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

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Toxic amyloid beta oligomers (AβOs) 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, AβOs 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 AβO antibodies, while Aducanumab, an antibody that targets AβOs as well as fibrillar Aβ, has provided cognitive benefit to humans in early clinical trials. AβOs have now been investigated in more than 3000 studies and are widely thought to be the major toxic form of Aβ. Although much has been learned about the downstream mechanisms of AβO action, a major gap concerns the earliest steps: How do AβOs 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 AβOs act as neurotoxins because they attach to particular membrane protein docks containing Na/K ATPase-α3, where they inhibit ATPase activity and pathologically restructure dock composition and topology in a manner leading to excessive Ca\textsuperscript{++} build-up. Better understanding of the mechanism that makes attachment of AβOs 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 AΒO HYPOTHESIS -- A MECHANISM WITH PRIME TARGETS FOR AD THERAPEUTICS

The amyloid beta oligomer (AβO) hypothesis for Alzheimer’s disease (AD), introduced in 1998 [1], says that dementia is the consequence of neural damage instigated by soluble, toxic AβOs. Earlier investigations had found AβOs in AD brain extracts [2], but their toxicity was not apparent. Discovery of methods to make soluble...
Table 1. NU-series antibodies that selectively bind AβOs. AβO-antibodies commonly used by our lab comprise NU 1, 2, and 4 [11]. They have low affinity for Aβ monomers and fibrils. Their affinities for our AβO preparations were determined here using an indirect ELISA with 20 pmols of AβOs per well (total Aβ 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 AβOs.

| Ab   | EC₅₀ (µg/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    |

AβOs without contaminating fibrils [3] opened the door to brain slice experiments that revealed AβOs 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. AβOs 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 AβOs with toxin receptors, a hypothesis still of current interest and one that will be discussed later in this article.

Toxic AβOs 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 E693Δ [8], Tg McGill-Thy1-APP [9], APP-Tg E693Q “Dutch” [10], and 5xFAD (unpublished data from WL Klein and R Vassar labs), AβOs 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 AβOs accumulate without amyloid plaques, which are absent despite an otherwise full complement of AD pathology. The plaque-free build-up of AβOs 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 AβOs are.

The subunits of synthetic AβOs in most instances are not covalently bonded, and assembly states of AβOs 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 AβOs with a peak near 110 kDa and another at about twice this mass. AβOs are larger in aqueous buffers than in buffers with detergents. In Western blots, synthetic AβOs typically break down to monomers and very low MW AβOs. 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 Aβ, for example, is common in brain-derived AβOs. 2D gel analysis shows structural homology between AβOs in aqueous extracts of AD-affected brain and toxic AβOs 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 Aβ*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 Aβ42 peptide is now widely used for experimentation because it is more closely associated with AD pathology than is Aβ40 [19]. The original fibril-free preparation of toxic AβOs was made using clusterin as a chaperone and also by using very low Aβ42 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 Aβ [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 AβOs [21]. A summary of preparations and structures can be found in our recent review [22]. Protocols for preparation and use of AβOs typically used in our laboratory can be accessed at our home page (www.kleinlab.org).

Synthetic and brain-derived AβO preparations cause a spectrum of AD-like, cell-specific neural damage. In CNS cultures, e.g., neurons with bound AβOs manifest AD-type phospho-tau, whereas neurons without bound AβOs show much less of this phospho-tau [23]. Overall
evidence strongly supports the role of AβOs in instigating tau pathology [24], which mediates some of the AβO toxic impact [24]. Because APP transgenes accelerate propagation of tau pathology in Tg mice [25,26], we hypothesize it is likely that AβOs may likewise play a role in this aspect of tau pathology. ICV injections of AβOs into wildtype animals likewise evoke AD neuropathology, including non-human primates [27]. Tg animals producing AβOs manifest equivalent neural damage [8,9,28]. Table 2 provides a short list of the wide-spread AD-like damage evoked by toxic AβO 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 AβOs, found in vitro and in brain, are potent CNS neurotoxins. AβOs, which have been investigated in more than 3000 studies, are now considered the major toxic form of Aβ.

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 Aβ pathology giving rise to tau pathology. In this context of disease progression, it is likely that brain damage begins with AβOs, which appear before plaques and comprise the major toxic forms of Aβ. Sometimes, as in the case of the Osaka mutation, AβOs appear even without plaques. Current finding are consistent with the hypothesis that AβOs provide a unifying mechanism for initiation of the neural damage underlying dementia. Evidence strongly points to the build-up of toxic AβOs as a seminal event in AD progression.

**TESTING THE AΒO HYPOTHESIS WITH CLINICAL TRIALS**

Continued interest in the AβO hypothesis will require successful clinical trials based on preventing AβOs from instigating neural damage. The most advanced approach is immunotherapy. AβO-specific antibodies, developed to verify the presence of toxic AβOs 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 AβO-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 Aβ going back to 2000. The latest disappointment occurred with Lilly’s Solanezumab, which targets monomeric Aβ (Figure 2). These failures, along with unsuccessful trials using small molecule treatments designed to prevent Aβ pathogenesis, have cost an estimated $18 billion. The extreme cost of past failures has virtually poisoned the well for new therapeutic strategies targeting Aβ.

However, after 15 years of failures, a new trial has
AβO antibodies also may provide companion diagnostics useful for tracking AβOs as biomarkers for efficacy of investigational new drugs (INDs). Measurements of AβOs in CSF showed strong AD-dependence, with greater accuracy than other CSF biomarkers in resolving AD from non-AD samples (Figure 3a). AβOs 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 AβOs are associated with acute traumatic brain injury [36], and a poor prognosis appears linked to elevated AβO levels (Figure 3b). CSF AβO levels, however, are extremely low and very difficult to assay [37]. Neuroimaging is emerging as a promising alternative. Molecular MRI detection of AβOs, e.g., can differentiate AD
eries that tau mediates aspects of damage instigated by AβOs [24]. In essence, AβOs act as the match, and tau is a fuse they light. It is likely that other fuses exist, including the AβO-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].

AβO-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 Aβ were made using mixed Aβ preps containing abundant fibrils [45]. Subsequent knockout data showed Fyn has a central role in the mechanism of AβO 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 AβO species to the cellular prion protein [46].

As mentioned, pathogenic tau species are induced by AβOs, and extensive efforts are underway to find treatments that protect against tau-induced neural damage. This downstream target is appealing given discoveries that tau mediates aspects of damage instigated by AβOs [24]. In essence, AβOs act as the match, and tau is a fuse they light. It is likely that other fuses exist, including the AβO-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].

Although an important goal, the discovery of therapeutic small organic molecules (SOMs) that block the impact of AβOs is limited by the gaps in our understanding of the AβO 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]).

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The mGluR5 receptor appears to act between AβO-affect ed 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 AβO exposure, ideally, a therapeutic...
A mechanism that fits well for the cell-based evidence obtained with most AβOs preparations is the receptor hypothesis. The idea that AβOs act by binding to specific proteins that act as toxin receptors was introduced to explain the sensitivity of AβO binding and toxicity to low amounts of trypsin [1]. Figure 4 illustrates aspects of the evidence supporting the toxin receptor hypothesis. Experiments investigating AβO 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 AβOs. The conclusion from these studies is that binding of AβOs is ligand-like and mediated adventitiously by proteins acting as toxin receptors. Such specific binding offers strategic routes to drug discovery, as

**Figure 3.** AβOs 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) AβO 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 Georgopoulou 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 AβO 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 AβO levels using the Biobarcode immunoassay. Higher AβO levels were associated with worsening prognosis. C: NU4 was covalently modified with the cation chelator DOTA and labeled with Cu^{64}. 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.
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 by 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].
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 lab 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.

INTERACTION BETWEEN AβOs AND NAK ATPASE A3 (NKAA3) 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 NKAa3; this is consistent with results presented later. Ohnishi et al identify the NKAa3 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, NKAa3 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...
The authors reported their synthetic AβOs show close homology with brain-derived AβOs they refer to as amylospheroids. They found NKAα3 showed little interaction with various synthetic preparations but bound tightly to AβOs made in F12 medium using 50 μM Aβ. 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 NKAα3 to the mechanism underlying a spectrum of AβO-induced neural damage.

NKAα3 acts, in the authors’ terms, as a death protein for AβOs. 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. AβO binding and toxicity were found to be linked to the abundance of NKAα3, both regionally and developmentally. The EC50 for ATPase inhibition and neurodegeneration correlated with the high affinity of AβO binding in vitro. The EC50 for binding was ~5nM based on a MW of 118 kDa. This EC50 is equal to 1.4 μM based on the commonly used monomer equivalents, used because of the difficulty in determining precise AβO structure. For toxicity experiments, the authors used 100 to 140 nM doses of the amylospheroids; these doses are 3 to 4 μM in total Aβ equivalents. The distribution of the NKAα3 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 NKAα3 in AβO 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 NKAα3 has high affinity for synthetic preparations of AβOs used by our group and others. Moreover, content-rich cell biology experiments provide support for our previous hypothesis that the tox-
pathogenicity of AβOs derives at least in part from a pathological redistribution of membrane proteins [50]. This hypothesis is in harmony with an intriguing “docking” function of NKAα3, discussed below.

To obtain AβO 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 AβO binding [56]. In this preparation, AβOs bind saturably to trypsin-sensitive proteins, and, assuming the ligands we prepare are 24mers (Figure 2, above), the EC50 for binding is ~4 nM. This is similar to that observed for amylospheroid binding to NKAα3. Our ligand also binds with approximately the same EC50 to synaptosomes, and the Bmax 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 AβOs, washed to remove unbound AβOs, and incubated with the AβO-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 AβO-dependent proteins in the isolated Nanodiscs showed a band at ~110 kDa, essentially the size of the NKAα3 subunit (Figure 5A). Another mouse brain preparation showed this prominent 110 kDa species and some faint bands where AβO trimers to pentamers would be present in SDS gels (Figure 5B). Peptide spectra from LC-MS/MS analysis confirmed that the isolated 110 kDa AβO binding protein was NKAα3 (Figure 5C). MS analysis of the human AβO binding protein was not done, but two separate analyses of mouse isolates and one of a rat isolate confirmed the presence of NKAα3. 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 NKAα3, as surmised by Ohnishi et al.

We previously found it also was possible to pull down AβOs 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 ~110 kDa. Detergent extraction using conditions that maintain AβO binding thus also maintain lateral interactions between membrane proteins. LC-MS/MS analysis of the isolates included NKAα3 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 a NKAα3 docking station [62] is unknown.

Cell biology experiments with hippocampal neurons showed AβOs co-distribute with NKAα3 (Figure 6). Mature hippocampal neuron cultures incubated with AβOs were fixed and double-labeled with the NU4 AβO-specific antibody and with an NKAα3 specific antibody. These data are consistent with the hypothetical role of NKAα3 as a toxin receptor for AβOs. High magnification shows that co-localization is evident in dendritic spines, consistent with previous experiments concerning AβO distribution (Figure 4c,d) [4].

Significantly, exposure of neurons to AβOs results in a profound alteration in NKAα3 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 AβOs, 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 NKAα3, also increased with time, indicating the NKAα3 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 AβO exposure. The data support the conclusion that normal NKAα3 membrane organization is greatly disrupted by AβOs.

This phenomenon of co-clustering and recruitment into expanding clusters was found previously in our studies of AβOs and mGluR5, a Ca++ mobilizing receptor whose activity is required for AβO toxicity. Based on this prior work, the co-clusters of AβOs and ATPase seen here can be inferred to also include mGluR5. This redistribution of NKAα3 and, putatively, of its docking station proteins is an important new facet of the mechanism of AβO toxicity.

AN INTEGRATED MECHANISM FOR ABO TOXICITY WITH DUAL PATHS TO PATHOGENICITY

Our new results substantiate and extend the discovery of Ohnishi and colleagues that NKAα3 is a binding protein for AβOs [57]. We have confirmed that AβOs bind to NKAα3 in vitro and co-localize with NKAα3 in
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 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].
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The current data are consistent with a central role for NKAα3 in the ectopic clustering associated with the mechanism of AβO toxicity. Because mGluR5 and NKAα3 each co-localize with cell-surface bound AβOs, we infer they are part of the same ectopic clusters. With respect to generation of these clusters, the role of the NKAα3 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 AβOs to NKAα3 and its impact on the topology of the NKAα3 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 AβO toxicity, whether provided as hypothesized in Figure 8 by NKAα3 or some other protein, was first evident in single particle tracking experiments. These experiments followed diffusion of individual AβOs and mGluR5 molecules on the surfaces of live neurons using quantum dots [50]. Both AβOs and mGluR5 at first diffuse like untethered membrane proteins. Within minutes of adding AβOs to the cells, however, both the AβOs and mGluR5 became immobilized, frequently at synapses. This immobilization is consistent with confocal imaging showing AβO clusters at dendritic spines in fixed cells. Recently, single particle tracking experiments have shown that NKAα3 becomes immobilized during exposure of hippocampal neurons to toxic assemblies of synuclein [67]. Results suggest a possible central role for ATPase as an immobilizing docking station for toxic oligomers found in multiple proteinopathies. It may be that AβOs 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 AβOs, the immobilized state appears to act as a seed to which certain proteins are rerouted, where they form expanding clusters containing AβOs, mGluR5 receptors, NKAα3, and in all likelihood, numerous other membrane proteins.

Results obtained with the AβO 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 AβOs and NKAα3 is clearly evident at dendritic spines (Figure 6). This distribution is consistent with dendritic spine localization of NKAα3 reported in experiments with super-resolution fluorescence microscopy [68,69]. Ohnishi and colleagues, however, in their study reported that binding of AβOs to NKAα3 occurred at presynaptic terminals. Another difference concerns the sensitivity of AβO toxicity to glutamate receptor antagonists. Our AβO 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 AβOs on NKAα3

**Figure 8. Hypothetical pathology of the NKAα3 docking station as an early event in AβO-induced neuronal damage.** AβOs are hypothesized to act as neurotoxins when they attach to neuron surfaces because they cause restructuring of NKAα3 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 AβOs also includes inhibition of NKAα3 transport function.
may render them more vulnerable to inflammatory cytokines. Ohnishi and colleagues found their AβOs 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 AβOs 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 µM Aβ monomer in F12 solutions. A possible salient difference in experimental conditions may be in concentrations of AβOs 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 AβO attachment to cell surfaces is transduced into a neurotoxic phenomenon by an altered membrane protein topography seeded by AβO binding to NKAα3. The seminal interactions between AβOs and NKAα3 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 NKAα3 [57]. This antagonist, which binds to the AβO 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 AβO binding (Figure 4e). Attachment of AβOs to NKAα3 is amenable to high throughput screening for antagonists using Nanodiscs [56]. Results from a preliminary screen showed that AβO 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 bepridil, a non-fibrillar beta-amyloid protein is associated with smooth muscle cells of vessel walls in Alzheimer disease. J Neuropathol Exp Neurol. 1994;53(6):637-45.

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