Single-channel Ca\(^{2+}\) imaging implicates A\(\beta\)1–42 amyloid pores in Alzheimer’s disease pathology

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Oligomeric forms of A\(\beta\) peptides are implicated in Alzheimer’s disease (AD) and disrupt membrane integrity, leading to cytosolic calcium (Ca\(^{2+}\)) elevation. Proposed mechanisms by which A\(\beta\) mediates its effects include lipid destabilization, activation of native membrane channels, and aggregation of A\(\beta\) into Ca\(^{2+}\)-permeable pores. We distinguished between these using total internal reflection fluorescence (TIRF) microscopy to image Ca\(^{2+}\) influx in Xenopus laevis oocytes. A\(\beta\)1–42 oligomers evoked single-channel Ca\(^{2+}\) fluorescence transients (SCCaFTs), which resembled those from classical ion channels but which were not attributable to endogenous oocyte channels. SCCaFTs displayed widely variable open probabilities (P\(\text{o}\)) and stepwise transitions among multiple amplitude levels reminiscent of subconductance levels of ion channels. The proportion of high P\(\text{o}\), large amplitude SCCaFTs grew with time, suggesting that continued oligomer aggregation results in the formation of highly toxic pores. We conclude that formation of intrinsic Ca\(^{2+}\)-permeable membrane pores is a major pathological mechanism in AD and introduce TIRF imaging for massively parallel single-channel studies of the incorporation, assembly, and properties of amyloidogenic oligomers.

Introduction

A seminal event in the pathogenesis of Alzheimer’s disease (AD) is the abnormal proteolytic processing of amyloid precursor protein (APP), resulting in increased production of a self-aggregating form of \(\beta\) amyloid (A\(\beta\)). Mutations in APP and in presenilins (secretase enzymes involved in APP processing) that are linked to early-onset familial forms of AD cause excessive production of A\(\beta\), resulting in massive accumulation of fibrillar A\(\beta\) in the amyloid plaques that are a hallmark of brains from AD patients (Cras et al., 1991). For many years, the plaques were thought to be the primary culprit of AD pathology, as formulated by the amyloid hypothesis (Hardy and Higgins, 1992). However, that hypothesis fails to explain several important pathological and clinical characteristics of AD. Markedly, there is little correlation between the amounts of fibrillar A\(\beta\) deposit at autopsy and the clinical severity of AD (Lemere et al., 1996). In contrast, a good correlation has been found between early cognitive dysfunction and levels of soluble forms of A\(\beta\) in the brain (Jensen et al., 2000; Shankar et al., 2008).

Aggregation of A\(\beta\) proceeds through several steps, including the formation of soluble low molecular weight spherical oligomers, before assuming a final and stable conformation as the insoluble fibrils from which plaques are constituted (Bucciantini et al., 2002; Kayed et al., 2003; Glabe, 2004). Growing evidence suggests that the neurotoxicity is associated with soluble aggregates of A\(\beta\)s rather than with the plaques themselves (Walsh et al., 2002; Kayed et al., 2003; Demuro et al., 2005; Deshpande et al., 2006). Moreover, soluble oligomeric forms of amyloidogenic peptides associated with diseases including Huntington’s and Parkinson’s have been shown to be similarly toxic, and this toxicity is directly related to the morphological structure of the aggregates rather than to their protein sequence (Kayed et al., 2003; Demuro et al., 2005). Consistent with this, antibodies raised against A\(\beta\) peptides in their oligomeric form recognize oligomeric species of other amyloidogenic proteins (\(\alpha\)-synuclein, polyglutamin, and lysozime) but not the monomeric or the fibrillar forms of A\(\beta\) and other peptides (Kayed et al., 2003).

Amyloid oligomers, but not monomers, exert a common pathophysiological action in disrupting the integrity of cell membranes, resulting in uncontrolled influx of extracellular Ca\(^{2+}\).
Figure 1. Western blot analysis of Aβ1–42 oligomer preparations and assay of potency to evoke Ca\(^{2+}\) influx in Xenopus oocytes. (A and B) Western blots showing aggregation profiles of Aβ1–42 at 30 min after initially dissolving Aβ peptide monomer (A) and after 48 h of incubation (B). 4 µg of samples was separated using gel electrophoresis on a 4–20% Tris-HCl gel. The membranes were probed separately with a monoclonal antibody, 6E10, which is sequence specific to Aβ, and with OC antibody, which recognizes a generic epitope associated with the fibrillar amyloid conformation independent of peptide sequence. Black lines indicate that intervening lanes have been spliced out. (C and D) Bioassay of the potency of Aβ1–42 oligomer preparations to induce membrane permeability to Ca\(^{2+}\). The traces in C show representative global Ca\(^{2+}\) fluorescence signals (indicated as F; top) and corresponding voltage-clamped membrane currents (indicated as I; bottom) evoked by 30 s voltage steps from 0 to –100 mV delivered at various times (indicated in minutes) before and after pipette application of Aβ1–42 oligomers to a nonpied oocyte loaded with fluo-4 dextran. (D) Measurements of inward currents (without leak subtraction) evoked by voltage steps from 0 to –100 mV at 5-min intervals after application of Aβ1–42 (final bath concentration of 1 µg/ml). Data show means ± 1 SEM from four oocytes using two different Aβ1–42 oligomer preparations.

with devastating consequences for cellular Ca\(^{2+}\) homeostasis (Mattson et al., 1992; Demuro et al., 2005; Deshpande et al., 2006). Major questions remain as to how amyloid oligomers exert their action on the cell membrane, and three distinct mechanisms have been proposed: (1) the fluidizing action hypothesis postulates that Aβ destabilizes membrane integrity via a nonreceptor-mediated mechanism that alters the physicochemical properties of membrane lipids and proteins (Hertel et al., 1997; Mason et al., 1999; Sokolov et al., 2006); (2) Aβ oligomers have been proposed to directly activate endogenous Ca\(^{2+}\)-permeable receptor/channels such as neuronal nicotinic and glutamate receptors (Wang et al., 2000; De Felice et al., 2007; Alberdi et al., 2010); and (3) Aβ and other amyloidogenic oligomers are proposed to incorporate into the cell membrane to form pores with high cation conductivity (Arispe et al., 1993; Pollard et al., 1993; Lin et al., 2001; Quist et al., 2005). Evidence in support of the pore-forming mechanism derives from electrophysiological recordings from both artificial and biological membranes (Lin et al., 2001; Lashuel et al., 2002; Quist et al., 2005; Capone et al., 2009) and from theoretical modeling (Durell et al., 1994; Jang et al., 2008). Moreover, EM reveals porelike Aβ structures in cell membranes of postmortem brains from AD but not control patients (Inoue, 2008), and similar Aβ globular structures have been visualized by atomic force microscopy in lipid membranes treated with synthetic Aβ peptides (Lin et al., 2001; Lashuel et al., 2002).

Studies of the molecular mechanisms by which amyloid oligomers induce membrane permeability have largely been confined to electrophysiological recordings of currents across artificial lipid bilayers (Kagan et al., 2004) or excised patches of neuronal membranes (Kawahara et al., 1997). Here, we used a technique (optical patch-clamping) we had previously developed for high-resolution imaging of Ca\(^{2+}\) flux through single ligand- and voltage-gated channels (Demuro and Parker, 2004a, 2005a) to study the functioning of individual Aβ1–42 oligomer pores in the plasma membrane of Xenopus laevis oocytes, which substantially lack endogenous Ca\(^{2+}\) channels (Weber, 1999). We find that extracellular application of Aβ1–42 oligomers induces punctate Ca\(^{2+}\) transients that closely resemble the single-channel Ca\(^{2+}\) fluorescence transients (SCCaFTs) generated by classical protein membrane channels. The results obtained with this novel approach support a pore-forming mechanism of Aβ toxicity and provide insights into the formation and functional characteristics of Aβ pores.

Results

Aβ oligomers induce Ca\(^{2+}\) influx across the oocyte membrane

We had previously shown that application of soluble Aβ oligomers (but not monomers or fibrils) to cultured neuroblastoma cells evoked large increases in cytosolic (Ca\(^{2+}\)) that arise largely through Ca\(^{2+}\) influx across the plasma membrane (Demuro et al., 2005). Our object here was to study this plasmalemmal Ca\(^{2+}\) flux at the single-channel level using total internal reflection fluorescence (TIRF) microscopy of near-membrane Ca\(^{2+}\) signals, which we had developed as a technique to resolve the gating of individual ion channels in the membrane of Xenopus oocytes (Demuro and Parker, 2005a). The oocyte offers several advantages for study of the actions of Aβ oligomers, including a paucity of native ligand- and voltage-gated channels (Bourinet et al., 1992) that have been proposed to be targets of Aβ modulation in neuronal cells (Wang et al., 2000; De Felice et al., 2007; Alberdi et al., 2010). We chose to investigate the molecular mechanisms of toxicity of the Aβ1–42 peptide, rather than the Aβ1–40 form, in light of the reported correlation between the early onset of familial AD and the increased level of Aβ1–42 in the AD brain (Jarrett and Lansbury, 1993; Kim and Hecht, 2005).

Aβ1–42 oligomers were prepared by incubating aqueous solutions of peptide monomer for up to 48 h. Western blots (Fig. 1, A and B) showed a wide range of oligomeric species with molecular masses ranging between ~35 and 300 kD, corresponding to roughly 5–40 peptide multimers, which were recognized by 6E10 and OC, sequence- and fibrillar-specific Aβ antibodies, respectively (Kayed et al., 2010). Aβ oligomer preparations were assayed for their ability to induce macroscopic Ca\(^{2+}\) influx in oocytes. Fig. 1 C shows representative membrane currents and fluorescence signals in an oocyte loaded with the Ca\(^{2+}\) indicator fluo-4 generated in response to voltage-clamped hyperpolarizing pulses from 0 to –100 mV to transiently increase the electrical driving force for Ca\(^{2+}\) entry. The pulses initially evoked only a small inward leakage current and a small rise in fluorescence, but both signals increased over
~40 min after adding Aβ1–42 oligomers to the bathing solution (Fig. 1 D), reflecting an increase in membrane Ca²⁺ permeability and consequent activation of a Ca²⁺-activated Cl⁻ conductance (Miledi and Parker, 1984). This action was specific to the oligomeric species of Aβ that aggregated after long-term incubation (12–48 h) because application of solutions of Aβ peptide that had been prepared shortly beforehand evoked little or no response.

**Imaging Ca²⁺ signals from individual Aβ1–42 pores**

To resolve Ca²⁺ influx through individual Aβ pores, oocytes were stripped of the vitelline envelope, allowing the cell membrane to appose closely to the cleaned cover glass of the imaging chamber so that TIRF microscopy of fluo-4 could be used to image Ca²⁺ signals restricted within the ~100-nm-deep evanescent field immediately adjacent to the plasma membrane. We selected regions with uniform background-resting fluorescence close to the edge of the membrane footprint, so as to maximize diffusional access of Aβ. Aβ1–42 oligomers dissolved at 1 µg/ml in Ringer’s solution were delivered by superfusion from a micropipette (30–50-µm tip) within <200 µm of the edge of the imaging field. A total volume of ~3 ml was delivered at 0.3–0.5 ml/min to a chamber initially containing 0.5 ml Ringer’s solution.

Before application of Aβ, hyperpolarizing steps to >100 mV usually evoked little or no change in resting fluorescence, and we discarded oocytes showing sustained or transient local Ca²⁺ elevations. In contrast, hyperpolarizing pulses evoked numerous localized, flickering bright spots when examined >20 min after Aβ application (Fig. 2 A). We used a custom software routine (CellSpecks) to locate the positions of these Ca²⁺ hotspots and to automatically generate plots of the fluorescence time course from localized regions of interest. For example, Fig. 2 B presents a representative map of all sites (n = 785) identified within the 40 × 40-µm imaging field during a 20-s hyperpolarization. The inhomogeneous distribution across Fig. 2 B is representative of eight similar experiments and probably reflects gradients of Aβ oligomer concentration away from the application pipette, which was located near the bottom right corner of the imaged region.

Fluorescence signals at individual sites were restricted within ~1 µm and flickered on and off at millisecond timescales (Fig. 2 C), closely resembling both patch-clamp recordings from ion channels and optical recordings of Ca²⁺ flux through Ca²⁺-permeable voltage- and ligand-gated ion channels (Demuro and Parker, 2003, 2004b, 2005a). In light of this similarity, together with other properties described later, we refer to them as SCCaFTs (Demuro and Parker, 2005a).

**Aβ SCCaFTs arise through local influx of Ca²⁺ across the plasma membrane**

SCCaFTs induced by Aβ1–42 oligomers disappeared after removal of external Ca²⁺ from the bathing solution (Ringer’s solution without added Ca²⁺ plus 3 mM EGTA), consistent with an extracellular source of Ca²⁺. Moreover, the mean amplitudes of SCCaFTs reduced at more positive membrane potentials (Fig. 3, A and B), showing a voltage dependence and extrapolated suppression potential (Fig. 3 A) mirroring that of SCCaFTs from Ca²⁺-permeable nicotinic acetylcholine receptors expressed in the oocyte (Demuro and Parker, 2005a), as expected from the electrical driving force for Ca²⁺ influx.

Zinc ions inhibit the Ca²⁺ conductance induced by Aβ oligomers (Arispe et al., 1996, 2007; Kawahara et al., 1997; Rhee et al., 1998). Application of 300 µM extracellular Zn²⁺ strongly attenuated the appearance of Aβ SCCaFTs (Fig. 3 C), and the mean fluorescence change across 10 × 10-µm membrane regions in response to hyperpolarization reduced ~10-fold from ΔF/F₀ = 0.14 ± 0.03 (n = 7 trials) to 0.016 ± 0.002 (n = 4), which is close to the mean value seen in the absence of Aβ (0.013 ± 0.002; n = 6).
whole-cell currents induced 40 min after application of 1 µg/ml.

Application of 5 µM of the specific stretch-activated channel CaOperating channels that mediate Ca2+ influx after depletion of ER Ca2+-permeable channels present at high density in oocytes (Weber, 1999). Neither is likely to account for our results. Moreover, during activation of store-operated influx in oocytes, we observed only a generalized rise in fluorescence Ca2+ signal without evidence of discrete SCCaFTs, suggesting that, like Ca2+ release–activated Ca2+ channels in other cells (Cahalan, 2009), these channels in the oocyte have an extremely low Ca2+ conductance, incompatible with our observation of the large SCCaFTs generated by Aβ.

Collectively, these results indicate that TIRF imaging of SCCaFTs provides a means to study the properties of pores directly constituted from Aβ oligomers, an approach with important advantages over electrophysiological techniques such as lipid bilayer reconstitution, in that measurements can be obtained simultaneously and independently from hundreds of pores within the membrane of intact cells.

**Figure 3. Ca2+ influx through Aβ1–42 pores is voltage dependent and blocked by Zn2+.** (A) Amplitudes of SCCaFTs from Aβ1–42 pores plotted as function of membrane potential. Points show mean amplitudes (ΔF/F0) ± 1 SEM from >30 events from 10 different pores. The fitted regression line extrapolates to 0 at about 30 mV. (B) Superimposed records of SCCaFTs recorded from 26 Aβ pore sites at the voltages indicated. (C) Representative traces from three regions of interest showing control records of SCCaFTs induced by Aβ1–42 (top) and records from the same regions 5 min after adding 300 μM Zn2+ to the bathing solution.

**SCCaFTs do not involve Aβ activation of endogenous ion channels in the oocyte membrane.**

Several studies indicate that Aβ may increase membrane conductance by activating diverse endogenous channels, including voltage-gated Ca2+ channels and nicotinic, AMPA, N-methyl-D-aspartic acid, and serotonin receptors (Rovira et al., 2002; De Felice et al., 2007; Alberdi et al., 2010). However, it is improbable that the SCCaFTs we observed arose from actions on any of these channels, as they are absent or present only occasionally and at very low density in oocytes (Weber, 1999). The only known Ca2+-permeable channels present at high density in the oocyte membrane are stretch-activated channels and store-operated channels that mediate Ca2+ influx after depletion of ER Ca2+ (Weber, 1999). Neither is likely to account for our results. Application of 5 μM of the specific stretch-activated channel blocker gsmtx-4 (Bode et al., 2001) failed to significantly inhibit whole-cell currents induced 40 min after application of 1 µg/ml Aβ1–42 oligomers (n = 3 oocytes; 2.1 ± 0.51 and 1.85 ± 0.7 μA before and after gsmtx-4, respectively). Moreover, during activation of store-operated influx in oocytes, we observed only a generalized rise in fluorescence Ca2+ signal without evidence of discrete SCCaFTs, suggesting that, like Ca2+ release–activated Ca2+ channels in other cells (Cahalan, 2009), these channels in the oocyte have an extremely low Ca2+ conductance, incompatible with our observation of the large SCCaFTs generated by Aβ.

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**Single Aβ1–42 pores exhibit multiple Ca2+-permeability levels.**

A characteristic feature of Aβ SCCaFTs was the appearance of stepwise transitions between multiple fluorescence levels (Fig. 4 A). The fluorescence signal recorded by TIRF microscopy closely reflects instantaneous Ca2+ flux (Ca2+ current) through single channels (Demuro and Parker, 2005a; Smith and Parker, 2009), and the stepwise changes are reminiscent of current recordings from lipid bilayer systems describing interconversion of Aβ1–40 pores between multiple conductance levels (Arispe et al., 2007). To further analyze this behavior, we measured dwell-state fluorescence amplitudes by visual inspection from 76 Aβ pore sites. The resulting multimodal amplitude distribution (Fig. 4 B) revealed several (five or more) peaks, which do not appear to recur at precise integer multiples.

**Variability in calcium permeability and gating kinetics among Aβ1–42 pores.**

In addition to the variability within and between individual SCCaFTs at a given site (e.g., Fig. 4 A), further variation in fluorescence amplitudes was apparent between different pores. We characterized this by using the CellSpecks program to derive the distribution of the maximal event amplitude detected at each of 2,820 sites (Fig. 4 C). A few sites gave SCCaFTs >2 ΔF/F0, whereas at others, no events larger than ~0.4 ΔF/F0 were observed. Moreover, the latter value likely represents an upper bound, owing to the inability of the automated routine to reliably detect yet smaller events.

An even greater variability among Aβ1–42 pores was apparent in their kinetic properties. This is illustrated in Fig. 5 A, showing a channel chip representation (Demuro and Parker, 2005a) of 172 pores presented in order of decreasing open probability (Popen, top to bottom), and in Fig. 5 B, showing representative traces from pores that showed frequent large amplitude SCCaFTs and those that gave only infrequent smaller events. The overall distribution of mean SCCaFT durations among 2,820 pores within a single 40 × 40-μm membrane region was well fitted by a double exponential function with time constants of 5 and 16 ms (Fig. 6 A); the distribution of mean closed times (intervals between successive SCCaFTs) for the same pores fitted a double exponential decay function with time constants of 5 and 16 ms (Fig. 6 B).
constants of 3.9 and 55 s (Fig. 6 B). The corresponding distribution of $P_o$ showed a very wide range (Fig. 6 C), which could again be fitted by two exponential components. A majority of sites had low (<0.005) $P_o$ values, but a few showed $P_o$ values as high as 0.2. This spread in $P_o$ primarily reflects differences in mean closed times among different pores and not differences in mean open times. After segregating the data into relatively low ($P_o < 0.02$) and high ($P_o ≥ 0.02$) populations, we found only a roughly twofold difference in mean open time between these groups (respective values derived from single-exponential fits of 4.33 ± 0.20 and 19 ± 0.36 ms), whereas the mean closed times differed more than 10-fold (respective time constants 521 ± 55 and 4,271 ± 333 ms). Interestingly, the $P_o$ of a pore correlated strongly with the amplitude of the largest event observed from that pore (Fig. 6 D).

Respective Ca$^{2+}$ load contributed by Aβ1–42 pores with differing $P_o$

The toxicity of Aβ pores will presumably vary in proportion to the amount of Ca$^{2+}$ that passes through them into the cytosol within a given time. Given the wide variation in permeability and gating properties among different pores, we were thus interested to determine which might represent the major toxic species: for example, whether a relatively small number of high $P_o$ pores might contribute a greater fraction of the total cellular Ca$^{2+}$ load than the more numerous low $P_o$ pores. To obtain a relative estimate of the amount of Ca$^{2+}$ entering the cell through a given pore in a fixed time, we analyzed fluorescence traces such as those in Fig. 5 B by integrating the local fluorescence signal over the time during which the pore was open ($\Delta F/F_o \times$ seconds). The blue bars in Fig. 7 A show the distribution of fluorescence...
To better understand the role of pores, we compared the mean fluorescence signals at given sites increased progressively with the number of pores (SCCaFT sites) in the imaging field increased only slightly (from 768 to 805), the relative contributions among different pores to the cumulative Ca$^{2+}$ load changed dramatically (Fig. 7 D). The proportion of pores with high fluorescence integrals (Fig. 7 D, blue bars) and high $P_o$ (Fig. 7 E) was greater than at the earlier time point, such that the top 5% of pores now contributed almost 80% of the total Ca$^{2+}$ load (Fig. 7 D, red curve), as compared with 50% 10 min earlier. Again, the $P_o$ of a pore was the main factor in determining its contribution to the Ca$^{2+}$ load (Fig. 7 E), whereas maximal SCCaFT amplitudes varied over only about a threefold range between pores (Fig. 7 F). Similar results were obtained in a total of four oocytes.

Discussion

Disruption of membrane integrity by soluble Aβ amyloids and consequent dysregulation of intracellular Ca$^{2+}$ homeostasis are implicated as important pathogenic factors in AD (Demuro et al., 2005, 2010; Arispe et al., 2007; Kawahara, 2010; Camandola and Mattson, 2011). Although the specific molecular mechanisms remain controversial (Demuro et al., 2010), there is good evidence that Aβ oligomers create Ca$^{2+}$-permeable pores in the cell membrane (Arispe et al., 2007). The structure (Lal et al., 2007) and electrophysiological properties of these pores have been studied in artificial lipid membrane systems (Arispe et al., 2007) and in excised patches of neuronal membranes (Kawahara et al., 1997). Here, we used TIRF microscopy (Demuro and Parker, 2004b, 2005a) to resolve the permeation of Ca$^{2+}$ through individual Aβ pores in the membrane of Xenopus oocytes. After bath application of Aβ1–42 oligomers, we observed that numerous localized transient hotspots of Ca$^{2+}$ influx, which were dependent on the electrochemical driving force for influx of extracellular Ca$^{2+}$, were blocked by Zn$^{2+}$ and closely resembled Ca$^{2+}$ signals previously seen from single classical Ca$^{2+}$-permeable channels (Demuro and Parker, 2006). Moreover, we did not observe any spatially diffuse Zn$^{2+}$-insensitive Ca$^{2+}$ elevations as expected if amyloid oligomers were to cause membrane thinning and reduction of the dielectric barrier to Ca$^{2+}$ ion translocation (Sokolov et al., 2006); Xenopus oocytes lack endogenous channels reported to interact with Aβ amyloids (De Felice et al., 2007; Alberdi et al., 2010). Therefore, we conclude that the local Ca$^{2+}$ transients (SCCaFTs) arise through openings of individual Aβ oligomer pores.

Thus, TIRF Ca$^{2+}$ imaging provides a novel and powerful means to study the permeation and gating properties of amyloid membrane pores. Specific advantages include the incorporation of pores in a native cell membrane rather than an artificial lipid environment and the ability to simultaneously and independently monitor the activity of hundreds of pores within a small region of the cell membrane. This combination of high-throughput and single-molecule resolution is of particular importance because preparations of amyloid oligomers typically contain a mix of widely differing aggregation states (Demuro et al., 2010). Population measurements (e.g., whole-cell assays) yield no information regarding this diversity, and existing single-pore assays...

![Figure 6. Gating kinetics of Aβ1–42 pores.](image-url)
of levels exhibited by a pore is determined by the number of $A_{3-42}$ pores colocalized within a region smaller than our limit of resolution; the stepwise behavior was seen even when the overall density of $A_{3-42}$ pores was very low, the amplitude levels were not distributed as integer multiples as expected if they represent the summatated $Ca^{2+}$ flux through multiple identical and independent pores, and we frequently observed apparently synchronous transitions across as many as four amplitude levels. Instead, we favor a mechanism wherein the multiple permeability levels represent different open states of a single pore, and the number of levels exhibited by a pore is determined by the number of $A_{3-42}$ peptides that have aggregated to form the pore. A recent study combining imaging of individual fluorescently labeled $A_{3}$ peptides into lipid bilayers together with conductance measurements led to a similar conclusion (Schauerte et al., 2010). Monomers and dimers were found to be nonconducting, whereas small oligomers (up to $\sim$14 mers) induced conductances of a few tens of picosiemens, and large aggregates (which formed only at high $A_{3}$ concentrations) gave conductances of hundreds of picosiemens. Schauerte et al. (2010) was unable to directly associate conductance measurements to individual fluorescent aggregates and instead segregated aggregation states into just three classes. Because the values from electrophysiological measurements are based on monovalent ion currents, it is not possible to make a direct comparison with our estimates of the specific $Ca^{2+}$ conductance of $A_{3}$ pores. Nevertheless, our findings of a wide spectrum of permeability and gating properties point to a more nuanced continuum of properties within a population of $A_{3}$ pores that likely correspond to the class II morphological aggregates of Schauerte et al. (2010).

A related question is whether the $A_{3}$ oligomers that create $Ca^{2+}$-permeable pores are preformed in solution or aggregate after interaction of smaller precursors within the membrane. The available evidence suggests involvement of both processes. Consistent with rapid membrane incorporation of preestablished and functional oligomeric complexes, we had found that extracellular application of oligomeric $A_{3}$ resulted in rapid induction of membrane $Ca^{2+}$ permeability, whereas monomers were ineffective (Demuro et al., 2005). On the other hand, Schauerte et al. (2010) described binding of individual fluorescently labeled $A_{3}$ monomers to lipid bilayers and subsequent appearance of ion-conducting oligomers at a rate much faster than the oligomerization rate (hours) in aqueous solution. Our finding that individual $A_{3}$ pores show progressive increases in开关
in P<sub>0</sub> and permeability over several minutes further supports the idea that pores already active in the membrane may grow and change their properties by continued accretion of monomers or small oligomers.

SCCaFTs arising from Aβ pores remained at fixed locations over several minutes. This greatly simplified analysis but is surprising because free diffusion of proteins in the lipid membrane would be expected to result in clearly detectable movements of several micrometers (Demuro and Parker, 2005a). We do not believe that the immotility of pores arose through mechanical interaction with the cover glass because a similar lack of motility was evident in oocytes with intact vitelline envelope. Instead, Aβ may bind to some static cytoskeletal structure.

Our results were obtained using Xenopus oocytes, and it remains to be determined how closely the actions of Aβ on this model cell system replicate those on neuronal membranes. Moreover, it is not clear how the progressive changes in gating and permeability that we observe over a timescale of minutes may relate to the progression of AD over years, during which time neurons are continually exposed to low but rising concentrations of soluble Aβ in the cerebrospinal fluid. Nevertheless, we speculate that the initial evolution of the disease may involve formation of small-conductance, low P<sub>0</sub> pores that, although not immediately triggering cell death, produce chronic pathological cell stress. A tipping point may then be reached when a sustainable level of Ca<sup>2+</sup> influx through these pores is abruptly exceeded by the appearance of even a few high-conductance, high P<sub>0</sub> pores that contribute a disproportionately large Ca<sup>2+</sup> load.

In summary, our application of high-resolution TIRF imaging provides strong support for a mechanism whereby Aβ amyloid oligomers form intrinsic Ca<sup>2+</sup>-permeable pores in the cell membrane and enables massively parallel characterization of the permeation and gating properties of thousands of pores. Our results reveal an enormous range in activities that may reflect concentration—and time dependent—differences in stoichiometry of Aβ pores and suggest a mechanism wherein continued aggregation of oligomers leads to the formation of highly active high-permeability pores that may play a disproportionate cytotoxic role in the pathogenesis of AD. Moreover, our single-channel Ca<sup>2+</sup> imaging approach would be equally applicable to studies of numerous other amyloid-related diseases, including Huntington’s, Parkinson’s, and prion disease, in which increased membrane Ca<sup>2+</sup> permeability is implicated as a major pathological mechanism (Demuro et al., 2005).

**Materials and methods**

**Preparation and characterization of Aβ<sub>1–42</sub> oligomers**

Soluble oligomers were prepared by dissolving 0.5 mg of human recombinant Aβ<sub>1–42</sub> peptide (hexafluorooisopropanol pretreated; RPeptide) in 20 µl of freshly prepared DMSO and were quickly diluted with 480 µl of double-distilled water in a siliconized Eppendorf tube. After a 10-min sonication, samples were incubated at room temperature for 10 min and then centrifuged for 15 min at 14,000 g. The supernatant fraction was transferred to a new siliconized tube and stirred at 500 rpm using a Teflon-coated microsir bar for 8–48 h at room temperature. Aliquots were taken at intervals and were assayed by pipette application to voltage-clamped oocytes at a final bath concentration of 1 µg/ml. Membrane currents were recorded 30 min after application in response to hyperpolarization from 0 to −100 mV. Aβ preparations incubated for <6 h evoked little or no current, indicating that the monomeric peptide was ineffective at inducing membrane Ca<sup>2+</sup> permeability. Preparations that evoked currents ≥1 µA were used immediately for TIRF imaging or were stored at −20°C before use (Demuro et al., 2005). Aβ1–42 samples were further characterized by Western blot analysis (Kayed et al., 2010). Samples containing 4 µg Aβ1–42 were diluted in SDS treatment buffer, boiled for 5 min, and separated on a 4–20% Tris-HCl gel (Bio-Rad Laboratories) at 4°C for 2 h at 85 V. Proteins were transferred onto a nitrocellulose membrane, which was then blocked for 1 h in 5% nonfat dry milk in TBS/Tween 20 buffer. The membranes were probed with the mouse monoclonal anti-Aβ antibody 6E10 (0.1 µg/ml; Sigma-Aldrich), and the conjugation-specific polyclonal antibody OC (0.4 µg/ml; a gift from C. Globe, University of California, Irvine, Irvine, CA) overnight at 4°C. The membranes were then incubated with anti-mouse IgG conjugated with HRP (1:10,000) for 1 h at room temperature. Blots were developed with a chemiluminescence kit (SuperSignal West Pico; Thermo Fisher Scientific).

**Oocyte preparation and electrophysiology**

Experiments were performed on defolliculated stage VI oocytes obtained from Xenopus. Oocytes were injected ~1 h before imaging with fluo-4 dextran (molecular mass of ~10 kD and Ca<sup>2+</sup> affinity of ~3 µM) to a final intracellular concentration of ~40 µM. For TIRF microscopy experiments, oocytes were placed in a hypertonic solution (200 mM K aspartate, 20 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM EGTA, and 10 mM Hepes, pH 7.2) to shrink so that the vitelline envelope could be manually torn apart using fine forceps. Oocytes were then placed on the flat-bottomed hemisphere down in a chamber whose bottom is formed by a fresh ethanol-washed microscope cover glass (type-545-M; Thermo Fisher Scientific) and were bathed in Ringer’s solution (110 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 2 mM KCl, and 5 mM Hepes, pH 7.2) at room temperature (~23°C) continually exchanged at a rate of ~0.5 ml/min by a gravity-fed superfusion system. The membrane potential was clamped at a holding potential of 0 mV using a two-electrode voltage clamp (Gene Clamp 500; Molecular Devices) and was stepped to more negative potentials (~100 mV unless otherwise indicated) when imaging Ca<sup>2+</sup> flux through amyloid pores to increase the driving force for Ca<sup>2+</sup> entry into the cytosol. Solutions containing Aβ42 oligomers were delivered from a glass pipette with a tip diameter of ~30 µm positioned near the edge of the membrane footprint of the oocyte membrane on the cover glass.

**TIRF microscopy, image acquisition, and processing**

Imaging was accomplished by using a custom-built TIRF microscope system based around a microscope (IX70; Olympus) equipped with a 60x TIRF microscopy objective (1.45 NA; Olympus; Demuro and Parker, 2005b). Fluorescence excited by a 488-nm laser was imaged using an electron-multiplied charge-coupled device camera (Cascade 128+; Roper Scientific) at full resolution (128 x 128 pixel; 1 pixel = 0.33 µm at the microscope) at a rate of 500 s<sup>−1</sup>. Image data were acquired using the MetaMorph software package (Universal Imaging) and were black-level corrected by subtracting the camera offset. To compensate for differential time dependent changes in baseline fluorescence across different locations in the image field, a strongly smoothed (10 x 10-pixel Gaussian blur) copy of each frame was calculated to create a running baseline (F<sub>0</sub>) image. The raw image stack was then divided, frame by frame, by the smoothed copy to create a baseline-corrected pseudoratio stack in which each pixel represents localized differences in fluorescence relative to the spatially averaged baseline fluorescence (ΔF/F<sub>0</sub>). In initial experiments, data were analyzed manually using MetaMorph to visually identify sites of pore activity, and traces of fluorescence versus time such as those in Figs. 2–5 were obtained as the maximum pixel intensity within fixed 3 x 3-pixel (~1 x 1 µm) regions of interest centered on putative pore locations. Fluorescence measurements were not confounded by movement of Aβ pores, as tracking the centroid locations of SCCaFTs with subpixel resolution revealed only slight random deviations throughout the imaging record (SD typically <0.3 pixel), which are negligible in comparison with the size of the regions of interest used to measure fluorescence intensities. The maximum observed fluorescence signals were small (maximum ΔF/F<sub>0</sub> <2.0) in comparison with the full dynamic range of fluo-4 (ΔF/F<sub>0</sub> >30 in saturating Ca<sup>2+</sup>) and are thus expected to be linearly proportional to Ca<sup>2+</sup> flux.

**Event detection algorithm and automated data analysis (CellSpexcs)**

TIRF microscopy recordings produce large (128 x 128 pixel x 15,000 frame) image stacks, which can represent the activity of >1,000 discrete Aβ pores. Thus, it was extremely laborious to compile and analyze population data by visual inspection, and we did not find existing software, including GIMP and SparkMaster (Mashanov and Molloy, 2007; Picht et al., 2007).
adequate for our purposes. Accordingly, we developed a Java-based program (CellSpecks) to identify active sites, generate idealized traces of pore gating, and derive parameters, including mean open and closed times and open probabilities as presented in Figs. 6 and 7. CellSpecks is coded in Java and accepts image files in MetaMorph stack format or using Sun Java standard libraries for opening directories of sequential images. Each frame is converted into a new 2D array and stored as a stack object in memory. The steps described in the next sections are then performed to identify fluorescence signals, associate these with pore locations, and output final data statistics.

**Fluorescence thresholding.** For each pixel, a 1D array of intensity values over the time is created and is corrected for baseline drift by subtraction of a heavily smoothed copy formed by filtering with a boxcar mean 51 frames in length. The modal value (most commonly observed value) of the resulting array is then taken as the baseline, and an estimate of baseline noise is calculated as the SD of only those source values that fall below the mode. A value of 2.5 times the negative SDs above the mode was selected as the noise threshold to minimize false positives, and a new stack is created in which all pixel values below the threshold are set to 0, and those above threshold are set to their original values relative to the mode.

**Event detection.** The event detection process is based on an object-oriented programming paradigm. For each pixel, a new 3D array (x,y,t) of event-part object pointers is represented to generate the pixel in space and time. Each event-part object contains the intensity value at that location in time as well as pointers to event-parts above, below, left, right, and before and after (in time) the current pixel. When these event-parts are loaded into each other’s pointers, contiguous signal pixels are then linked to each other and bounded by null pointers to nonsignal pixels. Events are built from these 3D groups of contiguous signal pixels by scanning the stack and recursively linking all contiguous event parts to a single event object (SCCAfT). At the end of this process, a list of all event locations in the stack is generated. After constructing a list of events, CellSpecks performs a series of steps to generate a list of pore locations.

**Channel attribution.** The events are then analyzed one by one and assigned to a designated pore location. To facilitate the location of pores, a weight array, w(x,y), is generated as a 2D array containing the sum of pixel values from the original stack over time. Local maxima are interpreted as likely pore locations and are used to create an initial list of putative pore objects. Events are then linked to pores one by one, either to the closest known pore or to a new pore location. Because the coordinates of a pore are dynamically generated from their constituent events, events early in the stack could bias the location of pores to which subsequent nearby larger events may be assigned. The algorithm avoids this problem by seeding the list of pores with the locations of larger events (whose fluorescence spread over a larger area and could therefore be more flexibly associated) regardless of their order, so that they are assigned their own putative pore locations at the outset. Whether or not an event is added to the existing pore list depends on two criteria. The first is binary overlap verification that checks whether or not the coordinates of the event and pore in consideration when rounded to the nearest pixel in space are equal. Event locations are calculated from their constituent event parts using a mean of the event-part coordinates weighted by intensity. The coordinate for the location of the pore is then calculated using a mean of the coordinates of the pore’s constituent events weighted by each event’s total intensity, calculated as the sum of the intensity measured in all the pixels involved in space and time. The second criterion for event-pore association takes into consideration the relative intensity, Ir, of the event’s pixels at the assigned pore location over the duration of the event. Ir is calculated as the ratio between the sums of the intensity of each pixel over the duration of the events versus the maximum intensity measured at this location. The relative intensity of an event at the center of the pore multiplied by the inverse of distance between the event and pore centers must be >0.2, a threshold value empirically determined for our experimental conditions.

Events that do not fulfill either of these criteria are assigned to new pore locations. Finally, putative pore locations from the initial list that are not associated with any events are discarded. By the end of the detection and attribution processes, a final list of designated pore locations has been produced, and each location is associated with a list of linked events.

**Data visualization, analysis, and export.** The list of events associated with each pore location is used to derive distributions of pore open and closed times (i.e., event durations and inter-event intervals), from which population statistics are then calculated. A graphical user interface allows on-screen viewing of raw and processed data (e.g., intermediate stack, baseline, signal, and noise information). Clicking on a map of locations brings up plots as these values as functions of time, and sampling can be modified to select a mean or maximum of pixel values over a user-specified region of interest. Data (including individual measurements of event durations, intervals, and fluorescence amplitudes together with population measurements of mean pore open/closed times, Po, and mean fluorescence) are exported as ASCII text files.

**Materials**

Fluo-4 dextran was purchased from Invitrogen, and human Aβ1–42 peptide was purchased from Millipore and rPeptide. gsmx-4 was a gift from F. Sachs (University at Buffalo, Buffalo, NY). All other reagents and antibodies were purchased from Sigma-Aldrich.

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### References

Alberdi, E., M.V. Sánchez-Gómez, F. Cavaliere, A. Pérez-Samartin, J.L. Zagaga, R. Traillas, M. Domeroç, and C. Mateo. 2010. Amyloid beta oligomers induce Ca2+ dysregulation and neuronal death through activation of ionotrophic glutamate receptors. *Cell Calcium*. 47:264–272. http://dx.doi.org/10.1016/j.ceca.2009.12.010

Arispe, N., H.B. Pollard, and E. Rojas. 1993. Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1-40)] in bilayer membranes. *Proc. Natl. Acad. Sci. USA*. 90:10573–10577. http://dx.doi.org/10.1073/pnas.90.22.10573

Arispe, N., H.B. Pollard, and E. Rojas. 1996. Zn2+ interaction with Alzheimer amyloid beta protein calcium channels. *Proc. Natl. Acad. Sci. USA*. 93:1710–1715. http://dx.doi.org/10.1073/pnas.93.4.1710

Arispe, N., J.C. Diaz, and O. Simakova. 2007. Abeta ion channels. Prospects for treating Alzheimer’s disease with Abeta channel blockers. *Biochim. Biophys. Acta*. 1768:1952–1965. http://dx.doi.org/10.1016/j.bbamem.2007.03.014

Bode, F., F. Sachs, and M.R.Franz. 2001. Tarantula peptide inhibits atrial fibrillation. *Nature*. 409:35–36. http://dx.doi.org/10.1038/35051165

Bourinet, E., F. Fournier, J. Nargeot, and P. Charnet. 1992. Endogenous Xenopus-oocyte Ca-channels are regulated by protein kinases A and C. *FEBS Lett*. 299:5–9. http://dx.doi.org/10.1016/0014-5793(92)80087-W

Bucciantini, M., E. Giannoni, F. Chiti, F. Baroni, L. Formigli, J. Zurdo, N. Taddei, G. Ramponi, C.M. Dobson, and M. Stefani. 2002. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature*. 416:507–511. http://dx.doi.org/10.1038/416507a

Cahalan, M.D. 2009. STIMulating store-operated Ca(2+) entry. *Nat. Cell Biol*. 11:669–677. http://dx.doi.org/10.1038/ncb1069-669

Camandola, S., and M.P. Mattson. 2011. Aberrant subcellular neuronal calcium regulation in aging and Alzheimer’s disease. *Biochim. Biophys. Acta*. 1813:965–973. http://dx.doi.org/10.1016/j.bbamer.2010.11.005

Capone, R., F.G. Quiroz, P. Prangkio, I. Saluja, A.M. Sauer, M.R. Bautista, R.S. Turner, J. Yang, and M. Mayer. 2009. Amyloid-beta-induced ion flux in artificial lipid bilayers and neuronal cells: Resolving a controversy. *Neurotox. Res*. 16:1–13. http://dx.doi.org/10.1007/s12640-009-9033-1

Cras, P., M. Kawai, D. Lowery, P. Gonzalez-DeWhitt, B. Greenberg, and G. Perry. 1991. Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein. *Proc. Natl. Acad. Sci. USA*. 88:7552–7556. http://dx.doi.org/10.1073/pnas.88.17.7552

De Felice, F.G., P.T. Velasco, M.P. Lambert, K. Viola, S.J. Fernandez, S.T. Ferreira, and W.L. Klein. 2007. Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *J. Biol. Chem*. 282:11590–11601. http://dx.doi.org/10.1074/jbc.M607483200

Demuro, A., and I. Parker. 2003. Optical single-channel recording: imaging Ca2+ flux through individual N-type voltage-gated channels expressed in Xenopus oocytes. *Cell Calcium*. 34:499–509. http://dx.doi.org/10.1016/S0143-4160(03)00154-4

Demuro, A., and I. Parker. 2004a. Imaging single-channel calcium microdomains by total internal reflection microscopy. *Biophys. Res. Lett*. 37:675–679. http://dx.doi.org/10.1007/S00716-97602004000040025

Demuro, A., and I. Parker. 2004b. Imaging the activity and localization of single voltage-gated Ca2+ channels by total internal reflection fluorescence microscopy. *Biophys. J*. 86:3250–3259. http://dx.doi.org/10.1016/S0006-3495(04)74373-8
Lemere, C.A., J.K. Blusztajn, H. Yamaguchi, T. Wisniewski, T.C. Saito, and Lashuel, H.A., D. Hartley, B.M. Petre, T. Walz, and P.T. Lansbury Jr. 2002. Amyloid beta ion channels: 3D structure and membranes prevents beta-amyloid-induced toxicity. Proc. Natl. Acad. Sci. USA. 100:2047–2052.

Liao, R., H. Lin, and A.P. Quist. 2007. Amyloid beta ion channel: 3D structure and relevance to amyloid channel paradigm. Biochim. Biophys. Acta. 1768:1966–1975. http://dx.doi.org/10.1016/j.bbamem.2007.04.021

Lashuel, H.A., D. Hartley, B.M. Petre, T. Walz, and P.T. Lansbury Jr. 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. Nature. 418:291. http://dx.doi.org/10.1038/418291a

Lemere, C.A., J.K. Blusztajn, H. Yamaguchi, T. Wisniewski, T.C. Saito, and D.J. Selkoe. 1996. Sequence of deposition of heterogeneous amyloid beta-peptides and APO E in Down syndrome: Implications for initial events in amyloid plaque formation. Neurobiol. Dis. 3:16–32. http://dx.doi.org/10.1006/nbd.1996.0003

Lin, H., R. Bhatia, and R. Lal. 2001. Amyloid beta protein forms ion channels: Implications for Alzheimer’s disease pathophysiology. FASEB J. 15:2433–2444. http://dx.doi.org/10.1096/fj.01-0377com

Mashanov, G.I., and J.E. Molloy. 2007. Automatic detection of single fluorophores in live cells. Biophys. J. 92:2199–2211. http://dx.doi.org/10.1529/biophysj.106.081117

Mason, R.P., R.F. Jacob, M.F. Walter, P.E. Mason, N.A. Avdulov, S.V. Chochuna, U. Ighbavwe, and W.G. Wood. 1999. Distribution and fluidizing action of soluble and aggregated amyloid beta-peptide in rat synaptic plasma membranes. J. Biol. Chem. 274:18801–18807. http://dx.doi.org/10.1074/jbc.M500977200

Mattson, M.P., B. Cheng, D. Davis, K. Bryant, I. Lieberberg, and R.E. Rydel. 1992. beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J. Neurosci. 12:157–165.

Miledi, R., and I. Parker. 1984. Chloride current induced by injection of calcium into Xenopus oocytes. J. Physiology. 357:173–183.

Picht, E.V., A.V. Zima, L.A. Blatter, and D.M. Bers. 2007. SparkMaster: Automated calcium spark analysis with ImageJ. Am. J. Physiol. Cell Physiol. 293: C1073–C1081. http://dx.doi.org/10.1152/ajpcell.00586.2006

Pollard, H.B., E. Rojas, and N. Arispe. 1993. A new hypothesis for the mechanism of amyloid toxicity, based on the calcium channel activity of amyloid beta protein (A beta P) in phospholipid bilayer membranes. Ann. NY Acad. Sci. 695:165–168. http://dx.doi.org/10.1111/j.1749-6632.1993.tb03046.x

Quist, A., I. Doudesvki, H. Lin, R. Azimova, D. Ng, B. Frangione, B. Kagan, J. Ghiso, and R. Lal. 2005. Amyloid ion channels: A common structural link for protein-misfolding disease. Proc. Natl. Acad. Sci. USA. 102:10447–10452. http://dx.doi.org/10.1073/pnas.0500266102

Rhee, S.K., A.P. Quist, and R. Lal. 1998. Amyloid beta protein (1-42) forms calcium-permeable, Zn2+-sensitive channel. J. Biol. Chem. 273:13379–13382. http://dx.doi.org/10.1074/jbc.273.22.13379

Rovira, C., N. Arbez, and G. Mariani. 2002. Abeta(25-35) and Abeta(1-40) act as calcium sparks in live cells. J. Biol. Chem. 277:67–75. http://dx.doi.org/10.1074/jbc.M500997200

Sainz, J., and I. Parker. 2005. Antibody current-induced calcium influx through individual ion channels with high temporal and spatial resolution. J. Biomed. Opt. 10:11002. http://dx.doi.org/10.1117/1.1846074

Selkoe, D.J. 2000. Quantification of Alzheimer amyloid beta peptides ending at residue 42. J. Neurosci. 20:1350612080252485

Shaw, T.A., P. Sung, R.C. Hesser, H. Ding, D.G. Steel, and A. Gafni. 2005. Simultaneous single-molecule fluorescence and conductivity studies reveal distinct classes of Abeta species on lipid bilayers. Biochemistry. 44:3031–3039. http://dx.doi.org/10.1021/bi041444w

Shank, G.M., S. Li, T.H. Mehta, A. Garcia-Munoz, N.E. Shepardson, I. Smith, F.M. Brett, M.A. Farrell, M.J. Rowan, C.A. Lemere, et al. 2008. Amyloid-beta protein dimers isolated directly from Alzheimer’s brains impair synaptic plasticity and memory. Nat. Med. 14:837–842. http://dx.doi.org/10.1038/nm1782

Smith, I.F., and I. Parker. 2009. Imaging the quantal substructure of single IP3R channel activity during Ca2+ influx in intact mammalian cells. Proc. Natl. Acad. Sci. USA. 106:6404–6409. http://dx.doi.org/10.1073/pnas.0810799106

Sokolov, Y., J.A. Kozak, R. Kayed, A. Chanturiya, C. Glabe, and J.E. Hall. 2006. Soluble amyloid oligomers increase bilayer conductance by altering dielectric structure. J. Gen. Physiol. 128:637–647. http://dx.doi.org/10.1529/jgp.200609533

Wals, D.M., I. Klyubin, J.V. Fadeeva, W.K. Cullen, R. Anwyl, M.S. Wolfe, M.J. Rowan, and D.J. Selkoe. 2002. Naturally secreted oligomers of amyloid beta protein potentially inhibit hippocampal long-term potentiation in vivo. Nature. 416:535–539. http://dx.doi.org/10.1038/416535a

Wang, H.Y., D.H. Lee, M.R. D’Andrea, P.A. Peterson, R.P. Shank, and A.B. Reitz. 2000. Amyloid beta-1-42 binds to alpha3 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer’s disease pathology. J. Biol. Chem. 275:5626–5632. http://dx.doi.org/10.1074/jbc.M50069533

Weber, W.M. 1999. Endogenous ion channels in oocytes of Xenopus laevis: Recent developments. J. Membr. Biol. 170:1–12. http://dx.doi.org/10.1007/s002329900532

Wang, H.Y., D.H. Lee, M.R. D’Andrea, P.A. Peterson, R.P. Shank, and A.B. Reitz. 2000. Amyloid beta-1-42 binds to alpha3 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer’s disease pathology. J. Biol. Chem. 275:5626–5632. http://dx.doi.org/10.1074/jbc.M50069533

Weber, W.M. 1999. Endogenous ion channels in oocytes of Xenopus laevis: Recent developments. J. Membr. Biol. 170:1–12. http://dx.doi.org/10.1007/s002329900532