Truncated β -amyloid peptide channels provide an alternative mechanism for Alzheimer's Disease and Down syndrome

Hyunbum Jang^{a,1}, Fernando Teran Arce^{b,1,2}, Srinivasan Ramachandran^{b,1,2}, Ricardo Capone^{b,2}, Rushana Azimova^c, Bruce L. Kagan^c, Ruth Nussinov^{a,d,3}, and Ratnesh Lal^{b,2,3}

^aCenter for Cancer Research Nanobiology Program, SAIC-Frederick, Inc., National Cancer Institute, Frederick, MD 21702; ^bCenter for Nanomedicine and Department of Medicine, University of Chicago, Chicago, IL 60637; ^cSemel Neuropsychiatric Institute, The David Geffen School of Medicine, University of California at Los Angeles and Greater Los Angeles Veterans Administration Health System, Los Angeles, CA 90024; and ^dDepartment of Human Molecular Genetics and Biochemistry, The Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

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Full-length amyloid beta peptides ($A\beta_{1-40/42}$) form neuritic amyloid plaques in Alzheimer's disease (AD) patients and are implicated in AD pathology. However, recent transgenic animal models cast doubt on their direct role in AD pathology. Nonamyloidogenic truncated amyloid-beta fragments ($A\beta_{11-42}$ and $A\beta_{17-42}$) are also found in amyloid plaques of AD and in the preamyloid lesions of Down syndrome, a model system for early-onset AD study. Very little is known about the structure and activity of these smaller peptides, although they could be the primary AD and Down syndrome pathological agents. Using complementary techniques of molecular dynamics simulations, atomic force microscopy, channel conductance measurements, calcium imaging, neuritic degeneration, and cell death assays, we show that nonamyloidogenic $A\beta_{9-42}$ and $A\beta_{17-42}$ peptides form ion channels with loosely attached subunits and elicit single-channel conductances. The subunits appear mobile, suggesting insertion of small oligomers, followed by dynamic channel assembly and dissociation. These channels allow calcium uptake in amyloid precursor protein-deficient cells. The channel mediated calcium uptake induces neurite degeneration in human cortical neurons. Channel conductance, calcium uptake, and neurite degeneration are selectively inhibited by zinc, a blocker of amyloid ion channel activity. Thus, truncated $A\beta$ fragments could account for undefined roles played by full length $A\beta$ s and provide a unique mechanism of AD and Down syndrome pathologies. The toxicity of nonamyloidogenic peptides via an ion channel mechanism necessitates a reevaluation of the current therapeutic approaches targeting the nonamyloidogenic pathway as avenue for AD treatment.

atomic force microscopy | molecular dynamics | cell calcium imaging | neurite degeneration and cell death assays | single-channel conductance

Amyloid-beta peptides ($A\beta_{1-40/42}$) produced by β - and γ -secretase processing of amyloid precursor protein (APP) in the amyloidogenic pathway are involved in Alzheimer's disease (AD) pathology. $A\beta_{1-40/42}$ peptides form β -sheet-rich ordered aggregates and soluble oligomers. Small oligomers are emerging as the predominant toxic species (1–3); the toxicity is believed to be a result of the loss of ionic homeostasis, presumably via ion channels formed in cellular membranes (4, 5). EM images of $A\beta$ oligomers show doughnut-like morphologies (6). Atomic force microscopic (AFM) images of $A\beta$ peptides reconstituted in lipid bilayers show heteromeric (rectangular to hexagonal) ion channel-like structures with a ~ 2.0 -nm central pore and 8- to 12-nm outer diameters (7, 8). Electrophysiological studies show heterodisperse cation-selective single-channel conductances (7–14) that are consistent with features of other amyloid ion channels (6–8).

On the other hand, when APP is cleaved by γ - and α -secretases, it forms the nonamyloidogenic pathway generating ~ 2.6 -kDa fragments ($A\beta_{17-40/42}$) known as the p3 peptides (15). Cleavage by γ and BACE between Tyr10 and Glu11 generates another non-amyloidogenic $A\beta$ peptide ($A\beta_{11-40/42}$) (16). Because of their

nonamyloidogenic nature, these peptides are assumed to be nonpathogenic and these pathways are even being targeted for AD therapeutics. Significantly, p3 peptides are present in AD amyloid plaques (17–20), are the main constituent of cerebellar preamyloid lesions in Down syndrome (21) and induce neuronal toxicity (22–24). However, their biophysical properties, mechanism of toxicity, and pathological significance in AD and Down syndrome remain unclear. Similarly, very little is known about $A\beta_{11-40/42}$ (16).

In this study, we have used molecular dynamics (MD) simulations, AFM, channel-conductance measurements, cell calcium imaging, neurite degeneration, and cell death assays to examine the biophysical properties and cellular effects of these truncated smaller $A\beta$ peptides. In the present study, we restricted our investigations to $A\beta_{17-42}$ (hereafter referred as p3) and $A\beta_{9-42}$ (hereafter referred as N9 to indicate that they are N-terminally truncated at position 9) (*SI Materials and Methods*). Although $A\beta_{11-42}$ is the physiological unit, its experiment-based coordinates are not available. ssNMR coordinates are available for $A\beta_{9-42}$ only. This fragment contains two more residues and is expected to have a similar structure to that of $A\beta_{11-42}$. Our results strongly suggest that nonamyloidogenic peptides N9 and p3 form ion channels and induce neuronal toxicity in dose-dependent fashion by altering cell calcium homeostasis and could provide additional mechanisms of AD and Down syndrome pathologies.

Results

Modeling N9 and p3 Channels in the Bilayer. Early MD simulations of amyloidogenic peptides (5, 25) consisting of U-shaped β -strand-turn- β -strand peptides in the bilayer predicted ion-permeable channels formed by loosely attached mobile subunits with morphologies and dimensions similar to the AFM-images of amyloid channels (7, 8). U-shaped motifs, first predicted by modeling of $A\beta_{16-35}$ (26), appear as a general feature of amyloid organization, suggesting that other U-shaped amyloid organizations may also

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¹H.J., F.T.A., and S.R. contributed equally to this work.

²Present address: Department of Bioengineering and Department of Mechanical and Aerospace Engineering, University of California, San Diego, La Jolla, CA 92093-0412.

³To whom correspondence may be addressed. E-mail: rlal@ucsd.edu or ruthnu@helix.nih.gov.

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form dynamic ion channels in the fluidic membrane (8). Because N9 and p3 have membrane-spanning segments, we modeled their 3D structures in the bilayer using the previous successful protocol (5, 25). Using the two available A β oligomer coordinate sets (27, 28), we constructed annular channels based on the U-shaped β -strand-turn- β -strand motif. Previously, U-shaped motifs were also observed in the ssNMR structure of a β_2 -microglobulin fragment (29) and in the CA150 WW domain (30), and they could also form ion channels similar to prion and to β_2 -microglobulin (31, 32). We constructed perfectly annular channels as the starting points for the atomistic simulations with 12 to 36 monomers per channel and lipid-favorable topology (5, 25) (*SI Materials and Methods*). For clarity, all modeling images presented in this article are made with 16 peptides. In the modeled channels, both N9 and p3 are U-shaped, although with different turn conformations. The p3 channel is embedded in the bilayer. In the pore, Glu22 side chains circularly cluster forming a negatively charged ring. The N9 channel protrudes from the bilayer surface, especially at the bottom leaflet, because the turn residues locate at the same height as the phosphate atoms at the top bilayer leaflet. In addition to the Glu22 cationic binding sites, positively charged His14 and Lys16 create anionic binding sites.

At $t > 5$ ns, these channels gradually relax through association or dissociation of the intermolecular backbone hydrogen bonds (H-bonds) between the β -strands. Consistent with earlier observations (5, 25), the channel outer β -sheet, absent in the initial structure because of the larger curvature at the channel periphery, is recovered at certain regions and the channel organizes into several small subunits with or without disordered monomers in between (Fig. 1*A* and *B*). Transient inner β -sheet H-bonds barely prevent subunit dissociation and the discontinuous β -sheet network can determine the boundary between the channel's ordered subunits (Fig. *S1A* and *B*).

The N9 channels obtain four or five ordered subunits (Fig. 1*A* and Fig. *S1C*). The outer N9 channel diameter is ~ 7.3 nm, and the pore diameter is ~ 1.5 to 1.7 nm. Similarly, the p3 channels also have four or five ordered subunits (Fig. 1*B* and Fig. *S1D*). In the averaged channel, the outer p3 channel diameter is ~ 6.8 to 6.9 nm and the pore diameter is ~ 1.7 nm. Our previous simulation for the p3 channel obtained three subunits (25), suggesting that even for the same channel (of differing size), subunit formations strongly depend on the fluidic bilayer dynamics. The p3 channel morphology is similar to the N9 channel, although the sequence lengths and detailed monomer conformations differ.

To test the predictive value of MD simulation that yields channel conformations, we introduced point mutations in the pore-lining residues. Our modeling indicates that the pore is lined by N-terminal β -strands; however, the C-terminal strands interact predominantly with the lipids. Two residues in the pore-lining region of p3 that could confer significant structural and functional changes are proline and cysteine. We selected proline, as it is a β -sheet breaker; its phi angle (-60° to 25°) is incompatible with β -sheet (-120° to -140°). Candidate locations for a proline mutation are Leu17, Phe19, Ala21, Asp23, and Gly25 in the N-terminal strand, with side-chains at the dry interface between the sheets. We selected Phe19 and disregarded Leu17, Ala21, Asp23, and Gly25, because Leu17 is the N terminus, and Ala21, Asp23, and Gly25 are at the hydrated cavity. In the pore, the Phe19 side-chains are π -stacked. Kinked N-terminal strands at the Pro residue point toward the interior solvated pore, and consequently block ions crossing through the pore. In the simulations, the p3-F19P mutant channels are tetrameric, with the outer diameter slightly less than the wild-type p3 channel (Fig. 1*C*).

Three-Dimensional Topography of N9 and p3 Channels. We examined the 3D topography of N9 and p3 using AFM (*SI Materials and Methods*). AFM images of freshly prepared N9 and p3 peptides show no fibril formation (Fig. *S2*), even after longer incubation time

Fig. 1. A β channel conformations by MD simulations. The simulated channel structures (Left) with highlighted subunits for the N9 (*A*), p3 (*B*), and p3-F19P mutant (*C*) channels are shown in the view along the membrane normal. (Center and Right) Averaged pore structures calculated by the HOLE program (50) embedded in the averaged channel conformations during the simulations. In the angle views of the pore structure (Center), whole channel structures are shown with the ribbon representation. In the lateral views of the pore structure (Right), cross-sectioned channels are given in the surface representation. For the pore structures in the surface representation, the degree of the pore diameter is indicated by the color codes in the order of red < green < blue. In the channel structures, hydrophobic residues are shown in white, polar, and Gly residues are shown in green, positively charged residues are shown in blue, and negatively charged residues are shown in red.

or for nonphysiological concentrations. AFM images of N9, p3, and p3-F19P mutant (Fig. 2*A–C*) reconstituted in lipid bilayers show annular structures protruding < 0.5 nm out of the membrane plane. In ~ 20 to 25% of these structures, a central pore-like feature could be resolved, indicating the formation of channel-like structures. At higher magnification (Fig. 2*D–G*), these annular structures look similar to the previously described A β_{1-40} or A β_{1-42} amyloid channels (7, 8). The outer and inner diameters of these structures are ~ 6 to 10 nm and ~ 1 to 2 nm, respectively, and in accord with our MD simulations. High-resolution AFM images of the p3-F19P mutant show pore-like structures with three to five subunits (Fig. 2*H* and *I*) and outer and inner pore diameters that are comparable to the normal p3 channels.

Annular structures of the type described above were visible in most reconstituted bilayers for all lipids studied (*SI Materials and Methods*). The image quality often varied among different preparations and even within different regions of the same image. In addition to tip-sample interactions (8), these variations could reflect inherent channel mobility, as predicted in our MD simulations. Upon closer examination of individual channel-like structures at higher resolution, several possible subunit arrangements were revealed, including rectangular with four and pentagonal with five subunits, respectively (Fig. 2). The differing multimeric structures and substructures of these peptides are

Fig. 2. AFM imaging. Error-mode AFM images of N9 (A), p3 (B), and p3-F19P mutant (C) reconstituted in lipid bilayers. Individual channels are enclosed by dotted circles and the white arrow in A indicates a mica region free of bilayer. High-resolution images are of individual channel structures. The number of subunits is indicated for each channel. Inner-pore sizes are typically 1 to 2 nm. Images sizes are 22 nm (D), 19 nm (E), 15 nm (F), 23 nm (G), 11 nm (H), and 9 nm (I).

consistent with our modeling (Fig. 1) and other amyloid channels (8). To assess the functionality of these channel structures, we recorded electrical conductance of reconstituted channels in planar bilayers.

Ionic Conductance of N9 and p3 Channels. Previous electrophysiological studies of reconstituted amyloid peptide ion channels show distinct multiple conductances, weak cation selectivity, voltage independence, inhibition by Congo red, and blockade by zinc (8, 11, 13, 14). We examined if the nonamyloidogenic A β peptides also have distinct channel conductances. Fig. 3 shows single-channel currents as a function of time across planar lipid bilayer membranes when N9 (Fig. 3A) and p3 (Fig. 3C) peptides were added to the aqueous solution. Heterogeneous single-channel conductances are observed (for details, see Fig. S3), suggesting that several distinct oligomeric species (or conformations) form distinct channel structures (8). The multiple subunit arrangements and varying inner pore diameters in AFM images combined with the multiple peptide contents could explain the experimentally observed multiple conductances. Preliminary results suggest that p3 activity is reproducible at concentrations as low as 0.44 μ M. The channel activity decreases in frequency with decreasing p3 concentration; this is to be expected because channel formation requires the oligomerization of monomers in the membrane. Channels were never observed in bilayers without the addition of amyloid peptides. Ion channel conductances were reversibly blocked by zinc for both N9 (Fig. 3B) and p3 (Fig. 3D), similar to the previous findings for A β _{1–40/42} (7, 8). In addition, a peptide made of p3-scrambled sequence showed no conductance (Fig. 3E) and no channel-like structures in AFM imaging.

MD simulation of the point mutation p3-F19P predicts that the proline substitution obstructs ion flux across the pore and, hence, loss of channel conductance (Fig. 1C). Electrophysiological recording of reconstituted p3-F19P peptide confirms the prediction: these peptides are nonconductive (Fig. 3F) over an extended period (average ~61 min, $n = 8$).

To connect the 3D structure and ion behavior in the pore, the potential of mean force (PMF) representing the relative free-energy profile for each ion during pore permeation across the bilayer was calculated. PMF in our simulation points to cationic binding sites in the pore (*SI Materials and Methods*). In the MD simulations, the channels preserve pores wide enough for con-

ducting water and ions. Both N9 (Fig. 3*G*) and p3 (Fig. 3*H*) channel structures confirm that cations are easily trapped by the negatively charged Glu22 side-chains at the top bilayer leaflet, creating a cationic ring (5, 25). In the p3 pore (Fig. 3*H*), Mg^{2+} and K^{+} are very mobile; however, Ca^{2+} and Zn^{2+} exhibit a low mobility at the binding site. Without Zn^{2+} , Ca^{2+} ions are dominantly trapped by the side chains (25), but in the presence of Zn^{2+} , Ca^{2+} exhibits greater mobility at the binding site. Experimentally, the ability to conduct calcium in bilayers has been conclusively addressed for A β 40/42 previously (7–14). The N9 pore has similar binding sites for cations as in the p3 pore, but has an additional binding site for Cl^{-} at the positively charged His14 and Lys16 side chains (Fig. 3*G*). The PMF of p3-F19P (Fig. 3*I*) shows that the binding site for Ca^{2+} is narrower than in the wild type. The p3-F19P channel has a collapsed pore that decreases the pore diameter, especially at the Pro regions. Although Ca^{2+} ions can interact with the negatively charged Glu22 side chains in the pore, they cannot further move because of the narrow pore. These PMF profiles predict no Ca^{2+} uptake for the p3-F19P mutant channel but significant cell Ca^{2+} uptake for p3 and N9 channels.

N9 and p3 Channels Alter Cellular Calcium Levels. Altered calcium homeostasis is a common denominator underlying many amyloid-related disorders (33), and amyloid ion channels allow cell calcium overload (7). To test for biological relevance of nonamyloidogenic peptide channels, we examined their role in cellular calcium homeostasis. Intracellular Ca^{2+} changes were measured in APP knockout cells with Fluo-4 Ca^{2+} -sensitive dye. Both p3 and N9, when added to cells bathed in normal Ca^{2+} -containing buffer, showed a time-dependent increase in Ca^{2+} , which stabilized to an elevated level (~ 15 min) in most cells (Fig. 4). Ca^{2+} uptake was not observed in cells bathed in nominally Ca^{2+} -free media (Fig. S4A, B, *M-P*) or cells treated with solvent alone (1% NH_4OH) (Fig. S4A–D), suggesting that Ca^{2+} comes mainly from external sources. The rate and the degree of Ca^{2+} increase for p3 was higher than N9 (Fig. 4B and Fig. S4E–H). Similarly, the loss of cellular Ca^{2+} was higher with p3 compared with N9 (Fig. S4M–P). These findings suggest that p3 forms pores more rapidly than N9. Pretreatment with zinc inhibits Ca^{2+} uptake, consistent with the single-channel conductance studies (Fig. 3 B and D and Fig. S4I–L), as well as with other A β -mediated Ca^{2+} -uptake findings (9, 10). Significantly, both

Fig. 3. Pore-forming activity of nonamyloidogenic peptides. Channel conductance measurements (A–F) and potential of mean forces (PMFs) for Mg^{2+} , K^+ , Ca^{2+} , Zn^{2+} , and Cl^- (G to I). Single channel currents induced by N9 (A) and p3 (C). Current jumps on traces correspond to single opening or closing of ion channels. Multilevel conductances can be observed. Blockade of N9 channels (B) and p3 channels (D) by 1 mM ZnCl_2 . Zinc addition is indicated by arrows. As a control, p3 scrambled sequence shows no membrane activity for extended periods of time (E). As predicted, structurally blocked p3 mutant F19P does not form conductive channels for extended periods of time (F). Planar lipid bilayers were made in electrolyte with 100 mM KCl, 10 mM hepes-K pH 7.4, and 1 mM MgCl_2 in all of the experiments. The bilayer membranes shown were made with asolectin lipids (soybean lecithin). The *cis* side is the virtual ground. The applied membrane potential was -50 mV. The current traces shown are representative of at least 7 and often more than 10 independent traces for each condition shown. PMF for each ion are shown for N9 (G), p3 (H), and p3-F19P mutant (I) channels. Both N9 and p3 channels preserve the pore; p3-F19P channel has a collapsed pore. ΔG_{PMF} was calculated using the equation $\Delta G_{\text{PMF}} = -k_B T \ln(\rho_z/\rho_{\text{bulk}})$, where k_B is the Boltzmann constant, T is the simulation temperature, ρ_z is the density of ion at the position z along the pore axis, and ρ_{bulk} is the density of ion in the bulk region, representing the relative free-energy profile for Mg^{2+} (green lines), K^+ (red lines), Ca^{2+} (blue lines), Zn^{2+} (cyan lines), and Cl^- (black lines) as a function of the distance along the pore center axis of each channel.

the p3 Pro mutant and the scrambled p3 peptide did not alter Ca^{2+} levels under similar conditions (Fig. 4). Quantification of the intracellular Ca^{+2} concentration resulting from calcium uptake via the p3 ion channel, although more relevant, was beyond the scope of the present study and not measured. Our aim here was to ascertain if indeed there is any relative change in intracellular calcium homeostasis because of p3-channel-mediated calcium uptake. Accordingly, we used qualitative calcium-measuring dye and measured a relative change in fluorescence intensity as a function of p3 and other controls. In general, the fluorescence results are in agreement with the simulations, AFM, and electrical behavior in model membranes. Moreover, inhibition by Zn^{2+} , an amyloid channel inhibitor, further suggests that these peptides form calcium channels directly. The above findings lend credence to our notion that nonamyloidogenic A β fragments form Ca^{2+} -permeable, zinc-sensitive pores that allow cell Ca^{2+} loading. We then examined the cellular response to increased cell calcium.

Fig. 4. Cell calcium imaging and relative concentration plot. (A) Non-amyloidogenic β -peptides form Ca^{2+} permeable, zinc-sensitive pores. Time-course measurement of Ca^{2+} change shows significant variation in the Ca^{2+} flux upon application of 5 μM N9 and p3 (first and second row, respectively). No increase in Ca^{2+} was seen in proline mutation and scrambled p3 peptides (third and fourth row, respectively). Pretreatment with zinc, a known A β -blocker, prevents such rise in intracellular Ca^{2+} (fifth row). (B) The plot summarizes the above findings for the entire span of the experiment in all of the cells under the field-of-view. The mean (average of Ca^{2+} changes in the field) ratio $[(F_t/F_0) \times 100]$ fluorescence intensity change was plotted against time. F_t is the fluorescence of the field at a given time t and F_0 is the fluorescence of the field at time $t = 0$. Error bar represents the SE of the mean of all of the blobs in the field and they are represented on any one side of the curve.

N9 and p3 Channels Mediate Neurite Degeneration and Cell Death.

Abnormal calcium loading has been reported to initiate synaptic degeneration and neuronal death in several neurodegenerative diseases (1, 2, 7). Consistent with this scenario, we observed a dose- and time-dependent degeneration of neurites in human cortical neurons after 24 h of incubation with p3, compared with the untreated groups (Fig. 5). Our results suggest that within 24 h, 20 μ M p3 induced neuritic degeneration that was too small to be observed under light microscopy but was readily visible in AFM images (Fig. 5*B*). In the same p3 incubation time (24 h), the degenerative effect of 40 and 100 μ M p3 was visible under light microscopy; cells probed with antitubulin (α/β) antibody show significant reduction in neurite density (Fig. 5 *C*: 40 μ M; *D*: 100 μ M). At the other extreme, 1 mM p3 induced a dramatic reduction in neuronal processes within 15 min (Fig. 5*E*). Leakage of Calcein dye (Fig. S5), which is otherwise impermeable from a cell with intact membrane, suggests loss of cell membrane integrity. Significantly, neurons pretreated with zinc did not show any significant neurite

Fig. 5. p3-induced dose-dependent neurite degeneration and cell death. Multimodal imaging of cells and processes: immunofluorescence imaging of microtubules (A, C, D), AFM imaging (B), Calcein AM dye (E), and apoptosis assay (F). After 24 h of incubation, 20 μ M p3 induced a small neuritic damage only visible in AFM images (B) and ≥ 40 μ M p3 induced loss of neurite density visible by light microscopy [white dotted lines in C (40 μ M) and D (100 μ M)]. On the other hand, 1 mM of p3 induced rapid neuritic degeneration within 15 min of incubation (E). Significantly, pretreatment with Zn^{2+} prevented p3-induced damage, even at such a large p3 dose (Fig. S5). The original fluorescence images (Fig. S5) were processed to reveal the neurites that were obscured by the leakage of Calcein dye. Laplacian of Gaussian (LoG) operator was applied on the image to detect the neurites faithfully. The bar chart represents mean fluorescence (see SI Materials and Methods for details) of apoptotic cells (F). Compared with untreated population, 100 μ M p3 causes significant ($*P < 0.01$) cell damage within 24 h of incubation.

degeneration or dye leakage, even when incubated with 1 mM (nonphysiological) p3 concentration (Fig. S5). The degree of neurite degeneration appears to mirror the rise in cellular calcium. P3-mediated cell degeneration leads to cell apoptosis. Apoptosis assay shows a clear dose-dependence of p3-induced apoptosis (Fig. S5F). We limited our cell-toxicity studies to only 24 h, although most of the published work report toxicity after or up to 7 days of incubation with amyloids. This could account for a lower level of synaptic degeneration and cell death observed in our study.

Discussion

Our simulations initiated with the NMR-based β -strand-turn- β -strand motif yield 12- to 36-mers dynamic nonamyloidogenic A β ion channels (5, 25) and provide atomic resolution models, which could aid in drug discovery. Interestingly, all AFM-imaged

amyloid channels (8, 34), including A β , α -synuclein, ABri, ADan, amylin SAA, and K3 have oligomeric subunit organizations. In the present study, both nonamyloidogenic A β fragment channels similarly self-organized into oligomeric subunits. Furthermore, our simulations of protegrin-1 made of β -hairpins also organize into loosely connected oligomeric subunits (35). Although the variability of the channel conformations, pore types, and the subunit interaction with the bilayer are lipid- and sequence-dependent, all modeled channels predict ion flux through the channel pore and between the twisted mobile subunits, and they all point to a common mechanism for protein misfolding diseases: ion channel-mediated cell pathophysiology.

Our findings of neurotoxicity, channel formation, and increased neuronal calcium uptake by p3 and N9 are consistent with the work of Pike et al. (24), who showed that these N-terminally truncated peptides demonstrated enhanced aggregation, neurotoxicity, and fibril-formation. p3 has been reported not to form “soluble oligomers” (36) and our present results support the notion that p3 oligomerizes directly in the membrane to cause toxicity. However, in a double transgenic mouse study with overexpressed ADAM10 and human APP that increased α -secretase activity, AD initiation and progression were limited (37). However, this protection could be caused by several factors, including (i) increased amounts of APP_{scs}, a neurotrophic and neuroprotective factor (38, 39) that could overwhelm the toxic effects of p3; (ii) diversion of more toxic A β _{1-40/42} to nonamyloidogenic pathway (37, 40); and (iii) potential additional neuroprotective effects mediated by many non-APP substrates (e.g., Notch, EGF, and β -cellulin) of ADAM10 (41). Significantly, transgenic-mouse models do not fully represent human AD pathology (42), thus complicating direct clinical conclusions.

Therefore, our findings are consistent with the idea that amyloid deposition, per se, is not key to amyloid peptide toxicity, but that membrane mediated action of truncated A β -peptide fragments, including p3 and N9, is critical. Because of its extreme hydrophobicity compared with full-length A β peptides, p3 may constitute a highly membrane-targeted pathway of neuronal toxicity. This unique finding can account for the conflicting roles played by the full-length β -amyloids (A β ₁₋₄₀, A β ₁₋₄₂). Our present work supports p3 and N9 channel-mediated toxicity and suggests that ion channel blocking strategies for these smaller amyloid fragments may present an important therapeutic avenue for treatments for sporadic AD and Down syndrome. Our work has far broader relevance beyond the boundaries of amyloid-related neurodegenerative diseases (43). The discovery that nonamyloidogenic peptides may be pathogenic when oligomerized and embedded in membranes is startling and may require a reevaluation of the pathophysiology of these diseases.

The amyloid hypothesis (44) proposed that β -amyloid (A β ₁₋₄₂) itself was the toxic element causing disease. A large body of subsequent evidence has indicated that it is not amyloid fibrils that are toxic, but a smaller species, perhaps consisting of channel-forming oligomers that might form in association with membranes. Because “nonamyloid-forming” peptides derived from APP were believed to be harmless, much effort has gone into trying to modify secretase activity to decrease the ratio of amyloidogenic to nonamyloidogenic peptides in the hope of ameliorating the disease. The present work suggests that this effort may be futile, because nonamyloidogenic peptides seem to possess a propensity comparable to that of amyloidogenic peptides in forming ion channels that can permit calcium influx, disrupt neurites, and kill neurons. Future approaches to AD therapeutic strategies should take this into account.

Materials and Methods

In the MD simulations, two A β fragments were used as monomers: p3 (26 residues, ~ 2.6 kDa/monomer, based on hydrogen/deuterium-exchange NMR data, side-chain packing constraints, ssNMR and EM; PDB code: 2BEG) (27), and N9 [34 residues, ~ 3.6 kDa/monomer, based on ssNMR model of A β ₉₋₄₀ (28)], adding Ile41 and Ala42 (45). For AFM experiments, liposomes reconstituted with p3 and N9 peptides

were allowed to form supported lipid bilayers on mica using procedures modified from refs. 7 and 8. Images were acquired with a Multimode AFM, using a Nanoscope IVA controller (Veeco). Single-channel conductance measurements were carried out using painted membranes as published previously (8, 13, 14, 46–49) (*SI Materials and Methods*). Cell (APP-deficient mouse fibroblast cell line) calcium imaging (Fluo-4 NW) was carried out for p3 and N9 (under normal and reduced extracellular calcium levels, as well as with and without preincubation with zinc chloride), p3-F19P mutant, and scrambled p3 peptides under normal extracellular calcium level. Fluorescence microscopic assays for neurite degeneration (assessed by antitubulin antibody staining) and cell death assays (apoptosis assay and Calcein leakage) were carried out for p3 peptide on human cortical neurons. More detailed methods are available in *SI Materials and Methods*.

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