Neuroserpin Binds Aβ and Is a Neuroprotective Component of Amyloid Plaques in Alzheimer Disease*

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Alzheimer disease is characterized by extracellular plaques composed of Aβ peptides. We show here that these plaques also contain the serine protease inhibitor neuroserpin and that neuroserpin forms a 1:1 binary complex with the N-terminal or middle parts of the Aβ1–42 peptide. This complex inactivates neuroserpin as an inhibitor of tissue plasminogen activator and blocks the loop-sheet polymerization process that is characteristic of members of the serpin superfamily. In contrast neuroserpin accelerates the aggregation of Aβ1–42 with the resulting species having an appearance that is distinct from the mature amyloid fibril. Neuroserpin reduces the cytotoxicity of Aβ1–42 when assessed using standard cell assays, and the interaction has been confirmed in vivo in novel Drosophila models of disease. Taken together, these data show that neuroserpin interacts with Aβ1–42 to form off-pathway non-toxic oligomers and so protects neurons in Alzheimer disease.

Alzheimer disease is the most common form of dementia. The pathological features are characterized by neurofibrillary tangles and extracellular Aβ plaques. The plaques are composed of 42 (Aβ1–42) and to a lesser extent 40 (Aβ1–40)-amino acid fragments of the amyloid precursor protein (1). Overproduction of the more aggregatory Aβ1–42 peptide is believed to cause neuronal dysfunction and death in most sporadic and familial forms of Alzheimer disease. Although insoluble plaques of Aβ1–42 are a classic feature of the brains of patients with Alzheimer disease, these plaques are also present in some healthy, elderly individuals (2). This discrepancy, and the observation that soluble Aβ is a better marker of cognitive decline (3), has led to the proposal that mature amyloid plaques are an end stage aggregation product and that the directly neurotoxic species occur earlier in the aggregation pathway (4, 5). The description of soluble oligomers of Aβ that can cause neuronal dysfunction and death (4–6) has emphasized the importance of understanding the pathways and kinetics of Aβ aggregation. The presence of ancillary proteins that interact with Aβ may stabilize particular aggregation intermediates or seed-specific patterns of aggregation that have distinct toxic properties. Of particular interest in this regard are several proteins that are associated with β amyloid plaques such as apolipoprotein E (7) and the serine protease inhibitor (serpin) α1-antichymotrypsin (8). The role of these proteins is underscored by the finding that the E4 polymorphism of apolipoprotein E is the most powerful genetic risk factor for the development of sporadic Alzheimer disease (9, 10), most likely because it accelerates the deposition of Aβ in the brain (11–13). α1-Antichymotrypsin is also found in the majority of senile plaques (8), with cerebrospinal fluid concentrations being consistently raised in patients with Alzheimer disease (14). Depending on the relative molar ratio, α1-antichymotrypsin may accelerate or inhibit the aggregation of the Aβ peptide in vitro (15, 16).

We have recently shown that mutants of the neuron-specific serpin, neuroserpin, underlie a novel inclusion body dementia that we have called familial encephalopathy with neuroserpin inclusion bodies (17–21). Wild-type neuroserpin is expressed throughout the nervous system and inhibits the serine protease tissue plasminogen activator (tPA)4 (19, 22, 23). tPA plays a critical role in neural development, synaptic plasticity, and memory (24), and its levels rise in animal models of both stroke and epilepsy (25–27). Neuroserpin expression is up-regulated in the neurons surrounding the ischemic core in experimental stroke, and its therapeutic administration in animal models reduces infarct size and apoptosis in the stroke penumbra (25). Neuroserpin administration has also been shown to attenuate seizure progression in animal models of epilepsy (27).

In view of the neuroprotective role of neuroserpin we have assessed whether it may be important in Alzheimer disease. We show here that neuroserpin is a plaque-associated protein in the brains of patients with Alzheimer disease and that it forms a specific binary complex with the Aβ peptide. The neuroprotective consequences of this interaction are demonstrated in cell culture and Drosophila models of disease.

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4 The abbreviations used are: tPA, tissue plasminogen activator; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; MITT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
EXPERIMENTAL PROCEDURES

Materials—Chemicals and buffers, Dulbecco’s modified Eagle’s medium (DMEM), horse serum, fetal bovine serum, penicillin and streptomycin, B27 supplement, and thioflavin T were all from Sigma. PC12 (rat pheochromocytoma) cells were from the American Type Culture Collection (Manassas, VA). Unless otherwise stated, all experiments were carried out in PBS. The Aβ1-40 peptides substituted with proline residues were obtained from the Keck Biotechnology Center, Yale University.

Immunohistochemistry—Immunostaining was performed on tissue fixed in 10% w/v buffered formalin for at least 2 weeks and embedded in paraffin before staining. Routine histological procedures were employed throughout, and staining used the streptavidin-biotin methodology and 3,3’-diaminobenzidine, with or without nickel enhancement, as the chromagen. The monoclonal 6F/3D (Novoceastra Laboratories Ltd., UK) was used to detect the Aβ peptides, and neuroserpin was detected using an affinity-purified, polyclonal rabbit antiseraum (23, 28).

Preparation of Aβ—To prepare monomeric Aβ, 1-mg aliquots of lyophilized Aβ1-40 or Aβ1-42 (Bachem Ltd., UK) were dissolved in 1 ml of anhydrous trifluoroacetic acid (Sigma, UK) and then dried. The peptides were then dissolved in 1 ml of 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma), divided into 40-μl aliquots, dried thoroughly in a rotary evaporator at room temperature, and stored at −80 °C.

Expression and Purification of Neuroserpin—Wild-type recombinant neuroserpin was expressed with a 6-histidine tag at the N terminus in the pQE81L vector in Escherichia coli SG13009 (pREP4) cells and purified to homogeneity as described previously (19–21). The resulting protein was dialyzed into PBS, or into DMEM for use in cell experiments, concentrated, and then stored at −80 °C.

Assessing the Interaction between Aβ Peptides and Neuroserpin—The effect of Aβ1-40 and Aβ1-42 peptides on neuroserpin polymerization was determined by incubating 0.8 mg/ml recombinant neuroserpin at 45 °C in the presence or absence of a 10- or 50-fold molar excess of Aβ1-40 or Aβ1-42. Neuroserpin polymerization was also assessed in the presence or absence of N- and C-terminal fragments of the Aβ1-42 peptide and with mutants of the Aβ1-40 peptide that contained proline substitutions (F20P, I31P, and M35P). Samples were taken at various time points and snap frozen before being assessed by 7.5% (w/v) non-denaturing PAGE.

The formation of an SDS-stable complex between neuroserpin and tPA was demonstrated by co-incubating 2 μM neuroserpin with 2 μM tPA (Calbiochem) in PBS for 5 min at room temperature. The effect of Aβ1-42 on complex formation was assessed by incubating neuroserpin with or without a 10X molar excess of Aβ1-42 for 24 h at 37 °C, followed by a 5-min incubation with tPA at room temperature. To control for any effect of Aβ1-42 on tPA, 2 μM neuroserpin, 2 μM tPA, and 20 μM Aβ1-42 were incubated together for 5 min. The reaction between neuroserpin and tPA was stopped by the addition of 1.5-dansyl-Glu-Gly-Arg-chloromethylketone (1 mM final concentration, Calbiochem) to inhibit any residual free tPA (29). Following the final incubation in each condition, the samples were snap frozen in liquid nitrogen and stored at −80 °C until the completion of the experiment. The samples were then mixed with SDS-PAGE loading buffer and boiled for 5 min, and the proteins were separated on a 10% w/v SDS-PAGE gel and visualized by silver staining.

N-terminal Amino Acid Sequencing—Recombinant neuroserpin (20 μM) was incubated with a 40X molar excess of Aβ1-42 for 30 h at 37 °C. The samples were then resolved by 7.5% (w/v) non-denaturing PAGE and electroblotted onto polyvinylidene difluoride (Immobilon, Millipore Corp.). Following transfer the polyvinylidene difluoride membrane was stained with Ponceau-S to visualize the bands. The bands containing the neuroserpin-Aβ1-42 complex was cut out and analyzed by quantitative N terminal amino acid sequencing.

Assessment of Aβ Aggregation with Thioflavin T—230 μM Aβ1-42 was incubated with a range of concentrations of neuroserpin (0, 2, 8, and 12 μM) in PBS at 37 °C. At various time points, up to 30 min, a 2-μl aliquot from each incubation mixture was diluted into 298 μl of 20 μM thioflavin T in 50 mM glycine, pH 8.5. Fluorescence was measured in a 2-mm path length quartz cuvette in an LS50B luminescence spectrophotometer (PerkinElmer Life Sciences). The excitation and emission wavelengths were 450 and 485 nm, respectively, with a 2.5 nm band pass for excitation and 15 nm for emission.

Electron Microscopy—Aβ1-42 and neuroserpin were incubated as described above for the thioflavin T experiments. At various time points aliquots were removed and either diluted 1:1 with buffer and placed directly on copper grids or centrifuged at 10,000 × g for 10 min, and the pellets were placed on the grids. The grids were negatively stained with 2% w/v uranyl acetate in water and then examined and photographed using a Hitachi H7100 microscope operated at 75 kV.

Cell Culture Experiments—Rat pheochromocytoma (PC12) cells were maintained in DMEM with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamate. The cells were plated at a density of 5000–7000 cells/well in a 96-well plate at 37 °C in 10% v/v CO2 until 70–80% confluency was achieved. Primary cortical neurons from E14 rat embryos were obtained as previously described (30) and cultured in B27 media (DMEM, 1% (w/v) penicillin/streptomycin, 2% v/v B27 supplement), plated at a density of 1000 cells/well in a 96-well plate and incubated at 37 °C in 5% v/v CO2 for 48 h. Aβ1-42 peptide (final concentration, 50 μM) and a range of concentrations of neuroserpin (both in DMEM) were then added to the cell media of the PC12 cells and the primary cortical rat embryo neurons followed by further incubation at 37 °C for 72 and 40 h, respectively. Mitochondrial activity of the cells was determined by the MTT assay. Quantification of cell death was performed using spectrophotometric measurement at 540 nm with a reference wavelength of 690 nm for the PC12 assays and 570 nm for the primary rat cortical neuronal culture assays.

Transgenic Flies—Wild-type neuroserpin with its human secretion signal peptide and the Aβ1-42 peptide, fused to a secretion signal peptide from the Drosophila necrotic gene (31) were cloned into the GAL4-responsive pUAST expression vectors. The lines expressing wild-type neuroserpin had the transgene inserted on chromosomes 2 (NS16) and 3 (NS9). In the
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Figure 1. Expression of Aβ₁₋₄₂ (a, d, and e) and neuroserpin (b, c, d, and f) in the CA1 region of the hippocampus (asterisks) of brain tissue from patients with Alzheimer disease. Using the 6F/3D monoclonal antibody, Aβ₁₋₄₂ was seen in amyloid plaques. Using a polyclonal antiserum against recombinant human neuroserpin (b, brown stain, main figure ×40 magnification, and inset ×200) we observed neuroserpin primarily at the periphery (arrowheads) of amyloid plaques (arrow). Double staining for both Aβ₁₋₄₂ (d, dark brown) and neuroserpin (d, light brown, arrowhead) demonstrated co-localization of neuroserpin and Aβ₁₋₄₂ expressing lines the inserts were on chromosomes 2 (Alz1) and 3 (Alz2). Flies co-expressing neuroserpin and Aβ₁₋₄₂ were generated by crossing the respective single transgenic lines. The lines containing the GAL4-responsive constructs were then crossed with various drivers (GAL4⁺d, GAL4⁺Sc, GAL4⁺GMR, GAL4⁺ve) to drive expression in various tissues (da and ActScc widespread, and GMR and sev primarily in the eye). Hatching frequency observation and scanning electron microscopy were both performed on flies cultured at 25 °C and 29 °C as described previously (32).

Results

Neuroserpin Antibodies Selectively Label Aβ Deposits in Alzheimer Disease—Neuroserpin was detected in the brains of seven patients with sporadic Alzheimer disease using a polyclonal antiserum to recombinant human neuroserpin. The relative distributions of neuroserpin and β-amyloid peptides were compared using the 6F/3D monoclonal antibody that detects Aβ peptides (Fig. 1). Both neuroserpin and Aβ were detected at high levels in the CA1 region of the hippocampus; Aβ was present as plaques (Fig. 1a); however, neuroserpin (Fig. 1b) demonstrated a widespread intra-neuronal distribution with some puncta of peri- and intra-neuronal staining. This discrete staining for neuroserpin was only seen in association with plaques in tissue from patients with Alzheimer disease and was not seen in control tissue. Neuroserpin (Fig. 1c, brown stain) was found at the periphery of the amyloid core (arrow) of plaques, associated with dystrophic neurites and swollen axonal processes (arrowheads). Staining of the amyloid plaques for both Aβ and neuroserpin (Fig. 1d) demonstrated co-localization of the Aβ peptide (darker stain) with neuroserpin (lighter stain, arrowheads) within the plaque. The co-localization of neuroserpin to plaques was confirmed by staining consecutive sections of Alzheimer disease brain tissue for neuroserpin and Aβ. Plaques were stained for neuroserpin (Fig. 1e, arrow and arrowhead), and the corresponding plaques were stained for Aβ in the adjacent section (Fig. 1f). At higher power the presence of Aβ and neuroserpin within a single plaque was clearly seen (Fig. 1, e and f, insets).

Aβ₁₋₄₂ Abolishes the Protease Inhibitory Activity of Neuroserpin by the Formation of a 1:1 Molar Complex—To determine whether the localization of neuroserpin to β-amyloid plaques represents a specific interaction between the serpin and the Aβ peptide, we investigated the functional consequences of co-incubating pure solutions of protein and peptide. The protease inhibitory activity of neuroserpin, in common with the other serine proteinase inhibitors (serpins), requires the insertion of the reactive center loop (Fig. 2, a and b, yellow) into β-sheet A (Fig. 2, a and b, red) following the initial interaction with the protease. Reactive loop peptide insertion is energetically favorable and allows the subsequent deformation and inhibition of the protease (Fig. 2, a and b, blue and purple) (33).
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Exogenous peptides such as a synthetic 12-mer peptide, corresponding to the reactive loop of the serpin (Fig. 2c, arrow) or the N terminus of the $\beta_{1-42}$ peptide (Fig. 2d), may also anneal to $\beta$-sheet A, resulting in the loss of protease-inhibitory activity (34). Consequently we investigated the effect of $\beta$-peptides on the inhibitory activity of neuroserpin toward its cognate protease tPA. Normally 2 $\mu$M neuroserpin (Fig. 3, lane 1) reacts with equimolar tPA (Fig. 3, lane 2) resulting in a high molecular mass SDS-resistant serpin-enzyme complex (Fig. 3, lane 3, arrowhead). In contrast, preincubation of the neuroserpin with a 10$\times$ molar excess of $\beta_{1-42}$ at 37 °C for 24 h before adding tPA abolished the serpin-enzyme complex (Fig. 3, lane 4). As a control we showed that tPA is not inhibited by $\beta_{1-42}$, because when tPA, neuroserpin, and a 10$\times$ molar excess of $\beta_{1-42}$ were mixed simultaneously, the high molecular weight neuroserpin-tPA complex was still observed (Fig. 3, lane 5). These data show that $\beta_{1-42}$ irreversibly inactivates neuroserpin. The migration profile of neuroserpin was also modified by preincubation with $\beta_{1-42}$ such that the tPA-cleaved neuroserpin species (Fig. 3, lanes 3 and 4, arrows) is no longer seen (Fig. 3, lane 5).

To determine the stoichiometry of the neuroserpin-$\beta_{1-42}$ complex, 20 $\mu$M neuroserpin was incubated with a 40$\times$ molar excess of the $\beta_{1-42}$ peptide for 30 h at 37 °C. The samples were resolved by non-denaturing PAGE, and the neuroserpin band was analyzed by quantitative N-terminal amino acid sequencing. Two sequences were identified: 3.0 pmol of MRGSHH (consistent with the N-terminal end of the His-tagged neuroserpin protein) and 3.5 pmol of DAEFRH (the first six amino acids of the $\beta_{1-42}$ peptide). Thus the neuroserpin-$\beta_{1-42}$ complex has a 1:1 stoichiometry.

$\beta_{1-42}$ Prevents the Loop-sheet Polymerization of Neuroserpin—The patency of $\beta$-sheet A is also required for a competing biophysical process, termed loop-sheet polymerization. As with other serpins, neuroserpin will form homopolymers when incubated at elevated temperatures (19−21) (Fig. 4, a and b). The insertion of an exogenous peptide into $\beta$-sheet A of serpins blocks the formation of loop-sheet polymers; however, to date, this has not been demonstrated for neuroserpin (19). Consequently we co-incubated a 50$\times$ molar excess of $\beta_{1-40}$ (Fig. 4a, arrowhead) or $\beta_{1-42}$ (Fig. 4b, arrowhead) with 0.8 mg/ml neuroserpin at 45 °C and assessed the effect on the rate of neuroserpin polymerization using non-denaturing PAGE (Fig. 4, a and b) with densitometry (Fig. 4, c and d). Both $\beta_{1-40}$ and $\beta_{1-42}$ significantly inhibited neuroserpin polymerization at
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![Image](image-url)

**FIGURE 4.** Aβ₁₋₄₀ and Aβ₁₋₄₂ inhibit the polymerization of neuroserpin. Neuroserpin (0.8 mg/ml) in PBS was incubated alone (--) or in the presence (+) of a 50 molar excess of Aβ₁₋₄₀ (a, arrowhead) or Aβ₁₋₄₂ (b, arrowhead) over a 10-h time period at 45 °C. Samples were taken at various time points and visualized by 7.5% non-denaturing PAGE. Co-incubation of neuroserpin with Aβ₁₋₄₀ or Aβ₁₋₄₂ slowed polymerization of neuroserpin and stabilized the monomer (arrows, comparing the -- lane with the + lane for each time point). Densitometry shows the intensity of the polymer bands as compared with the monomer band for neuroserpin incubated in the presence (□) and absence (△) of Aβ₁₋₄₀ (c) or Aβ₁₋₄₂ (d). The data are representative of three experiments.

all time points there was more monomeric neuroserpin (arrows) and less polymer in the presence of Aβ compared with neuroserpin alone (Fig. 4, a and b, + lanes versus -- lanes, and c and d, squares versus triangles). Polymerization was also inhibited by a 10× molar excess of Aβ₁₋₄₂, but the degree of inhibition was less marked (data not shown). The specificity of the Aβ₁₋₄₂-neuroserpin interaction was confirmed by co-incubating Aβ₁₋₄₂ with another serpin, α₁-antitrypsin. In contrast the polymerization of α₁-antitrypsin was not inhibited by a 50× molar excess of Aβ₁₋₄₂ at 50 °C and 55 °C (data not shown).

The β-sheet A of neuroserpin can only accommodate 12 amino acids (34, 35) of the Aβ peptide. N-terminal and middle fragments of Aβ (Aβ₁₋₁₆, Aβ₁₅₋₂₁, and Aβ₂₂₋₃二代) but not C-terminal fragments (Aβ₂₃₋₄₂) of the Aβ peptide were able to block neuroserpin polymerization when present in 50× molar excess of the Aβ peptides. Thus neuroserpin dramatically alters the course of Aβ polymerization so that the dominant product, although thioflavin T-positive, appears amorphous and not fibrillar.

**Neuroserpin Reduces the Cytotoxicity of Aβ**

*In Cell Culture*—The toxicity of Aβ is dependent on the size and conformation of its aggregated species. Mature amyloid is typically composed of unbranched, 10 nm diameter fibrils; however, oligomerization of Aβ is more toxic than its fibrillar form (36). Neuroserpin reduces the toxicity of Aβ in cell culture (Fig. 5b). Aβ is toxic to human neuroblastoma SH-SY5Y cells (37). Incubating SH-SY5Y cells with Aβ at 37 °C for 24 h results in a 50% reduction of cell viability, as measured by a fluorescein isothiocyanate (FITC) uptake assay (37). Incubation with neuroserpin at 37 °C for 24 h did not affect cell viability (Fig. 5b).

**Neuroserpin Modulates Aβ Polymerization**—The effect of neuroserpin on Aβ aggregation was assessed using the thioflavin T assay (37) that gives a fluorescence signal in the presence of cross-β structures and not with monomeric Aβ or amorphous aggregates. The co-incubation of neuroserpin with the Aβ₁₋₄₂ peptide at 37 °C increased the fluorescence signal over a 30-min incubation period (Fig. 6a). The increased thioflavin T fluorescence was dependent on the concentration of neuroserpin over a range of 0 and 12 μM. Control incubations with monomeric or polymeric neuroserpin alone did not give a fluorescence signal (data not shown), an expected result because serpin polymerization involves native-fold proteins and does not generate cross-β structure (38, 39). Confirmation that neuroserpin accelerates the rate of aggregation of Aβ was seen on non-denaturing PAGE gels as the more rapid loss of Aβ (Fig. 5, Aβ band, lane 4 versus 5).

Unexpectedly, electron microscopic inspection of the aggregates revealed that 12 μM neuroserpin markedly suppressed the generation of Aβ fibrils over a range of incubation times and sample preparation protocols (1 mg/ml Aβ, at 60 min for Fig. 6, b and c, applied directly to the grid; at 60 min for Fig. 6, d and e, centrifuged pellet applied to the grid). Thus neuroserpin dramatically alters the course of Aβ polymerization so that the dominant product, although thioflavin T-positive, appears amorphous and not fibrillar.
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Aβ has a variety of conformations, including beads-on-a-string, spheres, and rings (4–6, 40–42). Our finding that neuroserpin accelerates the aggregation of Aβ did not immediately predict either a neuroprotective or neurotoxic role for neuroserpin. Consequently, we used two cell culture models to determine the effect of neuroserpin on Aβ toxicity. Firstly, we treated cultures of PC12 rat pheochromocytoma cells with Aβ and used the MTT colorimetric assay to demonstrate impairment in metabolic activity. In the presence of 50 μM Aβ, PC12 cells showed a 40% reduction in the MTT signal; however, when we co-incubated the PC12 cells with 50 μM Aβ and increasing concentrations of neuroserpin (4–16 μM) we observed a concentration-dependent reduction in cytotoxicity (Fig. 7a). At a concentration of 16 μM neuroserpin the majority of the toxicity of Aβ was reversed (p < 0.01). In contrast neuroserpin alone had a mild toxic effect. We then confirmed the protective role of neuroserpin by repeating the experiments using primary cultures of E14 rat embryonic cortical neurons (Fig. 7b). In this system 50 μM Aβ resulted in a 60% reduction in the MTT signal and neuroserpin was able to rescue the cells in a concentration-dependent manner with 40% of the toxicity being reversed following treatment with 18 μM neuroserpin (p < 0.01). Neuroserpin alone was not toxic to rat embryonic cortical neurons.

In Vivo Demonstration of an Interaction between Neuroserpin and Aβ in D. melanogaster—We confirmed that the Aβ neuroserpin interaction occurs in vivo using transgenic Drosophila melanogaster. Flies expressing the wild-type human neuroserpin gene under the control of the GAL4-UAS system were generated and characterized. Expression of wild-type neuroserpin under control of the strong GAL4Act5c (strong ubiquitous expression) and GAL4Daughterless (moderate ubiquitous expression) drivers was lethal for developing embryos. Likewise, expression of wild-type neuroserpin in the retina using GAL4GMR at 29 °C resulted in a rough eye phenotype associated...
recombinant PP-neuroserpin expressed in E. coli demonstrated that, despite being correctly folded, it was no longer an inhibitor of its cognate serine protease, tPA (data not shown). Having determined that the embryonic lethality of wild-type neuroserpin was due to its protease inhibitory activity we then performed experiments to determine the effect of co-expressing neuroserpin with \( \text{A}\beta_{1-42} \).

Flies expressing \( \text{A}\beta_{1-42} \) as a secreted peptide were generated and characterized (32). Flies expressing \( \text{A}\beta_{1-42} \) under the control of GAL4\(^{\text{sevenless}} \) did not exhibit embryonic lethality, and so we determined the effect of co-expressing \( \text{A}\beta_{1-42} \) with neuroserpin. An interaction with neuroserpin would be expected to block the protease inhibitory activity of wild-type neuroserpin (Fig. 3) and consequently rescue the embryonic lethality. We established combinatorial crosses in which GAL4\(^{\text{sevenless}} \) was used to drive expression of \( \text{A}\beta_{1-42} \) and wild-type neuroserpin. By counting the genotypes of newly eclosed flies we determined the fractional lethality of expressing either \( \text{A}\beta_{1-42} \) or neuroserpin alone, assuming no lethality in flies expressing neither transgene. Assuming that each protein was acting independently to cause lethality we calculated the predicted eclosion (hatching) rates for flies, expressing both transgenes. We observed that co-expression of \( \text{A}\beta_{1-42} \) with wild-type neuroserpin using the GAL4\(^{\text{sevenless}} \) driver resulted in a 5× increase in the number of adult flies eclosing as compared with expected (Fig. 8b, bar NS+\( \text{A}\beta \)). Similarly, co-expression of the neuroserpin 12-mer reactive loop peptide, which inserts into the neuroserpin β-sheet A, had a similar rescuing effect (Fig. 8b, bar NS+\( \text{A}\beta_{1-42} \)).

DISCUSSION

The aggregation of the \( \text{A}\beta \) peptide is widely regarded as the primary toxic insult in Alzheimer disease. \( \text{A}\beta \) aggregates are composed of peptides arranged in a cross-β structure (43, 44) with the individual peptides stacked in a parallel orientation (45, 46). The process of assembling an ordered aggregate is a
highly co-operative process requiring a nucleation event but also permitting propagation of specific patterns of aggregation through time. The significance of propagating folding patterns is seen most clearly in the clinical consequences of the various prion strains (47). Despite being generated from a protein with identical primary structure the different strains cause different clinical consequences (48). The concept of aggregate strains is also relevant to Alzheimer disease, because propagation of differentially folded aggregates of Aβ, with varying cytotoxic potencies, has also been observed (49). In vitro the favored pathways of ordered protein aggregation may be altered by changing simple physicochemical parameters such as solvent composition or pH (50), however, in vivo other factors such as interacting proteins are likely to be involved.

We show here that neuroserpin is present in close proximity to the Aβ peptide in plaques of patients with Alzheimer disease. We show in vitro that there is a specific molecular interaction between the Aβ peptide and neuroserpin (Fig. 2d). Support for this interaction comes from the finding of a 1:1 stoichiometry in the Aβ-neuroserpin complex. The results show that the interaction fills the β-sheet A of neuroserpin, because, after co-incubation with Aβ, neuroserpin is no longer able to inhibit tPA and to undergo loop-sheet polymerization, both of which are dependent on a patent β-sheet A (Fig. 2, a and b). The interaction with neuroserpin is likely to occur with the N-terminal and middle fragments of Aβ, because Aβ1-16, Aβ15-21, and Aβ22-32 and Aβ40 peptides with proline substitutions in their mid and C-terminal portions were equally effective at preventing neuroserpin-induced Aβ fibrillization, favoring instead the formation of small amorphous aggregates (Fig. 6b). Previous studies have shown that oligomeric forms of Aβ are toxic to neurons (53); therefore, we undertook two independent cell experiments to assess the cytotoxicity of Aβ-neuroserpin aggregates. To our surprise we found a concentration-dependent cytoprotective effect of neuroserpin on both PC12 cells and primary embryonic rat neurons. This protective effect argues against neuroserpin accelerating the formation and stabilization of a toxic oligomer and argues in favor of neuroserpin changing the pathway of Aβ aggregation toward a less toxic end point.

The specific interaction between neuroserpin and the Aβ peptide was then shown to be significant in vivo using a D. melanogaster model system. The ubiquitous expression of wild-type neuroserpin in the fly during development was lethal. Expressing a mutant form of neuroserpin with a non-functional reactive loop abolished the toxicity, demonstrating that lethality was mediated by inappropriate protease inhibition. The formation of an inactive complex between β-sheet A of neuroserpin and Aβ explains the rescue of the serpin lethality. This was underscored by the demonstration that the lethality could only be rescued by peptides that insert into the β-sheet A of neuroserpin, specifically Aβ and the peptide representing the neuroserpin 12-mer reactive loop peptide. In contrast, the reactive loop 12-mer peptide from another serpin, which does not interact with neuroserpin (19), did not rescue the lethality.

FIGURE 8. Aβ1-42 rescues the toxic phenotype of neuroserpin expression in D. melanogaster. The expression of wild-type neuroserpin under the control of the eye-specific GAL4 driver at 29 °C (strong expression) results in a rough, deformed eye with frequent necrotic degeneration (black patch, a). Co-expressing Aβ1-42 rescued neuroserpin lethality, resulting in 5× more flies hatching as compared with the number predicted (b, bar NS + Aβ, 370 flies eclosed; ***, p < 0.001 by Chi-square, representative of triplicate experiments using two independent fly lines for both Aβ1-42 and neuroserpin). Similarly, the expression of another peptide that would be predicted to interact with neuroserpin (the neuroserpin reactive loop 12-mer) resulted in a 6.5× increase in the number of flies hatching (b, bar NS + NS-RLP, 140 flies eclosed; ***, p < 0.001 by Chi-square, representative of quadruplicate experiments using two independent fly lines for NS-RLP and one fly line for neuroserpin). In contrast the co-expression of neuroserpin with a non-reactive peptide (the antithrombin reactive loop 12-mer peptide) did not suppress neuroserpin toxicity (b, bar A. Tryp + Aβ). When flies were cultured at 25 °C the eye phenotype was less severe, consisting of micro-necrotic spots (c, bar = 20 μm). Micro-necrotic spots were significantly (p < 0.001) less frequent in flies co-expressing wild-type neuroserpin and the Aβ1-42 peptide (1 spot per 62 eyes) as compared with flies expressing wild-type neuroserpin alone (22 spots per 62 eyes).
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FIGURE 9. Biophysical data predict a structure for Aβ in fibrillar aggregates (a) that consists of two areas of β-structure (residues 12–24 and 30–40) that are preceded by an unstructured region (residues 1–10) and separated by a turn (25–29). Our hypothetical structure (b) of Aβ in a binary complex with neuroserpin (NS, gray box), forces the N terminus of Aβ into a β strand conformation. This results in a propagated change in the conformation of Aβ aggregates, resulting in reduced toxicity.

Our data suggest that neuroserpin incorporates the N-terminal part of Aβ as the sixth strand within β-sheet A. Solid-state NMR studies of conventional Aβ aggregates are consistent with an unstructured N terminus (residues 1–10) that is followed by two areas of β-structure (residues 12–24 and 30–40) separated by a turn (residues 25–29) (Fig. 9a) (45, 46, 54–56). We hypothesize that forcing the N terminus of Aβ into a β conformation will change the way in which the peptide folds (Fig. 9b) and result in the propagation of an off-pathway aggregate of reduced toxicity. In this hypothesis the binary complex of neuroserpin and Aβ seeds the aggregation of further Aβ into a non-canonical conformation with greater N-terminal β-structure. This conformation is predicted to be less stable, breaking frequently and seeding further non-canonical aggregates. This in turn prevents mature fibril formation and renders the Aβ peptide less toxic to neuronal cells in Alzheimer disease.

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