In Vivo Reversal of Amyloid-β Lesions in Rat Brain

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Abstract. Cerebral amyloid-β (Aβ) deposition is central to the neuropathological definition of Alzheimer disease (AD) with Aβ related toxicity being linked to its β-sheet conformation and/or aggregation. We show that a β-sheet breaker peptide (iAβ5) dose-dependently and reproducibly induced in vivo disassembly of fibrillar amyloid deposits, with control peptides having no effect. The iAβ5-induced disassembly prevented and/or reversed neuronal shrinkage caused by Aβ and reduced the extent of interleukin-1β positive microglia-like cells that surround the Aβ deposits. These findings suggest that β-sheet breakers, such as iAβ5 or similar peptidomimetic compounds, may be useful for reducing the size and/or number of cerebral amyloid plaques in AD, and subsequently diminishing Aβ-related histopathology.

Key Words: Alzheimer disease; Amygdala; Amyloid-β; Cell shrinkage; Interleukin-1β; Microglia; Therapy.

INTRODUCTION

The histopathological characteristics of Alzheimer disease (AD) include senile plaques, reactive astrocytes and microglia, neurofibrillary tangles, neuronal shrinkage, and cell loss in various brain regions. Senile plaques are associated with neurotoxicity in AD and their main component is the Aβ peptide (1, 2). Among the affected brain regions, the number of senile plaques has been reported to be highest in the amygdala (3).

Amino acid substitutions in the central hydrophobic region of Aβ, amino acids 17–21 (LVFFA), substantially alter the peptide’s conformation and its ability to make amyloid fibrils (4–6). Therefore, a peptide homologous to this region, with a less propensity to adopt a β-sheet conformation, may interfere with fibril formation and stability. A 5-residue peptide, iAβ5 (LPFFD), was designed based on this hypothesis, where valine was replaced by proline to decrease the peptide’s propensity to adopt a β-sheet conformation (5, 7), and a charged residue, aspartic acid, was added at the C-terminus to increase solubility. We previously demonstrated that iAβ5 was capable of blocking Aβ1-42 neurotoxicity in tissue culture, and amyloid fibril formation when Aβ1-42 was co-injected with iAβ5 into the rat amygdala and the animals killed 8 d later (8). These findings in the rat brain are in part an in vitro phenomenon because Aβ and the inhibitor were mixed in a test tube before injection. Furthermore, this preliminary study only addressed whether the iAβ5 can inhibit Aβ fibril formation, rather than if it can dissolve pre-existing fibrils in vivo. The latter is clinically a more relevant question.

In this study, we injected the iAβ5 8 d after Aβ1-42 was injected into the amygdala and allowed to form amyloid fibrils. The rats were killed 8 d later. The twofold aim of the study was to determine: a) if iAβ5 can induce disassembly of pre-existing Aβ fibrils in vivo, and b) whether this leads to a reversal or prevention of Aβ-induced histopathological changes. The long-term objective is to explore if this approach may reverse the pathology in AD patients, all of whom have pre-existing Aβ deposits.

MATERIALS AND METHODS

Animals and Surgery

Male Fischer-344 rats (Taconic, Georgetown, NY) weighed 250 g to 300 g and were 3–4 months of age at the time of arrival. The animals were maintained on a 12 h light-dark cycle, had access to food and water ad libitum, and were habituated to their new environment for 2–3 wk prior to surgery. The animal care was in accordance with institutional guidelines. Surgery was performed under sodium pentobarbital (50 mg/kg, intraperitoneally [i.p.]; Abbott Laboratories, Chicago, IL) anesthesia. Atropine sulfate (0.4 mg/kg; Sigma, St. Louis, MO) and ampicillin sodium salt (50 mg/kg; Sigma) were injected subcutaneously once the animals were anesthetized. The animals received a bilateral injection into the amygdala. A Kopf stereotaxic instrument was used with the incisor bar set at 3.3 mm below the interaural line. Injection coordinates measured from the bregma and the surface of the skull (anteroposterior −3.0, mediolateral ± 4.6, dorsoventral −8.8) were empirically determined based on the atlas of Paxinos and Watson (9). A volume of 3.0 μl was administered over 6 min (flow rate 0.5 μl/min) using a CMA/100 microsyringe pump (Carnegie Medicine, AD, Sweden). The cannula was left in situ for 2 min following injection, withdrawn 0.2 mm and left for 3 min, and then slowly withdrawn completely. Following surgery the animals were placed on a heating pad until they regained their righting reflex.

Peptides

Aβ1-42 (W. M. Keck Foundation, Yale University), iAβ5 [LPFFD], CP5 [ETRGD], CP10 [ISEVKMDAEF] (Bio-Synthesis Inc., Lewisville, TX) were supplied as trifluoroacetic acid
(TFA) salts. Aβ1-42 was dissolved in dimethylsulfoxide (DMSO; Sigma) and then diluted with Nanopure® water (H₂O) to 16.7% of DMSO. Under these conditions, the Aβ appeared to be soluble. No aggregation or fibrils were visible. iAβ5 and CP10 were dissolved in H₂O. The respective vehicles (VEH1: 0.1% TFA (Pierce, Rockford, IL) in 16.7% DMSO in H₂O; VEH2: 0.05% TFA in H₂O) were made in the same manner. CP5 was not completely soluble in H₂O at the concentration used (200.0 nmol) and was, therefore, dissolved in the same manner as Aβ1-42.

Histology

For histological analyses, animals were anesthetized with sodium pentobarbital (150 mg/kg, i.p.), perfused transaortically with phosphate buffer and 4% paraformaldehyde in phosphate buffer at 16 °C postoperatively, and the brains processed as previously described (10). Serial coronal sections (40 μm) were cut and five series of sections at 0.2 mm intervals were saved for histological analysis of cresyl violet, Congo red, EM-3, and IL-1β stained sections. Selected series were also stained for GFAP and with OX-42. The sections were placed in ethylene glycol cryoprotectant and stored at −20°C until used.

Cresyl Violet and Congo Red: Mounted sections were defatted in xylene and hydrated in a gradient of ethyl alcohol and water series. Cresyl violet and Congo red staining were performed as previously described (10, 11).

EM-3, IL-1β, GFAP, and OX-42: Staining was performed as previously described (8, 10). Briefly, sections (40 μm) were incubated in EM-3 primary antibody (generously provided by Dr. Enrique Mendez) that selectively binds to Aβ 1-42 (12) at a 1:1000 dilution for 24 h at room temperature. A goat anti-rabbit IgG secondary antibody (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) was used at a 1:2000 dilution. IL-1β (1:250; Endogen, Woburn, MA) and GFAP (1:500; Dako, Denmark) staining were performed the same way as the EM-3 staining, except the secondary antibody was diluted 1:1333. OX-42 (Biosource Int., Camarillo, CA) recognizes CD11b, a rat equivalent of the human C3bi receptor (13). This antibody was used at a 1:250 dilution, and a peroxidase-linked sheep anti-mouse IgG secondary antibody (Amersham, Arlington Heights, IL) was used at a 1:200 dilution.

Data Analysis

An image analysis system (NIH Image 1.60 or Scion Image β3b) was used to determine the size of the EM-3 stained Aβ deposits and neuronal shrinkage within the left and right amygdala. The area of the entire Aβ deposition (∼50 magnification) was measured in each fifth section (0.2 mm intervals). The data was first analyzed by two-way ANOVA (Sigmastat 1.01 or GraphPad Prism 2.01). No interhemispheric statistical difference was discerned by a Newman–Keuls’ multiple range test in the size of these deposits within each treatment group and they were, therefore, combined in the subsequent analysis by one-way ANOVA, followed by a Newman–Keuls’ test for post hoc comparisons. The basolateral nucleus within the amygdala can be divided into parvicellular and magnocellular subnuclei based on the cytoarchitecture of the neurons. The cells within these subnuclei have a relatively uniform size and appearance and their boundaries are easily identified (14). The magnocellular division is located mainly rostral and medial to the parvicellular division. The area of 20 of the largest magnocellular cells (∼400 magnification) within the left and right basolateral amygdala was measured in one cresyl violet stained section per animal slightly rostral to the injection site as described previously (11, 15). We carefully chose sections at a similar coronal level (approximately at bregma−2.3 mm [9]). The average area of these 20 cells was calculated. The same statistical analysis was used as for the Aβ deposits. The rating of the IL-1β stained sections was based on the extent of IL-1β positive microglia associated with the Aβ deposits (0, no or very few microglia; 1+, moderate number of microglia; 2+, several microglia). These data were analyzed using Kruskal–Wallis test followed by Dunn’s multiple comparison test for post hoc analysis (Graphpad Prism 2.01). The correlation between the size of the Aβ deposits and shrinkage of magnocellular neurons within the amygdala was analyzed using the Pearson product–moment correlation.

RESULTS

Disassembly of Aβ Fibrils in Rat Brain

Young male F344 rats were injected bilaterally into the amygdala as shown in Table 1. Image analysis was performed on brain sections stained with EM-3, a polyclonal antibody that recognizes Aβ (12). Two-way analysis of variance (ANOVA) revealed a treatment effect (p = 0.0001) on the size of the Aβ deposits (Fig. 1). We have previously verified in this model the fibrillar nature of the Aβ deposits by immunoelectroscopy (8). There was no difference between the hemispheres in deposit size and, therefore, the data from each hemisphere were combined for further analysis. All the rats injected with Aβ1-42 followed 8 d later by VEH2 or iAβ5 had Aβ deposits, but the size of the deposits was smaller in rats that were reinjected with iAβ5 (100.0 nmol: 29% reduction, p < 0.05, n = 9; 200.0 nmol: 67% reduction, p < 0.001, n = 8), than in animals reinjected with VEH2 (n = 9). Congo red positive deposits were observed in all groups.

<table>
<thead>
<tr>
<th>Number of rats (n)</th>
<th>Treatment 1 (Day 0)</th>
<th>Treatment 2 (Day 8)</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>Aβ1-42 (5.0 nmol)</td>
<td>VEH2</td>
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<tr>
<td>9</td>
<td>Aβ1-42 (5.0 nmol)</td>
<td>iAβ5 (100.0 nmol)</td>
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<tr>
<td>8</td>
<td>Aβ1-42 (5.0 nmol)</td>
<td>iAβ5 (200.0 nmol)</td>
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<td>4</td>
<td>VEH1</td>
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<td>6</td>
<td>VEH1</td>
<td>iAβ5 (200.0 nmol)</td>
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Treatment 1: The rats were injected bilaterally into the amygdala with Aβ1-42 (5.0 nmol/3.0 μl, n = 26), or vehicle 1 (VEH1) [3.0 μl of 0.1% trifluoroacetic acid (TFA) in 16.7% DMSO in H₂O, n = 10]. Treatment 2: The animals were reinjected into the amygdala 8 days postoperatively with iAβ5 [LPFFD; 100.0 nmol/3.0 μl, n = 9; 200.0 nmol/3.0 μl, n = 14], or VEH2 [3.0 μl of 0.05% TFA in H₂O, n = 13]. The rats were killed 8 days later.

that received Aβ injections, but the apple-green birefringence was less prominent in animals that had smaller deposits (Fig. 1). The total area of the Congo red positive Aβ deposits (1.44 × 10⁶ ± 1.3 × 10⁵ μm²; mean ± SEM) at 16 d postoperatively in rats that were not treated with iAβ5 is comparable to that observed in our previous study at 8 d postoperatively (1.20 × 10⁶ ± 1.9 × 10⁵ μm²). This observation shows the reproducibility of intracerebral Aβ deposition in this rat model and suggests that these deposits are not easily degraded. Further, iAβ5 appears to mediate disassembly of amyloid fibrils that presumably makes Aβ more susceptible to proteolytic degradation, which then leads to a reduction in the size of the Aβ deposits.

**Histological Effects of Aβ Deposits**

Cellular atrophy was examined in cresyl violet stained sections by measuring the cell area of the large magnocellular neurons within the basolateral amygdala (Fig. 2). Two-way ANOVA revealed a significant difference between treatment groups (p < 0.0001), and between hemispheres (p = 0.02). Because Newman–Keuls’ post hoc multiple comparison test did not reveal a significant difference between the hemispheres within individual treatment groups the data from each hemisphere were combined for further analysis. Post hoc analysis showed that the average neuronal cell area was significantly less in the Aβ/VEH2 treatment group (208 ± 6 μm²; mean ± SEM) relative to all other treatment groups; VEH1/VEH2 (251 ± 14 μm², p < 0.01), VEH1/iAβ5 (200.0 nmol) (257 ± 6 μm², p < 0.001), Aβ/iAβ5 (100.0 nmol) (256 ± 6 μm², p < 0.001), and Aβ/iAβ5 (200.0 nmol) (235 ± 11 μm², p < 0.05). Within individual animals, there was a significant negative correlation between the area of the Aβ deposits and the average area of the magnocellular neurons (r = −0.32, p = 0.01). Therefore, the neuronal shrinkage is related to Aβ deposition and can be prevented and/or reversed by iAβ5.

Injections of the high dose of iAβ5 (200.0 nmol) 8 d following VEH1 infusions resulted in Congo red- and EM-3-negative deposits that appeared to be smaller than the Aβ deposits and were associated with physical displacement of tissue. However, this did not appear to lead to histopathological changes greater than those caused by the cannula placement.

Other histopathological characteristics of AD are reactive astrocytes and microglia. Because of the experimental approach of using two bilateral injections per animal over 8 d and the killing of these animals 8 d later, an extensive nonspecific astrogliosis was expected due to the cannula placement. This was confirmed by staining for glial fibrillary acidic protein (GFAP) on selected series. Therefore, we did not attempt to evaluate if there was a difference in astrogliosis between the treatment...
groups. We focused instead on microglial activation because these cells are likely important for proteolytic degradation of Aβ (16). Sections were stained for interleukin (IL)-1β, which is a cytokine that is predominantly expressed in microglia within the CNS. Some IL-1β positive microglia-like cells were seen along the cannula track in all treatment groups. These cells were also always seen surrounding the Aβ deposits in rats that were reinjected with a vehicle solution, to a lesser extent in animals reinjected with 100.0 nmol of iAβ5, and were virtually absent in rats that were reinjected with 200.0 nmol of iAβ5 (Fig. 3). Kruskal-Wallis test revealed a significant difference between these three treatment groups in the extent of IL-1β positive microglia associated with the Aβ deposits (Fig. 3, p = 0.0005). Post hoc analysis with Dunn’s multiple comparison test showed a significant difference between Aβ/iAβ5-injected (100.0 nmol) rats (p < 0.05) and Aβ/iAβ5-injected (200.0 nmol) rats (p < 0.01), compared with Aβ/VEH2-treated animals. In addition to these findings, a few phagocyte-like anti-Aβ antibody (EM-3) positive cells were associated with the Aβ deposits and appeared to be in greater numbers in animals with the smaller deposits (data not shown). The above data suggest that in Aβ-treated rats reinjected with iAβ5, there is a dose-related effect on microglial phagocytosis and their expression of IL-1β. Following microglial activation there is a transformation of resting microglia into activated cells, which in their end stage resemble phagocytic cells. It is likely that expression of various proteins such as IL-1β changes during this activation process and may be at low levels in their phagocytic stage. This was confirmed by staining of selected sections with OX-42, which recognizes a complement receptor on microglia (13). Numerous OX-42 positive phagocytic cells were observed at the injection site in Aβ/iAβ5-treated (200.0 nmol) rats, and to a lesser extent in Aβ/iAβ5-treated (100.0 nmol) and Aβ/VEH2-treated rats. Virtually no OX-42 staining was observed in VEH1/VEH2-treated rats (data not shown). Thus, these observations suggest that disassembled Aβ fibrils are cleared by microglia, and that the neuronal shrinkage within the basolateral nucleus adjacent to the Aβ deposits may be related to the enhanced microglial expression of IL-1β.
Reproducibility and Specificity

To determine the reproducibility and specificity of the iAβ5-induced disassembly of Aβ fibrils in vivo, we injected Aβ1-42 (n = 35) or vehicle (n = 6) into rat amygdala (Table 2), followed 8 d later by vehicle (n = 9), iAβ5 (n = 9) or two control peptides, CP5 (ETRGD; n = 11) and CP10 (ISEVKMDAEF; n = 12). Multiple controls were used since the extent of iAβ5 inhibition of Aβ fibril formation and disassembly using the in vitro thioflavin fluorometric assay has shown some variance (8, unpublished observations). CP10 has the sequence of a portion of the amyloid precursor molecule, including the first four amino acids of Aβ1-42; therefore it has a sequence homology to a different portion of the Aβ molecule than iAβ5. Also CP10 does not contain any amino acids that can act as a β-sheet breaker. Our preliminary findings in vitro and in cell culture suggest that CP10 does not interfere with Aβ fibrillogenesis, fibril disassembly, or Aβ mediated toxicity. CP5 is 5 amino acids as iAβ5 and does not have a sequence homology to Aβ or any amino acids that can act as a β-sheet breaker. Two-way ANOVA revealed a treatment effect (p = 0.003) on the size of the Aβ deposits. There was no difference between the hemispheres in deposit size and, therefore, the data from each hemisphere were combined for further analysis (Fig. 4). The lack of a difference between hemispheres is an indication of the reproducibility of the methods. The iAβ5-induced disassembly of the Aβ deposits (60% reduction, p < 0.01) was comparable to our previous observation (67% reduction, Fig. 1G). Furthermore, the control peptides that do not have a sequence homology to the hydrophobic region of Aβ did not affect the size of the Aβ deposits. Sections from rats injected first with a vehicle followed 8 d later by either control peptide (n = 6) did not stain with EM-3, the antibody against Aβ. Overall, the effects of iAβ5 are highly reproducible and appear to be specific since the control peptides had no effect.

DISCUSSION

Our findings demonstrate that iAβ5 induces Aβ fibril disassembly in pre-existing Aβ deposits in this rat model of cerebral amyloidosis. Furthermore, we show that the effect is reproducible and appears to be specific. This data raises the possibility that peptides or peptidomimetics with properties similar to iAβ5 could be used as a therapeutic approach for the treatment of AD-related cerebral amyloidosis.

In vitro studies indicate that Aβ develops resistance to proteolytic degradation when polymerized into fibrils (17). Our in vivo findings support this because the size of the fibrillar Aβ deposits is similar at 8 d (8 vs 16 d post-injection (Figs. 1G, 4). Also fibrillar amygdaloid Aβ25-35 deposits are not cleared 8–128 d postoperatively (11). Given the importance of the central hydrophobic cluster (amino acids 17–21; LVFFA) of Aβ for fibril formation (4–6), this region may also be important for fibril stability. Aβ disassembly mediated by iAβ5 is presumably based on its ability to bind to this region of the Aβ molecule and thereby destabilizing the interaction between Aβ monomers and/or oligomers that is necessary for fibril stability. The subsequent loss of fibril integrity may then lead to exposure of cleavage sites that facilitates proteolytic processing and removal of Aβ. Alternatively, iAβ5 bound to Aβ may act as an immunocomplex that activates microglia to a greater extent than Aβ fibrils alone. This scenario may then result in enhanced phagocytosis and subsequent removal of Aβ.

The Aβ-induced neuronal shrinkage did not appear grossly to be associated with extensive neuronal loss resembling the effects of an Aβ fragment, Aβ25-35, within this same brain region (11). Neuronal shrinkage and cell loss is prominent in various brain regions in AD, including the amygdala. These findings show that Aβ deposition is associated with neuronal shrinkage and this may simulate the initial histopathological changes that occur.
in AD. Furthermore, this neuronal atrophy can be prevented and/or reversed by intracerebral injection of a β-sheet breaker peptide.

The phagocytes responsible for the clearance of Aβ in this particular model are likely to be both microglia and macrophages. Intracerebral cannula placement transiently disrupts the blood-brain barrier allowing infiltration of hematogenous macrophages into the brain. Macrophages have been implicated in the slow clearance of amyloid in AD brains with infarcts (18). Microglia are known to associate with amyloid plaques (19) and inflammatory cytokines found in senile plaque regions include IL-1β. Microglia seem to be the major source of IL-1 within the CNS but this cytokine is also expressed in astrocytes and neurons (20). Aβ in vitro enhances astroglial and microglial secretion of IL-1 and stimulates the proliferation and morphological transformation of microglia (21). In addition, Aβ25-35 in vivo appears to induce microglial IL-1β staining (11), similar to the effects of Aβ1-42 in the present study. On the other hand, IL-1β in vitro has been demonstrated to enhance Aβ cytotoxicity in PC12 cells (22), and activation of microglia with interferon-γ and Aβ leads to neuronal cell injury in vitro (23). Numerous OX-42 positive phagocyte-like cells were observed at the injection site in Aβ/iAβ5 (200.0 nmol)-treated rats, and to a lesser extent in Aβ/iAβ5-treated (100.0 nmol) and Aβ/VEH2-treated rats (data not shown). Therefore, it can be concluded that there is a dose-dependent reduction in IL-1β microglial expression, rather than a lack of microglial activation, following intramygdaloid iAβ5 injections in rats that were previously injected with Aβ1-42 into the same brain region. Overall, these findings suggest that the neuronal shrinkage within the basolateral nucleus adjacent to the Aβ deposits may be related to the enhanced microglial expression of IL-1β, although many other potential factors are likely to be involved.

Unfortunately, every model for amyloidosis is artificial to a degree. Here, we are focusing on the effects of a β-sheet breaker peptide (iAβ5) on Aβ fibrils in vivo. In our opinion, it is appropriate to first investigate the direct in vivo interaction of iAβ5 with Aβ fibrils alone before exploring if various amyloid associated compounds (that can be present in different proportions) interfere with this phenomenon. Plaque associated substances such as proteoglycans and transforming growth factor β1 have sometimes been used to enhance amyloid deposition when Aβ1-40 is infused for weeks into rat brain (24, 25). It is likely that under those conditions these compounds may be useful for deposition to occur and/or stabilization of the Aβ. Overall, factors such as injection time (min vs days), sequence of Aβ, dose, solvent, and injection site are likely to be important for Aβ deposition and persistence of the deposits. Under our conditions, we obtain (in a very reproducible manner) fibrillar Aβ deposits at the injection site (Figs. 1, 4; refs. 8, 10, 11, 15). The injection model that we employed does not have the widespread amyloid deposition observed in aged transgenic mice. However, identical Congo red positive Aβ fibrils are observed in this rat injection model as in the other models. Also, injection of a known quantity of Aβ into a confined region that is vulnerable in AD allows for an efficient and reliable quantification of the in vivo effect of various compounds on Aβ deposit size, conformation, and associated toxicity. Histopathology, such as gliosis, has been observed in transgenic mice overexpressing the amyloid precursor protein (APP) in the absence of Aβ deposition (26). This observation and other potential effects of transgene integration and APP overexpression that are not related to AD make it difficult to evaluate in transgenic models the true histopathological effects of Aβ. However, testing of β-sheet breaker peptides or their peptidomimetics both in transgenic and Aβ injection models will be useful in investigating the importance of Aβ cerebral deposits in the pathogenesis of AD.

Recently, it was reported that immunization with Aβ1-42 attenuates AD-like pathology in a transgenic mouse model of cerebral amyloidosis (27). Theoretically, β-sheet breaker peptides that have an extensive sequence homology to Aβ may have a similar immune system mediated effect in addition to their direct effect on Aβ fibril integrity and inhibition of fibrillogenesis.

In addition to Aβ, other amyloidogenic proteins are deposited in various diseases. The concept of β-sheet breaker peptides, tailored for related conformational disorders, provides a potentially general treatment strategy for defective protein folding. Using this approach, we have recently developed short β-sheet breaker peptides that in vitro prevent and revert the prion protein conformational changes implicated in the pathogenesis of spongiform encephalopathies (unpublished observations). For therapy of diseases affecting the CNS, these compounds should ideally cross the blood-brain barrier.

A variety of compounds interfere with Aβ fibril formation in vitro, an effect that may often be nonspecific. The findings of the present study are conceptually different and therapeutically more relevant than our previous report (8) because the inhibitor is administered several days following the in vivo Aβ fibril formation. Several studies have suggested that AD neuropathology progresses for years or decades before clinical symptoms become apparent. Therefore, compounds with these properties may be valuable to reduce the size and/or number of amyloid plaques in AD patients, which may result in less neuronal loss and subsequent memory impairment.

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