats to investigate the effect of neuroserpin in seizures (40). They came to the following

conclusions: (1) both tPA activity and neuroserpin are upregulated in the ipsilateral amygdala 10 minutes, in the ipsilateral hippocampus 30 minutes and in the contralateral hippocampus one hour after KA-injection; (2) neuroserpin administration in the ipsilateral hippocampus following KA-injection reduces tPA activity and hippocampal neuronal loss bilaterally. In tPA-deficient mice, seizure progression is delayed even without any neuroserpin treatment, whereas in plasminogen-deficient mice the situation is similar to that observed in wild-type mice, suggesting that tPA mediates seizure spreading in a plasminogen-independent way. Neuroserpin does not prevent seizure onset, but it delays seizure progression by blocking the activity of tPA and thus seizure propagation to the hippocampus. For this reason it was postulated to be an endogenous neuroprotective factor to increased tPA activity.

5.3. Is neuroserpin a cancer-associated gene?

Some evidence suggests that neuroserpin is a cancer-associated gene. Neuroserpin is a functional serine protease inhibitor that binds to and inhibits tPA *in vitro*, a protease also involved in tumorigenesis. Moreover, neuroserpin and pancpin, a pancreas-specific serpin that is down-regulated during cancer growth, share the same gene locus and organization. This prompted some investigators to study neuroserpin expression in tumors. Chang and colleagues detected neuroserpin mRNA in normal brain, but not in malignant brain tumors and in two brain cancer cell lines, U-87 MG and H4, suggesting that neuroserpin could play a role in suppressing the growth of brain tumors (55).

On the other hand, another study found neuroserpin to be overexpressed in prostate cancer, and in particular to be highly expressed in hormone refractory, recurrent, or high grade prostate tumors (56). Moreover, the relationship between neuroserpin expression and recurrence-free survival was negative. Therefore, based on these results neuroserpin was hypothesized to contribute to tumor cell survival.

In conclusion, whether the absence or overexpression of neuroserpin in cancer cells contribute to tumorigenesis or tumor spreading remains to be elucidated.

5.4. Familial encephalopathy with neuroserpin inclusion bodies

Familial encephalopathy with neuroserpin inclusion bodies (FENIB) is a pre-senile dementia first described in two Caucasian families living in the USA (57). The two families differed in both ages of onset and clinical manifestations: whereas in the first family patients presented symptoms around the fifth decade of life, the affected individuals of the second showed an earlier clinical onset, during the second to third decade of life. Moreover, patients from the first family showed cognitive decline including deficits in attention, concentration, controlled oral fluency, response regulation difficulties, impaired visuospatial skills, and memory impairment. Patients from the second family, in contrast, presented a progressive dementia that was accompanied by seizures and myoclonus epilepsy. Histologically, the brains of patients from the two families are characterized by the presence of round, eosinophilic neuronal inclusions, called Collins bodies. Collins bodies are about 5-50 micrometers in diameter, positive to periodic acid-Schiff, diastase resistant, and distinct from any previously described inclusions. After isolation, they were shown to contain a single protein of about 50-55 kDa in size that was identified as neuroserpin by amino acid sequencing and Western blot analysis. Electron microscopy of the inclusion bodies isolated from the patients showed entangled fibrils that were immunoreactive for neuroserpin.

DNA sequencing revealed a point mutation (T-to-C transition at nucleotide 226) resulting in a substitution of serine 49 to proline (S49P) in the first family and A-to-C transition resulting in serine 52 to arginine (S52R) substitution in the second. The mutations, called $PI12_{Syracuse}$ and $PI12_{Portland}$, respectively, are inherited in an autosomal dominant fashion, indicating that the expression of one mutant allele is sufficient to produce the disease.

Later, two other mutations of neuroserpin were discovered in humans with FENIB. They result from amino acid substitutions histidine to arginine at position 338 (H338R) (A-to-G at nucleotide 1013) and glycine to glutamic acid at position 392 (G392E) (G-to-A at nucleotide 1175) (58). They are characterized by even more severe symptoms and by an earlier manifestation. The H338R-patient developed progressive myoclonus epilepsy, dementia, tremor, and dysarthria at age 15 years. The G392E-patient developed, at age 13 years, progressive myoclonus epilepsy with seizures and dementia. She died at age 19 years in a status epilepticus.

5.4.1. Inclusion body distribution

In general, the distribution of the inclusion bodies seems to reflect the regional expression of neuroserpin. In fact, no intracellular accumulation of neuroserpin was found outside the nervous system.

Neuronal inclusion bodies are present throughout the gray matter of the cerebral cortex (primarily pyramidal layers III-IV) and in many subcortical nuclei, especially the substantia nigra. Inclusions were also localized in the spinal cord and, in addition to the central nervous system, in dorsal root ganglion cells, a finding that may correlate with the sensory abnormalities detected clinically (59). Moreover, because the frontal lobe and related frontal-subcortical structures are associated with attention, the patient's attention deficits probably correlate with the accumulation of inclusion bodies within these areas during the early stages of the disease (60). As the disease progresses, deficits become more global in nature, presumably because of a more widespread accumulation of inclusions.

Intracellularly, electron microscopy analysis showed accumulation of the inclusion bodies within the membranes of the rough endoplasmic reticulum. Occasionally, neuroserpin immunoreactivity was detected diffusely throughout the cytoplasm, without the presence of

well-defined bodies (59). A precise description of the spatial distribution of inclusion bodies is given by Davis et.al. (61) and Takao et.al. (59).

5.4.2. Characterization of the accumulated neuroserpin

An analysis of inclusion bodies revealed that the entire neuroserpin molecule (from amino acid 20 to 410) accumulates and that it was glycosylated (62). Thus, mutant neuroserpin is secreted past the endoplasmic reticulum membrane with cleavage of the signal peptide; it is glycosylated but not excreted. Normal neuroserpin was not identified within the inclusions, indicating that synthesis and processing of the non-mutated neuroserpin is not affected by the presence of the mutated protein.

The inhibitory activity of mutant neuroserpin is severely impaired. Whereas $PI12_{Syracuse}$ remains partly active, $PI12_{Portland}$ is inactive (63). In both cases, the complexes with tPA rapidly dissociate by favoring the substrate pathway. The reactive site loop of the mutant forms was proposed to be less accessible for protease binding than in wild-type neuroserpin, with the loop of $PI12_{Portland}$ being inserted further than in $PI12_{Syracuse}$. Inactivity of $PI12_{Portland}$ may result in an uncontrolled tPA activity, thereby explaining the epileptic seizures observed in patients with more severe forms of FENIB.

A cell model was established to study the processing of wild-type as well as Syracuse and Portland mutant of neuroserpin (64). In transiently transfected COS-7 cells, the punctate accumulation of mutant neuroserpin forms was observed. Polymers of the mutant proteins were detected both in the cell lysate and in the cell supernatant. Confocal microscopy analysis as well as deglycosylation study localized the inclusion bodies within the endoplasmic reticulum. In order to model the heterozygosity of the disease, the wild-type and mutant forms were co-transfected simultaneously in COS-7 cells. The presence of wild-type neuroserpin did not prevent the formation of polymers, which suggests that the two forms do not interact. Finally, in the cell model the mutant proteins are transported to the cytosol and degraded by the proteasome.

5.4.3. Mechanism of polymerization

All four mutations affect conserved amino acids in the key mobile regions of the molecules, i.e. the hinges of the reactive loop and the shutter region that underlies the focal opening of the beta-sheetA. Other serpins are mutated in the same region, and they are also characterized by polymer formation within inclusion bodies. The four mutations in neuroserpin result in instability of the protein. The predictable gradation of conformational instability of the different variants is the following: G392E>H338R>S52R>S49P.

The mutations are thought to be responsible for an opening of the main five-stranded sheet, thus perturbing the relationship between the reactive center loop and beta-sheetA. This allows the insertion of the reactive center loop of one molecule into the sheet of a second one, leading to intermolecular linkages and tissue deposition. The polymerization mechanism is corroborated by the following evidences: 1) Electron microscopy analysis and far-UV CD spectra are consistent with formation of loop-sheet polymers as observed for alpha1-antitrypsin (63, 65). 2) When incubating $PI12_{Syracuse}$, polymerization is already visible after two hours at 37°C, whereas for the wild-type form only after four hours. The rate of polymerization of $PI12_{Syracuse}$ at different temperatures was almost 13-fold higher than that obtained for neuroserpin at 37°C. $PI12_{Portland}$ forms polymers at an even faster rate than the wild-type and $PI12_{Syracuse}$ forms (63). 3) At 4°C neuroserpin forms dimers, whereas the mutants form higher order polymers.

In addition to polymerization, $PI12_{Syracuse}$ also forms a latent species, *in vitro* under physiological conditions and *in vivo*, as demonstrated by the isolation of latent $PI12_{Syracuse}$ from inclusion bodies of FENIB patients (66). The latent form is inactive, although the activity can be restored by refolding; it can form polymers, although less readily and through a different mechanism than native $PI12_{Syracuse}$. The author emphasizes the significance of this second inactivation pathway in FENIB, suggesting that it could exacerbate neuroserpin-deficiency caused by polymerization.

5.4.4. Comparison of the four variants

Autopsy- and biopsy-material from different patients carrying one of the four mutations was compared. In all four cases there was accumulation of neuroserpin in inclusion bodies. However, differences were noted in the rates of accumulation: interestingly, the amount of inclusions correlates with the predicted conformational instability of neuroserpin, with the age of onset and with the severity of the disease (58). In $PI12_{Syracuse}$-patients, the least severe phenotype, only a few neurons contain inclusions, with most affected cells having only one or two inclusions. The onset of the dementia is between the ages of 40 and 60 years. The most severely disruptive mutation (G392E) results in near ten times more inclusions, with most neurons being affected, many having grape-like clusters of ten or more inclusion bodies per cell. The onset of disease occurred at age 13 with more severe clinical manifestations (progressive myoclonus epilepsy, only observed in patients harboring this mutation). These data are confirmed by experiments performed with transiently transfected COS-7 cells, where it was shown that the secretion of neuroserpin carrying the Portland mutation was slower than neuroserpin carrying the Syracuse mutation (64). Therefore, a stronger accumulation within the cell lysate was detected for the Portland form. At the same time, the turnover of the Portland form by the proteasome was higher when compared to the Syracuse form, a fact that again suggests a higher degree of misfolding.

A similar correlation between mutations and rate of polymer formation is observed for alpha1-antitrypsin, a serpin whose mutations are also known to cause inclusion body formation in hepatocytes (67).

The symptomatic difference between the four neuroserpin variants can be explained not only by the amount of inclusions, which depend on the instability of

neuroserpin, but also by the different distribution of the inclusions. In the more severe case, inclusions are present in the cerebral cortex as well as in the cerebellum, with a well-defined accumulation in the cerebellar dentate nucleus, a focus that has been implicated in the development of progressive myoclonus epilepsy.

5.4.5. Is the absence of neuroserpin or its accumulation responsible for the neurodegeneration?

The analysis of biopsy material from patients where the disease is still in progression suggests that the development of the disease is accompanied by an increase in neuroserpin inclusions. In fact biopsy samples of early stages show fewer and more dispersed inclusions. At early stages, patients have also less severe clinical manifestation. These findings provide evidence that inclusion body formation is in itself, or through the consequences that follow from it, a sufficient cause of neurodegeneration (58). Similarly, the aggregation of alpha1-antitrypsin, and not its accompanying deficiency, is responsible for hepatocyte damage and the eventual development of cirrhosis.

In summary, the biochemical and histological findings as well as the homologies to other serpinopathies suggest that FENIB is a neurodegenerative disease caused by mutations in the neuroserpin gene that result in polymerization of the protein. The mutations result in a "gain-of-function". The disease shows a clear genotype-phenotype correlation, with the severity of the disease correlating with the propensity of the mutated neuroserpin to form polymers (67).

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Abbreviations: BBB: brain-blood-barrier; ECM: extracellular matrix; FENIB: familial encephalopathy with neuroserpin inclusion bodies; KA: kainic acid; LRP: low-density lipoprotein receptor-related protein; MCAO: middle cerebral artery occlusion; MD: monocular deprivation; NGF: nerve growth factor; $Ns^{-/-}$**:** homozygous neuroserpin-deficient mice; $Ns^{+/-}$**:** heterozygous neuroserpin-deficient mice; PA: plasminogen activator; PAI-1: plasminogen activator inhibitor-1; PC12: pheochromocytoma cells of rat adrenal medulla; $PI12_{Portland}$: neuroserpin protein carrying the Portland (S52R) mutation; $PI12_{Syracuse}$: neuroserpin protein carrying the Syracuse (S49P) mutation; SPC: subtilisin-like proprotein convertase; tPA: tissue-type plasminogen activator; uPA: urokinase plasminogen activator; UTR: untranslated region

Key Words: Serpin, Serine protease inhibitor, Familial Encephalopathy With Neuroserpin Inclusion Bodies, FENIB, Neurodegeneration, Conformational disease, Neural plasticity, Review

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