to a single second-chromosome gene (*23*), and mutagenesis has produced other *D. simulans* alleles with large effects on hydrocarbon profiles (*24*). [Such polymorphism may explain the observation in *Drosophila* of intraspecific genetic variation in the propensity to initiate courtship with other species (*14*).] All this suggests that the evolution of sexual isolation in this group may sometimes be caused by changes in only a few genes (*2*). This in turn militates against the evolution of sexual isolation by long-term runaway processes, which might produce polygenic sexual isolation (*1*) and, in conjunction with the phylogenetic data, suggests scenarios beginning with the evolution of female traits or mating preferences. It is important to note, however, that other cues besides pheromones, such as auditory and visual signals, almost certainly play a role in sexual isolation among *Drosophila* species.

Finally, the mapping of the hydrocarbon differences to a chromosome does not support recent ideas that speciation is often caused by the acquisition of symbionts (*25*) but adds instead to the considerable data implicating changes in nuclear genes. *Drosophila* is a valuable group for isolating and characterizing these genes.

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26. The dried extract (*23*) was redissolved in 5 μl of n-hexane, 3 μl of which was injected into a HP 5890 Series II gas chromatograph connected to a HP 3396 Series II integrator. The temperature was increased from 210° to 250°C at 3°C/min, each run lasting 20 min. The split ratio was 50:1. Hydrocarbon peaks were identified by comigration with known standards and analysis on a VG Analytical 70–70 mass spectrometer. Depending on the sample, we detected between 15 and 25 peaks, with 7-T constituting ~48% of the total sample in *D. simulans* and 7,11-HD constituting ~25% of the total sample in *D. sechellia*. These proportions are similar to those obtained in a previous study (*9*). Absolute quantities of hydrocarbons were estimated by comparison of peak areas with those of an internal *N*-hexacosane standard added to each sample. All samples contained extract from either two flies (eight backcross genotypes) or four flies (all other samples).
27. Courtships were defined as any episode which began with a male orienting toward the female and vibrating his wings (*10*); such episodes ended when the male no longer oriented toward the female. Attempted copulations were defined as any instance in which the male curled his abdomen ventrally and attempted to mount the female. Copulations with dead females were never seen.
28. We also measured courtship duration in a separate experiment with single *D. simulans* males confined with single females from the two groups in treatment 3. We watched 45 pairs of vials for 10 min each; scoring was again blind. Pure *D. simulans* females raised under crowded conditions (controls) were courted 72 times with mean courtship length of 1.12 min (SD = 1.66). *D. simulans* females crowded with *D. sechellia* females were courted 26 times with mean courtship length of 0.33 min (SD = 0.47). Therefore, co-rearing with *D. sechellia* females significantly shortened the duration of courtship bouts (Mann-Whitney *U* test, $Z = 3.00$, two-tailed $P = 0.003$), giving further evidence for a transferable substance inhibiting courtship by *D. simulans* males.

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Rapid Induction of Alzheimer Aβ Amyloid Formation by Zinc

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$A\beta_{1-40}$, a major component of Alzheimer's disease cerebral amyloid, is present in the cerebrospinal fluid and remains relatively soluble at high concentrations (less than or equal to 3.7 mM). Thus, physiological factors which induce Aβ amyloid formation could provide clues to the pathogenesis of the disease. It has been shown that human Aβ specifically and saturably binds zinc. Here, concentrations of zinc above 300 nM rapidly destabilized human $A\beta_{1-40}$ solutions, inducing tinctorial amyloid formation. However, rat $A\beta_{1-40}$ binds zinc less avidly and is immune to these effects, perhaps explaining the scarcity with which these animals form cerebral Aβ amyloid. These data suggest a role for cerebral zinc metabolism in the neuropathogenesis of Alzheimer's disease.

The role of Aβ amyloid formation in the pathogenesis of Alzheimer's disease (AD) has been underscored by the discovery of mutations in the Alzheimer amyloid protein precursor (APP) close to or within the Aβ domain that are linked to familial AD (*1*). Aβ is found as a 40-residue peptide ($A\beta_{1-40}$) in cerebrospinal fluid (CSF) but is not found at elevated concentrations in sporadic AD cases (*2*). Synthetic $A\beta_{1-40}$ remains soluble in neutral phosphate buffer at concentrations of up to 16 mg/ml (*3*), indicating that overproduction of soluble Aβ cannot sufficiently explain Aβ precipitation. Therefore, biochemical mechanisms that promote Aβ amyloid formation in sporadic cases may be relevant to the pathogenesis of AD. We have shown that Aβ specifically and saturably binds Zn, manifesting both high-affinity binding (K_A = 107 nM) compatible with normal CSF Zn concentrations, and low-affinity binding (K_A = 5.2 μM) (*4*). Cerebral Zn homeosta-

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sis, which has been reported to be abnormal in AD, may be important for the metabolic fate of Aβ because increased concentrations of Zn promote the peptide's adhesiveness and resistance to proteolytic digestion (*4*). Moreover, the safety of oral Zn supplementation in the at-risk population has been brought into question by a study that demonstrated adverse effects on cognition in AD subjects but not in age-matched controls (*5*).

For these reasons, we studied the effects of physiological concentrations of Zn on the stability of synthetic human $A\beta_{1-40}$ in solution and compared its effects to those of the rat-mouse species of the peptide (rat Aβ). Soluble $A\beta_{1-40}$ is produced by rat neuronal tissue (*6*); however, Aβ amyloid deposition is not a feature of aged rat brains. Aβ amyloidogenesis occurs in other aged mammals with the human Aβ sequence (*7*), which is strongly conserved in all reported animal species, except rat and mouse (*8*). The rat-mouse Aβ substitutions [Arg→Gly, Tyr→Phe, and His→Arg at positions 5, 10, and 13, respectively (*9*)] appear to cause a change in the peptide's physicochemical properties sufficient to confer on the peptide its relative immunity to amyloid formation. Because Zn binding to human $A\beta_{1-40}$ is mediated by histidine (*4*), rat Aβ may be expected to manifest altered Zn-binding properties.

We studied the binding affinity of Zn to rat $A\beta_{1-40}$ (Fig. 1) in a ^{65}Zn competitive assay system used (*4*) to measure the K_A of Zn binding to human $A\beta_{1-40}$. In contrast to human $A\beta_{1-40}$ (*4*), the Scatchard analysis of Zn binding to rat $A\beta_{1-40}$ reveals only one binding association (K_A = 3.8 μM), with 1:1 stoichiometry.

We have observed that the recovery of human $A\beta_{1-40}$ in filtration chromatography is reduced in the presence of Zn, due, in part, to increased adhesiveness of Aβ (*4*). To determine whether the aggregation of human $A\beta_{1-40}$ was also enhanced in the presence of Zn, we incubated the peptide in various concentrations for 30 min with Zn^{2+} (25 μM) or EDTA and then filtered the solutions through 0.2-μm filters. The Zn caused up to 80% of the available peptide to aggregate into >0.2-μm particles (Fig. 2A) (*10*). There appears to be a shallow, negative log-linear relation between human Aβ peptide concentration and the proportion of filterable peptide in 25 μM Zn^{2+}, but even at the lowest concentration tested (0.8 μM), >70% of the human $A\beta_{1-40}$ solution aggregated. In contrast, there appeared to be no effect of Zn^{2+} on rat $A\beta_{1-40}$, with no aggregation of a 0.8-μM peptide solution detected under the same conditions and only 25% aggregation of a 4-μM solution. In the presence of EDTA, human and rat $A\beta_{1-40}$ solutions behaved indistinguishably, with no detectable aggregation observed in a peptide solution of 0.8 μM and an ~15% aggregation at higher peptide concentrations.

We next titrated the formation of >0.2-μm Aβ particles against increasing Zn concentrations (Fig. 2B) and observed a shallow response curve for human $A\beta_{1-40}$ (1.6 μM) until the Zn concentration reached 300 nM, corresponding to the saturation of high-affinity binding (*4*). At Zn concentrations above 300 nM, corresponding to low-affinity binding (*4*), human $A\beta_{1-40}$ aggregated. In contrast, rat $A\beta_{1-40}$ remained sta-

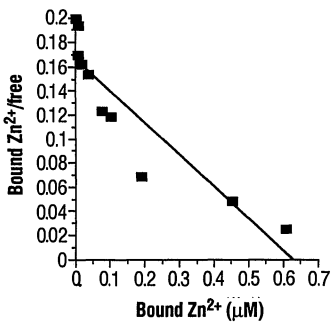

Fig. 1. Scatchard analysis of ^{65}Zn binding to rat $A\beta_{1-40}$. Dissolved peptides (1.2 nmol) were dot blotted onto 0.20-μm polyvinylidene fluoride (PVDF) membrane (Pierce) and a competition analysis was performed as described (*4*). Rat $A\beta_{1-40}$ and human $A\beta_{1-40}$ were synthesized by solid-phase fluorenyl methoxy carbonyl (FMOC) chemistry. Purification by reverse-phase high-pressure liquid chromatography and amino acid sequencing confirmed the synthesis. The regression line indicates a K_A of 3.8 μM. Stoichiometry of binding is 1:1. Although the data points for the Scatchard curve are slightly suggestive of a biphasic curve, a biphasic iteration yields association constants of 2 and 9 μM, which do not justify an interpretation of physiologically separate binding sites (these data are derived from the above Scatchard analysis).

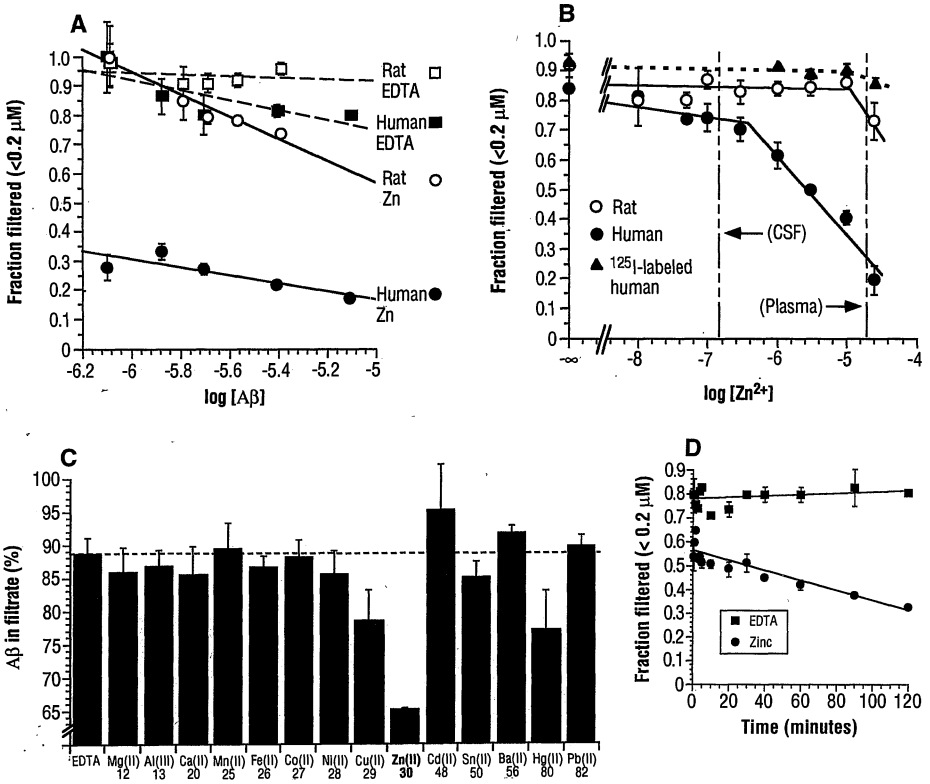

Fig. 2. Effect of Zn on human, ^{125}I-labeled human, and rat $A\beta_{1-40}$ aggregation into >0.2-μm particles. Stock human and rat $A\beta_{1-40}$ peptide solutions (16 μM) in water were filtered (Spin-X, Costar) on 0.2-μm cellulose acetate at 700*g*, brought to 100 mM NaCl and 20 mM tris-HCl at pH 7.4 (buffer 1) with or without EDTA (50 μM) or metal chloride salts, incubated (30 min, 37°C), and then filtered again (700*g*, 4 min). The fraction of the $A\beta_{1-40}$ in the filtrate was calculated by the ratio of the filtrate OD_{214} (*33*) relative to the OD_{214} of the unfiltered sample. All data points are means ± SD, n = 3, unless indicated otherwise here. (**A**) Proportion of $A\beta_{1-40}$, incubated with or without Zn^{2+} (25 μM) or EDTA (50 μM) and then filtered through a 0.2 μm filter, titrated against peptide concentration. (**B**) Proportion of $A\beta_{1-40}$ (1.6 μM) filtered through 0.2-μm filter, titrated against Zn^{2+} concentration. The ^{125}I-labeled human $A\beta_{1-40}$ (*34*) (15,000 cpm) was added to unlabeled $A\beta_{1-40}$ (1.6 μM) as a tracer, incubated, and filtered as described above. The counts per minute in the filtrate and retained on the excised filter were measured by a γ counter. Dashed lines indicate the normal physiological plasma and CSF Zn concentrations. (**C**) Proportion of $A\beta_{1-40}$ (1.6 μM) filtered through a 0.2-μm filter after incubation with various metal ions (3 μM). The atomic numbers of the metal species are indicated below each species. (**D**) Effects of Zn^{2+} (25 μM) or EDTA (50 μM) on the kinetics of human $A\beta_{1-40}$ aggregation measured by 0.2-μm filtration. Data points are means ± SD, n = 2.

ble in the presence of up to 10 μM Zn, and only at 25 μM Zn was aggregation observed.

To determine the effects of Zn on $A\beta_{1-40}$ at physiological peptide concentrations requires an assay more sensitive than spectroscopy (*11*). Thus, we attempted to characterize the effects of Zn on human ^{125}I-labeled $A\beta_{1-40}$ used as a tracer in the presence of unlabeled peptide. Unlike its unlabeled precursor, ^{125}I-labeled $A\beta_{1-40}$ (at 1.6 μM total peptide) remained stable in the presence of increasing Zn concentrations, indicating that ^{125}I-labeled $A\beta_{1-40}$ is not a suitable tracer (Fig. 2B). The tracer is iodinated on the tyrosine residue at position 10, which is a phenylalanine in the rat peptide. Thus, the tyrosine residue may be critical to the stability of the human peptide. These data may also explain why another study required relatively high concentrations of Zn^{2+} (1 mM) to precipitate ^{125}I-labeled human $A\beta_{1-40}$ in the centrifuge (*12*). Extrapolation of the curve in Fig. 2A to 0.6 nM provided an estimate of the effect of Zn on physiological Aβ concentrations (*2*) and indicated that 25% of the peptide would aggregate into >0.2 μm particles under these conditions. The specific vulnerability of human $A\beta_{1-40}$ for Zn^{2+} is indicated by the observation that Zn^{2+} is the only one of several metal ions tested on an equimolar basis, including Al^{3+}, that induced significant aggregation of human $A\beta_{1-40}$ in this system (Fig. 2C).

We next investigated the kinetics of the assembly of Zn-induced human $A\beta_{1-40}$ aggregates (Fig. 2D) (*13*). Our data indicate that after the addition of stock $A\beta_{1-40}$ in water (15.9 μM, pH 5.6) to Zn^{2+} (25 μM) in saline buffer (pH 7.4) there is a near-instantaneous destabilization of the peptide (1.6 μM final concentration) into filterable particles with two phases observed over 2 hours. The initial phase is rapid, with a half-maximal particle assembly rate of ~0.4 μM/min (Fig. 2D). The steady state of the second phase is achieved in 2 min (Fig. 2D), whereupon particle assembly proceeds at a rate of 3.2 nM/min with no evidence of saturation in 2 hours. At this rate, the available peptide would be exhausted in 5 hours of initiation. Although the addition of EDTA buffer caused the near-instantaneous destabilization of 20% of the 1.6 μM $A\beta_{1-40}$ solution into >0.2-μm particles, no further particle assembly was observed over the time course of the experiment. In comparison, human $A\beta_{1-40}$ [20 μM in phosphate-buffered saline (PBS), pH 7.4] has been reported to be stable for 10 days (*14*), and seeding the solution with $A\beta_{1-42}$ (2 μM), the more amyloidogenic Aβ species, induced aggregation of this solution that was half-maximal only after 4 to 5 days (*14*). Thus, the findings presented here represent a significantly accelerated reaction for inducing amyloid formation in vitro with the wild-type form of the main species of secreted Aβ ($A\beta_{1-40}$).

To estimate the size of the Aβ aggregates formed in the presence of Zn, we incubated $A\beta_{1-40}$ (1.6 μM) with Zn^{2+} (25 μM) or EDTA and then passed the mixture through filters with various pore sizes (Fig. 3, A and B). After incubation in EDTA, human $A\beta_{1-40}$ assembled into populations of heterogeneous particle sizes as follows: >0.1 μm, 47%; >0.22 μm, 40%; and >0.65 μm, 32%. The comparable proportions of filtered rat $A\beta_{1-40}$ particles were >0.1 μm, 36%; >0.22 μm, 27%; and >0.65 μm, 25%. After incubation with Zn^{2+} (25 μM), the proportion of >0.65-μm rat peptide particles increased only slightly; however, the proportion of >0.65-μm human peptide particles increased markedly, recruiting 82% of the available peptide. The proportions of >0.1-μm and >0.22-μm particles formed from the human $A\beta_{1-40}$ also increased by 50 and 55%, respectively, after incubation with Zn^{2+}. However, the same reaction induced only a 20 and 30% increase, respectively, in the amounts of these particles assembled from rat peptide. Only 4% of the human $A\beta_{1-40}$ incubated with Zn^{2+} remained in solution after 0.1-μm filtration. These data indicate that the human species of $A\beta_{1-40}$ differs from the rat species both in the extent and

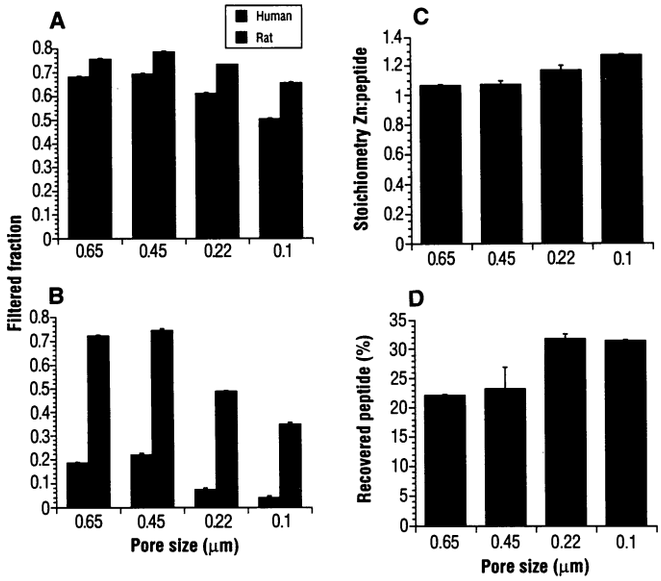

Fig. 3. Size estimation of Zn-induced Aβ aggregates. Proportion of $A\beta_{1-40}$ (1.6 μM in buffer 1) incubated (**A**) with EDTA (50 μM) or (**B**) with Zn^{2+} (25 μM) and then filtered through filters of the indicated pore sizes (35). (**C**) The $^{65}ZnCl_2$ (130,000 cpm, 74 nM) was used as a tracer of the assembly of the Zn-induced aggregates of human $A\beta_{1-40}$ produced in (A). By determining the amounts of $A\beta_{1-40}$ and ^{65}Zn in the filtrate, we determined the quantities retarded by the filters and estimated the stoichiometry of the Zn:Aβ assemblies. (**D**) After this procedure, the filters, retaining Zn:Aβ assemblies, were washed with buffer 1 and EDTA (50 μM, 300 μl at 700*g* for 4 min). The amounts of Zn-precipitated $A\beta_{1-40}$ resolubilized in the filtrate fraction were determined by OD_{214} and expressed as a percentage of the amount originally retained by the respective filters. The ^{65}Zn released into the filtrate was measured by γ counting. All data points are means ± SD, $n = 3$.

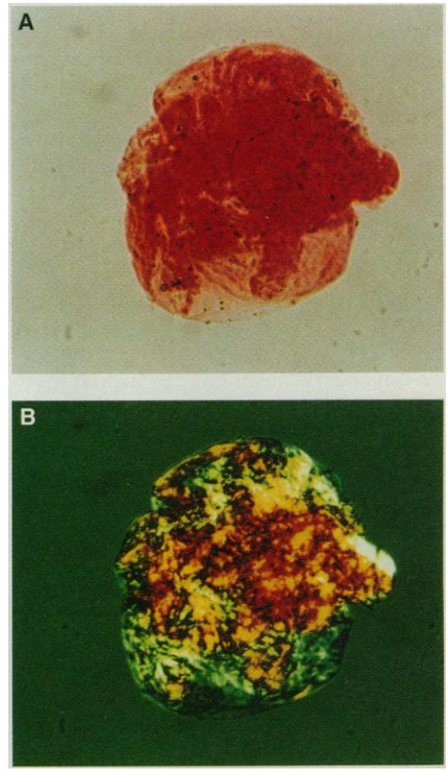

Fig. 4. Zn-induced tinctorial amyloid formation. (**A**) Zn-induced human $A\beta_{1-40}$ precipitate stained with Congo Red. The particle diameter is 40 μm. $A\beta_{1-40}$ (200 μl, 25 μM, in buffer 1) was incubated (30 min at 37°C) in the presence of 25 μM Zn^{2+}. The mixture was then centrifuged (16,000*g* for 15 min), the pellet was washed in buffer 1 and EDTA (50 μM), the mixture was pelleted again, and then the pellet was resuspended in Congo Red (1% in 50% ethanol for 5 min). Unbound dye was removed, and the pellet was washed with buffer 1 and mounted for microscopy. (**B**) The same aggregate visualized under polarized light manifested green birefringence. The experiment was repeated with EDTA (50 μM) substituted for Zn^{2+} and yielded no visible material (*16*).

size of Zn-induced particle formation.

The stoichiometry of Zn:human Aβ in these aggregates is at least 1:1 (Fig. 3C) but increases to 1.3:1 with the smaller (0.1-μm) pore size filters. Because the stoichiometries for high- and low-affinity Zn:Aβ binding are ~1:1 and ~2:1, respectively (*4*), these data indicate that formation of >0.65-μm Aβ aggregates is mediated by the high-affinity Zn interaction, whereas the low-affinity Zn interaction most likely contributes to the formation of smaller (<0.22-μm) aggregates. When the retained aggregates were washed with EDTA, only 22% of the peptide was recovered from >0.65-μm aggregates, although the complexed Zn (using ^{65}Zn as tracer) was completely recovered (Fig. 3D). These data indicate that Zn-induced Aβ aggregation is largely irreversible by chelation. The amount of ≤0.22-μm peptide resolubilized by EDTA treatment was 7% greater, which may reflect the increased contribution of low-affinity Zn binding to the smaller Aβ particle formation that is reversible by chelation.

Sedimentation of Zn-induced Aβ particles by centrifugation resulted in a precipitate of human $Aβ_{1–40}$ that stained with Congo Red (Fig. 4A) and manifested green birefringence under polarized light (Fig. 4B), meeting the criteria for tinctorial amyloid formation (*15*). However, after incubation with Zn^{2+} under the same conditions, the rat peptide formed significantly fewer and smaller particles, with minimal birefringence (*16*). No rat Aβ amyloid was induced by Zn^{2+} concentrations of less than 10 μM, whereas, by tinctorial criteria, human Aβ amyloid was induced by Zn^{2+} concentrations as low as 3 μM. In neither case was Congo Red–stained material detected after incubation with EDTA-containing buffer alone (*16*).

Taken together, our data indicate that soluble human $Aβ_{1–40}$ has a greater propensity than rat $Aβ_{1–40}$ to form amyloid in the presence of physiological Zn concentrations. The tinctorial amyloid aggregates are frequently as large as the amorphous amyloid plaque cores purified from AD brain tissue (*17*). The small degree (10 to 20%) of >0.2-μm $Aβ_{1–40}$ particle assembly observed after the incubation of $Aβ_{1–40}$ with EDTA probably reflects the relatively slow aggregation that occurs in the presence of neutral pH (*3*) and NaCl (*18*). Hence, the specific vulnerability of human Aβ to Zn-induced amyloid formation is an explanation for aspects of the pathology of AD.

The cerebral cortex, especially the hippocampus, contains the highest concentrations of Zn in the body (*19*) and is exposed to fluctuations of extracellular Zn concentrations [0.15 to 300 μM, (*20*), for example, during synaptic transmission (*21*)]. The cortical vasculature contains an intraluminal Zn concentration of 20 μM (*22*), but the perivascular interstitial Zn concentration is 0.15 μM (*20*). Both sites of high Zn concentration gradients are severely and consistently affected by the pathological lesions of AD (*23*). A neurochemical deficit in AD is cholinergic deafferentation of the hippocampus, which raises the concentration of Zn in this region (*24*). Additional evidence for altered cerebral Zn metabolism in AD include decreased temporal lobe Zn concentrations (*25*), elevated (80%) CSF concentrations (*26*), increased extracellular Zn^{2+}-metalloproteinase activities in the hippocampus of individuals with AD (*27*), and decreased concentrations of astrocytic growth inhibitory factor, a metallothionein-like protein that chelates Zn (*28*). Collectively, these reports indicate that there may be an abnormality in the uptake or distribution of Zn in the AD brain. Pervasive abnormalities of Zn metabolism (*29*) and premature AD pathology (*30*) are also common clinical complications of Down syndrome.

The clinical utility of Zn supplementation in the treatment of dementia has been advocated (*31*) despite the lack of evidence demonstrating benefit in AD subjects. In fact, a study of the effects of daily oral Zn supplementation in individuals with AD, as compared with supplementation in age-matched controls, demonstrated deleterious effects on cognition in the AD group in days, correlating with an increase in plasma concentrations of 130-kD APP (*5*, *32*). If verified, these data may indicate that the brain is vulnerable to dietary Zn exposure in AD.

Our data indicate that different stabilities in the presence of physiological concentrations of Zn are what distinguish the propensity of human and rat $Aβ_{1–40}$ peptide species to form amyloid. The rapid induction of tinctorial human Aβ amyloid, under physiologically relevant conditions, at peptide concentrations more than an order of magnitude lower than the lowest concentrations achieved before for $Aβ_{1–40}$ aggregation, and within 2 min of incubation, establishes an assay system for the study of Aβ amyloidosis. These findings carry implications for the potential role of Zn in Alzheimer-associated neuropathogenesis.

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32. The effects of oral Zn supplementation on neuropsychometric, eye-movement, and plasma APP responses in a larger series of AD and control individuals has been assessed (J. Currie *et al.*, in preparation).
33. We determined that the response of the OD_{214}, titrated against human and rat $Aβ_{1–40}$ concentrations (up to 20 μM in the buffers used in these experiments), was linear.
34. Human ^{125}I-labeled $Aβ_{1–40}$ was prepared as described (*12*).
35. Durapore filters (Ultrafree-MC, Millipore) were used for this study; hence, there is a slight discrepancy between the values obtained with the 0.22-μm filters used in Fig. 3, as compared with the values obtained in Fig. 2 with 0.2-μm Costar filters.

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