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Permalink
https://escholarship.org/uc/item/1zx5n343

Journal
Proceedings of the National Academy of Sciences of the United States of America, 112(32)

ISSN
0027-8424

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Publication Date
2015-08-11

DOI
10.1073/pnas.1421182112

Peer reviewed
Na, K-ATPase α3 is a death target of Alzheimer patient amyloid-β assembly

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Edited by Thomas C. Südhof, Stanford University School of Medicine, Stanford, CA, and approved July 6, 2015 (received for review November 6, 2014)

Neurodegeneration correlates with Alzheimer’s disease (AD) symptoms, but the molecular identities of pathogenic amyloid β-protein (Aβ) oligomers and their targets, leading to neurodegeneration, remain unclear. Amylospheroids (ASPD) are AD patient-derived 10- to 15-nm spherical Aβ oligomers that cause selective degeneration of mature neurons. Here, we show that the ASPD target is neuron-specific Na+/K+-ATPase α3 subunit (NAKα3). ASPD-binding to NAKα3 impaired NAKα3-specific activity, activated N-type voltage-gated calcium channels, and caused mitochondrial calcium dyshomeostasis, tau abnormalities, and neurodegeneration. NMR and molecular modeling studies suggested that spherical ASPD contain N-terminal-Aβ-derived “thorns” responsible for target binding, which are distinct from low molecular-weight oligomers and dodecamers. The fourth extracellular loop (Ex4) region of NAKα3 encompassing Asn279 and Trp380 is essential for ASPD-NAKα3 interaction, because tetrapeptides mimicking this Ex4 region bound to the ASPD surface and blocked ASPD neurotoxicity. Our findings open up new possibilities for knowledge-based design of peptidomimetics that inhibit neurodegeneration in AD by blocking aberrant ASPD-NAKα3 interaction.

NMR | computational modeling | abnormal protein–protein interaction in synapse | hyperexcitotoxicity | protein–protein interaction inhibitors

Alzheimer’s disease (AD) brains characteristically display fibrillar and nonfibrillar (oligomeric) protein assemblies composed of the amyloid β-protein (Aβ) (1–6). Aβ has been shown to bind to postsynaptic receptors, such as α7-nicotinic acetylcholine receptor (α7nAChR) (7), receptor for advanced glycation end products (RAGE) (8), receptor tyrosine kinase EPHB2 (9), and cellular prion protein PrP8-30 (10). These “Aβ receptors,” except for RAGE, have been reported to mediate toxicity of Aβ oligomers through modulating NMDA receptors (NMDAR) (11). Aβ oligomers, including dimers from AD brains (12, 13), dodecamers (Aβ8-56) from AD model mice (14), and in vitro-generated Aβ-derived fusible ligands (ADDLs) (15, 16), induce synaptic impairment by affecting NMDAR (11). Thus, NMDAR are a common target for synaptic impairment in AD. However, these oligomers do not cause neuronal death (12, 14). The atomic resolution structures of neurotoxic Aβ oligomers and their in vivo targets leading to neuronal death in AD remain unclear (6), even though neuronal death is the central mechanism responsible for symptomatic onset in AD (17).

We previously isolated neurotoxic Aβ oligomers, termed amylospheroids (ASPD), from the brains of AD patients (18–20). ASPD appear in transmission electron microscopic (TEM) images as spheres of diameter ~1.9 ± 1.7 nm (19). ASPD appear to be unique Aβ assemblies, as determined immunochemically. These structures are recognized strongly by ASPD-specific antibodies (Kd ~ pM range), but not with the oligomer-specific polyclonal antisera A11 (19). ASPD are distinct from Aβ dimers, ADDLs, dodecamers, and other A11-reactive entities (19).

ASPD cause severe degeneration of mature human neurons (19). ASPD levels in the cortices of AD patients correlate well with the degree of Alzheimer disease (AD) symptoms and loss of functions. This brain damage is thought to be caused by a small protein, the amyloid β-protein (Aβ), which forms aggregates that are neurotoxic. This neurotoxicity has been explained by multiple mechanisms. We reveal here a new neurotoxic mechanism that involves the interaction between patient-derived Aβ assemblies, termed amylospheroids, and the neuron-specific Na+/K+-ATPase α3 subunit. This interaction causes neurodegeneration through pre-synaptic calcium overload, which explains earlier observations that such neuronal hyperactivation is an early indicator of AD-related neurodegeneration. Importantly, amylospheroid concentrations correlate with disease severity and progression in AD patients. Amylospheroid-neuron-specific Na+/K+-ATPase α3 subunit interactions may be a useful therapeutic target for AD.


Conflict of interest statement: M. Hoshi has served as a technical advisor to TAO, a Kyoto University–derived biotech venture, with the permission of the conflict-of-interest committee of Kyoto University. T. Sasahara, Y.A., H.K., K.S., M.I., E.S., S.S., and N.T. are employees of TAO.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1421182112/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1421182112
with disease severity (19). In contrast, ASPD-like oligomers were minimally detectable in the brains of transgenic mice expressing human amyloid precursor protein (APP), in which no significant neuronal loss is observed (19). These findings suggest that ASPD are an important effector of neuronal death in AD patients. We sought to elucidate mechanisms of ASPD-induced neurotoxicity. We report here that ASPD interact with the α-subunit of neuron-specific Na⁺/K⁺-ATPase (NAKα3), resulting in presynaptic calcium overload and neuronal death.

Results

ASPD Bind To NAKα3 in Mature Neurons. ASPD caused degeneration of mature rat hippocampal neurons, but not immature neurons or nonneuronal HEK293 (Fig. L4). ASPD toxicity required binding to mature neurons, because ASPD-specific mouse monoclonal amylospheroid (mASD)3 antibody blocked binding and toxicity of ASPD (Fig. 1A and B). [The Kₐ for ASPD binding was 5.43 ± 0.27 nM (n = 3) (Fig. 1C). ASPD concentration was determined using an average ASPD molecular weight of 128 kDa (20).] See summary of the characteristics of patient and synthetic ASPD in Table S1. See also SI Discussion for an updated definition of ASPD.] Blockers of known Aβ receptors (21), including glutamate receptors (NMDA, non-NMDA, and metabotropic types) and voltage-gated sodium channels, did not affect ASPD neurotoxicity (Fig. S1). These findings suggested ASPD exert their toxicity through binding to novel cell-surface molecules specific to mature neurons.

To identify ASPD-binding proteins on mature neurons, Far-Western ligand-binding assays were performed in a physiological medium. We used ASPD isolated (19) from the soluble brain extracts of the two AD patients displaying the most severe neurodegeneration and the highest ASPD concentrations among those shown in Fig. 2 (Fig. 2B). These ASPD were A11-negative (Fig. 2C), composed predominantly of Aβ1–42 and Aβ1–40

![Fig. 1](image1.png)

**Fig. 1.** Mature neuron-specific binding and toxicity of ASPD. (A) ASPD neurotoxicity was determined by measuring apoptotic DNA fragmentation in HEK293 cells, 2 DIV immature or 19 DIV mature rat hippocampal neurons after overnight treatment with 140 nM synthetic ASPD, with or without 2-h pre-treatment of ASPD with each antibody (0.1 mg/mL for ASPD-specific mASD3 antibody, 0.4 mg/mL for Aβ1–40 antibody 6E10) (mean ± SD; *P < 0.001 Games–Howell post hoc test, n = 6). The antibody remained during overnight incubation with ASPD. As shown previously (19), mASD3 inhibited ASPD-induced neuronal death, but 6E10, targeting Aβ1–40, did not. ASPD concentration is expressed in terms of the average ASPD mass 128 kDa (20). (B) Cells were treated for 30 min with 140 nM synthetic ASPD as in A. Images are representative of ASPD binding detected by mASD3 (red). Green represents anti-actin for HEK293 or anti-MAP2 for neurons. Neuronal 2D images were made by subtracting from the z stack to show neurites (19). Neurons (19–27 DIV) gave essentially the same results as to binding and toxicity of ASPD. (C) Binding of synthetic ASPD was performed as in B, quantified (10), and shown as ASPD concentration in 400 μL per well. Scatchard analysis gave Kₛ = 5.43 ± 0.27 nM (n = 3). Bₐₕₐₜₜ of ASPD binding was 8.00 ± 1.0 nM for 30 min, from which the maximum level of ASPD binding was calculated to be 14 ± 0.7 pmol of ASPD/mg membrane protein.

![Fig. 2](image2.png)

**Fig. 2.** Characterization of AD patient-derived ASPD. (A) Levels of ASPD in soluble extracts of the cerebral cortex are shown according to disease duration and the level of neurodegeneration (see SI Materials and Methods, Human Brain Pathology and ISH; reanalysis of the samples used as in ref. 19). ASPD are undetectable in most of the age-matched NCI cases, but are present even in the very early AD cases. ASPD levels in patients increase markedly in parallel with the severity and progression of the disease. The 100-kDa retentates of the extracts from the two patients containing the highest level of ASPD were used for isolation of patient ASPD in this study. In a severe AD patient 20 y after onset, ASPD levels were very low, because such widespread neuronal death had already occurred. (B–D) Synthetic ASPD and patient ASPD (see above in A) were purified by IP using ASPD-specific haASD1 antibody as in ref. 19. Soluble extracts from NCI brains and normal mouse IgG were used as controls for patient extracts and haASD1 antibody, respectively. Silver staining in B, dot blotting (SI Materials and Methods) along with a TEM image of negatively stained patient ASPD (see SI Materials and Methods, TEM for particle analysis) in C, and MALDI-TOF/MS analyses in D were performed as per ref. 19. Representative data are shown. In the silver-stained gels, a band corresponding to Ap1–42 or Ap1–40 (red asterisk) was detected only in haASD1-immunopurified patient ASPD. Consistently, in MS of patient ASPD, significant peaks corresponding to Ap1–40 (4313.1 Da, centroid) and Ap1–42 (4515.5 Da, centroid) were reproducibly detected. Less-intense peaks at lower mass than Ap1–40 (e.g., see the asterisk in D, Lower Left) were occasionally, but not reproducibly, detected. The mass of the peak (3319.9 Da, centroid) is consistent with that of [pyroglutamyl-Aβ1–42 + H⁺]. Peaks at higher mass than Ap1–40 or Ap1–42 monomer were not detected (Right). These findings showed that Ap1–40 and Ap1–42 are the predominant components of patient ASPD. (B) Fractionation of patient ASPD (6 pmol) in a 15–30% linear glycerol gradient (see SI Materials and Methods, Glycerol Gradient Sedimentation) (18). Protein standards (aldolose, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; and thyroglobulin, 669 kDa) were centrifuged at the same time as a reference and used for molecular mass determination (Upper). Fractions were collected and immediately assayed for neurotoxicity (Lower) (18). One unit of toxicity induces apoptosis in 1% of cells (see SI Materials and Methods, Glycerol Gradient Sedimentation). Data were obtained from three independent experiments and normalized to Ap1 concentration (mean ± SD; Scheffe’s post hoc test, *P < 0.0001 compared with vehicle alone). Inset shows a representative TEM image of the sample recovered in fraction 2 (∼11.6 ± 2.2-nm spheres; n = 54).

Fig. 2 B and D), had molecular masses of 123 ± 20 kDa (Fig. 2E), and appeared as ∼11.7 ± 1.6-nm spheres (n = 49) in TEM (Fig. 2C), consistent with previous data (18–20). We also used in vitro-reconstituted synthetic ASPD, which share essential characteristics with patient ASPD (19) (Table S1), as an analog.

Binding of ASPD was detected with ASPD-specific hamster monoclonal (haASD)1 antibody Kₐ for ASPD ~ 0.5 mM (19). ASPD bound to a 105-kDa band in extracts from mature neurons cultured for 21 d in vitro (DIV) (red arrowhead in Fig. 3 A, Center), but not immature 2 DIV neurons or HEK293 cells. This band was also recognized by synthetic ASPD (Fig. S2A, red arrow on the left), indicating that ASPD bind directly to this band, because purified synthetic ASPD contained no protein other than ASPD (19).
MS and MS/MS analyses of the 105-kDa band (red arrow in Fig. S2A, Right) identified various NAKα-derived peptides (Table S2). We confirmed that NAKα appeared at 105 kDa (Fig. 3A, Right). ASPD-binding bands from immature neurons or HEK293 (asterisks in Fig. 3A, Center, and Fig. S2A, Left) were identified as intracellular proteins (see legend to Fig. S2A). Thus, NAKα, which has a molecular mass of 112–113 kDa, is likely to be the 105-kDa band entity.

NAKα is an essential catalytic subunit of the NAK pump (Fig. 3B), which is responsible for keeping the neuron resting membrane potential at about −70 mV (22). The functional NAK pump also requires a β-subunit (22) (Fig. 3B). In adult brains, NAKα1 is ubiquitously expressed, whereas NAKα3 is expressed exclusively in neurons, and NAKα2 is found in astrocytes (22). We found that NAKα3 was abundant in extracts of mature neurons only (Fig. 3C). Western blotting showed that NAKα3 became detectable at 7 DIV and that its levels continued to increase until 21 DIV in our culture (Fig. S2C). This developmental increase in NAKα3 level correlated well with that of the 105-kDa band in Far-Western blotting using synthetic ASPD (Fig. S2B). To establish whether NAKα3 is the sole ASPD-binding isofrom, we performed coimmunoprecipitation experiments. First, NAKα3 in mature neuron extracts was coimmunoprecipitated directly with purified synthetic ASPD using haASD1 antibody (Fig. 3D). Second, biotin-labeled ASPD were incubated with mature hippocampal neurons, and MS/MS analysis confirmed that the NAKα3-specific form was selectively coimmunoprecipitated as the 105-kDa band with biotin-labeled ASPD (red arrows in Fig. S2D). We then confirmed that ASPD and NAKα3 were essentially colocalized within the neuropil of mature hippocampal neurons double-stained for synthetic ASPD and NAKα3 (Fig. 3E, Left Insert). Furthermore, surface plasmon resonance spectroscopy (SPR) demonstrated that rat NAKα3 directly bound to synthetic ASPD (Kd = 7.8 ± 2.5 nM, n = 3) (Fig. 3F). The Kd value obtained from this SPR data was almost identical with that obtained from ASPD-binding to mature neurons (Fig. 1C) (Kd = 5.43 ± 0.27 nM, as described above). We confirmed that human and mouse NAKα3 bound directly to synthetic ASPD using SPR (human Kd = 28.6 ± 6.6 nM, n = 5; mouse Kd = 4.0 ± 1.9 nM, n = 3). Notably, most mature hippocampal neurons were stained for NAKα3, whereas most mature cerebellar neurons were NAKα3-immunonegative (Fig. S2E). This finding was consistent with Western blotting showing that the amount of membrane NAKα3 in cerebellar neurons was only ~20% of that in hippocampal neurons (Fig. 3G, Left). Synthetic ASPD bound to NAKα3-immunopositive neurons among hippocampal or cerebellar neurons (Fig. 3E) and induced death in those neurons at levels correlating with their membrane NAKα3 amount (Fig. 3G). Stable knockout of NAKα3 expression with microRNA (miR)-expressing virus vector decreased NAKα3-expressing neurons to 19.8 ± 14% (n = 3; P < 0.0001 compared with the untreated neurons using Scheffe’s post hoc test, n = 3) in hippocampal neurons and abolished ASPD-binding and ASPD-induced neurodegeneration (Fig. 3H). These data support the conclusion that NAKα3 is the mature neuron-specific ASPD-binding protein that is linked to ASPD neurotoxicity.

**ASPD Impair NAKα3 Activity, Leading to Ca2+ Dyshomeostasis and Neuron Death.** We next examined the effect of ASPD-binding on NAKα3 activity. As shown in Fig. 4A, exposure of membrane preparations from mature hippocampal neurons to 110 nM synthetic ASPD rapidly (~70% in 1 h) impaired NAKα3-specific activity in those membrane preparations. Overnight treatment of intact mature hippocampal neurons with 140 nM synthetic ASPD caused a ~90% decrease in NAKα3-specific activity (Fig. 4B).

This NAKα3 impairment should cause a failure in active transport of Na+ and K+ ions (Fig. 3B). Indeed, we found that cytoplasmic Na+ levels were increased immediately after exposure of neurons to synthetic ASPD (at the orange arrow in Fig. S3A), which reached a maximum after ~18 min (at the yellow arrow in
This process would lead to an increase in intracellular Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)) through the plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) or voltage-gated Ca\(^{2+}\) channels (VGCC) activated by depolarization (22). We therefore monitored [Ca\(^{2+}\)]\(_i\) using Fura-PESAM staining. Vehicle treatments did not affect [Ca\(^{2+}\)]\(_i\), for at least 2 h (Fig. 4C1 and Fig. S3B), but ASPD increased the number of Ca\(^{2+}\)-responsive cells dose-dependently (Fig. 4C2 and Fig. S3B), with a plateau at ~40 nM synthetic ASPD (Fig. 4D). The dose-dependent effect of ASPD on the number of Ca\(^{2+}\)-responsive cells correlated well with the level of ASPD-binding (Fig. 1C, Left), as well as that of ASPD neurotoxicity (EC\(_{50}\) = 18 ± 2.4 nM, n = 5), suggesting that ASPD-binding to mature neurons caused increased [Ca\(^{2+}\)]\(_i\), leading to eventual neuronal death.

Depending on the ASPD concentration, two types of Ca\(^{2+}\)-responses were observed (see SI Discussion for a plausible mechanistic explanation). With 18 nM ASPD, repetitive Ca\(^{2+}\) spikes were observed ~3 min after the treatment (Fig. S3B). In this case, [Ca\(^{2+}\)]\(_i\) increased gradually and led to a sustained increase after 2 h (e.g., Fig. S3C1). With higher concentrations of ASPD (e.g., 42 nM in Fig. 4C2), [Ca\(^{2+}\)]\(_i\) increased ~2 min after the treatment, maintained a sustained increase during 0–40 min, and then rose precipitously before plateauing at ~40–70 min. Much higher concentrations of ASPD accelerated the process, with [Ca\(^{2+}\)]\(_i\) plateauing earlier (within 1 h); compare 88 nM as in Fig. S3B, with 42 nM synthetic ASPD in Fig. S3B). Unlike ASPD, 1.4 μM 20–30-nm Aβ42 aggregates (TEM in Fig. 4E), which were obtained by making a 20-fold concentration and then a 10-fold dilution of ASPD (SI Materials and Methods, Preparation of 20- to 30-nm Aβ Aggregates), were rarely detected by ASPD-specific rabbit polyclonal (r-pASD1) antibody in dot blotting (Fig. 4E) and did not cause such a persistent increase in [Ca\(^{2+}\)]\(_i\), (Fig. 4C3), and the neurons remained intact (inset bright-field images in Fig. 4C3).

This ASPD-induced increase in [Ca\(^{2+}\)]\(_i\), required extracellular Ca\(^{2+}\), because it was blocked by addition of EGTA to the medium (Fig. S3C). Furthermore, blockade of [Ca\(^{2+}\)]\(_i\) overload by chelation with BAPTA-AM suppressed ASPD toxicity (Fig. 5A). We then asked which plasma membrane calcium conductors, VGCC or NCX, were involved in ASPD toxicity. Inhibitor effects on ASPD neurotoxicity showed that N-type VGCC were involved in ASPD-induced neuronal death, whereas the other VGCC subtypes and NCX were not involved (Fig. 5A). We confirmed that an inhibitor specific to N-type VGCC blocked ASPD-induced [Ca\(^{2+}\)]\(_i\) increase (Fig. S3D). Notably, N-type VGCC are found primarily at presynaptic terminals of neurons (23), consistent with presynaptic binding of ASPD (19).

Next, we examined whether the two major intracellular Ca\(^{2+}\) stores, mitochondria and endoplasmic reticulum (ER), could be involved in ASPD neurotoxicity. A mitochondrial NCX inhibitor and an inhibitor of mitochondrial permeability transition (MPT) pore opening both blocked ASPD-induced apoptosis, whereas an inhibitor of Ca\(^{2+}\) influx in ER (mediated by a Ca\(^{2+}\) sensor, STIM1), an IP3 receptor antagonist, or a ryanodine receptor (RYR) antagonist, failed to block ASPD neurotoxicity (Fig. 5A and B). We found that treatment with both CGP37157 (a mitochondrial NCX inhibitor) and cyclosporin A (an inhibitor of mitochondrial MPT pore opening) inhibited the ASPD-induced sharp [Ca\(^{2+}\)]\(_i\) increase that followed the initial sustained increase in [Ca\(^{2+}\)]\(_i\) more strongly than the single treatments (Fig. S3E). This finding suggests that mitochondrial NCX and MPT pore opening are both involved in Ca\(^{2+}\) release from mitochondria that is linked to ASPD neurotoxicity (see Fig. S3E for more details).

These findings indicate that impairment of NAKα3 activity by ASPD binding increases cytoplasmic Na\(^{+}\) levels [Fig. S3A], activates N-type VGCC, and causes continuous Ca\(^{2+}\) influx into cytoplasm. This in turn results in Ca\(^{2+}\) overload in mitochondria (observed as the sustained [Ca\(^{2+}\)]\(_i\) increase) and subsequent Ca\(^{2+}\) overload.

![Figure 4](image_url)
release from mitochondria through mitochondrial NCX and MPT pore opening (observed as the sharp increase [Ca\(^{2+}\)]), which results in the eventual death of neurons.

Previously we have shown involvement of tau protein kinase I/ glycogen synthase kinase-3β (TPKI/GSK3β) in ASPD neurotoxicity (18). We also found that tau protein kinase II/cyclin-dependent protein kinase 5 (TPKII/CDK5) was activated after ASPD treatment (Fig. 5 C and D) and tau phosphorylation increased at Ser\(^{396}\) and Ser\(^{404}\), which are known to be phosphorylated in AD brains by TPKII/CDK5/TPKII/CDK5 and TPKII/CDK5, respectively (24) (Fig. 5C). Furthermore, tau and microtubule-associated protein 2 (MAP2) were rapidly lost after ASPD treatment (Fig. 5F). Tau phosphorylation and loss, together with MAP2 loss, could destabilize microtubules and promote neurodegeneration.

Next, time-lapse DIC images of mature neurons after ASPD treatment were taken. Because of osmotic imbalance, neurons started swelling at \(\sim 30\) min after exposure to 140 nM synthetic ASPD, and cell shrinkage, a ubiquitous feature of apoptosis (25), took place in \(\sim 3-5\) h (Fig. 6A, Fig. S4A, and Movie S1). Subsequently, DNA fragmentation became detectable after \(5\) h (Fig. 6B). Scanning electron microscopic (SEM) images clearly showed ASPD-induced neuronal swelling and shrinkage (Fig. 6C). The movie shows that the surface of the ASPD-treated neurons became rough within \(30\) min after the ASPD treatment (Movie S1) and holes appeared within \(1\) h (see arrows in Fig. 6C). Similar swelling and shrinkage of neurons were induced by 100 nM ouabain (Fig. 6A, Fig. S4A, and Movie S2), which selectively blocks rodent NAK\(^{α3}\) activity (26). Unlike ASPD and ouabain, staurosporine, an apoptosis inducer that inhibits multiple kinases, induced shrinkage but not swelling (Fig. 6A, Fig. S4A, and Movie S3). Vehicle treatment induced neither swelling nor shrinkage (Fig. 6A, Fig. S4A, and Movie S4). Ouabain also elicited the events triggered by ASPD (summarized in Fig. 5B; see also Fig. S3F). Our data are consistent with the conclusion that NAK\(^{α3}\) is a death target for ASPD toxicity. We summarize a possible ASPD-induced sequence of events leading to neuronal death in Fig. 6.

We next examined NAK\(^{α3}\) localization in human brains. In nonclinically demented individual (NCI) cerebrum, punctate staining by anti-NAK\(^{α3}\) antibody surrounded the cell body of pyramidal neurons (arrows in Fig. 6D, Upper Left) and diffuse staining was detected on axons and in neuropils. Intense staining was found in basket cells surrounding Purkinje cells in NCI cerebellum (Fig. 6D, arrowheads, Lower Left). NAK\(^{α3}\) localization was consistent with that in adult mouse brain (27).

We found that NAK\(^{α3}\) staining was essentially lost in AD cerebrum, whereas it was not decreased in AD cerebellum (Fig. 6D, Right). Quantitative dot blotting with an ASPD-specific antibody, rpASD1, showed patient ASPD levels in NCI cerebrum, AD cerebrum, and AD cerebellum of 0.7 ± 0.4, 55.7 ± 7.0, and 0.7 ± 0.7 pmol/mg soluble brain extracts, respectively (a representative blot in Fig. 6D), suggesting that decreased NAK\(^{α3}\) staining correlated with patient ASPD levels in AD brains. Comparison of quantitative dot blotting results with rpASD1 and those with anti-A\(^{β}\) antibody 82E1 indicated that patient ASPD...
accounted for 62 ± 3% (n = 3) of soluble Aβ. Unlike decreased NAK3 staining, anti-NAK1 antibody showed diffuse staining throughout NCI and AD cerebrum (Fig. S4B). Results of in situ hybridization (ISH) of adjacent sections of the same AD or NCI cases with NAK3 mRNA (ATP1A3) or NAK1 mRNA (ATP1A1) (n = 3 each) showed the same correlation with the histological data (compare Fig. 6E and Fig. S4C with Fig. 6D and Fig. S4B). With NAK3 ISH, the signal intensity of ATP1A3 was clearly lower in layers of pyramidal neurons in the hippocampus in AD compared with that observed in NCI. No obvious differences were detected in the cerebellum between AD and NCI. With NAK3 ISH, the signal intensity of ATP1A1 was not changed in both regions between AD and NCI cases. These results are consistent with a previous quantitative ISH study on AD patients (28) and suggest NAK3-expressing neurons are preferentially lost in AD-susceptible brain regions where ASPD concentrations are high.

**Structural Characteristics of ASPD and Their Target, Ex4 of NAK3.** To examine structural features of ASPD relevant to binding with NAK3, we acquired solution NMR spectra of 15N-labeled ASPD. Although we detected 39 heteronuclear single quantum coherence (HSQC) signals in the case of freshly dissolved Aβ, we detected 14 of 39 HSQC signals representing peptide backbones of Aβ in ASPD, and these were nearly superimposable on the signals of the Aβ1-13, Aβ15-16, and Aβ21 regions of monomer (Fig. 7A). Remaining NMR-invisible amino acids presumably form a magnetically non-equivalent core. In other words, the NMR-visible amino acids, although derived from different Aβ regions, may exist in close proximity to the ASPD surface (Fig. 7B), in accordance with our finding that specific binding of monoclonal mASD3 antibody to ASPD was blocked by pentapeptides covering Aβ2-8, Aβ15-19, or Aβ19-23 (19). Because mASD3 antibody neutralizes ASPD toxicity (19), the NMR-identified ASPD surface is likely involved in binding to NAK3. The HSQC spectra of ASPD were different from those of precursors of synaptotoxic dodecamers (termed neutralizes ASPD toxicity (29), and of LMW oligomers (30). Thus, ASPD appear to be structurally distinct from preglobularomers and LMW oligomers. This finding is consistent with our finding that ASPD are immunologically distinct from synaptotoxic oligomers (19). We obtained the same HSQC spectra of ASPD in all experiments (n = 3), suggesting that there is one defined structure that is dominant within the ASPD sample. Taken together with our previous immunological data using ASPD-specific antibodies (details in Table S1), this finding supports the notion that ASPD consist of closely related structures. Our recent analysis of ASPD structure using solid-state NMR also supports the notion (31).

The above findings suggest that the unique ASPD surface has a key role in ASPD-NAK3 interaction. Because protein–protein interaction inhibitors can be designed based on the protein surface region essential for binding, we sought to determine which extracellular region of NAK3 serves as the ASPD target site. NAK3 and NAK3 share 96% sequence identity. Given ASPD’s preferential binding to NAK3, the NAK3-specific region, either the first extracellular loop (Ex1) or the fourth extracellular loop (Ex4) (Figs. 3B and 7C), would be the key ASPD target. We found that a chemically synthesized octapeptide from Ex4 (RLNWDDRKT) significantly blocked ASPD toxicity, whereas Ex1 peptides neither bound to ASPD, as determined by SPR (Fig. S3A), nor blocked ASPD toxicity (Fig. 7D). Scrambled Ex4 peptides failed to block ASPD toxicity (Fig. 7D). We confirmed that Ex4 interfered with binding of both patient ASPD and synthetic ASPD to mature hippocampal neurons (Fig. 7E and Fig. S5B), in addition to blocking the subsequent [Ca2+]i increases (Fig. S3D) and activation of the kinases (Fig. S3C). Notably, with 15-min patient ASPD treatment, neurites began to degenerate (Fig. 7E, arrowheads). This degeneration was almost completely blocked by Ex4 (Fig. 7E). These findings suggest that Ex4 is the main ASPD target site (pink in Fig. 7C).

We then planned to develop ASPD-binding peptides with toxicity-neutralizing activity by using phage display (PD) analysis. Three independent screenings of a randomized 12-mer peptide library detected ~21 kinds of ASPD-binding peptides with high His and Trp contents. Among them, many showed similarity to Ex4 of NAK3. Chemically synthesized PD-identified dodecapeptides (PD2, -50, -68) bound to ASPD (Kd = 5.8–8.9 × 10⁻⁸ M by SPR). These dodecapeptides, which commonly contain a four-amino acid sequence (H**W (lacking NAK3-specific Asn)) were ineffective (Fig. 7F). Notably, ASPD-binding tetraepitope PD2-11 (HFNW) was enough to significantly block binding and neurotoxicity of ASPD (Fig. 7F and Fig. S5B). These findings suggest that the NAK3-specific region in Ex4 encompassing residues Asn879 and Trp880 is essential for ASPD-NAK3 interaction (Fig. 7 C and G).

We built a 3D homology model of human NAK3 based on the pig NAK1 structure (32), as no crystal structure of human NAK3 is available. Ex4 appeared to form a cavity and Asn879 and Trp880 are exposed on the NAK3 surface, to which ASPD can gain access (Fig. 7C). Molecular modeling suggested that the mature NAK pump has a 9.9-nm-wide opening (Fig. S6). Solution atomic force microscopy showed that ASPD are 7.2 ± 2.6 nm in height (20). The maximum number concentration of bound ASPD, calculated from Bmax was 14 ± 0.7 pmol of ASPD per milligram of membrane protein (Fig. 1C). This concentration was 40% of the concentration of NAK3 (35.2 ± 10 pmol/mg membrane protein; n = 4) that we measured (as shown in Fig. 3G using prequantified, cell-free synthesized NAK3 as the quantification standard; for details see SI Materials and Methods) and it was ~37–88% of the concentrations found in the literature (16–38 pmol/mg membrane protein) (33, 34). Simple arithmetic calculations thus give an ASPD:NAK3 ratio ranging from 1.1:1–1.3. However, because past
studies have shown that the NAK pump exists predominantly as (NAKα3)2(NAKβ1)2 heterotetramer in membranes (35, 36) and ASPD appeared to bind almost all NAKα3-containing NAK pumps in membranes (Fig. 3E), we infer, using Occam’s razor (37), that the most likely ratio is 1:2 (i.e., 1:1 binding of ASPD with NAKα3 dimer). This inference is consistent with other ASPD binding data (Fig. S6). Because the tetrapeptide segment in Ex4 of NAKα3 is conserved in rodents and humans (Fig. 7G), this opens up possibilities for knowledge-based design of inhibitors of neuronal death in AD.

**Discussion**

Past studies have reported the loss of the NAK pump activity, and the protein itself, in the brains of AD patients (28, 38, 39). In particular, NAKα3 expression, but not NAKα1 expression, is reduced in the frontal cortices of AD patients and this reduction correlates inversely with levels of diffuse plaques in that region (28). No such reduction is observed in the unaffected cerebellum of the same patients (28). NAKα3 activity was reported to decrease in Aβ1–40-treated neuronal cultures (40) and the NAK pump activity was reported to decrease in 17- to 18-mo-old APP and presenilin-1 transgenic mice (41). These previous observations suggest a potential link between Aβ and NAKα3 impairment. However, until now, the question of whether NAKα3 impairment is the cause or the result of neuronal death has remained unanswered. We present here direct evidence that the neuron-specific α-subunit of the NAK pump, NAKα3, is the neuronal death-inducing target of ASPD.

SPR data demonstrated direct binding of ASPD to NAKα3 (Fig. 3F). ASPD-binding to NAKα3 impaired rapidly NAKα3-specific NAK pump activity (Fig. 4A). Cytoplasmic Ca2+ overload was highly correlated with ASPD-binding (compare Figs. 1C and 4D), which eventually led to mitochondrial defects and neuronal death (Fig. 5A), as observed in AD (42). Stable knockout of NAKα3 expression with a microRNA-expressing virus vector decreased NAKα3-expressing neurons and abolished ASPD-binding and ASPD-induced neurodegeneration (Fig. 3H). ASPD binding peptides inhibited the ASPD binding to NAKα3 and protected...
mature neurons from ASPD neurotoxicity (Fig. 7 D–F, and Figs. S3D and S5 B and C). In addition to these cell-based data, we found that NAKα3-expressing neurons were lost in AD-susceptible regions, such as the cortex and the hippocampus in patients (where ASPD levels were high), but were not lost in the far less affected cerebellum of the same patients (where ASPD levels were low) (Fig. 6 D and E and Fig. S4 B and C). The explanation for these regional differences in ASPD concentrations must await further experimentation, but these observations further support our conclusion that NAKα3 serves as the specific death-inducing target of ASPD.

Recent studies have shown that Aβ forms numerous structurally distinct oligomers that may contribute differently to disease pathogenesis (6, 19). Aβ receptor/ligand systems may be organized into three categories (Fig. 8 and Table S3). The first category involves regulating the CNS concentration of Aβ monomer available for assembly. The second category involves Aβ impairment of synaptic connections by indirectly affecting NMDAR activity (see SI Discussion for more details about the first two categories). Postsynaptic Sigma-2/PGRMC1 (progesterone receptor membrane component 1) has been reported to serve as a receptor for 50–75 kDa Aβ1–42 oligomers (43). We report here a third system involving presynaptic neurons. Presynaptic neuronal Ca2+ hyperactivation has been reported to occur near amyloid plaques in AD model mice (44). Interestingly, such hyperactivation of neurons in hippocampus has been associated with the cortical thinning in mild cognitive impairment patients and has been considered to be an early indicator of AD-related neurodegeneration (45). However, the underlying mechanisms were largely unknown. We have previously shown that Aβ colocalize with presynaptic Bassoon (19). Among different NAKα subunits, only NAKα3 is present in the presynaptic side of neurons (27, 46–48). Electrophysiological studies have also shown the importance of NAKα3 in the presynaptic function (49, 50). Our data are consistent with ASPD binding to presynaptic NAKα3 of mature neurons, leading to activation of presynaptic N-type VGCC, and eventual death of NAKα3-expressing neurons. Notably, NAKα3 appears as punctate patterns restricted to layers III and V of the neocortex (27), where N-type VGCC are present (51). These layers are particularly vulnerable in AD brains (52). It is an intriguing speculation that the 100-kDa band sometimes observed by Gong et al. in ligand overlay assays with ADDLs was NAKα3 (53).

The human NAKα3 gene ATPIA3 is located on chromosome 19q13.31, an AD linkage region, but at present there are no reports that mutations in ATPIA3 are linked to AD. The mutation rate in ATPIA3 is very low in humans (54), suggesting that people with mutations in ATPIA3 might be at high risk of neurodevelopmental/neuropsychiatric diseases and develop these diseases before they become old. Indeed, genetic studies have shown that mutations in the protein-coding region of ATPIA3 cause rapid-onset dystonia parkinsonism, alternating hemiplegia of childhood, and cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss syndrome (54). Notably, although a direct linkage of ATPIA3 mutations to AD is difficult to detect, AD and ATPIA3-mutated diseases share features such as convulsive seizures (derived from hyperactivity of neurons). A recent clinical study of AD patients has revealed that convulsive seizures occurred early in the course of AD, which often began around the same time when symptoms of neurodegeneration first appeared (55). Levetiracetam, which inhibits presynaptic calcium channels (56), suppresses neuronal hyperactivation, and reverses cognitive deficits in their AD model mice (57). These results are consistent with our findings (Fig. 8) and suggest that hyperactivation of presynaptic neurons occurs in AD patients contemporaneously with neurodegeneration.

AD is a progressive disease with risk highly correlated with aging. As shown in Fig. 24, ASPD are not detectable in brains of most healthy old people, suggesting that accumulation of ASPD may not begin in the very early phase of Aβ accumulation. This notion is consistent with the fact that ASPD are minimally present in the brains of APP-overexpressing mice, which accumulate dodecamers and retain the early features of AD development (such as spine loss) but not the features of symptomatic human AD (such as neurodegeneration) (19). These observations suggested to us that formation of ASPD might require an age-related “facilitating factor,” in addition to APP overexpression. A prospective study of ASPD formation relative to AD development and progression could be of interest. We think this study may be particularly significant in the light of our prior work showing that ASPD levels correlate with disease severity (19) and the data presented here (Fig. 24) demonstrating that AD patient ASPD levels correlate with the severity of neurodegeneration based on Braak staging and duration of disease.

Fine structural analysis of ASPD by solution NMR suggests that amino acid residues Ala14–His21, Glu15–Lys26; and Asp85, are involved in forming a portion of the ASPD surface, a portion likely involved in neutralizing ASPD neurotoxicity, we speculate that H-bonding interactions are involved in the ASPD–NAKα3 interaction. Indeed, an ASPD-binding tetrapeptide that mimics this Ex4 region blocked ASPD neurotoxicity. Because Asn879 is essential for neutralizing ASPD neurotoxicity, we speculate that H-bonding interactions are involved in the ASPD–NAKα3 interaction. Interestingly, in the case of human NAKα2, mutation at this tryptophan (W887R; Trp887 in NAKα2 corresponds to Trp880 in NAKα3), which is highly conserved among all NAKα isoforms (Fig. 7G), leads to complete loss of catalytic activity and causes familial hemiplegic migraine type 2 (58). This finding suggests the importance of this conserved Trp for native NAKα function and it may be that ASPD binding to the region containing this Trp causes impairment of NAKα3 function.

Except for the NAKβ subunit and agrin, all other NAK-binding proteins have been reported to bind to the intracellular region of NAKα (59). Because this NAKβ-binding region is located 10 amino acids more C-terminally than Trp880 (Fig. 7G), the Ex4-mimicking tetrapeptide is unlikely to interfere with the NAKα–NAKβ interaction. In the case of the NAKα2 mutation W887R, loss of NAK pump activity occurs without interfering with the NAKα–NAKβ interaction because the NAK pump containing the NAKα and NAKβ subunits is present at substantial levels in the plasma membrane (58). With respect to agrin, although its binding region in NAKα3 has not been

![Fig. 8. Major Aβ ligand/receptor systems previously detected and the system identified in this work (Table S3).](Image)
determined, it has been suggested to take the place of NAKβ, and accordingly, it is reasonable to consider that agrin and NAKβ share the same binding region (59). Taken together, these observations suggest that peptidomimetics would not disrupt interactions between NAKα3 and proteins other than ASPD. The Ex4-mimicking tetrapeptide (PD2–11, MW602.6) that inhibits ASPD neurotoxicity is sufficiently small that the molecule could serve as a lead compound for knowledge-based design of peptidomimetics. This class of compounds is expected to offer superior stability and pharmacokinetics compared with anti- Aβ antibodies. Given the essential functions of the NAK pump in neurons (22), direct modulation of the NAK pump would be a risky approach. We therefore propose a new strategy to treat AD by blocking ASPD–NAKα3 interaction through masking the Aβ oligomer surface with specific peptidomimetics, as illustrated in Fig. 6. In summary, our data support the conclusion that ASPD–NAKα3 interaction is a cellular basis for neuronal loss induced by ASPD and that blocking this interaction could be a useful strategy for AD treatment.

Materials and Methods

Ethics. The Bioethics Committee and the Biosafety Committees of MITeLS, Niigata University, Kyoto University, FBRi, and TAO approved experiments using human subjects. The Animal Care and Experimentation Committees of MITeLS, Kyoto University, FBRi, and TAO approved animal experiments.

ASPD Preparation. ASPD are neurotoxic, spherical Aβ oligomers of 10- to 15-nm diameter (measured by TEM) that passed through 0.22-μm filters, and contain x15 oligomers of 10- to 30-nm Aβ (measured by F12 buffer without l-cysteine and phenol red by slowly rotating the solution of 15-nm diameter (measured by TEM) that are recognized by ASPD-specific antibodies (19, 20). Patient ASPD were purified from the 100-kDa retentates (essential for obtaining ASPD) (19).

Additional information can be found in SI Materials and Methods, SI Discussion, Figs. S1–S5, Tables S1–S3, and Movies S1–S4. Fig. S1 shows glutamate receptor antagonists did not change ASPD neurotoxicity. Fig. S2 shows data on ASPD interactions with NAKα3, development changes in NAKα3 levels, commonpreparations, and immunostaining. Fig. S3 shows ASPD-induced Na+ increase, Ca2+ overload induced by different concentrations of ASPD, which were abolished by EGTA pretreatment, as well as ouabain-induced Ca2+ overload and tau phosphorylation/distabilization. Fig. S4 shows time-lapse shots and immunohistochemical studies of human brains using anti-NAKα1 antibody and IF. Fig. S5 shows ASPD-binding peptides inhibit ASPD-binding to NAKα3 and other ASPD-induced downstream phenomena. Fig. S6 presents a model of ASPD-NAKα3 interactions and how masking the ASPD surface with specific masking peptides or peptidomimetics could be a new therapeutic strategy.

ACKNOWLEDGMENTS. We thank S. Kikuchi, Y. Matsumura, and K. Takatsuka for technical assistance; Masatoshi Takeuchi and Shigenobu Yonemoto for help in using the transmission electron microscope instrument; and Dinmanch for comments. This work was supported by grants from the Ministry of Health, Labor and Welfare (Research on Nanotechnological Medical and Initiative for Accelerating Regulatory Science in Innovative Drug, Medical Device, and Regenerative Medicine); the Ministry of Education, Culture, Sports, Science and Technology [Grant-in-Aid for Scientific Research B and Grant-in-Aid for Scientific Research on Innovative Areas (Comprehensive Brain Science Network)]; the Collaborative Research Project of the Brain Research Institute, Niigata University; The Takeda Science Foundation (M. Hoshi); and National Institutes of Health Grants NS038328 and AG041295 (to D.B.T.).