

Alzheimer's Toxic Amyloid Beta Oligomers: Unwelcome Visitors to the Na/K ATPase alpha3 Docking Station

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Toxic amyloid beta oligomers (A β O_s) are known to accumulate in Alzheimer's disease (AD) and in animal models of AD. Their structure is heterogeneous, and they are found in both intracellular and extracellular milieu. When given to CNS cultures or injected ICV into non-human primates and other non-transgenic animals, A β O_s have been found to cause impaired synaptic plasticity, loss of memory function, tau hyperphosphorylation and tangle formation, synapse elimination, oxidative and ER stress, inflammatory microglial activation, and selective nerve cell death. Memory loss and pathology in transgenic models are prevented by A β O antibodies, while Aducanumab, an antibody that targets A β O_s as well as fibrillar A β , has provided cognitive benefit to humans in early clinical trials. A β O_s have now been investigated in more than 3000 studies and are widely thought to be the major toxic form of A β . Although much has been learned about the downstream mechanisms of A β O action, a major gap concerns the earliest steps: How do A β O_s initially interact with surface membranes to generate neuron-damaging transmembrane events? Findings from Ohnishi et al (PNAS 2005) combined with new results presented here are consistent with the hypothesis that *A β O_s act as neurotoxins because they attach to particular membrane protein docks containing Na/K ATPase- α 3, where they inhibit ATPase activity and pathologically restructure dock composition and topology in a manner leading to excessive Ca⁺⁺ build-up*. Better understanding of the mechanism that makes attachment of A β O_s to vulnerable neurons a neurotoxic phenomenon should open the door to therapeutics and diagnostics targeting the first step of a complex pathway that leads to neural damage and dementia.

THE ABO HYPOTHESIS -- A MECHANISM WITH PRIME TARGETS FOR AD THERAPEUTICS

The amyloid beta oligomer (A β O) hypothesis for

Alzheimer's disease (AD), introduced in 1998 [1], says that *dementia is the consequence of neural damage instigated by soluble, toxic A β O_s*. Earlier investigations had found A β O_s in AD brain extracts [2], but their toxicity was not apparent. Discovery of methods to make soluble

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†Abbreviations: A β O_s, Amyloid beta oligomers; AD, Alzheimer's disease; CNS, central nervous system; CSF, cerebrospinal fluid; ER, endoplasmic reticulum; INDs, investigational new drugs; LTP, long-term potentiation; mGluR5, metabotropic glutamate receptor 5; MW, molecular weight; NMDA-R, N-methyl-D-aspartate receptor; NaKA α 3, Sodium potassium ATPase alpha3; ROS, reactive oxygen species; Tg, transgenic; SOM, small organic molecule.

Keywords: Abeta oligomers, Alzheimer's, sodium-potassium ATPase, NaK ATPase, toxicity, synapse, spine loss, receptor

Table 1. NU-series antibodies that selectively bind A β O. A β O-antibodies commonly used by our lab comprise NU 1, 2, and 4 [11]. They have low affinity for A β monomers and fibrils. Their affinities for our A β O preparations were determined here using an indirect ELISA with 20 pmols of A β O per well (total A β monomer equivalents). For comparison, affinities were determined for other commonly used antibodies that have varying degrees of selectivity for monomers, fibrils, and different forms of A β O.

Ab	EC ₅₀ (ug/mL Ab)
NU2	0.15
NU1	0.29
NU4	0.40
6E10	0.67
4G8	1.85
OC	4.66
A11	No signal
MOAB-1	No signal

A β O without contaminating fibrils [3] opened the door to brain slice experiments that revealed A β O are potent CNS neurotoxins, capable of rapidly inhibiting hippocampal LTP at low doses and, with longer exposures, causing cell death in vulnerable neuron populations [1]. Cell-selective impact was evident, as only subpopulations of neurons were lost. Death, moreover, was signaling-dependent, as knockout of the protein tyrosine kinase Fyn was neuroprotective. A β O did not act as “molecular shrapnel.” These were toxins that were cell-selective and required signal transduction. The mechanism was hypothesized to depend on the interaction of A β O with toxin receptors, a hypothesis still of current interest and one that will be discussed later in this article.

Toxic A β O are now known to be salient features of AD neuropathology. They accumulate early in the disease process, in humans and in transgenic (Tg) animal AD models [4,5]. In many Tg models, including hAPP [6], 3xTg-AD [7], APP-Tg E693 Δ [8], Tg McGill-Thy-1-APP [9], APP-Tg E693Q “Dutch” [10], and 5xFAD (unpublished data from WL Klein and R Vassar labs), A β O accumulate before the emergence of plaques. Detection is possible using oligomer-specific antibodies for histology and sensitive dot immunoblots and sandwich ELISAs for solution assays [11]. Table 1 shows the affinities of commonly used research antibodies, several of which are oligomer-specific. In some cases, as in the Osaka mutation [12], toxic A β O accumulate without amyloid plaques, which are absent despite an otherwise full complement of AD pathology. The plaque-free build-up of A β O and other AD pathology is recapitulated in a mouse model of the Osaka mutation [8]. This suggests that the old definition of AD as dementia with plaques and tangles may be misdirected. Amyloid plaques are not required for dementia; toxic A β O are.

The subunits of synthetic A β O in most instances are not covalently bonded, and assembly states of A β O are heterogeneous. While dynamic, the major assembly states are stable enough to be detected. Figure 1, e.g., shows an

FPLC-SEC profile of synthetic A β O with a peak near 110 kDa and another at about twice this mass. A β O are larger in aqueous buffers than in buffers with detergents. In Western blots, synthetic A β O typically break down to monomers and very low MW A β O. Breakdown is reduced by crosslinking procedures, particularly for smaller species [13]. Larger species are stabilized by extended incubation [14]. It is likely that the brain environment modifies the oligomeric state, as the inflammatory molecule levuglandin stabilizes larger species [15], and pyroglutamylated A β , for example, is common in brain-derived A β O. 2D gel analysis shows structural homology between A β O in aqueous extracts of AD-affected brain and toxic A β O made *in vitro*. Prominent dodecamers (54 kDa) are present in AD and synthetic preparations but not control brain [16]. A dodecamer found in Western blots, referred to as A β *56, accumulates in Tg2576 mouse brain roughly at the onset of memory dysfunction [17]. 24mers also have been found in SDS extracts of AD affected human and animal brain tissue [16,18].

The A β 42 peptide is now widely used for experimentation because it is more closely associated with AD pathology than is A β 40 [19]. The original fibril-free preparation of toxic A β O was made using clusterin as a chaperone and also by using very low A β 42 concentrations *sans* clusterin [1]. Because of the expense of clusterin, most preparations currently are made in simple buffers. Oligomerization is highly influenced by concentration, temperature, buffer, and presence of non-monomeric seeds; even vortexing affects the outcome. It also has become clear that there are naturally-occurring alternative pathways of self-assembly. These alternative pathways produce relatively stable toxic oligomers greater than 50 kDa (on Western blots) and oligomers that assemble further into fibrillar A β [14]. The products have been referred to as Type 1 and Type 2 oligomers, respectively [20]. A variety of preparations have been developed and used for experimentation, including use of pyroglutamylated N-terminal to generate highly toxic A β O [21]. A summary of preparations and structures can be found in our recent review [22]. Protocols for preparation and use of A β O typically used in our laboratory can be accessed at our home page (www.kleinlab.org).

Synthetic and brain-derived A β O preparations cause a spectrum of AD-like, cell-specific neural damage. In CNS cultures, e.g., neurons with bound A β O manifest AD-type phospho-tau, whereas neurons without bound A β O show much less of this phospho-tau [23]. Overall

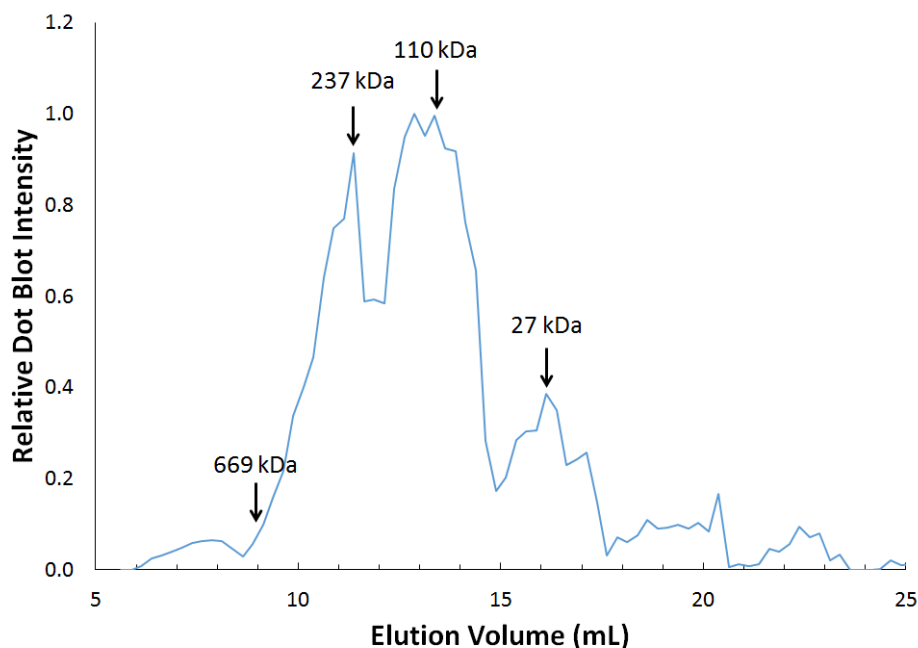


Figure 1. Prominent peaks in FPLC-SEC of synthetic A β O (110 kDa and 237 kDa). Preparations of A β O were made by incubating 200 nM A β monomer in F12 and centrifuging to remove traces of fibrils according to published protocols [22]. Chromatography was as described [72]. Fractions were collected off the column and immunoreactivity to NU2 determined via dot immunoblotting. Mass of 110 kDa corresponds approximately to a 24mer of A β 42.

evidence strongly supports the role of A β O in instigating tau pathology [24], which mediates some of the A β O toxic impact [24]. Because APP transgenes accelerate propagation of tau pathology in Tg mice [25,26], we hypothesize it is likely that A β O may likewise play a role in this aspect of tau pathology. ICV injections of A β O into wildtype animals likewise evoke AD neuropathology, including non-human primates [27]. Tg animals producing A β O manifest equivalent neural damage [8,9,28]. Table 2 provides a short list of the wide-spread AD-like damage evoked by toxic A β O preparations. Even though structure-function details vary between laboratories, and some effects may have been found at pharmacological rather than pathogenic doses, the take-home lesson is that certain species of A β O, found *in vitro* and in brain, are potent CNS neurotoxins. A β O, which have been investigated in more than 3000 studies, are now considered the major toxic form of A β .

The stages in AD progression have been summarized by Jack and colleagues (Figure 2, right). Brain damage is now understood to begin decades before dementia, with A β pathology giving rise to tau pathology. In this context of disease progression, it is likely that brain damage begins with A β O, which appear before plaques and comprise the major toxic forms of A β . Sometimes, as in the case of the Osaka mutation, A β O appear even without plaques. Current findings are consistent with the hypothesis that A β O provide a unifying mechanism for initia-

tion of the neural damage underlying dementia. Evidence strongly points to the build-up of toxic A β O as a seminal event in AD progression.

TESTING THE ABO HYPOTHESIS WITH CLINICAL TRIALS

Continued interest in the A β O hypothesis will require successful clinical trials based on preventing A β O from instigating neural damage. The most advanced approach is immunotherapy. A β O-specific antibodies, developed to verify the presence of toxic A β O in AD pathology, can prevent pathology and memory loss in transgenic AD animals. An early success used the pan-oligomer specific A11 polyclonal to lower tau pathology in the 3xTg-AD mouse model [7]. Other A β O-targeting antibodies have rescued behavior as well as neural health in Tg AD models [29-32]. The critical question is whether these successes can be translated to humans. There have been multiple clinical trial failures of immunotherapy related to A β going back to 2000. The latest disappointment occurred with Lilly's Solanezumab, which targets monomeric A β (Figure 2). These failures, along with unsuccessful trials using small molecule treatments designed to prevent A β pathogenesis, have cost an estimated \$18 billion. The extreme cost of past failures has virtually poisoned the well for new therapeutic strategies targeting A β .

However, after 15 years of failures, a new trial has

Table 2. Alzheimer's-like neural damage instigated by A β O.

A comprehensive discussion of A β O-instigated neural damage can be found in recent reviews [22,40].

Neuronal damage induced by A β O	References
AD-type aberrant tau hyperphosphorylation	De Felice et al, 2008 [23]; Ma et al, 2009 [77]; Tomiyama et al, 2010 [8]; Zempel et al, 2010 [78]
Plasticity dysfunction (LTP/LTD)	Lambert et al, 1998 [1]; Walsh et al, 2002 [79]; Wang et al, 2002 [80]; Townsend et al, 2006 [81]
Memory failure	Selkoe, 2008 [82]; Shankar et al, 2008 [83]; Freir et al, 2011 [84]; Lesne et al, 2008 [85]; Poling et al, 2008 [86]; Xiao et al, 2013 [29]
Synapse loss	Zhao et al, 2006 [87]; Lacor et al, 2007 [55]; Shankar et al, 2007 [88]; Townsend et al, 2010 [89]
Disrupted Ca ⁺⁺ homeostasis	Demuro et al, 2005 [90]; De Felice et al, 2007 [49]; Alberdi et al, 2010 [91]
Oxidative, ER stress	Longo et al, 2000 [92]; Sponne et al, 2003 [93]; Tabner et al, 2005 [94]; De Felice et al, 2007 [49]; Resende et al, 2008 [95]; Nishitsuji et al, 2009 [96]
Synaptic receptor trafficking abnormalities	Snyder et al, 2005 [97]; Roselli et al, 2005 [98]; Lacor et al, 2007 [55]; Zhao et al, 2008 [52]
Inhibition of ChAT	Heinitz et al, 2006 [99]; Nunes-Tavares et al, 2012 [100]
Insulin resistance	Zhao et al, 2008 [52]; Zhao et al, 2009 [101]; Ma et al, 2009 [77]; De Felice et al, 2009 [102]
Inhibition of axonal transport	Pigino et al, 2009 [103]; Poon et al, 2011 [104]; Decker et al, 2010 [105]
Aberrant astrocytes, microglia	Hu et al 1998 [106]; Jimenez et al, 2008 [107]; Tomiyama et al, 2010 [8]
Cell cycle re-entry	Varvel et al, 2008 [108]; Bhaskar et al, 2009 [109]
Selective nerve cell death	Lambert et al, 1998 [1]; Kim et al, 2003 [54]; Ryan et al, 2009 [110]

provided positive results, and these are in harmony with predictions of the A β O hypothesis. Aducanumab, a therapeutic monoclonal from Biogen Idec was found to slow cognitive deterioration in early stage clinical trials [33]. Aducanumab binds A β O and fibrillar A β but not monomeric A β , although there appears to be a problem with dosage. The need for high levels can be explained by nonproductive association of Aducanumab with senile plaques. More specific antibodies could prove beneficial. ACU193, a humanized antibody developed by Acumen, engages A β O without binding monomers or the fibrillar A β of amyloid plaques (Figure 2) [34,35]. If successful in clinical trials, ACU193 would provide definitive substantiation of the A β O hypothesis.

A β O antibodies also may provide companion diagnostics useful for tracking A β O as biomarkers for efficacy of investigational new drugs (INDs). Measurements of A β O in CSF showed strong AD-dependence, with greater accuracy than other CSF biomarkers in resolving AD from non-AD samples (Figure 3a). A β O may be important, too, for certain types of neural damage in younger individuals, not due to AD. It has been found, e.g., that CSF A β O are associated with acute traumatic brain injury [36], and a poor prognosis appears linked to elevated A β O levels (Figure 3b). CSF A β O levels, however, are extremely low and very difficult to assay [37]. Neuroimaging is emerging as a promising alternative. Molecular MRI detection of A β O, e.g., can differentiate AD

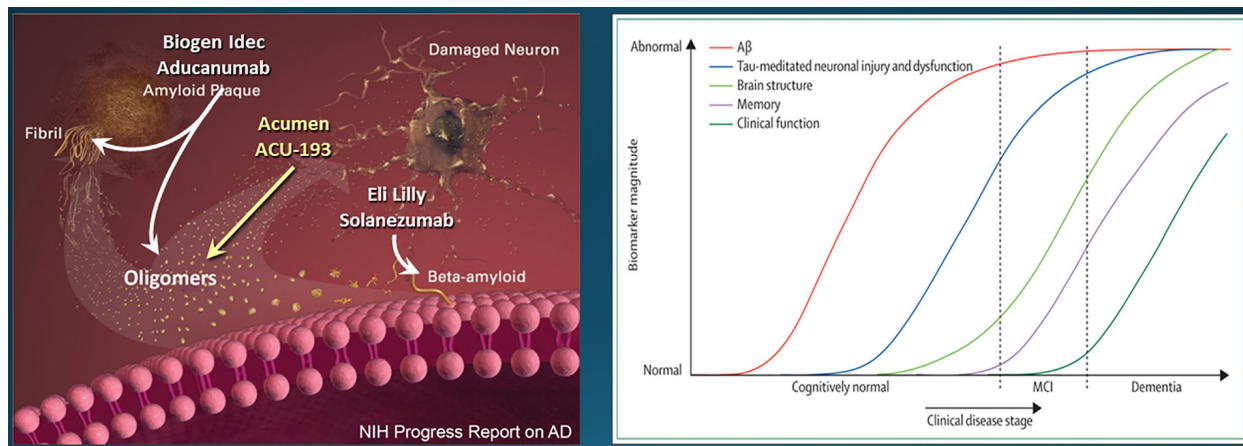


Figure 2. Targeting A β O for Alzheimer's immunotherapy. Left: Potentially therapeutic antibodies from Biogen Idec, Acumen, and Lilly show specificity for different forms of A β and A β assemblies. The major pathogenic form of A β is thought to be oligomeric (Reprinted with Jannis Productions permissions from the "Progress Report on Alzheimer's Disease 2004-2005" (ed. AB Rodgers), NIH Publication Number: 05-5724. Digital images produced by Stacy Jannis and Rebekah Fredenburg of Jannis Productions.) [73]. Right: Neural damage begins decades before the onset of clinical dementia. Pathology in A β and tau are regarded as instigating the neural damage. The timing and inter-relationship of the two pathologies remains under investigation (Reprinted from *The Lancet Neurology*, v. 9. CR Jack Jr, DS Knopman, WJ Jagust, LM Shaw, PS Aisen, MW Weiner, RC Petersen, and JQ Trojanowski. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade, pp 119-128, (2010), with permission from Elsevier.) [74].

from control mice [38]. The MRI probe in this study was provided by A β O-specific antibodies covalently linked to magnetic nanostructures, which provide a strong contrast agent. New evidence suggest that A β O antibodies also can be modified to provide ultrasensitive PET probes useful for early AD diagnostics (Figure 3c).

AN ALTERNATIVE TO THERAPEUTIC ANTIBODIES: SEARCHING FOR SMALL MOLECULES THAT BLOCK ABOS FROM INSTIGATING NEURAL DAMAGE

Although an important goal, the discovery of therapeutic small organic molecules (SOMs) that block the impact of A β O is limited by the gaps in our understanding of the A β O mechanism. The better understood steps occur downstream in the toxic pathway. These intracellular abnormalities include excessive Ca⁺⁺ mobilization by hyperactive mGluR5 receptors; stimulation of Fyn protein tyrosine kinase; hyper-activation of NMDA-Rs, which exacerbates Ca⁺⁺ build-up and causes ROS accumulation; pathological phosphorylation of tau; and, ultimately, bifurcating pathways leading to multiple pathological outcomes (for reviews, see [22,39-41]).

As mentioned, pathogenic tau species are induced by A β O, and extensive efforts are underway to find treatments that protect against tau-induced neural damage. This downstream target is appealing given discov-

eries that tau mediates aspects of damage instigated by A β O [24]. In essence, A β O act as the match, and tau is a fuse they light. It is likely that other fuses exist, including the A β O-induced build-up of excessive Ca⁺⁺ levels. Anti-tau strategies include development of antibodies against pathological tau as well as SOMs designed to prevent pathological tau build-up [42,43].

A β O-activation of the protein tyrosine kinase Fyn also is being targeted for therapeutics. As the case for AD-type tau phosphorylation [44], initial discoveries that Fyn is germane to the impact of toxic A β were made using mixed A β preps containing abundant fibrils [45]. Subsequent knockout data showed Fyn has a central role in the mechanism of A β O toxicity, with Fyn implicated in deteriorating synapse plasticity as well as neuron death [1]. Most recently, the role of Fyn has been substantiated by experiments showing Fyn is an effector of the binding of toxic A β O species to the cellular prion protein [46]. The mGluR5 receptor appears to act between A β O-affected prion protein and Fyn [47]. A re-targeted Fyn inhibitor developed for cancer is now under investigation in an Alzheimer's clinical trial [48].

TARGETING THE EARLIEST STEPS IN THE TOXIC PATHWAY--NOT ENOUGH IS KNOWN

Although a great deal is known about the cellular consequences of A β O exposure, ideally, a therapeutic

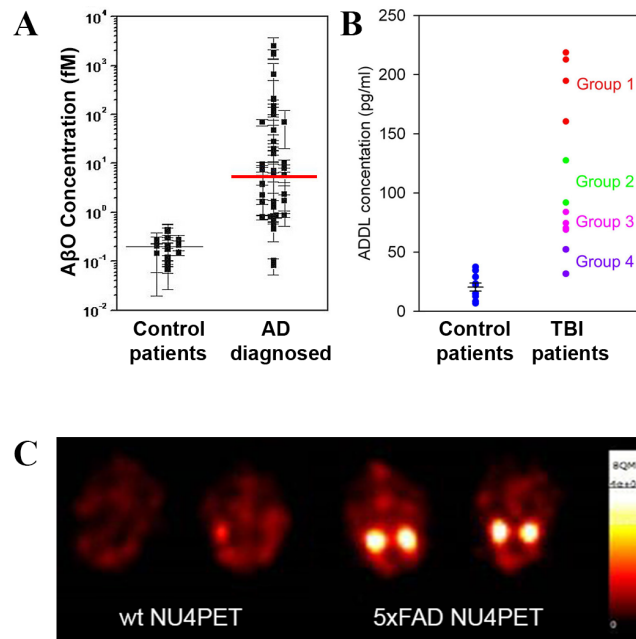


Figure 3. A β O_s in CSF of AD and TBI patients detected by Biobarcode and in AD mouse brain by PET imaging. A: An ultrasensitive nanotechnology-based immunoassay (Biobarcode) was used to determine cerebral spinal fluid (CSF) A β O levels in AD patients compared to controls (adapted and reprinted with permission from “Nanoparticle-based detection in cerebral spinal fluid of a soluble pathogenic biomarker for Alzheimer’s disease” by Georganopoulou DG, Chang L, Nam JM, Thaxton CS, Mufson EJ, Klein WL, and Mirkin CA. This was published in Proc Natl Acad Sci U S A. 2005 Feb 15;102(7):2273-6. Epub 2005 Feb 4. Copyright (2005) National Academy of Sciences, U.S.A.) [75]. Error bars are for individual samples, which required replicate assays for accuracy because of the low A β O levels. There is minimal overlap between AD and control patients, and the median difference is 30-fold. **B:** CSF from emergency room patients were assayed for relative A β O levels using the Biobarcode immunoassay. Higher A β O levels were associated with worsening prognosis. **C:** NU4 was covalently modified with the cation chelator DOTA and labeled with Cu⁶⁴. Two 5XFAD mice and two wildtype littermates (age 8 to 9 months) received probe (NU4PET) through tail-vein injection. After 30 hours of periodic whole animal scans, animals were sacrificed and the brains removed for final imaging. Final scans show robust PET signal is still present in AD but not wildtype samples.

inhibitor would act before A β O_s induce intracellular pathology. The current AD drug Namenda is an open channel inhibitor of NMDA-Rs, and it reduces the ability of A β O_s to upregulate Ca⁺⁺ and ROS levels [49], but its efficacy in patients diminishes with time. The mechanism for NMDA-R hyperactivity likely involves Fyn, and Fyn appears stimulated by Ca⁺⁺, which is elevated by mGluR5 hyperactivity [46,47,50]. Ca⁺⁺ appears to be central to the mechanism [51,52]. While Ca⁺⁺ is elevated by A β O-induced hyperactivity of NMDA-R and mGluR5 receptors, there also is evidence suggesting elevation is due to a pore-like action of A β O_s, inserted directly into lipid bilayers [53]. Action as a pore may be the mechanism for certain structural forms of A β O_s. However, a non-selective action as Ca⁺⁺ pores is difficult to reconcile with A β O species that show cell-specific responses. An example considered above was the dependence of AD-type tau phosphorylation on cell surface A β O clusters of synthetic or brain-derived A β O_s [23]. In cell and brain slice cultures, moreover, many neurons are resistant to A β O-toxicity [1,54].

A mechanism that fits well for the cell-based evidence obtained with most A β O preparations is the receptor hypothesis. The idea that A β O_s act by binding to specific proteins that act as toxin receptors was introduced to explain the sensitivity of A β O binding and toxicity to low amounts of trypsin [1]. Figure 4 illustrates aspects of the evidence supporting the toxin receptor hypothesis. Experiments investigating A β O binding have established (A) saturation and high-affinity binding to cultured neurons and synaptosome preparations; (B) specificity for particular neurons and particular brain regions; (C) targeting of synapses; (D) accumulation at dendritic spines; (E) sensitivity to low doses of antagonist; (F) binding to trypsin-sensitive proteins; (G) association with small patches of isolatable membranes; (H) specificity in Far Western blots for a small number of proteins [4,16,38,55,56]. These findings generally apply to brain-derived as well as synthetic A β O_s. The conclusion from these studies is that binding of A β O_s is ligand-like and mediated adventitiously by proteins acting as toxin receptors. Such specific binding offers strategic routes to drug discovery, as

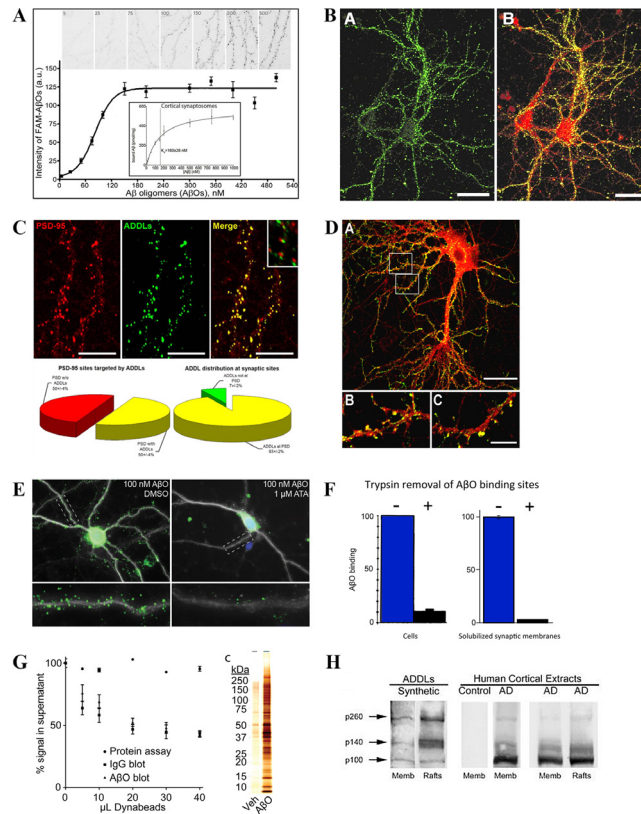


Figure 4. Evidence in harmony with the AβO toxin receptor hypothesis. Panels **A-D**: Mature hippocampal neuron cultures (21 to 24 days *in vitro*) were incubated with AβOs and imaged for distribution using AβO specific immunofluorescence and relevant markers. The first four panels illustrate, respectively - **A**: saturable, high-affinity binding (Reprinted with permission from Macmillan Publishers Ltd: Nature Nanotechnology. Viola KL, Sbarboro J, Sureka R, De M, Bicca MA, Wang J, Vasavada S, Satpathy S, Wu S, Joshi H, Velasco PT, MacRenaris K, Waters EA, Lu C, Phan J, Lacor P, Prasad P, Dravid VP, Klein WL. Towards non-invasive diagnostic imaging of early-stage Alzheimer's disease. *Nat Nanotechnol.* 10(1):91-8., 2015.) [38]; **B**: specificity for particular neurons (double-labeled for CaM kinase II in red and AβOs in green) (B-D Reprinted with permission from "Synaptic Targeting by Alzheimer's Related Amyloid β Oligomers" by Pascale N. Lacor, Maria C. Buniel, Lei Chang, Sara J. Fernandez, Yuesong Gong, Kirsten L. Viola, Mary P. Lambert, Pauline T. Velasco, Eileen H. Bigio, Caleb E. Finch, Grant A. Krafft and William L. Klein, published in *J Neurosci* 2004 24(45):10191-200.) [4]; **C**: specificity for synapses (double-labeled for PSD95 in red and AβOs in green) [4]; and **D**: accumulation at dendritic spines (double-labeled for CaM kinase II in red and AβOs in green) [4]. Panels **E-H**: The second 4 panels illustrate, respectively - **E**: binding of AβOs to spines of hippocampal neuron cultures is blocked by low doses of aurintricarboxylic acid (ATA) (Reprinted with permission under the Creative Commons Attribution License from Wilcox KC, Marunde MR, Das A, Velasco PT, Kuhns BD, Marty MT, et al. (2015) Nanoscale Synaptic Membrane Mimetic Allows Unbiased High Throughput Screen That Targets Binding Sites for Alzheimer's-Associated Aβ Oligomers. *PLoS ONE* 10 (4): e0125263.) [76]; **F**: binding to cells or membrane fractions is to trypsin-sensitive proteins (Reprinted with permission from "Diffusible, nonfibrillar ligands derived from Aβ1-42 are potent central nervous system neurotoxins" by M. P. Lambert, A. K. Barlow, B. A. Chromy, C. Edwards, R. Freed, M. Liosatos, T. E. Morgan, I. Rozovsky, B. Trommer, K. L. Viola, P. Wals, C. Zhang, C. E. Finch, G. A. Krafft, and W. L. Klein. This was published in *Proc Natl Acad Sci U S A.* 1998 May 26;95(11):6448-53. Copyright (1998) National Academy of Sciences, U.S.A.) [1]; **G**: AβO-dependent immune-pulldown of partially solubilized synaptosomes yields a highly selective protein complex (Reprinted with permission under the Creative Commons Attribution License from Wilcox KC, Marunde MR, Das A, Velasco PT, Kuhns BD, Marty MT, et al. (2015) Nanoscale Synaptic Membrane Mimetic Allows Unbiased High Throughput Screen That Targets Binding Sites for Alzheimer's-Associated Aβ Oligomers. *PLoS ONE* 10 (4): e0125263.) [76]; and **H**: Far Western blots using synthetic AβOs and human AD brain extracts show selective, ligand-like binding (Reprinted with permission from "Alzheimer's disease-affected brain: presence of oligomeric A beta ligands (ADDLs) suggests a molecular basis for reversible memory loss" by Gong Y, Chang L, Viola KL, Lacor PN, Lambert MP, Finch CE, Krafft GA, Klein WL. This was published in *Proc Natl Acad Sci U S A.* 2003 Sep 2;100(18):10417-22. Epub 2003 Aug 18. Copyright (2003) National Academy of Sciences, U.S.A.) [16].

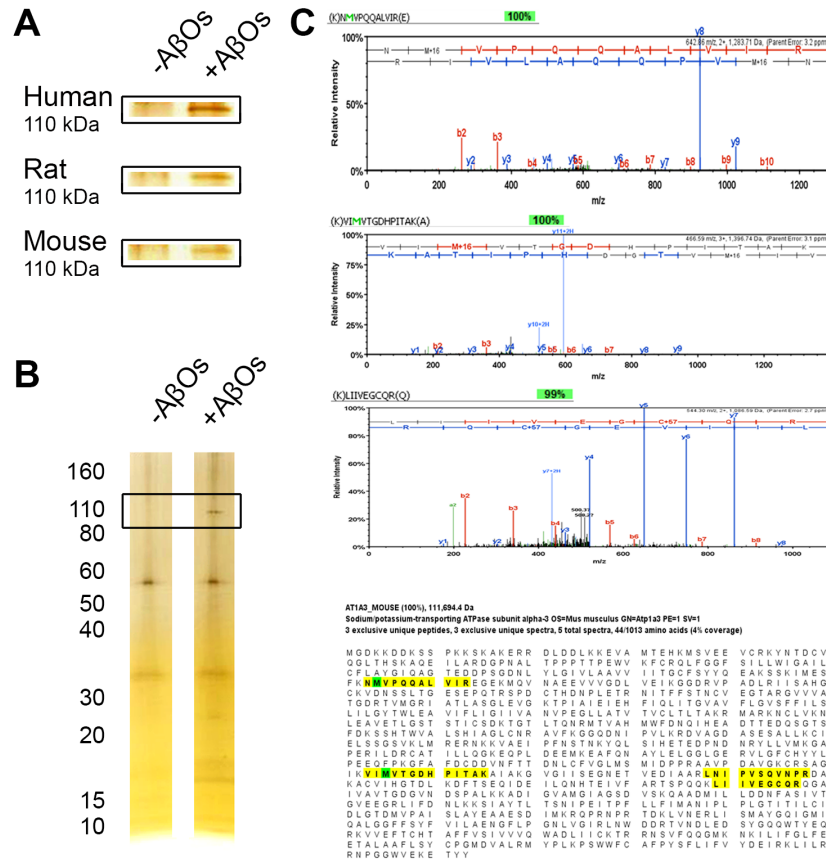


Figure 5. 110 kDa AβO binding protein from Nanodisc-solubilized synaptosomes is NaK ATPase α3. **A.** Crude synaptosomes prepared from human, rat, and mouse brain were solubilized and membrane proteins reconstituted in soluble Nanodiscs as described for rat [56]. Nanodiscs were incubated +/- 500 nM AβOs, washed, incubated with the NU2 AβO-specific antibody, washed, and antibody positive Nanodiscs isolated using magnetic beads as described [85]. Comparison of isolates analyzed by SDS PAGE and silver stain showed an AβO-dependent band at ~110 kDa for all species. **B.** Crude synaptosomes from adult wildtype mice were solubilized, membrane proteins reconstituted in soluble Nanodiscs, and treated as in (A) to identify AβO-dependent binding proteins. Silver stained SDS gels, in addition to the prominent 110 kDa band, show faint bands where SDS-sensitive AβO peptides are expected. **C.** LC-MS/MS spectra of three peptides of solubilized AβO-dependent isolates from mouse synaptosomes show identity with sequences unique to NKAα3. A fourth peptide (not shown) showed 90 percent identity. These amino acid sequences distribute across the coding region (bottom panel, yellow highlight) and provide a 100 percent confidence level that the isolated AβO binding protein is NKAα3. In three separate preparations, this level of confidence was observed twice for mouse and once for rat.

most common drugs interact with cell surfaces. It would be ideal if analogous targets could be found for AD therapeutics.

INTERACTION BETWEEN ABOS AND NAK ATPASE A3 (NKAα3) MAY BE THE FIRST CELL SURFACE STEP WITH PATHOGENIC CONSEQUENCES

A number of AβO binding proteins have been identified, and many have properties that make them promising candidates as toxin receptors (reviewed in [22,40]). An intriguing new candidate recently was described in

a comprehensive study by Ohnishi et al. [57]. They reported that the α3 subunit of the NaK ATPase is a toxin receptor for synthetic and AD brain-derived AβOs. In their study they speculated that the p100 band our laboratory observed in Far Western blots (Figure 4H) was likely NKAα3; this is consistent with results presented later. Ohnishi et al identify the NKAα3 as a “death target” for AβOs, which inhibit sodium pump activity. As we describe below, AβOs have a second impact that could be central to their pathogenic mechanism.

In the Ohnishi study, NKAα3 was shown to have high affinity for AβOs derived from AD brain tissue as well as for what appears to be a widely used synthetic AβO

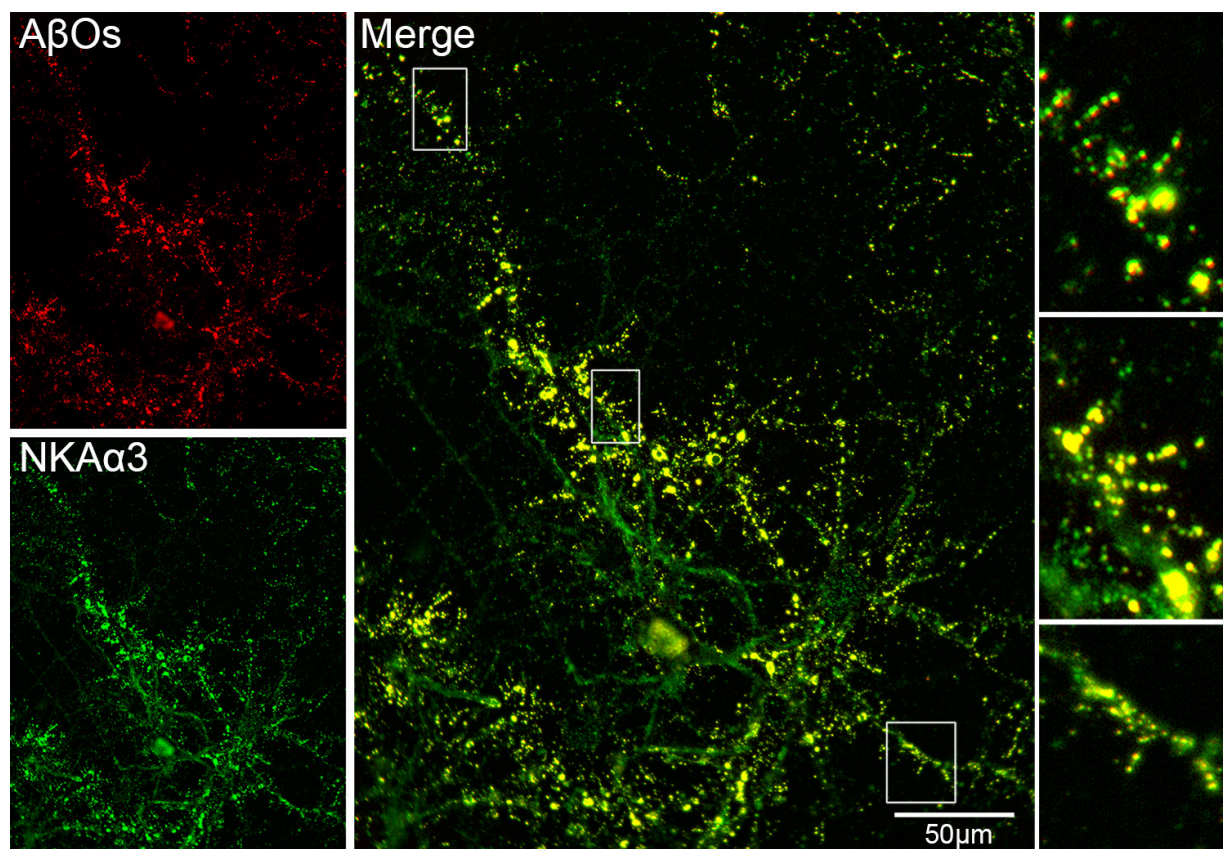


Figure 6. A β O binding sites co-localize with NKA α 3 in hippocampal neuron cultures. Primary rat hippocampal neurons were obtained from E18 embryos and cultured for 18 days before use in immunofluorescence studies. Mature cultures were incubated with 200 nM A β O_s (in total A β monomer equivalents, which equals 8 nM of 24mers). Neurons were fixed and double-labeled for A β O_s (red) and NKA α 3 (green). Overlays show prominent co-localization (gold), consistent with NKA α 3 being a major A β O binding protein. Controls with labeled f-actin established that superposition of signals is not caused by bleed-through (not shown). Inset shows co-localization at dendritic spines.

preparation. The authors reported their synthetic A β O_s show close homology with brain-derived A β O_s they refer to as amylospheroids. They found NKA α 3 showed little interaction with various synthetic preparations but bound tightly to A β O_s made in F12 medium using 50 μ M A β . What adds interest to the ATPase discovery is that their method for preparing synthetic ligand is virtually the same as used by our lab and many others [58]. These preparations have been shown to instigate AD-like in a large number of cell and animal experiments. Findings from Ohnishi and colleagues thus open the door to connecting NKA α 3 to the mechanism underlying a spectrum of A β O-induced neural damage.

NKA α 3 acts, in the authors' terms, as a death protein for A β O_s. They found that binding leads to a slow, time-dependent inhibition of ATPase activity, Ca⁺⁺ build-up via N-VSCC and mitochondrial channels, and apoptosis. Various glutamate receptor antagonists were not neuroprotective. A β O binding and toxicity were found to be linked to the abundance of NKA α 3, both re-

gionally and developmentally. The EC₅₀ for ATPase inhibition and neurodegeneration correlated with the high affinity of A β O binding *in vitro*. The EC₅₀ for binding was ~ 5nM based on a MW of 118 kDa. This EC₅₀ is equal to 1.4 μ M based on the commonly used monomer equivalents, used because of the difficulty in determining precise A β O structure. For toxicity experiments, the authors used 100 to 140 nM doses of the amylospheroids; these doses are 3 to 4 μ M in total A β equivalents. The distribution of the NKA α 3 toxin receptor was inferred to be presynaptic, and they cited their prior work as supporting this inference [59].

Our new findings strongly support involvement of NKA α 3 in A β O toxicity, but with several differences in detail from the Ohnishi study, and they provide new insight into the molecular mechanism. As presented below, our data demonstrate that NKA α 3 has high affinity for synthetic preparations of A β O_s used by our group and others. Moreover, content-rich cell biology experiments provide support for our previous hypothesis that the tox-

icity of A β O_s derives at least in part from a pathological redistribution of membrane proteins [50]. This hypothesis is in harmony with an intriguing “docking” function of NKA α 3, discussed below.

To obtain A β O binding proteins in an unbiased way using classic affinity isolation, we used nanoscale artificial membranes to reconstitute the solubilized synaptic membrane proteome. The nanoscale membranes are referred to as Nanodiscs [60,61]. Nanodiscs are self-assembling discoid monolayers that have diameters of approximately 15 nm, and each nanoscale membrane disc is expected to have one or zero incorporated proteins. In other words, they are virtually soluble membranes. We recently described use of Nanodiscs with the reconstituted synaptic membrane proteome for investigating A β O binding [56]. In this preparation, A β O_s bind saturably to trypsin-sensitive proteins, and, assuming the ligands we prepare are 24mers (Figure 2, above), the EC₅₀ for binding is \sim 4 nM. This is similar to that observed for amylospheroid binding to NKA α 3. Our ligand also binds with approximately the same EC₅₀ to synaptosomes, and the B_{max} for synaptosomes is 24 pmols/mg protein, roughly equivalent to the numbers obtain by Ohnishi and colleagues.

In a new set of experiments, crude synaptosomes from human, rat, and mouse brains were solubilized in a nonionic detergent and the solubilized proteins reconstituted in Nanodiscs as described [56]. Reconstituted synaptosome proteomes were incubated with A β O_s, washed to remove unbound A β O_s, and incubated with the A β O-specific monoclonal antibody (NU2). Washes were done to remove unbound antibody and the NU2-positive Nanodiscs were collected according to our published protocol [56]. Silver stain of the A β O-dependent proteins in the isolated Nanodiscs showed a band at \sim 110 kDa, essentially the size of the NKA α 3 subunit (Figure 5A). Another mouse brain preparation showed this prominent 110 kDa species and some faint bands where A β O trimers to pentamers would be present in SDS gels (Figure 5B). Peptide spectra from LC-MS/MS analysis confirmed that the isolated 110 kDa A β O binding protein was NKA α 3 (Figure 5C). MS analysis of the human A β O binding protein was not done, but two separate analyses of mouse isolates and one of a rat isolate confirmed the presence of NKA α 3. A 260 kDa band (not shown) was identified as an intracellular protein which will be considered in another publication. The p100 band observed in Far Western blots (Figure 4H) also was found to be NKA α 3, as surmised by Ohnishi et al.

We previously found it also was possible to pull down A β O_s associated with partially solubilized synaptic membranes (Figure 4G). Although the isolates contained an immeasurably small fraction of the starting material, they nonetheless contained a large array of associated

proteins, as seen in the figure, including a prominent band at \sim 110 kDa. Detergent extraction using conditions that maintain A β O binding thus also maintain lateral interactions between membrane proteins. LC-MS/MS analysis of the isolates included NKA α 3 as one of 43 proteins identified with 100 percent confidence. The extent to which the isolated membrane fragments contained what has been referred to as an NKA α 3 docking station [62] is unknown.

Cell biology experiments with hippocampal neurons showed A β O_s co-distribute with NKA α 3 (Figure 6). Mature hippocampal neuron cultures incubated with A β O_s were fixed and double-labeled with the NU4 A β O-specific antibody and with an NKA α 3 specific antibody. These data are consistent with the hypothetical role of NKA α 3 as a toxin receptor for A β O_s. High magnification shows that co-localization is evident in dendritic spines, consistent with previous experiments concerning A β O distribution (Figure 4c,d) [4].

Significantly, exposure of neurons to A β O_s results in a profound alteration in NKA α 3 distribution (Figure 7). This is a time-dependent phenomenon. As seen in the panels of Figure 7, the size of ATPase puncta, which co-distribute with surface bound A β O_s, increases markedly by 15 to 60 minutes. Quantitation shows a 4-fold increase by 60 minutes. The intensity of the punctate signal, which reflects abundance of NKA α 3, also increased with time, indicating the NKA α 3 molecules did not just spread out but in fact were still present in high density. Total puncta number was unchanged at 15 minutes, but showed a possible decrease by 60 minutes. The puncta are most likely at dendritic spines, which have previously been shown to show time-dependent changes in morphology and abundance due to A β O exposure. The data support the conclusion that normal NKA α 3 membrane organization is greatly disrupted by A β O_s.

This phenomenon of co-clustering and recruitment into expanding clusters was found previously in our studies of A β O_s and mGluR5, a Ca⁺⁺ mobilizing receptor whose activity is required for A β O toxicity. Based on this prior work, the co-clusters of A β O_s and ATPase seen here can be inferred to also include mGluR5. This redistribution of NKA α 3 and, putatively, of its docking station proteins is an important new facet of the mechanism of A β O toxicity.

AN INTEGRATED MECHANISM FOR A β O TOXICITY WITH DUAL PATHS TO PATHOGENICITY

Our new results substantiate and extend the discovery of Ohnishi and colleagues that NKA α 3 is a binding protein for A β O_s [57]. We have confirmed that A β O_s bind to NKA α 3 *in vitro* and co-localize with NKA α 3 in

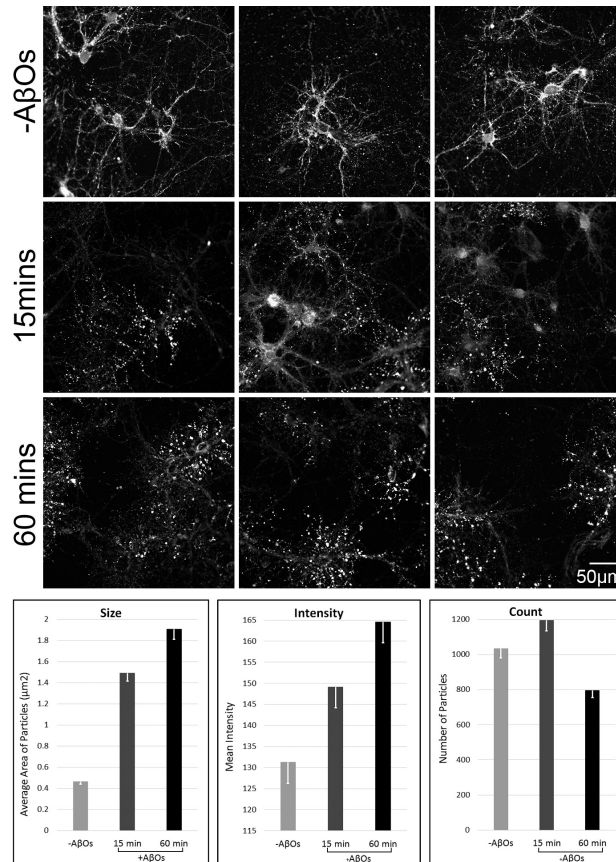


Figure 7. AβO-induced disruption of NKAα3 topology. Mature hippocampal neuron cultures were incubated +/- 200 nM AβOs (8 nM of 24mer) for 15 or 60 minutes. Cells were fixed and labeled for NKAα3. Results show that by 15 minutes, AβOs had caused a redistribution of NKAα3 into enlarged clusters along dendrites. These clusters co-localize with AβOs (Figure 6), which accumulate at dendritic spines (Figures 4 and 6). For quantitation, raw images were normalized to an 8-bit range and inverted before thresholding with the Intermodes method in Image J. The Analyze Particles function was used to calculate the total number of particles within a 786x888 pixel region of interest (ROI). Size, mean intensity, and total count of particles within ROIs from each image were averaged for Vehicle control (-AβO), 15 min AβO, and 60 minute AβO exposures (n=3 for each condition). Induction of enlarged clusters of NKAα3 resembles the AβO-induced clustering of mGluR5 [50].

mature hippocampal cultures. In a finding we consider mechanistically significant, our data show striking changes induced in the topology of the NKAα3 docking station. Within minutes of exposure to AβOs, NKAα3 became accumulated in dense clusters along dendrites, a pathological redistribution of NKAα3 molecules in the membranes of vulnerable hippocampal neurons. This is a newly found impact of AβOs that extend findings regarding inhibition of sodium transport function. Besides cation transport, NKAα3 plays a role as a docking station for multiple membrane proteins [62], including neurotransmitter receptors linked to AβO-induced neuronal damage [63]. The function of ATPase docking stations normally is in signaling [64,65], somewhat analogous to the protein-organizing role of focal adhesions in integrin signaling. Altered topology of these signaling clusters would be expected to contribute to neuronal dysfunction and damage.

In addition, these images implicate ATPase docking stations in the mechanism by which AβOs become clustered at cell surfaces. As we previously showed, this clustering is particularly prominent at dendritic spines ([4]; Figure 4). The pathological significance of AβO clusters is indicated by experiments in which tau pathology induced by AβOs is restricted to neurons that manifest these clusters [23].

The clustering of NKAα3 is in harmony with our earlier observation that AβOs induce the clustering of mGluR5 [50]. mGluR5 is a Ca⁺⁺ mobilizing receptor, and it is regarded as a key mediator of AβO-elevated Ca⁺⁺ build-up and the damage that ensues [47]. Importantly, clustering of mGluR5 molecules can also be induced by receptor antibodies [50]. This antibody-mediated mGluR5 clustering mimics the toxic impact of AβOs. Clustering itself thus appears to be a seminal step for the

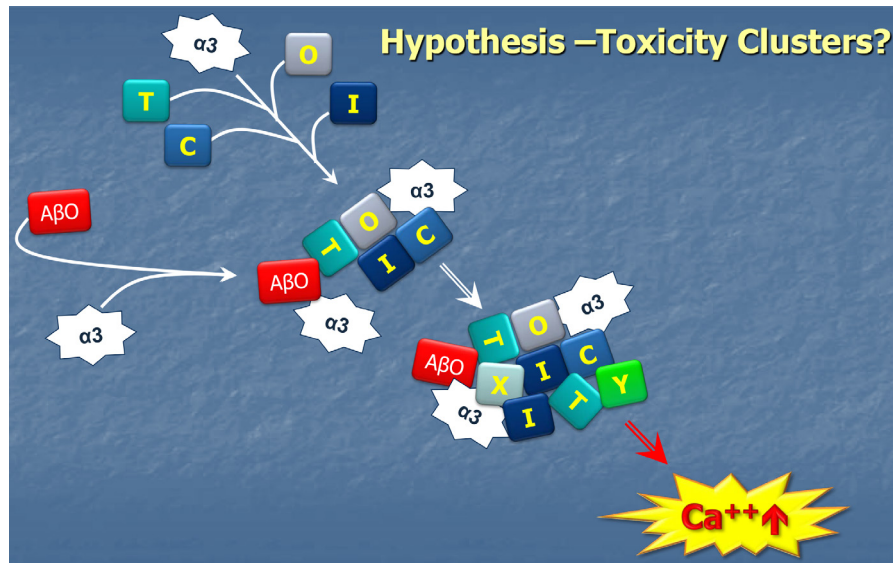


Figure 8. Hypothetical pathology of the NKA α 3 docking station as an early event in A β O-induced neuronal damage. A β O_s are hypothesized to act as neurotoxins when they attach to neuron surfaces because they cause restructuring of NKA α 3 docking stations, with pathogenic clustering of various membrane proteins such as mGluR5 contributing to toxic Ca⁺⁺ build-up. As described by Ohnishi et al (2015) [57], the impact of A β O_s also includes inhibition of NKA α 3 transport function.

mechanism.

The current data are consistent with a central role for NKA α 3 in the ectopic clustering associated with the mechanism of A β O toxicity. Because mGluR5 and NKA α 3 each co-localize with cell-surface bound A β O_s, we infer they are part of the same ectopic clusters. With respect to generation of these clusters, the role of the NKA α 3 docking station relative to roles played by mGluR5, or other membrane domain-organizing proteins such as PrP [66], is not yet clear. Hypothetically, it would seem, however, that the direct binding of A β O_s to NKA α 3 and its impact on the topology of the NKA α 3 docking station would cause major disruption in the distribution of many other membrane proteins, with one important consequence being build-up of Ca⁺⁺ to pathogenic levels (Figure 8).

The need for a docking station in the mechanism of A β O toxicity, whether provided as hypothesized in Figure 8 by NKA α 3 or some other protein, was first evident in single particle tracking experiments. These experiments followed diffusion of individual A β O_s and mGluR5 molecules on the surfaces of live neurons using quantum dots [50]. Both A β O_s and mGluR5 at first diffuse like untethered membrane proteins. Within minutes of adding A β O_s to the cells, however, both the A β O_s and mGluR5 became immobilized, frequently at synapses. This immobilization is consistent with confocal imaging showing A β O clusters at dendritic spines in fixed cells. Recently, single particle tracking experiments have shown that NKA α 3 becomes immobilized during exposure of hippocampal neurons to toxic assemblies of synuclein [67]. Results suggest a pos-

sible central role for ATPase as an immobilizing docking station for toxic oligomers found in multiple proteinopathies. It may be that A β O_s can be brought to docking stations by different protein shuttles, and that at docking stations, there may be a need for co-receptors to mediate docking, or for additional scaffolding proteins to stabilize the pathogenic docking station itself. For A β O_s, the immobilized state appears to act as a seed to which certain proteins are rerouted, where they form expanding clusters containing A β O_s, mGluR5 receptors, NKA α 3, and in all likelihood, numerous other membrane proteins.

Results obtained with the A β O ligands prepared for the current study show both differences and similarities with respect to the findings of Ohnishi and colleagues. First of all, the co-localization of our A β O_s and NKA α 3 is clearly evident at dendritic spines (Figure 6). This distribution is consistent with dendritic spine localization of NKA α 3 reported in experiments with super-resolution fluorescence microscopy [68,69]. Ohnishi and colleagues, however, in their study reported that binding of A β O_s to NKA α 3 occurred at presynaptic terminals. Another difference concerns the sensitivity of A β O toxicity to glutamate receptor antagonists. Our A β O preparations elicit an array of AD-like pathology, and these responses are significantly lowered or fully blocked by antagonists of NMDA and mGluR5 receptors [49,50]. Most AD-like pathology is evident in cultures containing almost exclusively neurons, but cell death is minimal; neuron death likely requires the presence of factors released by glia [70]. We speculate that the impact of A β O_s on NKA α 3

may render them more vulnerable to inflammatory cytokines. Ohnishi and colleagues found their A β O_s evoked cell death, and this was resistant to glutamatergic antagonists. Although this might be attributable to unique structural features of their preparations, this seems unlikely, as their size and shape in AFM, aspects of their immunoreactivity, and the MW obtained by biochemical assays are very much like those of the A β O_s used in the current study. This is consistent with the fact that the synthetic amylospheroids are prepared in much the same manner as our preparations, using 50 μ M A β monomer in F12 solutions. A possible salient difference in experimental conditions may be in concentrations of A β O_s used. The concentrations employed for amylospheroid experiments are at least 10 times greater than in our experiments.

Overall, the current data are consistent with the hypothesis that A β O attachment to cell surfaces is transduced into a neurotoxic phenomenon by an altered membrane protein topography seeded by A β O binding to NKA α 3. The seminal interactions between A β O_s and NKA α 3 molecules at the cell surface may prove to be suitable targets for new drug discovery strategies. Detailed structural analysis of the binding site by Ohnishi and colleagues has yielded neuroprotective peptides based on the amino acid sequence of an external loop of the NKA α 3 [57]. This antagonist, which binds to the A β O ligand, is now being exploited for rational drug design. A successful result would provide, in essence, a small molecule equivalent of a therapeutic antibody. In another approach, proof of concept has been obtained that small molecules can bind to the toxin receptor at the cell surface and prevent A β O binding (Figure 4e). Attachment of A β O_s to NKA α 3 is amenable to high throughput screening for antagonists using Nanodiscs [56]. Results from a preliminary screen showed that A β O binding to spines can be blocked by low doses of a small organic molecule, albeit one with promiscuous binding precluding its use for therapeutics. Nonetheless, McGeer and colleagues have shown that behavior in a transgenic AD model could be safely rescued using this same compound [71]. Future investigations of the docking station hypothesis are expected to open the door to therapeutics targeting the first step of a complex pathway that leads to neural damage and dementia.

Author contributions: Thomas DiChiara, – designed, executed, and analyzed experiments & data; Nadia DiNunno – designed, executed, and analyzed experiments & data; Jeffrey Clark – designed, executed, and analyzed experiments & data; Riana Lo Bu – designed, executed, and analyzed experiments & data; Erika N. Cline – designed, executed, and analyzed experiments & data; Madeline G. Rollins – designed, executed, and analyzed experiments and data; Yuesong Gong – designed, executed, and analyzed experiments & data; David L. Brody – provided key materials and expertise for experimental

design & analysis; Stephen G. Sligar - provided key materials and expertise for experimental design & analysis; Pauline T. Velasco – designed, executed, and analyzed experiments & data; Kirsten L. Viola – designed, executed, and analyzed experiments & data; William L. Klein – wrote manuscript, supervised and assisted with the design, execution, and analysis of experiments & data.

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