Neuroserpin, a Brain-associated Inhibitor of Tissue Plasminogen Activator Is Localized Primarily in Neurons

IMPLICATIONS FOR THE REGULATION OF MOTOR LEARNING AND NEURONAL SURVIVAL

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A cDNA clone for the serine proteinase inhibitor (serpin), neuroserpin, was isolated from a human whole brain cDNA library, and recombinant protein was expressed in insect cells. The purified protein is an efficient inhibitor of tissue type plasminogen activator (tPA), having an apparent second-order rate constant of 6.2 × 10^5 M^-1 s^-1 for the two-chain form. However, unlike other known plasminogen activator inhibitors, neuroserpin is a more effective inactivator of tPA than of urokinase-type plasminogen activator. Neuroserpin also effectively inhibited trypsin and nerve growth factor-γ but reacted only slowly with plasmin and thrombin. Northern blot analysis showed a 1.8 kilobase messenger RNA expressed predominantly in adult human brain and spinal cord, and immunohistochemical studies of normal mouse tissue detected strong staining primarily in neuronal cells with occasionally positive microglial cells. Staining was most prominent in the ependymal cells of the choroid plexus, Purkinje cells of the cerebellum, select neurons of the hypothalamus and hippocampus, and in the myelinated axons of the commissura. Expression of tPA within these regions is reported to be high and has previously been correlated with both motor learning and neuronal survival. Taken together, these data suggest that neuroserpin is likely to be a critical regulator of tPA activity in the central nervous system, and as such may play an important role in neuronal plasticity and/or maintenance.

Neuroserpin is a newly identified member of the serpin family that is primarily expressed in brain (1, 2). Sequence analysis of the neuroserpin cDNA has suggested that it is likely to be a functional serine proteinase inhibitor with specificity for trypsin-like enzymes. However, until now no functional activity has been shown for neuroserpin. In the present study we show that neuroserpin is a fully functional inhibitory serpin that reacts preferentially with tPA, a serine proteinase implicated in both CNS development and maintenance. Consistent with this, tPA gene expression is prominent in the developing embryonic and neonatal brain (3–5), and tPA activity is higher within the adult brain relative to most other tissues (6). Specific expression of tPA has been shown in adult rodent brains in cells of the hippocampus and dentate gyrus, as well as the Purkinje neurons of the cerebellum, neurons in the hypothalamus, and cells of the ventricular ependyma (3, 7, 8).

Although the functional role of tPA in these cells is not known, recent studies have implicated tPA in the processes of neuronal plasticity and degeneration. A role in synaptic remodeling has been suggested for tPA since elevated mRNA and protein levels were detected specifically in the cerebellar Purkinje cells of rats retrained to negotiate a pegged runway (9). Long term potentiation, another form of synaptic plasticity thought to be important for long term memory, has also been correlated with the induction of tPA mRNA expression in granular cells of the dentate gyrus (10). tPA has also been implicated in the regulation of neuronal survival in response to excitotoxin. In these studies, neural degeneration of the CA1-CA3 pyramidal cells of the hippocampus appeared to be mediated by tPA, since mice deficient in tPA were protected from neural degeneration (11, 12). Moreover, plasminogen, which is the only known target substrate for tPA, also appears to be required for excitotoxin-mediated neuronal cell death, since plasminogen-deficient mice are similarly protected from neuronal death (13). This latter result suggests that a proteolytic cascade may be involved in the excitotoxin-induced neural degeneration. That disregulation of tPA activity has the potential for such a severe outcome implies that its regulation in the brain is critical, and for this reason there is likely to be a need for balancing tPA’s proteolytic activity with the opposing activity of an inhibitor.

In this report we suggest that neuroserpin may provide that inhibitory activity within the CNS. Our data demonstrate that neuroserpin rapidly inhibits both the single- and two-chain forms of tPA, and show by immunohistochemistry it’s localization within neurons of the normal mouse brain. Moreover, most of the regions within the brain where neuroserpin is observed are also sites were either tPA message or protein have been found previously. Together, these data imply that neuroserpin may be an important inhibitor of tPA activity within the CNS.
and as such could play a significant role in regulating either neuronal plasticity and/or survival.

MATERIALS AND METHODS

Proteins and Reagents—Human tPA was either from Genentech (Activase, South San Francisco, CA) or from BioPool (Ventura, CA). Human high molecular weight uPA (Ukidan, Serono A.B., Aubonne, Switzerland) was a generous gift of Dr. T. Ny, and low molecular weight uPA (Abbkinaise, Abbott Laboratories, North Chicago, IL) was a generous gift of Dr. J. Henkin. Human α-thrombin and bovine β-trypsin were generous gifts from Dr. S. T. Olson, and NGF-γ was a generous gift from Dr. S. Gonas. Human plasmin was from Enzyme Research Laboratories (South Bend, IN). Antibodies against neuroserpin were produced in rabbits by standard procedures and affinity purified on neuroserpin-Sepharose as described (14).

Northern Blot Analysis—Expression of mRNA was determined by hybridizing polymerase chain reaction-amplified whole neuroserpin cDNA insert probes to poly(A)+ RNA multitissue Northern blots. The blots were prehybridized in ExpressHyb solution (CLONTECH, Palo Alto, CA) at 65 °C for 30 min and then hybridized overnight in the same buffer at 65 °C. The blots were washed in 0.2 × SSC, 0.1% SDS at 65 °C twice for 15 min each. Following autoradiography, the blots were stripped in 0.5% SDS at 100 °C, cooled for 15 min at room temperature, and then checked for consistent mRNA levels by hybridizing with the β-actin probe supplied by the manufacturer.

Expression and Purification of Recombinant Neuroserpin in Insect Cells—The protein-coding region of neuroserpin was amplified from a full-length neuroserpin cDNA with KpnI polymerase, and the product was ligated to the BamHI and XbaI sites of the pA2 baculovirus expression vector (15). Following transformation into DH5α Escherichia coli, plasmid DNA was isolated, and the entire coding region sequenced to exclude any polymerase chain reaction generated errors. Plasmid DNA was then purified and transfected into Sf9 insect cells to generate a recombinant virus as described previously (15). For protein production, Sf9 cells were seeded in serum-free media at a density of 1.5 × 10^6 cells/ml in spinner flasks. The cells were infected at a multiplicity of 10 plaque-forming units per cell, and then checked for consistent mRNA levels by hybridizing with the β-actin probe supplied by the manufacturer.

Expression and SDs-Stable Complexes between Neuroserpin and Various Enzymes—Expression and purification of recombinant neuroserpin was described previously (15). For these assays, 20 nM enzyme was added to neuroserpin at the concentrations shown and incubated at 23 °C for 30–3600 s, depending on the time frame being assessed, after which chromogenic substrate was added to a final concentration of 1 mM, and the change in absorbance at 405 nm was monitored for 2 min. The slope of this curve was then compared with the slope of the same enzyme in the absence of neuroserpin, and the amount of inhibition by neuroserpin was determined from the difference. In all cases the curves were linear over the 2 min time interval indicating that there was no additional inhibition by neuroserpin following addition of the chromogenic substrate. The standard errors for each point calculated are indicated in Fig. 1 by the error bars, and the reduced y² value for each fit was less than 0.6. The substrates used were as follows: for tPA, thrombin, plasmin, and NGF-γ, Flavagen tPA (BioPool, Ventura, CA), high molecular weight, and low molecular weight uPA were with S2444 (Chromogenix, Franklin, OH), and trypsin was with Chymozyme TRY (Boehringer Mannheim).

Analysis of SDs-stable complexes between neuroserpin and various enzymes was determined by non-reducing SDS-PAGE on a Phastgel system with 12.5% homogenous gels. Briefly, 5 μM neuroserpin was reacted with 5 μM of each enzyme for 30 min at 23 °C in Tris-buffered saline pH 7.5 followed by the addition of SDS to 1% and heated to 68 °C for 3 min prior to SDS-PAGE. Gels were then stained with Coomassie Brilliant Blue in 50% methyl alcohol, 10% acetic acid.

Immuno blot Analysis of Normal Murine Brain Extracts—A fresh murine brain was homogenized in 1 ml of extraction buffer (0.5 M NaCl, 50 mM Hepes, pH 7.4, containing 1% Triton X-100, 0.05% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 1 mM d-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, 200 ng/ml leupeptin, and 6.8 ng/ml pepstatin) by repeated passage through a 22-gauge needle. The extracted material was then treated with a 1.5 volume excess of Sorelocel (Calbiochem) by vortexing for 1 min, followed by centrifugation for 10 min at 10,000 rpm in a microcentrifuge. The supernatant was then transferred into a fresh tube, and the protein concentration was determined by BCA assay (Pierce) prior to SDS-PAGE. Extracts (1 μl) or purified neuroserpin (2 μg or 500 μg) was then subjected to SDS-PAGE on 10% gel (Novex, San Diego, CA), followed by either staining with Coomassie Brilliant Blue or transfer to nitrocellulose. Immunoblots were washed once with 2% SDS at 68 °C for 30 min, followed by 3 washes with Tris-buffered saline containing 0.05% Tween 20 (TBST) at 23 °C. All subsequent steps were performed at 23 °C. The immunoblots were then blocked with 3% nonfat dry milk in TBST for 1 h. Next, 1 μg/ml of either affinity purified antineuroserpin IgG or normal rabbit IgG in TBST containing 1% bovine serum albumin and 5% normal goat serum was added and the immunoblots incubated for 1 h, followed by washing with TBST three times. These were then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase in TBST containing 1% bovine serum albumin, and 5% normal goat serum, for 30 min, washed three times with TBST and developed with chemiluminescence (Pierce).

Immunohistochemical Staining of Tissue Sections—Neuroserpin, a Brain-associated Inhibitor of tPA 33063

RESULTS AND DISCUSSION

Expression and Functional Analysis of Neuroserpin—To determine if neuroserpin is a functional inhibitory serpin, and to characterize its target specificity, the cDNA for neuroserpin was isolated from human whole brain library (data not shown). The putative protein coding sequence was then ligated into the
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Fig. 1. Kinetic analysis of the inhibition of various enzymes by neuroserpin. Panel A shows the plot of the observed first-order rate constant with each enzyme versus the concentration of neuroserpin, and the inset shows an expanded view of the origin of each curve. Panel B shows an expanded view of the curves with plasmin and thrombin only. Each point represents the average of at least two separate determinations, and standard errors are shown where they exceed the size of the symbol. □, human two-chain tPA; ◊, human single-chain tPA; △, human high molecular weight uPA; *+, human low molecular weight uPA; †, bovine β-trypsin; ▼, rat nerve growth factor-γ; ◊, human plasmin; ×, human thrombin.

To test if neuroserpin has inhibitory activity, a number of proteinases with trypsin-like specificities were tested. Fig. 1, panel A, shows the pseudo first-order rate constants obtained with each enzyme plotted as a function of neuroserpin concentration. These data demonstrate that neuroserpin is inhibitory toward each enzyme tested, and that the $k_{obs}$ for each proteinase approaches a limiting rate consistent with the serpin inhibitory mechanism (17, 22). The concentration of neuroserpin required for half-maximal saturation was similar with each enzyme, varying by no more than 10-fold. However, the relative limiting rates of neuroserpin’s reaction with each enzyme varied by more than 3 orders of magnitude. These values, calculated from the data in Fig. 1, are shown in Table I together with the calculated apparent second-order rate constants ($k_i$) for the inhibition of each enzyme by neuroserpin. These results indicate that the two-chain form of tPA is inhibited most efficiently by neuroserpin, compared with the other proteinases examined, with a rate of nearly 10$^6$ M$^{-1}$ s$^{-1}$. This is $10–100$-fold greater than the rates of inhibition toward uPA, trypsin, and NGF-γ, which ranged from 0.65–5.9 × 10$^4$ M$^{-1}$ s$^{-1}$. In contrast, the inhibition of plasmin and thrombin by neuroserpin was significantly slower, yielding $k_i$ less than 10$^3$ M$^{-1}$ s$^{-1}$. Finally, unlike the serpins antithrombin III, heparin cofactor II, protein C inhibitor, PN-1, or PAI-1 (23), the inhibition of thrombin by neuroserpin was not enhanced by heparin (data not shown).

SDS-PAGE analysis of neuroserpin-proteinase complexes was also performed. These results are shown in Fig. 2, and indicate that neuroserpin does form SDS-stable complexes with each enzyme except plasmin and trypsin. The observation that neuroserpin can form SDS-stable complexes with proteinases further indicates that the mechanism whereby neuroserpin inhibits a serine proteinase is similar to other serpins and involves the formation of a covalent acyl-enzyme complex (19–21, 24, 25). In addition to the SDS-stable complex observed in each case there is also a lower molecular weight species of neuroserpin present. This has been shown to occur with other serpins and is due to the turnover of some of the serpin by the enzyme in a substrate reaction (18, 21). The reason that neuroserpin does not show SDS-stable complexes with trypsin and plasmin, is likely to be due to the degradation of the neuroserpin-trypsin or neuroserpin-plasmin complex by free enzyme prior to the SDS-PAGE analysis. Consistent with this, only a small amount of high molecular weight complex can be seen with plasmin (Fig. 2, lane 14). Furthermore, with both trypsin and plasmin the cleaved form of neuroserpin observed after reaction with the proteinase is smaller than that seen with the other enzymes (Fig. 2, lanes 10 and 14). This suggests that both trypsin and plasmin are able to cleave neuroserpin at a site other than the reactive center loop, and this secondary cleavage may account for the lower apparent stability of the complex. Finally, NH$_2$-terminal sequencing of the neuroserpin-tPA complex demonstrated that formation of the stable complex resulted in cleavage of the predicted P1-P1’ Arg$_{362}$-Met$_{363}$ reactive center bond (data not shown) as expected for serpin inhibition (19, 20). Taken together with the inhibitory results presented in Fig. 1 and Table I, these data suggest that neuroserpin is a typical inhibitory serpin with a primary reactivity toward tPA.

### Table I

| Enzyme                        | $k$     | $K$     | $k_i$ |
|-------------------------------|---------|---------|-------|
| Human two-chain tPA           | 0.078   | 0.12    | 6.2 × 10$^5$ |
| Human single-chain tPA        | 0.17    | 2.1     | 8.0 × 10$^4$ |
| Trypsin                       | 0.0096  | 0.16    | 5.9 × 10$^4$ |
| Human high molecular weight uPA | 0.0050  | 0.20    | 2.5 × 10$^4$ |
| Human low molecular weight uPA | 0.013   | 1.4     | 9.2 × 10$^3$  |
| NGF-γ                         | 0.0086  | 1.3     | 6.5 × 10$^3$  |
| Plasmin                       | 0.000052| 0.15    | 3.6 × 10$^2$ |
| Thrombin                      | 0.00013 | 0.64    | 2.1 × 10$^2$ |

Tissue Distribution of Neuroserpin—Northern blot analysis was performed to determine the tissue-specific expression of neuroserpin. As previously shown (2), neuroserpin is strongly expressed in adult brain and to a lesser extent in pancreas but not in lung, liver, skeletal muscle, kidney, or placenta (Fig. 3). Extending these studies, neuroserpin mRNA is also seen at low

baculovirus protein expression vector, pA2 (15), and recombinant protein was expressed and purified. The purified recombinant neuroserpin migrated as a doublet with relative molecular masses of 49 and 47 kDa on SDS-PAGE in close agreement with the predicted molecular mass of 44 kDa. NH$_2$-terminal sequence analysis showed two overlapping amino termini of approximately equivalent yields beginning at either alanine 19 or threonine 20, confirming that the signal sequence was removed from both polypeptides. Thus, the differences in apparent molecular mass of the two species are most likely due to alternative glycosylation of the three putative Asn-linked glycosylation sites present in the protein.
levels in heart and testis but not significantly in other adult tissues examined (Fig. 3). High levels of neuroserpin message are also observed in human fetal brain, and in contrast to the adult tissues, lower but detectable amounts are seen in human fetal liver and kidney. Analysis of specific areas of the adult human CNS demonstrate that neuroserpin is highly expressed throughout the CNS, including the cerebral cortex, frontal and temporal lobes, putamen, occipital pole, medulla, and spinal cord. Expression in the cerebellum appears to be significantly lower compared with other regions of the brain. The predominant neuroserpin message is ~1.8 kilobases, however, as previously noted (2), an additional ~3-kilobase message is also seen in brain, suggesting that there may be alternatively spliced or polyadenylated transcripts in some tissues. The chicken neuroserpin gene is also expressed as two different transcripts that result from alternative usage of polyadenylation signals in the 3′-untranslated region (1). The significance of the alternative transcript for either the human or chicken genes is unknown.

**Immunoblot Analysis of Murine Brain**—To see if neuroserpin protein was also expressed in the brain and to demonstrate the specificity of the antineuroserpin antibodies, immunoblot analysis was performed on an extract of normal murine brain. These results are shown in Fig. 4 and indicate that neuroserpin protein is present in murine brain, and that it exists in three discrete molecular weight forms. The molecular sizes of these three forms were calculated to be 47, 52, and 106 kDa. This pattern is remarkably similar to that seen in Fig. 2 for neuroserpin in complex with tPA (Fig. 2, lane 2) and suggests that the three bands represent neuroserpin in a cleaved (47 kDa), a native (52 kDa), and a proteinase-complexed (106 kDa) form. If this interpretation is correct then the 52-kDa native form would indicate that murine neuroserpin is slightly larger than the 47–49 kDa forms of the native recombinant human neuroserpin expressed in the baculovirus system. More importantly, if the 106-kDa form represents neuroserpin in complex with an enzyme, these results suggest that in normal murine brain neuroserpin is an active proteinase inhibitor. Furthermore, since the brain extract was isolated in the presence of proteinase inhibitors, it implies that the proteinase-complex with neuroserpin existed prior to the extraction procedure, and thus suggests that in normal brain tissue there may be a balance between the activity of neuroserpin and a putative proteinase. Finally, the size of this complex is approximately that expected for a complex with tPA and is similar in size to a high molecular weight species of tPA (110 kDa) identified in both human and rat pheochromocytoma cells, which was suggested to be tPA in complex with an unknown proteinase inhibitor (26). However, the exact nature of each of these antineuroserpin reactive proteins is unknown.
bands is purely speculative, and further experiments will be required to demonstrate that neuroserpin is an active inhibitor in normal brain, and if so, what its specific target enzyme is.

**Immunohistochemical Analysis of Neuroserpin Protein**—To more precisely examine the expression of neuroserpin protein, immunohistochemical staining of adult mouse tissue sections was performed. Consistent with the mRNA distribution, only brain and spinal cord tissues demonstrated significant reactivity, and these results are shown in Figs. 5 and 6. Neuroserpin protein is widely distributed throughout the brain but is primarily localized to neurons. The major exceptions to this pattern are expression in the ependymal cells of the choroid plexus, and the brush border of the cells lining the ventricles (Fig. 5, panels A and B, respectively). These cells are thought to be of microglial origin and are important for maintaining the cerebrospinal and ventricular fluid. Other regions of the brain with high neuroserpin immunoreactivity are the Purkinje cells of the cerebellum, which show strongly positive staining of the cell body as well as the axons (Fig. 5E). Most neurons of the spinal cord are also strongly positive (Fig. 5D), as are the axons in the myelinated tracts of the commissura (Fig. 5E). This latter staining is most evident when seen in cross section where the axons stain strongly, but the myelin sheath surrounding the axons are negative (Fig. 5E, large arrow). This panel also shows that neuroserpin is generally not present in endothelial cells that show only the blue hematoxylin counterstain (Fig. 5E, small arrow). Another region of strong staining is the hypothalamus where most of the neurons appear to show significant amounts of neuroserpin immunoreactivity within the cell body (Fig. 5F). Finally, neuroserpin was also present in the large motor neurons of medulla oblongata and in scattered neurons throughout the cortex (data not shown).

**Fig. 4. Neuroserpin immunoblot analysis of normal murine brain extracts.** Lanes 1 and 2 show a Coomassie stained 10% SDS-PAGE, and lanes 3–6 show immunoblots developed with chemiluminescence. The primary antibody in lanes 3 and 4 was rabbit antineuroserpin, and in lanes 5 and 6 was normal rabbit IgG. Lanes 1, 3, and 5 were loaded with 1 μl (18 μg) of brain extract, lane 2 was loaded with 500 ng of purified neuroserpin, and lanes 4 and 6 were loaded with 2 ng of purified neuroserpin.

**Fig. 5. Immunohistochemical staining of different brain sections.** Panel A, the ventricular ependyma shows strong cytoplasmic staining of the epithelial cells (large arrow), magnification 190 ×. Panel B, the staining of the ventricular lining cells is confined to their brush borders (large arrows), magnification 480 ×. Panel C, Purkinje cells (large arrow) and their processes (small arrows) display the most intense staining within the cerebellum, magnification × 190. Panel D, most large neurons in the spinal cord/medulla oblongata are positive for neuroserpin. The large arrow indicates the tangentially cut central channel, magnification × 48. Panel E, extensive positive staining in neuronal cytoplasm and axons in the region of the commissura. Cross-sections of the myelinated axons appear as brown dots in a white circle, indicating the positive cytoplasmic yet negative myelin staining (large arrow). Blood vessels appear empty due to the perfusion fixation and show only the blue hematoxylin counterstain of their endothelial cell nuclei without a detectable signal for neuroserpin (small arrow), magnification × 190. Panel F, strong cytoplasm staining of many granular cells in the hypothalamus. Smaller cells and vascular endothelial cells are negative for neuroserpin, showing only the blue/purple nuclear color of the hematoxylin counterstain, magnification × 480.

**Fig. 6. Immunohistochemical staining of the hippocampus and dentate gyrus.** Panel A, specific strong staining for neuroserpin in the hippocampus with decreasing intensity and frequency from the CA1 to CA3 regions. Dentate gyrus (DG) is negative except for a few individual cells that stain similarly to the other scattered individual cells in the non-granular region, magnification × 48. Panel B, higher magnification of the CA1 region of the hippocampus shows very strong staining of an individual large cell (also found in the non-granular region), cribriform staining of most granular cells, and a few smaller negative cells, magnification × 480. Panel C, higher magnification of the dentate gyrus shows weak positive staining in only a few cells, magnification × 480.
Comparison of Neuroserpin Activity and Expression to tPA Activity and Expression—A comparison of neuroserpin inhibitory activity and expression with that of PAI-1 and PN-1, suggests that neuroserpin may have a biological role distinct from these other serpins. Whereas neuroserpin reacts ~30-fold slower with tPA than does PAI-1, its rate of 6.2×10^5 M⁻¹ s⁻¹ is ~20-fold faster than that of PN-1. In addition, neuroserpin’s primary target enzyme would appear to be tPA, since its rate of inhibition of tPA is approximately 25-fold faster than its rate of inhibition of uPA (Fig. 1 and Table I). In contrast, PAI-1 inhibits uPA and tPA with essentially the same rate, whereas PN-1 reacts with uPA ~5-fold faster than it does with tPA (27). Finally, unlike PAI-1 and PN-1, neuroserpin’s inhibition of thrombin is not stimulated by heparin (28, 29) (data not shown). The tissue distribution of neuroserpin is also very different from that of PAI-1 and PN-1. For example, PAI-1 expression is rarely detectable in the adult mouse brain (30), whereas PN-1 expression is generally similar to that of neuroserpin (7), this apparent overlapping expression may not extend to discrete cell types within the brain. Neuroserpin expression is primarily restricted to neuronal cells (Fig. 5), whereas PN-1 expression appears diffuse throughout the entire adult mouse brain in a pattern that was suggested to be due to glial cell transcription (31). Moreover, the highest levels of neuroserpin protein do not correlate with PAI-1 and PN-1, but instead appear to be found in sites of tPA expression. For example, tPA is expressed at high levels in the ventricular ependyma, brain stem, hypothalamus, hippocampus, and Purkinje neurons of the cerebellum in adult rodents (7–9, 11, 32), whereas tPA message has been detected in all of the fields of the hippocampus, suggesting the existence of a specific tPA inhibitor in CA1 (7, 11, 32). Fig. 6A shows that the highest levels of neuroserpin in the hippocampus are in the CA1 field suggesting that neuroserpin may be this unidentified inhibitor.

The role of neuroserpin in such complex processes as learning and neuronal survival is not yet clear. However, it seems likely that proteolytic activity in general, and tPA in particular, can contribute to neuronal plasticity and/or death through various mechanisms including cell motility and synaptic remodeling and that in these dynamic processes there is likely to be a critical balance between the proteolytic and inhibitory activities. Given its specificity and localization, our data suggest that neuroserpin may be central to the regulation of these complex processes.

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