



TITLE:

Na, K-ATPase $\alpha 3$ is a death target of Alzheimer patient amyloid- β assembly.

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1 *Biological Sciences: Biochemistry, Neuroscience*

2 **Na, K-ATPase $\alpha 3$ Is a Death Target of Alzheimer Patient Amyloid- β Assembly**

3

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- 6

1 Abstract

2 Neurodegeneration correlates with Alzheimer's disease (AD) symptoms, but the molecular
3 identities of pathogenic amyloid β -protein ($A\beta$) oligomers and their targets leading to
4 neurodegeneration remain unclear. Amylospheroids (ASPD) are AD patient-derived 10-to 15-nm
5 spherical $A\beta$ oligomers that cause selective degeneration of mature neurons. Here, we show that
6 the ASPD target is neuron-specific Na^+/K^+ -ATPase $\alpha 3$ subunit (NAK $\alpha 3$). ASPD-binding to
7 NAK $\alpha 3$ impaired NAK $\alpha 3$ -specific activity, activated N-type voltage-gated calcium channels,
8 and caused mitochondrial calcium dyshomeostasis, tau abnormalities, and neurodegeneration.
9 NMR and molecular modeling studies suggested that spherical ASPD contain
10 N-terminal- $A\beta$ -derived "thorns" responsible for target binding, which are distinct from
11 low-molecular-weight oligomers and dodecamers. The fourth extracellular loop (Ex4) region of
12 NAK $\alpha 3$ encompassing Asn⁸⁷⁹ and Trp⁸⁸⁰ is essential for ASPD-NAK $\alpha 3$ interaction, because
13 tetrapeptides mimicking this Ex4 region bound to the ASPD surface and blocked ASPD
14 neurotoxicity. Our findings open up new possibilities for knowledge-based design of
15 peptidomimetics that inhibit neurodegeneration in AD by blocking aberrant ASPD-NAK $\alpha 3$
16 interaction.

1 **Significance statement** (up to 120 words understandable for undergraduates)

2 Alzheimer's disease (AD) involves neuron dysfunction and loss. This brain damage is thought to
3 be caused by a small protein, the amyloid β -protein ($A\beta$), which forms aggregates that are
4 neurotoxic. This neurotoxicity has been explained by multiple mechanisms. We reveal here a
5 new neurotoxic mechanism that involves the interaction between patient-derived $A\beta$ assemblies
6 termed amylospheroids, and the neuron-specific Na^+/K^+ -ATPase $\alpha 3$ subunit. This interaction
7 causes neurodegeneration through pre-synaptic calcium overload, which explains earlier
8 observations that such neuronal hyperactivation is an early indicator of AD-related
9 neurodegeneration. Importantly, amylospheroid concentrations correlate with disease severity
10 and progression in AD patients. Amylospheroid:neuron-specific Na^+/K^+ -ATPase $\alpha 3$ subunit
11 interactions may be a useful therapeutic target for AD.
12

1 ¥body
 2 Alzheimer's disease (AD) brains characteristically display fibrillar and non-fibrillar (oligomeric)
 3 protein assemblies composed of the amyloid β -protein ($A\beta$)(1-6). $A\beta$ has been shown to bind to
 4 postsynaptic receptors, such as $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7nAChR$)(7), receptor for
 5 advanced glycation end products (RAGE)(8), receptor tyrosine kinase EPHB2 (9), and cellular
 6 prion protein PrP^C (10). These 'A β receptors', except for RAGE, have been reported to mediate
 7 toxicity of A β oligomers through modulating NMDA receptors (NMDAR)(11). A β oligomers,
 8 including dimers from AD brains (12, 13), dodecamers (A β *56) from AD model mice (14), or *in*
 9 *vitro*-generated A β -derived diffusible ligands (ADDLs)(15, 16), induce synaptic impairment by
 10 affecting NMDAR (11). Thus, NMDAR are a common target for synaptic impairment in AD.
 11 However, these oligomers do not cause neuronal death (12, 14). The atomic resolution structures
 12 of neurotoxic A β oligomers and their *in vivo* targets leading to neuronal death in AD remain
 13 unclear (6), even though neuronal death is the central mechanism responsible for symptomatic
 14 onset in AD (17).

15 We previously isolated neurotoxic A β oligomers, termed amylospheroids (ASPD), from the
 16 brains of AD patient (18-20). ASPD appear in transmission electron microscopic (TEM) images
 17 as spheres of diameter $\sim 11.9 \pm 1.7$ nm (19). ASPD appear to be unique A β assemblies, as

1 determined immunochemically. These structures are recognized strongly by ASPD-specific
 2 antibodies ($K_d \sim \text{pM}$ range), but not with the oligomer-specific polyclonal antiserum A11 (19).
 3 ASPD are distinct from $A\beta$ dimers, ADDLs, dodecamers, and other A11-reactive entities (19).
 4 ASPD cause severe degeneration of mature human neurons (19). ASPD levels in the cortices
 5 of AD patients correlate well with disease severity (19). In contrast, ASPD-like oligomers were
 6 minimally detectable in the brains of transgenic mice expressing human amyloid precursor
 7 protein (*APP*), in which no significant neuronal loss is observed (19). These findings suggest that
 8 ASPD are an important effector of neuronal death in AD patients. We sought to elucidate
 9 mechanisms of ASPD-induced neurotoxicity. We report here that ASPD interact with the α -
 10 subunit of neuron-specific Na^+/K^+ -ATPase ($\text{NAK}\alpha 3$), resulting in presynaptic calcium overload
 11 and neuronal death.

12

13 **Results**

14 **ASPD Bind To $\text{NAK}\alpha 3$ In Mature Neurons.** ASPD caused degeneration of mature rat
 15 hippocampal neurons, but not immature neurons or nonneuronal HEK293 (Fig. 1A). ASPD
 16 toxicity required binding to mature neurons, because ASPD-specific mouse monoclonal

1 amylospheroid (mASD)³ antibody blocked binding and toxicity of ASPD (Fig. 1 *A* and *B*). [The
 2 K_d for ASPD binding = 5.43 ± 0.27 nM ($n = 3$; Fig. 1*C*). ASPD concentration was determined
 3 using an average ASPD molecular weight of 128 kDa (20). See summary of the characteristics of
 4 patient and synthetic ASPD in Table S1. See also *SI* Discussion for updated definition of ASPD.]
 5 Blockers of known A β receptors (21), including glutamate receptors (NMDA, non-NMDA, and
 6 metabotropic types) and voltage-gated sodium channels, did not affect ASPD neurotoxicity (Fig.
 7 S1). These findings suggested ASPD exert their toxicity through binding to novel cell-surface
 8 molecules specific to mature neurons.

9 To identify ASPD-binding proteins on mature neurons, Far-Western ligand-binding assays
 10 were performed in physiological medium. We used ASPD isolated (19) from the soluble brain
 11 extracts of the two AD patients displaying the most severe neurodegeneration and the highest
 12 ASPD concentrations among those shown in Fig. 2*A* (Fig. 2*B*). These ASPD were A11-negative
 13 (Fig. 2*C*), composed predominantly of A β_{1-42} and A β_{1-40} (Fig. 2*B* and *D*), had molecular masses
 14 of 123 ± 20 kDa (Fig. 2*E*), and appeared as $\sim 11.7 \pm 1.6$ nm spheres ($n = 49$) in TEM (Fig. 2*C*),
 15 consistent with previous data (18-20). We also used *in vitro*-reconstituted synthetic ASPD, which
 16 shares essential characteristics with patient ASPD (19)(Table S1), as an analogue.

1 Binding of ASPD was detected with ASPD-specific hamster monoclonal (haASD)1 antibody
2 (K_d for ASPD ~ 0.5 pM (19)). ASPD bound to a 105-kDa band in extracts from mature neurons
3 cultured for 21 days *in vitro* (DIV) (red arrow in Fig. 3A Center), but not immature 2 DIV
4 neurons or HEK293 cells. This band was also recognized by synthetic ASPD (Fig. S2A, red
5 arrow on the left), indicating that ASPD bind directly to this band, because purified synthetic
6 ASPD contained no protein other than ASPD (19). This band was not detected when we used
7 haASD1 alone or synthetic ASPD presaturated with haASD1 (Fig. S2B). Unlike ASPD, freshly
8 dissolved $A\beta_{1-42}$, which consisted of monomers, dimers, and other low-molecular-weight (LMW)
9 oligomers, did not bind to the 105-kDa band, but bound to 55-kDa and 45-kDa bands, likely
10 corresponding to 54.2 kDa $\alpha 7nAChR$ and 40.4 kDa RAGE, respectively (Fig. 3A, Left). These
11 findings showed that the 105-kDa band corresponds to ASPD-specific binding proteins present
12 only in mature neurons.

13 MS and MS/MS analyses of the 105-kDa band (red arrow in Fig. S2A Right) identified
14 various NAK α -derived peptides (Table S2). We confirmed that NAK α appeared at 105 kDa (Fig.
15 3A, right). ASPD-binding bands from immature neurons or HEK293 (* in Fig. 3A middle and

1 Fig. S2A left) were identified as intracellular proteins (see Fig. S2A legend). Thus, NAK α , which
 2 has a molecular mass of 112-113 kDa, is likely to be the 105-kDa band entity.

3 NAK α is an essential catalytic subunit of the NAK pump (Fig. 3B), which is responsible for
 4 keeping the neuron resting membrane potential at about -70 mV (22). The functional NAK pump
 5 also requires β subunit (22)(Fig. 3B). In adult brains, NAK α 1 is ubiquitously expressed, whereas
 6 NAK α 3 is expressed exclusively in neurons, and NAK α 2 is found in astrocytes (22). We found
 7 that NAK α 3 was abundant in extracts of mature neurons only (Fig. 3C). Western blotting
 8 showed that NAK α 3 became detectable at 7 DIV and that its levels continued to increase until
 9 21 DIV in our culture (Fig. S2C). This developmental increase in NAK α 3 level correlated well
 10 with that of the 105-kDa band in Far-Western blotting using synthetic ASPD (Fig. S2B). To
 11 establish whether NAK α 3 is the sole ASPD-binding isoform, we performed
 12 co-immunoprecipitation experiments. First, NAK α 3 in mature neuron extracts was
 13 co-immunoprecipitated directly with purified synthetic ASPD using haASD1 antibody (Fig. 3D).
 14 Second, biotin-labelled ASPD were incubated with mature hippocampal neurons, and MS/MS
 15 analysis confirmed that the NAK α 3 isoform was selectively co-immunoprecipitated as the
 16 105-kDa band with biotin-labelled ASPD (red arrows in Fig. S2D). We then confirmed that

1 ASPD and NAK α 3 were essentially co-localized within the neuropil of mature hippocampal
 2 neurons double-stained for synthetic ASPD and NAK α 3 (*Inset* in Fig. 3*E* left). Furthermore,
 3 surface plasmon resonance spectroscopy (SPR) demonstrated that rat NAK α 3 directly bound to
 4 synthetic ASPD ($K_d = 7.8 \pm 2.5$ nM, $n=3$, Fig. 3*F*). The K_d value obtained from this SPR data
 5 was almost identical with that obtained from ASPD-binding to mature neurons (Fig. 1*C*, $K_d =$
 6 5.43 ± 0.27 nM as described above). We confirmed that human and mouse NAK α 3 bound
 7 directly to synthetic ASPD using SPR (human $K_d = 28.6 \pm 6.6$ nM, $n=5$; mouse $K_d = 4.0 \pm 1.9$
 8 nM, $n=3$). Notably, most mature hippocampal neurons were stained for NAK α 3, whereas most
 9 mature cerebellar neurons were NAK α 3-immunonegative (Fig. S2*E*). This finding was
 10 consistent with Western blotting showing that the amount of membrane NAK α 3 in cerebellar
 11 neurons was only ~20% of that in hippocampal neurons (Fig. 3*G* left). Synthetic ASPD bound to
 12 NAK α 3-immunopositive neurons among hippocampal or cerebellar neurons (Fig. 3*E*) and
 13 induced death in those neurons at levels correlating with their membrane NAK α 3 amount (Fig.
 14 3*G*). Stable knockout of NAK α 3 expression with microRNA (miR)-expressing virus vector
 15 decreased NAK α 3-expressing neurons to $19.8 \pm 14\%$ ($n = 3$; $p < 0.0001$ compared with the
 16 untreated neurons using Scheffé's *post-hoc* test, $n = 3$) in hippocampal neurons and abolished

1 ASPD-binding and ASPD-induced neurodegeneration (Fig. 3H). These data support the
 2 conclusion that NAK α 3 is the mature neuron-specific ASPD-binding protein that is linked to
 3 ASPD neurotoxicity.

4
 5 **ASPD Impair NAK α 3 Activity, Leading To Ca²⁺ Dyshomeostasis And Neuron Death.** We
 6 next examined the effect of ASPD-binding on NAK α 3 activity. As shown in Fig. 4A, exposure
 7 of membrane preparations from mature hippocampal neurons to 110 nM synthetic ASPD rapidly
 8 (~70% in 1 hr) impaired NAK α 3-specific activity in those membrane preparations. Overnight
 9 treatment of intact mature hippocampal neurons with 140 nM synthetic ASPD caused a >90%
 10 decrease in NAK α 3-specific activity (Fig. 4B).

11 This NAK α 3 impairment should cause a failure in active transport of Na⁺ and K⁺ ions (Fig.
 12 3B). Indeed, we found that cytoplasmic Na⁺ levels were increased immediately after exposure of
 13 neurons to synthetic ASPD (at the orange arrow in Fig. S3A), which reached a maximum after
 14 ~18 min (at the yellow arrow in Fig. S3A₃ and A₄; see legends). ASPD-induced impairment of
 15 NAK α 3 activity thus increased cytoplasmic Na⁺ levels and likely induced depolarization in the
 16 treated neurons.

1 This process would lead to an increase in intracellular Ca^{2+} level ($[\text{Ca}^{2+}]_i$) through the plasma
 2 membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) or voltage-gated Ca^{2+} channels (VGCC) activated by
 3 depolarization (22). We therefore monitored $[\text{Ca}^{2+}]_i$ using Fura-PE3AM staining. Vehicle
 4 treatments did not affect $[\text{Ca}^{2+}]_i$ for at least 2 hrs (Fig. 4C1 and Fig. S3B₁), but ASPD increased
 5 the number of Ca^{2+} -responsive cells dose-dependently (Fig. 4C2 and Fig. S3B_{2~4}), with a
 6 plateau at ~40 nM synthetic ASPD (Fig. 4D). The dose-dependent effect of ASPD on the number
 7 of Ca^{2+} -responsive cells correlated well with the level of ASPD-binding (Fig. 1C left), as well as
 8 that of ASPD neurotoxicity ($\text{EC}_{50} = 18 \pm 2.4$ nM, $n = 5$), suggesting that ASPD-binding to mature
 9 neurons caused increased $[\text{Ca}^{2+}]_i$, leading to eventual neuronal death.

10 Depending on the ASPD concentration, two types of Ca^{2+} -responses were observed (*SI*
 11 Discussion for a plausible mechanistic explanation). With 18 nM ASPD, repetitive Ca^{2+} spikes
 12 were observed ≈ 3 min after the treatment (Fig. S3B₂). In this case, $[\text{Ca}^{2+}]_i$ increased gradually
 13 and led to a sustained increase after 2 hrs (e.g. Fig. S3C₁). With higher concentrations of ASPD
 14 (e.g., 42 nM in Fig. 4C2), $[\text{Ca}^{2+}]_i$ increased ~ 2 min after the treatment, maintained a sustained
 15 increase during 0-40 min, and then rose precipitously before plateauing at ~ 40 -70 min. Much
 16 higher concentrations of ASPD accelerated the process, with $[\text{Ca}^{2+}]_i$ plateauing earlier (within 1

1 h; compare 88 nM synthetic ASPD in Fig. S3B₄ with 42 nM synthetic ASPD in Fig. S3B₃).
 2 Unlike ASPD, 1.4 μM 20-30-nm Aβ₁₋₄₂ aggregates (TEM in Fig. 4E), which were obtained by
 3 making a 20-fold concentration and then a 10-fold re-dilution of ASPD (*SI Materials and*
 4 *Methods, Preparation of 20- to 30-nm Aβ aggregates*), were rarely detected by ASPD-specific
 5 rabbit polyclonal (rpASD)1 antibody in dot blotting (Fig. 4E) and did not cause such a persistent
 6 increase in [Ca²⁺]_i (Fig. 4C₃), and the neurons remained intact (inset bright-field images in Fig.
 7 4C₃).

8 This ASPD-induced increase in [Ca²⁺]_i required extracellular Ca²⁺, because it was blocked by
 9 addition of EGTA to the medium (Fig. S3C₂). Furthermore, blockade of [Ca²⁺]_i overload by
 10 chelation with BAPTA-AM suppressed ASPD toxicity (Fig. 5A). We then asked which plasma
 11 membrane calcium conductors, VGCC or NCX, were involved in ASPD toxicity. Inhibitor
 12 effects on ASPD neurotoxicity showed that N-type VGCC were involved in ASPD-induced
 13 neuronal death, whereas the other VGCC subtypes and NCX were not involved (Fig. 5A). We
 14 confirmed that an inhibitor specific to N-type VGCC blocked ASPD-induced [Ca²⁺]_i increase
 15 (Fig. S3D₃). Notably, N-type VGCC are found primarily at pre-synaptic terminals of neurons
 16 (23), consistent with pre-synaptic binding of ASPD (19).

1 Next, we examined whether the two major intracellular Ca^{2+} stores, mitochondria and
 2 endoplasmic reticulum (ER), could be involved in ASPD neurotoxicity. A mitochondrial NCX
 3 inhibitor and an inhibitor of mitochondrial permeability transition (MPT) pore opening both
 4 blocked ASPD-induced apoptosis, whereas an inhibitor of Ca^{2+} influx in ER (mediated by a Ca^{2+}
 5 sensor, STIM1), an IP3 receptor antagonist, or a ryanodine receptor (RyR) antagonist, failed to
 6 block ASPD neurotoxicity (Fig. 5A). Thus, Ca^{2+} dyshomeostasis in mitochondria was involved
 7 in ASPD neurotoxicity, but ER Ca^{2+} stores were not (Fig. 5 A and B). We found that treatment
 8 with both CGP37157 (a mitochondrial NCX inhibitor) and cyclosporin A (an inhibitor of
 9 mitochondrial MPT pore opening) inhibited the ASPD-induced sharp $[\text{Ca}^{2+}]_i$ increase that
 10 followed the initial sustained increase in $[\text{Ca}^{2+}]_i$ more strongly than the single treatments (Fig.
 11 S3E). This finding suggests that mitochondrial NCX and MPT pore opening are both involved in
 12 Ca^{2+} release from mitochondria that is linked to ASPD neurotoxicity (see the legend of Fig. S3E
 13 for more details).

14 These findings indicate that impairment of $\text{NAK}\alpha 3$ activity by ASPD binding increases
 15 cytoplasmic Na^+ levels (Fig. S3A), activates N-type VGCC, and causes continuous Ca^{2+} influx
 16 into cytoplasm. This in turn results in Ca^{2+} overload in mitochondria (observed as the sustained

1 [Ca²⁺]_i increase) and subsequent Ca²⁺ release from mitochondria through mitochondrial NCX
2 and MPT pore opening (observed as the sharp increase [Ca²⁺]_i), which results in the eventual
3 death of neurons.

4 Previously we have shown involvement of tau protein kinase I/glycogen synthase kinase-3β
5 (TPKI/GSK3β) in ASPD neurotoxicity (18). We also found that tau protein kinase
6 II/cyclin-dependent protein kinase 5 (TPKII/CDK5) was activated after ASPD treatment (Fig. 5
7 *C* and *D*) and tau phosphorylation increased at Ser³⁹⁶ and Ser⁴⁰⁴, which are known to be
8 phosphorylated in AD brains by TPKI/GSK3β/TPKII/CDK5 and TPKII/CDK5, respectively
9 (24)(Fig. 5*E*). Furthermore, tau and microtubule-associated protein 2 (MAP2) were rapidly lost
10 after ASPD treatment (Fig. 5*F*). Tau phosphorylation and loss, together with MAP2 loss, could
11 destabilize microtubules and promote neurodegeneration.

12 Next, time-lapse DIC images of mature neurons after ASPD treatment were taken. Because
13 of osmotic imbalance, neurons started swelling at ~30 min after exposure to 140 nM synthetic
14 ASPD, and cell shrinkage, a ubiquitous feature of apoptosis (25), took place in ~3-5 h (Video S1,
15 Fig. 6*A* and S4*A*). Subsequently, DNA fragmentation became detectable after 5 h (Fig. 6*B*).
16 Scanning electron microscopic (SEM) images clearly showed ASPD-induced neuronal swelling

1 and shrinkage (Fig. 6C). The video showed that the surface of the ASPD-treated neurons became
 2 rough within 30 min after the ASPD treatment (Video S1) and holes appeared within 1 h (see
 3 arrows in Fig. 6C). Similar swelling and shrinkage of neurons were induced by 100 nM ouabain
 4 (Video S2, Fig. 6A and S4A), which selectively blocks rodent NAK α 3 activity (26). Unlike
 5 ASPD and ouabain, staurosporine, an apoptosis inducer that inhibits multiple kinases, induced
 6 shrinkage but not swelling (Video S3, Fig. 6A and S4A). Vehicle treatment induced neither
 7 swelling nor shrinkage (Video S4, Fig. 6A and S4A). Ouabain also elicited the events triggered
 8 by ASPD (summarized in Fig. 5B; Fig. S3F). Our data are consistent with the conclusion that
 9 NAK α 3 is a death target for ASPD toxicity. We summarize a possible ASPD-induced sequence
 10 of events leading to neuronal death in Fig. 6A.

11 We next examined NAK α 3 localization in human brains. In non-clinically demented
 12 individual (NCI) cerebrum, punctate staining by anti-NAK α 3 antibody surrounded the cell body
 13 of pyramidal neurons (arrows in Fig. 6D upper left) and diffuse staining was detected on axons
 14 and in neuropils. Intense staining was found in basket cells surrounding Purkinje cells in NCI
 15 cerebellum (arrows in Fig. 6D lower left). NAK α 3 localization was consistent with that in adult
 16 mouse brain (27).

1 We found that NAK α 3 staining was essentially lost in AD cerebrum, whereas it was not
2 decreased in AD cerebellum (Fig. 6D right). Quantitative dot blotting with an ASPD-specific
3 antibody, rpASD1, showed patient ASPD levels in NCI cerebrum, AD cerebrum, and AD
4 cerebellum of 0.7 ± 0.4 , 55.7 ± 7.0 , and 0.7 ± 0.7 pmol/mg soluble brain extracts, respectively (a
5 representative blot in Fig. 6D), suggesting that decreased NAK α 3 staining correlated with
6 patient ASPD levels in AD brains. Comparison of quantitative dot blotting results with rpASD1
7 those with and anti-A β antibody 82E1 indicated that patient ASPD accounted for 62 ± 3 % (n
8 =3) of soluble A β . Unlike decreased NAK α 3 staining, anti-NAK α 1 antibody showed diffuse
9 staining throughout NCI and AD cerebrum (Fig. S4B). Results of *in situ* hybridization (ISH) of
10 adjacent sections of the same AD or NCI cases with NAK α 3 mRNA (*ATPIA3*) or NAK α 1
11 mRNA (*ATPIA1*)(n=3 each) showed the same correlation with the histological data (compare
12 Figs. 6E and S4C with Figs. 6D and S4B). With NAK α 3 ISH, the signal intensity of *ATPIA3*
13 was clearly lower in layers of pyramidal neurons in the hippocampus in AD compared with that
14 observed in NCI. No obvious differences were detected in the cerebellum between AD and NCI.
15 With NAK α 1 ISH, the signal intensity of *ATPIA1* was not changed in both regions between AD
16 and NCI cases. These results are consistent with a previous quantitative ISH study on AD

1 patients (28) and suggest NAK α 3-expressing neurons are preferentially lost in AD-susceptible
 2 brain regions where ASPD concentrations are high.
 3
 4 **Structural Characteristics of ASPD And Their Target, Ex4 of NAK α 3.** To examine
 5 structural features of ASPD relevant to binding with NAK α 3, we acquired solution NMR spectra
 6 of ^{15}N -labelled ASPD. Although we detected 39 heteronuclear single quantum coherence
 7 (HSQC) signals in the case of freshly dissolved A β , we detected 14 of 39 HSQC signals
 8 representing peptide backbones of A β in ASPD, and these were nearly superimposable on the
 9 signals of the A β ₁₋₁₃, A β ₁₅₋₁₆, and A β ₂₃ regions of monomer (Fig. 7A). Remaining NMR-invisible
 10 amino acids presumably form a magnetically nonequivalent core. In other words, the
 11 NMR-visible amino acids, although derived from different A β regions, may exist in close
 12 proximity to form the ASPD surface (Fig. 7B), in accordance with our finding that specific
 13 binding of monoclonal mASD3 antibody to ASPD was blocked by pentapeptides covering A β ₂₋₈,
 14 A β ₁₅₋₁₉, or A β ₁₉₋₂₃ (19). Since mASD3 antibody neutralizes ASPD toxicity (19), the
 15 NMR-identified ASPD surface is likely involved in binding to NAK α 3. The HSQC spectra of
 16 ASPD were different from those of precursors of synaptotoxic dodecamers (termed

1 globulomer)(29) and of LMW oligomers (30). Thus, ASPD appear to be structurally distinct
2 from pre-globulomers and LMW oligomers. This finding is consistent with our finding that
3 ASPD are immunologically distinct from synaptotoxic oligomers (19). We obtained the same
4 HSQC spectra of ASPD in all experiments ($n = 3$), suggesting that there is one defined structure
5 that is dominant within the ASPD sample. Taken together with our previous immunological data
6 using ASPD-specific antibodies (details in Table S1), this finding supports the notion that ASPD
7 consist of closely related structures. Our recent analysis of ASPD structure using solid-state
8 NMR also supports the notion (31).

9 The above findings suggest that the unique ASPD surface has a key role in ASPD-NAK α 3
10 interaction. Because protein-protein interaction inhibitors can be designed based on the protein
11 surface region essential for binding, we sought to determine which extracellular region of
12 NAK α 3 serves as the ASPD target site. NAK α 1 and NAK α 3 share 96% sequence identity.
13 Given ASPD's preferential binding to NAK α 3, the NAK α 3-specific region, either the first
14 extracellular loop (Ex1) or the fourth extracellular loop (Ex4)(Figs. 3B and 7C), would be the
15 key ASPD target. We found that a chemically synthesized octapeptide from Ex4 (RLNWDDRT)
16 significantly blocked ASPD toxicity, whereas Ex1 peptides neither bound to ASPD, as

1 determined by SPR (Fig. S5A), nor blocked ASPD toxicity (Fig. 7D). Scrambled Ex4 peptides
 2 failed to block ASPD toxicity (Fig. 7D). We confirmed that Ex4 interfered with binding of both
 3 patient ASPD and synthetic ASPD to mature hippocampal neurons (Figs. 7E and S5B), in
 4 addition to blocking the subsequent $[Ca^{2+}]_i$ increases (Fig. S3D₄) and activation of the kinases
 5 (Fig. S5C). Notably, with 15-min patient ASPD treatment, neurites began to degenerate (Fig. 7E,
 6 arrowheads). This degeneration was almost completely blocked by Ex4 (Fig. 7E). These findings
 7 suggest that Ex4 is the main ASPD target site (pink in Fig. 7C).

8 We then planned to develop ASPD-binding peptides with toxicity-neutralizing activity by
 9 utilizing phage display (PD) analysis. Three independent screenings of a randomized 12-mer
 10 peptide library detected ~21 kinds of ASPD-binding peptides with high His and Trp contents.
 11 Among them, many showed similarity to Ex4 of NAK α 3. Chemically synthesized PD-identified
 12 dodecapeptides (PD2, 50, 68) bound to ASPD ($K_d = 5.8\text{-}8.9 \times 10^{-8}$ M by SPR). These
 13 dodecapeptides, which commonly contain a four-amino-acid sequence (H**NW*) similar to Ex4
 14 (RL*NW*; corresponding to positions 877-880), inhibited ASPD toxicity towards mature neurons
 15 as effectively as the Ex4-derived peptide, whereas other peptides containing H***W* (lacking
 16 NAK α 3-specific Asn) were ineffective (Fig. 7F). Notably, ASPD-binding tetrapeptide PD2-11

1 (HFNW) was enough to significantly block binding and neurotoxicity of ASPD (Fig. 7F and Fig.
 2 S5B). These findings suggest that the NAK α 3-specific region in Ex4 encompassing residues
 3 Asn⁸⁷⁹ and Trp⁸⁸⁰ is essential for ASPD-NAK α 3 interaction (Fig. 7 C and G).

4 We built a 3D homology model of human NAK α 3 based on the pig NAK α 1 structure (32), as
 5 no crystal structure of human NAK α 3 is available. Ex4 appeared to form a cavity and Asn⁸⁷⁹ and
 6 Trp⁸⁸⁰ are exposed on the NAK α 3 surface, to which ASPD can gain access (Fig. 7C). Molecular
 7 modeling suggested that the mature NAK pump has a 9.9-nm-wide opening (Fig. S6). Solution
 8 atomic force microscopy showed that ASPD are 7.2 ± 2.6 nm in height (20). The maximum
 9 number concentration of bound ASPD, calculated from B_{\max} , was 14 ± 0.7 pmol of ASPD per
 10 milligram of membrane protein (Fig. 1C). This concentration was 40% of the concentration of
 11 NAK α 3 (35.2 ± 10 pmol/mg membrane protein; n=4) that we measured (as shown in Fig. 3G
 12 using pre-quantified, cell-free synthesized NAK α 3 as the quantification standard; for details see
 13 *SI Materials and Methods*) and it was ~37-88% of the concentrations found in the literature
 14 (16-38 pmol/mg membrane protein)(33, 34). Simple arithmetic calculations thus give an
 15 ASPD:NAK α 3 ratio ranging from 1:1 to 1:3. However, since past studies have shown that the
 16 NAK pump exists predominantly as (NAK α 3)₂:(NAK β 1)₂ heterotetramer in membranes (35, 36)

1 and ASPD appeared to bind almost all NAK α 3-containing NAK pump in membranes (Fig. 3E),
2 we infer, using Occam's razor (37), that the most likely ratio is 1:2, (i.e., 1:1 binding of ASPD
3 with NAK α 3 dimer). This inference is consistent with other ASPD binding data (Fig. S6).
4 Because the tetrapeptide segment in Ex4 of NAK α 3 is conserved in rodents and humans (Fig.
5 7G), this opens up possibilities for knowledge-based design of inhibitors of neuronal death in
6 AD.

8 Discussion

9 Past studies have reported the loss of the NAK pump activity, and the protein itself, in the brains
10 of AD patients (28, 38, 39). In particular, NAK α 3 expression, but not NAK α 1 expression, is
11 reduced in the frontal cortices of AD patients and this reduction correlates inversely with levels
12 of diffuse plaques in that region (28). No such reduction is observed in the unaffected cerebellum
13 of the same patients (28). NAK α 3 activity was reported to decrease in A β ₁₋₄₀-treated neuronal
14 cultures (40) and the NAK pump activity was reported to decrease in 17- to 18-mo-old *APP* and
15 *presenilin-1* transgenic mice (41). These previous observations suggest a potential link between
16 A β and NAK α 3 impairment. However, until now, the question of whether NAK α 3 impairment

1 is the cause or the result of neuronal death has remained unanswered. We present here direct
2 evidence that the neuron-specific α subunit of the NAK pump, NAK α 3, is the neuronal
3 death-inducing target of ASPD.

4 SPR data demonstrated direct binding of ASPD to NAK α 3 (Fig. 3F). ASPD-binding to
5 NAK α 3 impaired rapidly NAK α 3-specific NAK pump activity (Fig. 4A). Cytoplasmic Ca²⁺
6 overload was highly correlated with ASPD-binding (compare Figs. 1C and 4D), which
7 eventually led to mitochondrial defects and neuronal death (Fig. 5A), as observed in AD (42).

8 Stable knockout of NAK α 3 expression with a microRNA-expressing virus vector decreased
9 NAK α 3-expressing neurons and abolished ASPD-binding and ASPD-induced neurodegeneration
10 (Fig. 3H). ASPD binding peptides inhibited the ASPD binding to NAK α 3 and protected mature
11 neurons from ASPD neurotoxicity (Figs. 7D-F, S3D₄, S5B, and S5C). In addition to these
12 cell-based data, we found that NAK α 3-expressing neurons were lost in AD-susceptible regions
13 such as the cortex and the hippocampus in patients (where ASPD levels were high), but were not
14 lost in the far less affected cerebellum of the same patients (where ASPD levels were low) (Figs.
15 6 D, E, S4B and S4C). The explanation for these regional differences in ASPD concentrations

1 must await further experimentation, but these observations further support our conclusion that
2 NAK α 3 serves as the specific death-inducing target of ASPD.

3 Recent studies have shown that A β forms numerous structurally distinct oligomers that may
4 contribute differently to disease pathogenesis (6, 19). A β receptor/ligand systems may be
5 organized into three categories (Fig. 8, Table S3). The first category involves regulating the CNS
6 concentration of A β monomer available for assembly. The second category involves A β
7 impairment of synaptic connections by indirectly affecting NMDAR activity (see *SI Discussion*
8 for more details about the first two categories). Post-synaptic Sigma-2/PGRMC1 has been
9 reported to serve as a receptor for 50~75 kDa A β ₁₋₄₂ oligomers (43). We report here a third
10 system involving pre-synaptic neurons. Pre-synaptic neuronal Ca²⁺ hyperactivation has been
11 reported to occur near amyloid plaques in AD model mice (44). Interestingly, such
12 hyperactivation of neurons in hippocampus has been associated with the cortical thinning in mild
13 cognitive impairment patients and has been considered to be an early indicator of AD-related
14 neurodegeneration (45). However, the underlying mechanisms were largely unknown. We have
15 previously shown that ASPD co-localize with pre-synaptic Bassoon (19). Among different
16 NAK α subunits, only NAK α 3 is present in the pre-synaptic side of neurons (27, 46-48).

1 Electrophysiological studies have also shown the importance of NAK α 3 in the pre-synaptic
2 function (49, 50). Our data are consistent with ASPD binding to pre-synaptic NAK α 3 of mature
3 neurons, leading to activation of pre-synaptic N-type VGCC, and eventual death of
4 NAK α 3-expressing neurons. Notably, NAK α 3 appears as punctate patterns restricted to layers
5 III and V of the neocortex (27), where N-type VGCC are present (51). These layers are
6 particularly vulnerable in AD brains (52). It is an intriguing speculation that the 100-kDa band
7 sometimes observed by Gong *et al.* in ligand overlay assays with ADDLs was NAK α 3 (53).

8 The human NAK α 3 gene *ATPIA3* is located on chromosome 19q13.31, an AD linkage
9 region, but at present there are no reports that mutations in *ATPIA3* are linked to AD. The
10 mutation rate in *ATPIA3* is very low in humans (54), suggesting that people with mutations in
11 *ATPIA3* might be at high risk of neurodevelopmental/neuropsychiatric diseases and develop
12 these diseases before they become old. Indeed, genetic studies have shown that mutations in the
13 protein-coding region of *ATPIA3* cause rapid-onset dystonia parkinsonism, alternating
14 hemiplegia of childhood, and cerebellar ataxia, areflexia, pes cavus, optic atrophy, and
15 sensorineural hearing loss syndrome (54). Notably, although a direct linkage of *ATPIA3*
16 mutations to AD is difficult to detect, AD and *ATPIA3*-mutated diseases share features such as

1 convulsive seizures (derived from hyperactivity of neurons). A recent clinical study of AD
2 patients has revealed that convulsive seizures occurred early in the course of AD, which often
3 began around the same time when symptoms of neurodegeneration first appeared (55).
4 Levetiracetam, which inhibits pre-synaptic calcium channels (56), suppresses neuronal
5 hyperactivation and reverses cognitive deficits in their AD model mice (57). These results are
6 consistent with our findings (Fig. 8) and suggest that hyperactivation of pre-synaptic neurons
7 occurs in AD patients contemporaneously with neurodegeneration.

8 AD is a progressive disease with risk highly correlated with ageing. As shown in Fig. 2A,
9 ASPD are not detectable in brains of most healthy old people, suggesting that accumulation of
10 ASPD may not begin in the very early phase of A β accumulation. This notion is consistent with
11 the fact that ASPD are minimally present in the brains of *APP*-overexpressing mice, which
12 accumulate dodecamers and retain the early features of AD development (such as spine loss) but
13 not the features of symptomatic human AD (such as neurodegeneration)(19). These observations
14 suggested to us that formation of ASPD might require an age-related "facilitating factor," in
15 addition to *APP* overexpression. A prospective study of ASPD formation relative to AD
16 development and progression could be of interest. We think this study may be particularly

1 significant in the light of our prior work showing that ASPD levels correlate with disease
 2 severity (19) and the data presented here (see Fig. 2A) demonstrating that AD patient ASPD
 3 levels correlate with the severity of neurodegeneration based on Braak staging and duration of
 4 disease.

5 Fine structural analysis of ASPD by solution NMR suggests that amino acid residues
 6 Ala¹-His¹³, Gln¹⁵-Lys¹⁶, and Asp²³ are involved in forming a portion of the ASPD surface, a
 7 portion likely involved in NAK α 3-binding (Fig. 7B). Furthermore, molecular modeling of
 8 NAK α 3 indicated that the region in Ex4 of NAK α 3 encompassing residues Asn⁸⁷⁹ and Trp⁸⁸⁰ is
 9 essential for the ASPD-NAK α 3 interaction. Indeed, an ASPD-binding tetrapeptide that mimics
 10 this Ex4 region blocked ASPD neurotoxicity. Because Asn⁸⁷⁹ is essential for neutralizing ASPD
 11 neurotoxicity, we speculate that H-bonding interactions are involved in the ASPD-NAK α 3
 12 interaction. Interestingly, in the case of human NAK α 2, mutation at this tryptophan (W887R;
 13 Trp⁸⁸⁷ in NAK α 2 corresponds to Trp⁸⁸⁰ in NAK α 3), which is highly conserved among all NAK α
 14 isoforms (Fig. 7G), leads to complete loss of catalytic activity and causes familial hemiplegic
 15 migraine type 2 (58). This suggests the importance of this conserved Trp for native NAK α

1 function and it may be that ASPD binding to the region containing this Trp causes impairment of
2 NAK α 3 function.

3 Except for the NAK β subunit and agrin, all other NAK α -binding proteins have been reported
4 to bind to the intracellular region of NAK α (59). Because this NAK β -binding region is located
5 ten amino acids more C-terminally than Trp⁸⁸⁰ (Fig. 7G), the Ex4-mimicking tetrapeptide is
6 unlikely to interfere with the NAK α -NAK β interaction. In the case of the NAK α 2 mutation
7 W887R, loss of NAK pump activity occurs without interfering with the NAK α -NAK β
8 interaction because the NAK pump containing the NAK α and NAK β subunits is present at
9 substantial levels in the plasma membrane (58). With respect to agrin, although its binding
10 region in NAK α 3 has not been determined, it has been suggested to take the place of NAK β , and
11 accordingly, it is reasonable to consider that agrin and NAK β share the same binding region (59).
12 Taken together, these observations suggest that peptidomimetics would not disrupt interactions
13 between NAK α 3 and proteins other than ASPD. The Ex4-mimicking tetrapeptide (PD2-11,
14 MW602.6) that inhibits ASPD neurotoxicity is sufficiently small that the molecule could serve as
15 a lead compound for knowledge-based design of peptidomimetics. This class of compounds is
16 expected to offer superior stability and pharmacokinetics compared with anti-A β antibodies.

1 Given the essential functions of the NAK pump in neurons (22), direct modulation of the NAK
2 pump would be a risky approach. We therefore propose a new strategy to treat AD by blocking
3 ASPD-NAK α 3 interaction through masking the A β oligomer surface with specific
4 peptidomimetics, as illustrated in Fig. S6. In summary, our data support the conclusion that
5 ASPD-NAK α 3 interactions is a cellular basis for neuronal loss induced by ASPD and that
6 blocking this interaction could be a useful strategy for AD treatment.

7

8 **Materials and Methods**

9 **Ethics.** The Bioethics Committees and the Biosafety Committees of MITILS, Niigata University,
10 Kyoto University, FBRI, and TAO approved experiments using human subjects. The Animal
11 Care and Experimentation Committees of MITILS, Kyoto University, FBRI, and TAP approved
12 animal experiments.

13 **ASPD Preparation.** ASPD are neurotoxic, spherical A β oligomers of 10-15-nm diameter
14 (measured by TEM) that are recognized by ASPD-specific antibodies (19, 20). Patient ASPD
15 were purified from the 100-kDa retentates of soluble AD brain extracts (devoid of LMW
16 oligomers, prepared as in (19)) by IP using haASD1 (“IP” in *SI* Materials and Methods).

1 Synthetic ASPD were formed in 50 μ M $A\beta_{1-42}$ solution (with or without 2 μ M biotinylated
2 $A\beta_{1-40}$) using in-house-prepared highly soluble $A\beta$ peptides (essential for obtaining ASPD; see
3 “ $A\beta$ synthesis”) in F12 buffer without L-glutamine and phenol red by slowly rotating the
4 solution for ~16 hrs at 4 °C (19). The level of $A\beta_{1-42}$ -ASPD in this $A\beta_{1-42}$ solution after slow
5 rotation is usually ~30 %. Synthetic ASPD were obtained in the fraction that passed through
6 0.22- μ m filters, but was retained on 100-kDa MWCO filters (Sartorius) (19). 15 N-Labelled
7 ASPD were prepared in the same way using purified *E. coli*-expressed 15 N-labelled $A\beta_{1-40}$ (60).
8 ASPD quality was confirmed by dot blotting, TEM, amino acid analysis, and toxicity assays
9 (20).

10 **Statistical Analyses.** Statistical analysis was performed using StatView[®] 5.0 (SAS Institute Inc.).

11 **Other Methods.** Methods for $A\beta$ Synthesis, Preparation of 20- to 30-nm $A\beta$ aggregates, Primary
12 neuronal cultures, ASPD Toxicity, ASPD binding, Immunocytochemistry, MicroRNA of
13 *ATPIA3*, Western and Far-western blotting and MS/MS, Cell-free NAK α 3 production, SPR, IP,
14 NAK activity, Ca^{2+} and Na^{+} Imaging, Time-lapse, Dot blotting, TEM, SEM, Human brain
15 pathology and ISH, Glycerol gradient sedimentation, PD, NMR, and Molecular modeling are
16 described in *SI* Materials and Methods.

1

2 **Online Supporting Information.** This includes *SI* Materials and Methods, *SI* Discussions, 6
 3 figures, 3 tables, and 4 videos. Fig. S1 shows that glutamate receptor antagonists did not change
 4 ASPD neurotoxicity. Fig. S2 shows data on ASPD interactions with NAK α 3, developmental
 5 changes in NAK α 3 levels, co-immunoprecipitations, and immunostaining. Fig.S3 shows
 6 ASPD-induced Na⁺ increase, Ca²⁺ overload induced by different concentrations of ASPD, which
 7 were abolished by EGTA pretreatment, as well as ouabain-induced Ca²⁺ overload and tau
 8 phosphorylation/destabilization. Fig. S4 shows time-lapse shots and immunohistochemical
 9 studies of human brains using anti-NAK α 1 antibody and ISH. Fig. S5 shows ASPD-binding
 10 peptides inhibit ASPD-binding to NAK α 3 and other ASPD-induced downstream phenomena.
 11 Fig. S6 presents a model of ASPD-NAK α 3 interactions and how masking the ASPD surface with
 12 specific masking peptides or peptidomimetics could be a new therapeutic strategy.

13

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- 14
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3

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22

23 Figure Legends

24 **Fig. 1.** Mature Neuron-Specific Binding And Toxicity of ASPD. (A) ASPD neurotoxicity was

25 determined by measuring apoptotic DNA fragmentation in HEK293 cells, 2 DIV immature or 19

26 DIV mature rat hippocampal neurons after overnight treatment with 140 nM synthetic ASPD,

27 with or without 2-hr pretreatment of ASPD with each antibody (0.1 mg/ml for ASPD-specific

1 mASD3 antibody; 0.4 mg/ml for A β_{3-8} antibody 6E10) (mean \pm SD; *, $p < 0.001$ Games-Howell
2 *post-hoc* test, $n = 6$). The antibody remained during overnight incubation with ASPD. As shown
3 previously (19), mASD3 inhibited ASPD-induced neuronal death, but 6E10, targeting A β_{3-8} , did
4 not. ASPD concentration is expressed in terms of the average ASPD mass 128 kDa (20). (B)
5 Cells were treated for 30 min with 140 nM synthetic ASPD as in A. Images are representative of
6 ASPD binding detected by mASD3 (red). Green represents anti-actin for HEK293 or anti-MAP2
7 for neurons. Neuronal 2D images were made from the z stack to show neurites (19). Neurons
8 (19-27 DIV) gave essentially the same results as to binding and toxicity of ASPD. (C) Binding of
9 synthetic ASPD was performed as in B, quantified (10), and shown as ASPD concentrations in
10 400 μ L/well. Scatchard analysis gave $K_d = 5.43 \pm 0.27$ nM ($n=3$). B_{max} of ASPD binding was
11 8.00 ± 1.0 nM for 30 min, from which the maximum level of ASPD binding was calculated to be
12 14 ± 0.7 pmol of ASPD/mg membrane protein.

13
14 **Fig. 2.** Characterization of AD Patient-Derived ASPD. (A) Levels of ASPD in soluble extracts of
15 the cerebral cortex are shown according to disease duration and the level of neurodegeneration
16 (see “Human brain pathology and ISH” in *SI* Materials and Methods; reanalysis of the samples

1 used in (19)). ASPD are undetectable in most of the age-matched non-clinically impaired (NCI)
2 cases, but are present even in the very early AD cases. ASPD levels in patients increase
3 markedly in parallel with the severity and progression of the disease. The 100-kDa retentates of
4 the extracts from the two patients containing the highest level of ASPD were used for isolation of
5 patient ASPD in this study. In a severe AD patient 20 years after onset, ASPD levels were very
6 low, likely because such widespread neuronal death already had occurred. (*B-D*) Synthetic
7 ASPD and patient ASPD (see above in *A*) were purified by immunoprecipitation (IP) using
8 ASPD-specific haASD1 antibody as in (19). Soluble extracts from NCI brains and normal mouse
9 IgG were used as controls for patient extracts and haASD1 antibody, respectively. Silver staining
10 in *B*, dot blotting (*SI* Materials and Methods) along with a TEM image of negatively stained
11 patient ASPD (see “TEM” for particle analysis) in *C*, and MALDI-TOF/MS analyses in *D* were
12 performed (19). Representative data are shown. In the silver-stained gels, a band corresponding
13 to $A\beta_{1-42}$ or $A\beta_{1-40}$ (red asterisk) was detected only in haASD1-immunoisolated patient ASPD.
14 Consistently, in MS of patient ASPD, significant peaks corresponding to $A\beta_{1-40}$ (4331.3 Da,
15 centroid) and $A\beta_{1-42}$ (4515.5 Da, centroid) were reproducibly detected. Less intense peaks at
16 lower mass than $A\beta_{1-40}$ (e.g. see “*” in *D* lower left) were occasionally, but not reproducibly,

1 detected. The mass of the peak (3319.9 Da, centroid) is consistent with that of
2 [pyroglutamyl-A β ₁₁₋₄₂ + H]⁺. Peaks at higher mass than A β ₄₀ or A β ₄₂ monomer were not
3 detected (right). These showed that A β ₁₋₄₀ and A β ₁₋₄₂ are the predominant components of patient
4 ASPD. (E) Fractionation of patient ASPD (~16 pmol) in a 15-30% linear glycerol gradient (see
5 “Glycerol gradient sedimentation” in *SI Materials and Methods*) (18). Protein standards (aldolase,
6 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; and thyroglobulin, 669 kDa) were centrifuged at
7 the same time as a reference and used for molecular mass determination (upper panel). Fractions
8 were collected and immediately assayed for neurotoxicity (lower panel) (18). One unit of toxicity
9 induces apoptosis in 1% of cells (see “Glycerol gradient sedimentation”). Data were obtained
10 from three independent experiments and normalized to A β concentration (mean \pm SD; Scheffé’s
11 *post-hoc* test, *, $p < 0.0001$ compared with vehicle alone). Inset shows a representative TEM
12 image of the sample recovered in fraction 2 ($\approx 11.6 \pm 2.2$ nm spheres; $n = 54$).

13
14 **Fig. 3.** Patient ASPD Bind To NAK α 3. (A) Far-western blotting of RIPA extracts (15 μ g/lane)
15 was done using 6.6 nM patient ASPD (Center; detected with ASPD-specific haASD1 antibody)
16 or 100 nM freshly dissolved A β ₁₋₄₂ containing monomers and dimers (left; detected with anti-A β

1 antibody 6E10). Bands marked an asterisk represent intracellular proteins (legend of Figure S2A).
 2 Western blotting with anti-NAK α 3 antibody (excised from *D*) showed the mass of NAK α 3
 3 (right). Red arrow indicates a 105-kDa mature neuron-specific ASPD-binding protein, NAK α 3.
 4 (*B*) Schematic representation of NAK $\alpha\beta$ and its function. (*C*) Western blotting of RIPA extracts
 5 (used in *A*). (*D*) Co-immunoprecipitation (CoIP) of NAK α 3 with 250 nM synthetic ASPD. (*E*)
 6 Representative Z-stack images of 27 DIV hippocampal or 22 DIV cerebellar neurons, or
 7 HEK293, treated with 13 nM synthetic ASPD for 15 min. ASPD-binding sites (red) on neuropil
 8 co-localize with anti-NAK α 3 staining (green)(high-power view in *Inset*; see
 9 “Immunocytochemistry” in *SI* Materials and Methods). (*F*) Direct interaction between ASPD on
 10 the tip and rat NAK α 3 in micelles using SPR ($K_d = 7.8 \pm 2.5$ nM, $n=3$). NAK α 3-binding to the
 11 tip without ASPD was subtracted as background. NAK β 1 did not interact with ASPD in the
 12 AlphaScreen system. (*G*) Rat hippocampal (19-20 DIV) or cerebellar (23 DIV) neurons were
 13 treated overnight with 110 nM ASPD. ASPD neurotoxicity was determined as in Fig. 1A and
 14 normalized to 0.2 μ M staurosporine toxicity. NAK α 3 levels in membrane fractions of these
 15 neurons before ASPD treatment were determined by Western blotting. Mean \pm SD; *, $p = 0.002$;
 16 **, $p < 0.0001$ compared with hippocampal neurons, $n = 3\sim 4$ by Scheffé’s *post-hoc* test. (*H*)

1 Representative images of 27 DIV hippocampal neurons, with or without selective knockout of
2 NAK α 3 (see “miR of *ATP1A3*” in *SI* Materials and Methods), treated with 13 nM synthetic
3 ASPD for 15 min. ASPD-binding sites (red) and anti-NAK α 3 staining (green) were detected as
4 in *E*. ASPD neurotoxicity was assayed as in Fig. 1*A* (mean \pm SD; Scheffé’s *post-hoc* test, *, $p <$
5 0.0001 compared with vehicle alone, $n = 3$).

6
7 **Fig. 4.** ASPD Impair NAK Activity And Cause Ca²⁺ Dyshomeostasis. (*A*) Membrane fraction of
8 25-26 DIV hippocampal neurons (30 μ g/assay) was incubated with 110 nM synthetic ASPD and
9 NAK α 3-specific ATPase activity ($K_d = 45$ nM for ouabain (61-63)) was determined by
10 subtracting the 100 nM-ouabain-sensitive activity from overall Mg²⁺-ATPase activity (46). Mean
11 \pm SD; *, Scheffé’s *post-hoc* test $p < 0.0001$ compared with 0 hr, $n = 4$. (*B*) NAK α 3-specific
12 ATPase activity of 25-26 DIV hippocampal neurons treated overnight with 140 nM synthetic
13 ASPD was determined as in *A*. Mean \pm SD; *, $p < 0.0001$ Scheffé’s *post-hoc* test, $n = 3$. (*C*)
14 Representative [Ca²⁺]_i changes of cell bodies of 19-20 DIV hippocampal neurons after treatment
15 with each sample using Fura-PE3AM. The ratio of responsive cells was obtained as in *D*.
16 20-30-nm A β ₁₋₄₂ aggregates were obtained by concentration and re-dilution processes (see

1 “Preparation of 20-30-nm A β aggregates” in *SI* Materials and Methods). For treatment with
 2 ASPD, the circulating buffer was replaced with the buffer containing ASPD. After the
 3 replacement, it took \approx 110 sec until the ASPD-containing fluid reached the assay chamber (at the
 4 orange arrow) and it took \approx 17.5 min until all the solution in the assay chamber was replaced by
 5 the ASPD solution (at the yellow arrow). Treatment with 20-30-nm A β ₁₋₄₂ aggregates was done
 6 similarly. (D) The ratio of neurons that exhibited repetitive Ca²⁺ spikes or reached a saturated
 7 [Ca²⁺]_i within 2 hrs after the treatment was calculated from [Ca²⁺]_i data from three independent
 8 experiments. Mean \pm SD; *, $p < 0.0001$ Scheffé’s *post-hoc* test. (E) TEM images and dot blotting
 9 of the samples used in C.

10

11 **Fig. 5.** Downstream Signals Leading To Neuronal Death After ASPD Binding To NAK α 3. (A)
 12 Inhibitor effect on 140 nM synthetic ASPD neurotoxicity examined as in Fig. 1A. (B) Schematic
 13 illustration of the mechanism of ASPD-induced neurodegeneration revealed in this study. (C)
 14 p25 generation from p35, a neuron-specific TPKII/CDK5 activator, after 110 nM synthetic
 15 ASPD treatment (right; representative Western blot). Data were normalized to actin and
 16 presented relative to total p35 and p25 amount at time 0. Mean \pm SD; *, $p < 0.02$ compared with

1 data at time 0 (Scheffé's *post-hoc* test, $n = 4\sim 6$). (D) TPKI/GSK3 β activation as shown by
 2 phospho-Tyr²¹⁶ increase and phospho-Ser⁹ decrease after 110 nM synthetic ASPD treatment
 3 (right; representative Western blot). Data were normalized to total TPKI/GSK3 β and presented
 4 relative to each phospho-protein amount at time 0. Mean \pm SD; *, $p < 0.002$ compared with data
 5 at time 0 (Scheffé's *post-hoc* test, $n = 4\sim 6$). (E) Time-dependent increases in tau phosphorylation
 6 after 110 nM synthetic ASPD treatment. Data were normalized to total tau and presented relative
 7 to each phospho-protein amount at time 0. Vehicle controls are shown as inverted triangles in
 8 black for pSer³⁹⁶ and white for pSer⁴⁰⁴ (Mean \pm SD; *, $p < 0.0001$ compared with data at time 0
 9 (Scheffé's *post-hoc* test, $n = 3\sim 4$). (F) Time-dependent decreases in MAP2 and tau after 110 nM
 10 synthetic ASPD treatment (right; representative Western blot). Data were normalized to actin
 11 and presented relative to each protein amount at time 0. Mean \pm SD; *, $p < 0.002$ compared with
 12 data at time 0 (Scheffé's *post-hoc* test, $n = 4\sim 6$).

13

14 **Fig. 6.** ASPD Effects On NAK α 3-Expressing Neurons In Rat Neurons And Human Brains. (A)
 15 Representative time-lapse images of mature rat hippocampal neurons treated with each reagent
 16 (see Videos S1-4). (B) Time-dependent increase in apoptotic DNA fragmentation (determined as

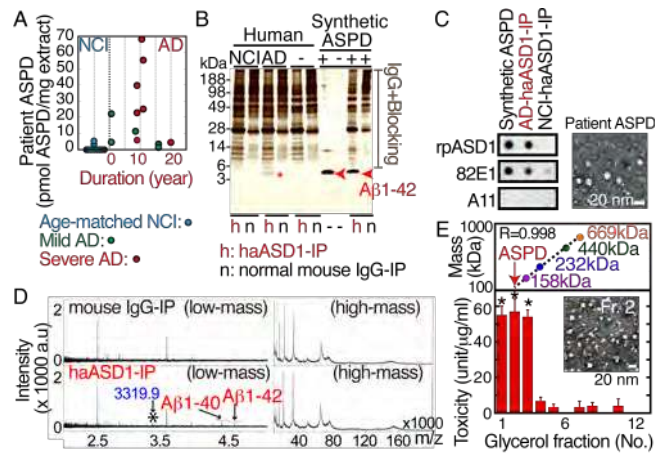
1 in Fig. 1A) induced by 140 nM synthetic ASPD. (C) SEM showed the surface and morphology of
2 mature rat hippocampal neurons treated with 35 nM synthetic ASPD. (D), (E)
3 Immunohistochemical studies using anti-NAK α 1 or anti-NAK α 3 antibody on 4- μ m
4 paraffin-embedded sections in D and ISH analyses using probes for *ATPIA1* and *ATPIA3* on
5 10- μ m paraffin-embedded sections in E. Soluble extracts were obtained from these brains and
6 amounts of patient ASPD (representative blots in D) and A β in the extracts were determined by
7 dot blotting as 429 ± 50 and 695 ± 104 (μ g/g brain, n = 3), respectively.

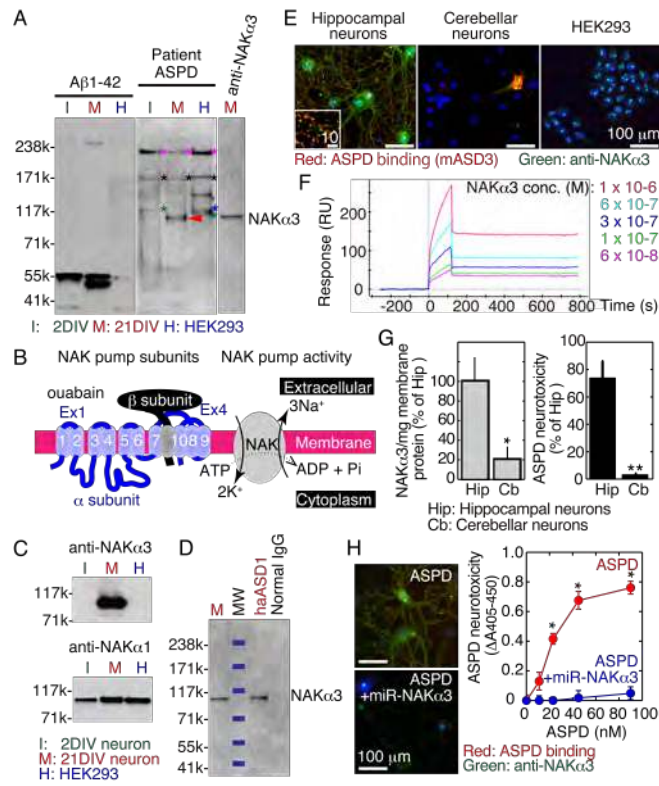
8
9 **Fig. 7.** Structural Basis For ASPD Binding To NAK α 3. (A) ^1H - ^{15}N HSQC spectra of ~ 350 nM
10 ^{15}N -ASPD and ~ 100 μM ^{15}N -A β_{1-40} monomers in 0.5x PBS (supplemented with 5% (vol/vol)
11 D $_2$ O) were recorded at 298K on a 600 MHz Bruker *AVANCE*-III spectrometer equipped with a
12 cryogenic TCI probe. (B) Schematic diagram of the NMR-derived ASPD surface. (C) A
13 three-dimensional homology model of human NAK α 3 was constructed using the Prime program
14 (Schrödinger) based on the structure of pig NAK α 1 (32) as a template (PDB code 3B8E). (D)
15 The effect of each NAK α 3-derived Ex1, Ex4, or scrambled Ex4 peptides on 140 nM synthetic
16 ASPD neurotoxicity was examined as in Fig. 1A. Mean \pm SD; Scheffé's *post-hoc* test, *, $p <$

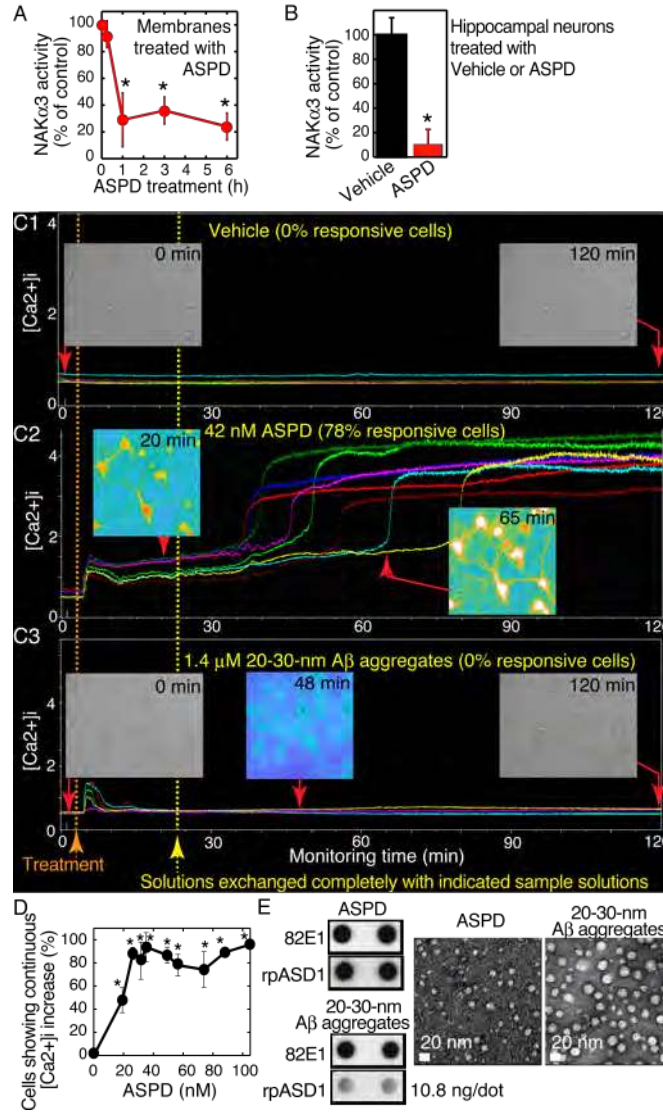
1 0.001 compared with vehicle, $n = 3$. (E) Representative Z-stack images of 26 DIV hippocampal
 2 neurons, after 15 min treatment with 7 nM patient ASPD (immunoisolated from the patient
 3 extracts as in Fig. 2B), with or without 30-min pretreatment with Ex4 (20 μ M in final
 4 concentration). IP eluates of NCI brain extracts, in which ASPD were not detected by dot
 5 blotting, were used as a control. ASPD-binding sites (red) and anti-NAK α 3 staining (green) were
 6 detected as in Fig. 3E. (F) The effect of each Ex4-mimicking ASPD-binding peptides on 140 nM
 7 synthetic ASPD neurotoxicity. All peptides used here contained a free N-terminal amino group
 8 and a carboxylic acid at the C-terminus. Mean \pm SD; Scheffé's *post-hoc* test, *, $p < 0.001$
 9 compared with vehicle, $n = 3$. (G) Alignment of Ex4 regions.

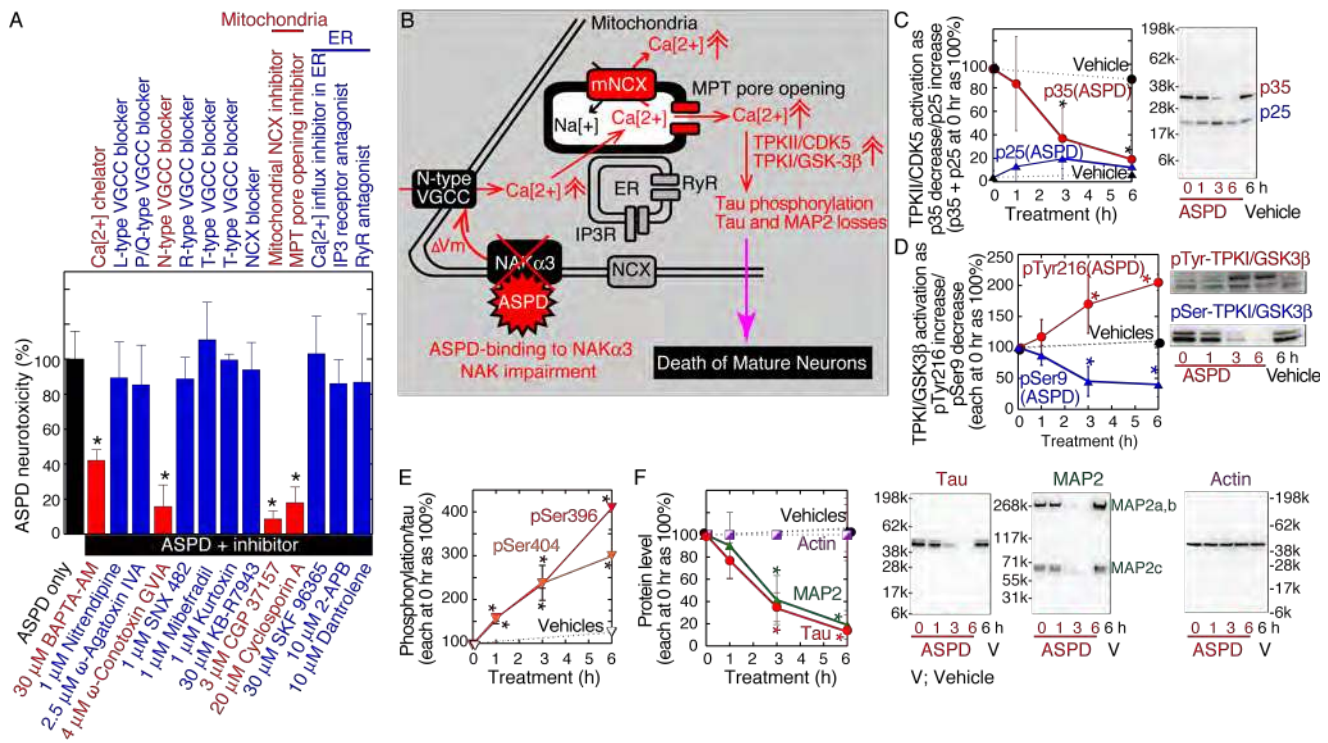
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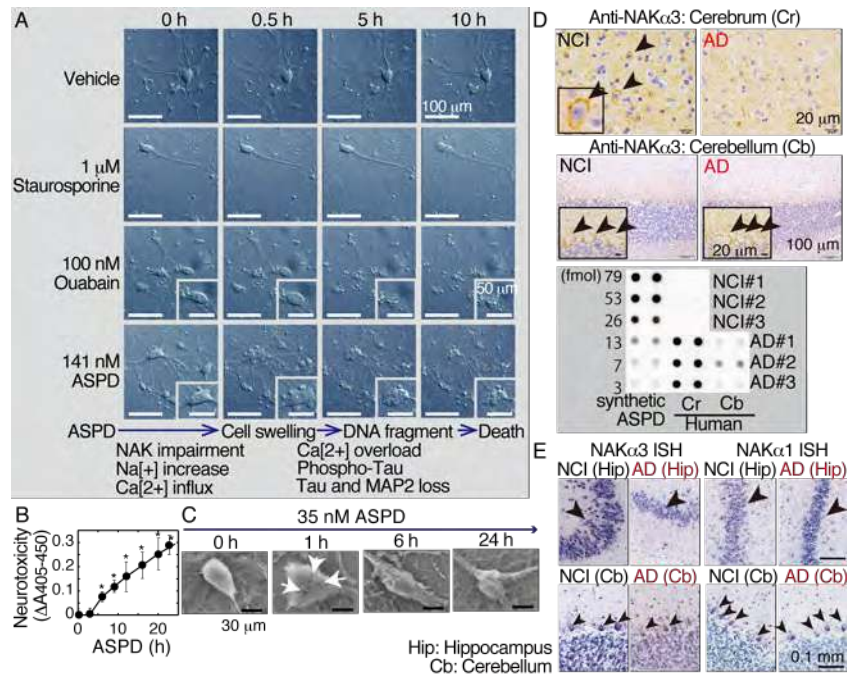
11 **Fig. 8.** Major A β ligand/receptor systems previously detected and the system discovered in this
 12 work (see Table S3).

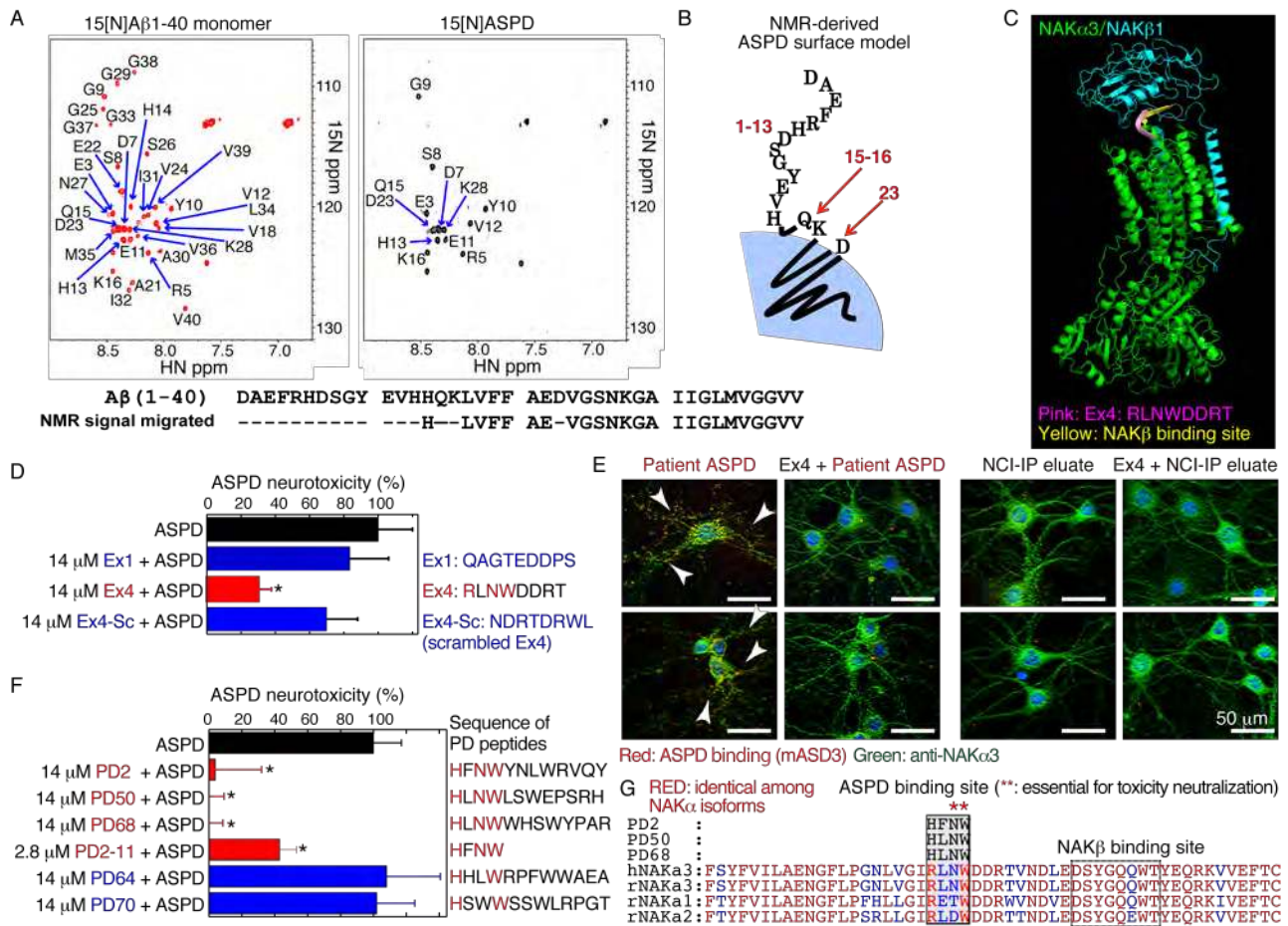


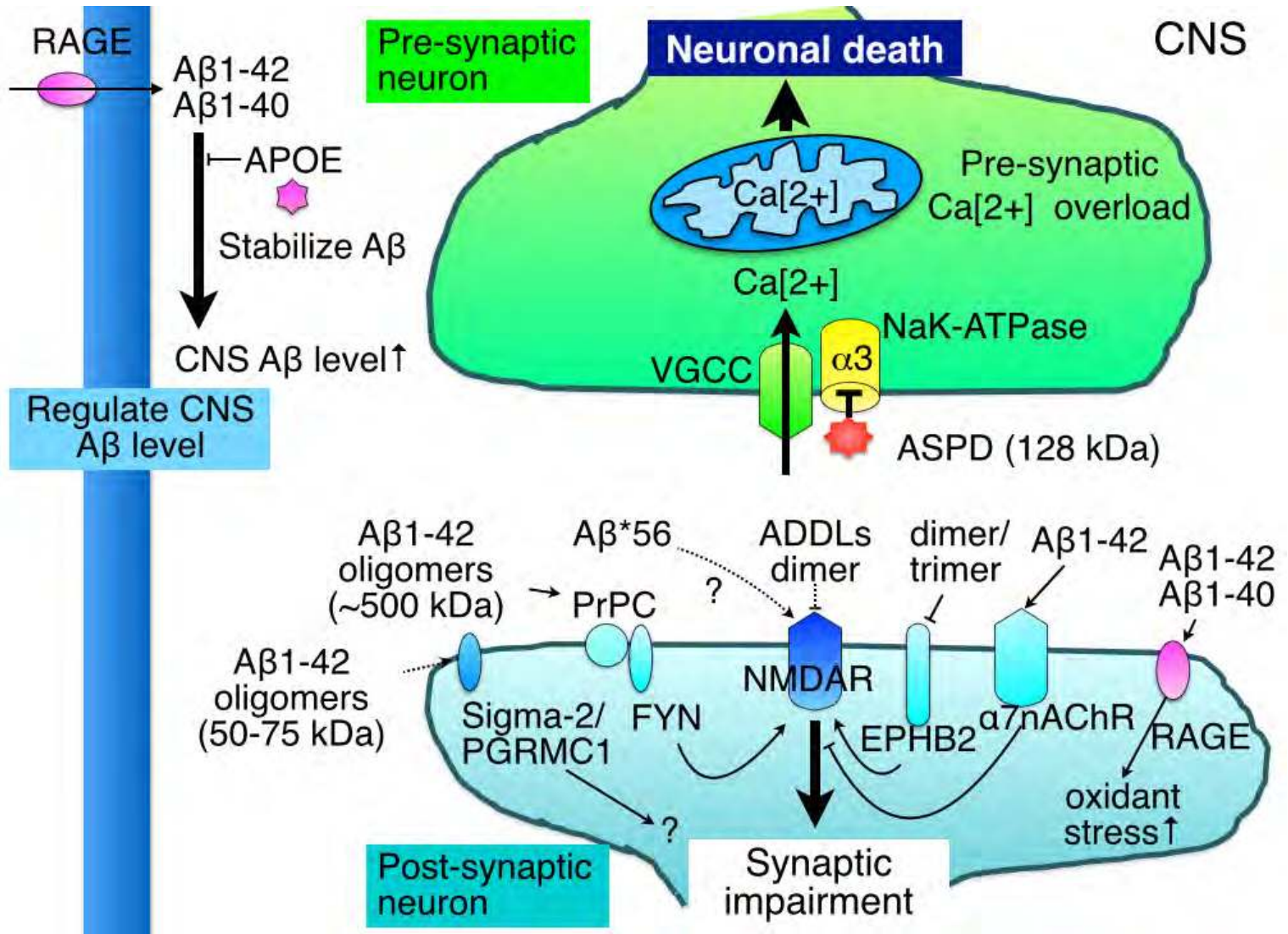












Supporting Information

Na,K-ATPase α 3 Is a Death Target of Alzheimer Patient Amyloid- β Assembly

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SI Materials and Methods

A β synthesis. Highly soluble A β_{1-42} and biotinylated A β_{1-40} peptides were in-house synthesized and purified as described below. For A β_{1-42} , peptides were synthesized on an Applied Biosystems model 433A peptide synthesizer by solid-phase *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry using Fmoc-Ala-NovaSyn-TGA resin (0.19 mmol/g; Novabiochem) (>75% yield), and were cleaved and deprotected in a solution containing phenol (0.15 g), trifluoroacetic acid (TFA, 1.65 ml), Milli Q water (0.05 ml), thioanisole (0.1 ml), and ethanedithiol (0.05 ml) (2 ml/200 mg resin). Crude peptides were precipitated by adding 30 ml of ice-cold diethyl ether, and the precipitates were washed twice, air-dried for 20 min, further dried in a vacuum for 1 hr, then completely dissolved in a solution containing 0.1% (vol/vol) TFA and 30% (vol/vol) acetonitrile (ACN) on ice, and lyophilized. The A β_{1-42} peptides were purified by ZORBAX 300 Extend-C18 (21.2 mm \times 250 mm, 5 μ m, Agilent) reversed-phase chromatography using linear gradient separation (8-32 % (vol/vol) ACN in 70 mM NH₄OH). The purified peptides were immediately lyophilized, redissolved in a solution containing 0.1% (vol/vol) TFA and 30% (vol/vol) ACN on ice (~150 μ M), lyophilized again, and kept at -30 °C until used. On average, 40-80 mg of purified A β_{1-42} peptide was obtained in the 0.1 mmol scale synthesis. For biotinylated A β_{1-40} , the peptides were synthesized as described above, except for the use of pre-loaded Fmoc-Val-NovaSyn-TGA resin (0.21 mmol/g; Novabiochem) (>85% yield), and were labelled at the N-terminus with *N*-(D-biotinyloxy)succinimide. Crude peptides were obtained as described above. Biotinylated A β_{1-40} peptides were first purified by Inertsil CN-3 (10 \times 150 mm, 5 μ m) reversed-phase chromatography using a linear gradient separation (8-80 % (vol/vol) ACN in 0.1% (vol/vol) TFA), followed by ZORBAX 300 Extend-C18 as described above. Analytical HPLC, quantitative amino acid analysis, and matrix-assisted laser desorption/ionization time of flight/mass spectrometry (MALDI-TOF/MS) confirmed the purity of the peptides.

Before ASPD preparation, A β_{1-42} (with or without 1/25 biotinylated A β_{1-40}) was completely dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP for HPLC; Kanto Chemical Co., Inc.) at 80-100 μ M by incubating the peptide solution overnight at 4 °C and for another 3 hrs at 37 °C, and finally lyophilized (~40 nmol/tube). The A β_{1-42} concentration in this step must be kept below 100 μ M in order to maintain the monomeric state. This step was repeated three times. The lyophilized peptide was kept at -30 °C. We had long used HFIP from Sigma-Aldrich for lyophilization, and we recently found that this usually contained ~1.3 mM bis(2-ethylhexyl)phthalate (DEHP). This means that solutions of peptide lyophilized in Sigma-Aldrich HFIP usually contained ~0.65 mM DEHP when the final peptide concentration was 50 μ M. Therefore, when we used HFIP in which DEHP was undetectable, we added DEHP (0.65 mM final

concentration; Tokyo Chemical Industry Co., Ltd.) when the lyophilized peptide was initially dissolved in anhydrous dimethyl sulfoxide (Sigma-Aldrich) in order to ensure consistency with our previous conditions (18-20).

Preparation of 20- to 30-nm A β aggregates. First, synthetic ASPD were prepared from 50 μ M A β ₁₋₄₂ and the flow-through fraction of 0.22- μ m filters was obtained as usual. Then, the flow-through fraction was concentrated (to \sim 1/20 volume) by recovering highly condensed retentates of Sartorius 100-kDa MWCO filters. These concentrated retentates were immediately diluted \sim 10 times with 0.5x Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS(-)). The A β aggregates thus produced were usually much larger than ASPD (mostly 20-30 nm in diameter), and showed low reactivity with ASPD-specific antibodies.

Primary neuronal cultures. Primary rat hippocampal cultures were prepared from embryonic day 17 pups and plated into 0.02% (w/v) polyethyleneimine (PEI; Sigma-Aldrich P3143)-coated wells at a density of 4.6×10^4 cells/cm² (Flexiperm), 4.3×10^4 cells/cm² (48-well plates), or 3.6×10^4 cells/cm² (Celldesk[®]; Sumitomo Bakelite) in neurobasal medium containing B27 supplement and 2.5 μ M L-glutamine (18-20). After 3 days, half of the medium was replaced once a week with the above media additionally containing 10% (vol/vol) astrocyte-derived conditioned medium (ACM, Sumitomo Bakelite). Primary rat cerebellar neuronal cultures were prepared from embryonic day 20 pups essentially as reported (64) with some modifications. Briefly, cerebella, free of meninges and blood vessels, were prepared, cut into small pieces, and incubated in Ca²⁺-Mg²⁺-free Hanks' balanced salt solution (HBS; Invitrogen) containing 1% (wt/vol) trypsin (Invitrogen) and 0.05% (wt/vol) DNase I (Sigma-Aldrich) for 6 min at 37 °C. The pieces were washed three times with HBS, and tissues were dissociated by trituration with a fire-polished Pasteur pipette in HBS containing 0.05% (wt/vol) DNase I and 12 mM MgSO₄. Cells were collected by centrifugation for 5 min at 1000 \times g at room temperature (r.t.), resuspended in culture medium containing basal medium Eagle (BME) (Sigma-Aldrich B9638) containing 10% (vol/vol) equine serum (HyClone) supplemented with 1 mg/ml bovine serum albumin (BSA; Sigma-Aldrich A3156), 10 μ g/ml insulin (Sigma-Aldrich I1882), 0.1 nM L-thyroxine (Sigma-Aldrich T0397), 100 μ g/ml transferrin (Sigma-Aldrich T1283), 1 μ g/ml trypsin inhibitor, aprotinin (Sigma-Aldrich A1153), 30 nM selenium (Sigma-Aldrich S9133), 0.25% (wt/vol) glucose (Sigma-Aldrich G7021), 0.21% (wt/vol) NaHCO₃ (Sigma-Aldrich S5761), and 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco 15140-122), and plated into 0.01% (wt/vol) poly-D-lysine (Sigma-Aldrich P6407)-coated 48-well plates (Corning

CellBIND[®]) at a density of 2.5×10^5 cells/cm². After 48 hrs, the culture medium was completely changed to serum-free culture medium additionally containing 10 μ M Ara-C. Thereafter, the medium was completely replaced twice a week with the above serum-free culture medium containing 10 μ M Ara-C and the cells were grown in a 10% CO₂ atmosphere until ~23 DIV.

ASPD Toxicity. Toxicity was quantified by estimating apoptotic DNA fragmentation (Cell Death Detection ELISA^{plus}, Roche Diagnostics)(19) and was expressed as absorbance difference ($\Delta A_{405-450}$). To examine toxicity neutralization by ASPD-specific mASD3 antibody or inhibitors, neurons were preincubated for 2 hrs with mASD3 or for 30 min with each inhibitor before ASPD treatment. Inhibitors used are; tetrodotoxin (TTX), D-(-)-2-amino-5-phosphonopentanoic acid (D-APV), LY341495, nitrendipine, mibefradil dihydrochloride, KB-R7943 mesylate, CGP 37157, 2-APB, and SKF 96365 hydrochloride from Tocris; (+)-MK-801 maleate (MK801) from Calbiochem; 6,7-dinitroquinoxaline-2,3-dione (DNQX), and (RS)- α -methyl-4-carboxyphenylglycine (MCPG) from ALEXIS; ω -agatoxin IVA, ω -conotoxin GVIA, kurotoxin, and SNX 482 from Peptide Institute; cyclosporin A, dantrolene, and BAPTA-AM from Merck Millipore.

ASPD binding. Rat primary hippocampal neuronal cultures (19 DIV) were incubated with synthetic ASPD (400 μ L/each well) for 30 min at 37 °C. After extensive washing with PBS at 37 °C, the cells were fixed and stained with rpASD1 (1.25 μ g/ml), essentially as described under “Immunocytochemistry”. Unbound ASPD in the medium were estimated by dot blotting (19, 20). Bound alkaline phosphatase was visualized by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate reaction (Pierce) in the presence of 1 mM levamisole and quantified by measuring the absorbance at 405 nm after solubilization (10).

Immunocytochemistry. Cells were rinsed with PBS at 37 °C, either fixed with 1% (wt/vol) paraformaldehyde (PFA) in 0.1 M sodium acetate buffer, pH 6.0, for 20 min at 37 °C (for NAK α 2 and NAK α 3 staining), or fixed with 2% (wt/vol) PFA in PBS/neurobasal media (1:1) for 20 min at 37 °C and then with 4% (wt/vol) PFA in PBS for another 20 min at 37 °C (for other staining), and rinsed three times with PBS. The fixed cells were treated with 1 mg/ml NaBH₄ for 20 min at room temperature (r.t.), rinsed three times with PBS, permeabilized with 0.2% (vol/vol) Triton X-100 for 5 min at r.t., and rinsed three times with PBS. The cells were then pretreated with PBS containing 3% (wt/vol) BSA (Sigma-Aldrich) and 10% (vol/vol) normal goat serum (NGS) (IBL Co., Ltd.) for 45 min at r.t., and incubated overnight

with a primary antibody against ASPD (1.25 $\mu\text{g/ml}$ rpASD1 or 0.51 $\mu\text{g/ml}$ mASD3), MAP2 (2 $\mu\text{g/ml}$; mouse monoclonal HM-2, Sigma M4403), actin (1:250; mouse monoclonal clone C4, Millipore MAB1501R), NAK α 2 (1:100; rabbit polyclonal, Upstate 07-674), or NAK α 3 (0.4 $\mu\text{g/ml}$; rabbit polyclonal, Santa Cruz sc-16051-R) at 4 °C. After three washes with PBS, the cells were incubated with a highly cross-adsorbed secondary antibody in PBS containing 3% (wt/vol) BSA and 10% (vol/vol) NGS (1:1000; Alexa Fluor 568 anti-rabbit IgG, 1:1000; Alexa Fluor 488 anti-mouse IgG; Molecular Probes) for approximately 90 min at r.t. The cells were rinsed three times with PBS and mounted with Prolong Gold anti-fade reagent (Invitrogen). Fluorescence images were acquired using a cooled CCD camera CoolSNAP HQ (Roper Scientific) on a Zeiss inverted microscope Axiovert 200M with MetaMorph imaging software (Molecular Device Corp.) with laser intensity and signal detection settings held constant (19). Neuronal images were collected at 0.4 μm intervals to create a stack in the z-axis and a 2D rendering was made from the z stack to show neurites that would otherwise be out of focus (19).

To elucidate the specificity of anti-NAK α 3 antibodies, NAK α 3 expression was selectively knocked down in rat primary hippocampal neuronal cultures by infection at 5 DIV with AAV-miR-*ATPIA3* vector that contained an antisense sequence for rat *ATPIA3* and an enhanced *GFP* sequence (see “MicroRNA (miR) of *ATPIA3*” below) at the virus titer of 1×10^4 particle/cell for 5 days. The cultures were maintained in virus-free medium for another 15-20 days before experiments, with half of the medium replaced once a week (see “Primary neuronal cultures”). NAK α 3 protein level, determined by Western blotting, was decreased to 19.8% of that of the mock control at 19-20 DIV, while NAK α 1 protein level was not decreased (increased to 233%), by the AAV-miR-*ATPIA3* treatment. Consistent with this large decrease in NAK α 3 protein level, little NAK α 3 staining was observed and almost all the neurons infected by AAV-miR-*ATPIA3* vector (judged from GFP expression) died, except for a huge cell, also transfected with AAV-miR-*ATPIA3* vector, yet which was heavily stained by anti-NAK α 3 antibodies and remained alive. Unlike the other mature hippocampal neurons, this AAV-miR-*ATPIA3* vector-resistant huge cell showed high levels of NAK α 2 and MAP2, suggesting that it is likely to have immature neuronal characteristics. These results strongly suggest that AAV-miR-*ATPIA3* vector-resistant NAK α 3 staining reflects cross-reactivity of anti-NAK α 3 antibodies with NAK α 2. We confirmed that the anti-NAK α 2 antibody used here did not cross-react with NAK α 3 in Western blotting. Consistent with this, ASPD did not bind to this AAV-miR-*ATPIA3* vector-resistant huge cell. As a result, as shown in Fig. 3E, punctate signals for ASPD and for NAK α 3 were detected on neuropil and were almost exclusively co-localized with each other under the established staining conditions. Within z-stack image sets obtained from specimens prepared according to this protocol, we carefully examined whether these punctate signals

occurred on the plasma membrane or intracellularly and confirmed that individual puncta co-stained for ASPD and NAK α 3 appeared to lie on the neuronal surface. In hippocampal neurons, NAK α 3-staining was also observed diffusely and strongly in the soma, which was likely to reflect intracellular NAK α 3 expression in ER. Binding of anti-NAK α 3 antibody to nucleolus-like structures in the nuclei was observed occasionally in both NAK α 3-expressing mature hippocampal neurons and NAK α 3-non-expressing HEK293 cells (see Western blot in Fig. 3C), indicating non-specific binding of the antibody. Thus, it is reasonable that NAK α 3-staining on the soma or the nucleolus-like structures did not co-localize with ASPD signals. Taking these results together, we conclude that ASPD and NAK α 3 essentially co-localize on the neuronal surface, in particular on neuropil (see Fig. 3E Inset).

MicroRNA (miR) of *ATPIA3*. For the selective knockout of rat *ATPIA3* expression, miR-*ATPIA3* vector expressing an antisense sequence for rat *ATPIA3* (TTTACTGTGTGTCAGACCCTG) was generated based on murine miR-155 using the Invitrogen BLOCK-iT™ system. As a control, miR-Mock vector containing Mock sequence (TATTGCGTCTGTACTCACC) was produced. The above miR sequence was inserted into an expression cassette in which a human cytomegalovirus immediate-early promoter was followed by an enhanced *GFP* sequence, either miR-*ATPIA3* or miR-Mock vector, woodchuck hepatitis virus posttranscriptional regulatory element (nucleotides 1093-1684, GenBank accession number J04514), and simian virus 40 polyadenylation signal sequence. This expression cassette was inserted between the inverted terminal repeats of the AAV3 genome. This miR-expressing AAV3 vector was introduced into HEK293 cells with two helper plasmids, pAAV-2R1C (harboring the AAV2 rep and AAV1 cap genes) and pHelper (harboring *E2A*, *E4*, and *VA1* genes of the adenovirus genome; Agilent Technologies), using the calcium phosphate co-precipitation method. AAV particles (AAV-miR-*ATPIA3* vector or AAV-miR-Mock vector) were harvested and purified by two-sequential continuous CsCl gradient ultracentrifugation. Vector titers were determined by quantitative PCR of the DNase-I-treated vector stocks and estimated at 10¹²-10¹³ vector genome.

Western and Far-Western Blotting and MS/MS. Whole-cell RIPA extracts (2 DIV or 19-25 DIV of rat primary neuronal cultures or HEK293 cells, 10 or 15 μ g/lane) were separated under denaturing conditions on reducing NuPAGE 3-8% Tris-Acetate gels (Invitrogen) and bands were transferred onto 0.45 μ m or 0.2 μ m nitrocellulose membranes. For the detection of tau- or GSK3 β -phosphorylation and p35/25, whole-cell RIPA extracts were obtained using PhosSTOP (Roche Diagnostics) and were separated under

denaturing conditions on reducing NuPAGE 12% Bis-Tris gels (Invitrogen). Bands were transferred onto 0.2 μm nitrocellulose membranes.

For western blotting, the membranes were blocked with 5% (wt/vol) skim milk for 1 h at r.t. and probed with a primary antibody against NAK α 1 (0.1 $\mu\text{g/ml}$, US Biological A4000-52), NAK α 2 (1/2,000; Upstate 07-674), NAK α 3 (1 $\mu\text{g/ml}$, Santa Cruz sc16052), actin (1:500, Millipore MAB1501R), MAP2 (1:2000, Sigma M4403; 1:1000, Millipore AB5622), tau (1:1000, Millipore 577801 or Merck Millipore MAB2239), phospho-tau (Ser³⁹⁶, 1:1000, Invitrogen 44752G; Ser⁴⁰⁴, 1:1000, Sigma-Aldrich T7444-1VL), p35/25 (1:1000, Cell Signaling 2680), GSK3 β (1:5000, Santa Cruz sc-9166), or phospho-GSK3 β (Ser⁹, 1:5000, Biosource 44-600G; Tyr²¹⁶, 1:5000, Millipore 05-413). Bands were detected with SuperSignal West Femto chemiluminescent substrates (Pierce) and quantified using a LAS-1000 Plus or LAS-4000 Mini (18).

For Far-western blotting, the membranes were blocked with 5% (wt/vol) skim milk for 1 h at r.t., washed three times with ice-cold F12 buffer without L-glutamine and phenol red, and incubated with either AD patient-derived ASPD (6.6 nM) or the purified synthetic ASPD (3.5 nM) as a ligand for 3 hrs at 4 °C. They were probed with haASD1 antibody (0.02 $\mu\text{g/ml}$) overnight at 4 °C, washed with TBS-T and incubated with highly cross-adsorbed anti-hamster antibody (1:5000) (Zymed Laboratories) for 1 h at r.t. Immunoreactions were detected and quantitated as described above (18). Far-western blotting was also performed using freshly dissolved A β ₁₋₄₂ (100 nM) as a ligand, which was detected with anti-A β N-terminal antibody 6E10 (0.1 $\mu\text{g/ml}$) as described above.

For MS/MS analysis, the samples separated on NuPAGE gels were silver-stained using Silver Quest Silver Staining kit (Invitrogen). Silver-stained bands were excised, destained, and in-gel digested with trypsin. The proteins were reduced with 25 mM NH₄HCO₃-50% (vol/vol) acetonitrile and digested with trypsin (10 $\mu\text{g/ml}$) at 37 °C overnight. The tryptic peptides were recovered from the gel slices by incubating twice in 50% (vol/vol) acetonitrile-0.1% (vol/vol) trifluoroacetic acid at r.t. for 20 min and then by incubating in 100% (vol/vol) acetonitrile at r.t. for 5 min. At each incubation, the supernate was harvested and pooled. The pooled peptide extracts were desalted, evaporated to dryness, and subjected to MS/MS analysis (Bruker Ultraflex III MALDI-TOF/TOF mass spectrometer). Spectra were collected from 400 shots per spectrum over an m/z range of 1000–5000 and calibrated by 7-point internal calibration (m/z 1046.5418, 1296.6848, 1347.7354, 1619.8223, 2093.0862, 2465.1983, and 3147.4710). Monoisotopic peptide masses were assigned and used for Swiss-Prot primary sequence database searches with BioTools 3.1 software (Bruker-Daltonics) and the MASCOT search engine (Matrix Science). Search parameters were set as follows: a maximum allowed peptide mass error of 50 ppm and consideration of 1

incomplete cleavage per peptide. The known peptide masses of keratins, internal standards, and trypsin were excluded. For MS/MS, the most intense precursor ions with a signal/noise ratio of >25 were selected after exclusion of the common background signal.

Cell-free NAK α 3 Production. NAK α 3 protein for SPR analysis and for use as standards for quantifying neuronal NAK α 3 was synthesized with the wheat germ cell-free protein synthesis system in the presence of asolectin liposomes (65). *ATPIA3* ORF DNA was cloned into pEU-E01-MCS vector, which was used as a template for *in vitro* transcription. Translation reaction was performed using the lipid bilayer method. A reaction mixture (25 μ l) containing 10 μ l (60 OD) of WEPRO7240 wheat germ extract (Cell-Free Sciences), 10 μ l of mRNA, 40 μ g/ml creatine kinase, and 10 mg/ml asolectin liposomes (65), was overlaid with 125 μ l of SUB-AMIX SGC solution (Cell-Free Sciences) in flat-bottomed 96-well titer-plates. The reaction was performed for 16 hrs at 26 °C. To quantify NAK α 3 levels, radiolabelled and unlabelled NAK α 3 proteins were simultaneously prepared, except for addition of [14 C]leucine to both the upper and bottom layers in translation reaction of radiolabelled NAK α 3. Each NAK α 3/liposome complex was purified by centrifugation at 20,000 \times g for 10 min at 4 °C and then resuspended into HEPES-buffered saline (HBS). This procedure was repeated 3 times. A portion of the purified (~60 %) 14 C-labelled NAK α 3 was spotted on 3MM filter paper and 14 C-decay was measured by liquid scintillation (Hitachi-Aloka Medical). Based on the [14 C]leucine level of radiolabelled NAK α 3, the concentration of simultaneously prepared unlabeled NAK α 3 was accurately determined and used as a standard for quantifying neuronal NAK α 3 levels in Western blotting. For SPR analysis, NAK α 3 micelles were prepared as described below with the cell-free protein synthesis system. Large-scale translation was conducted by the dialysis method. Five-hundred μ l of reaction mixture (60 OD of WEPRO7240 wheat germ extract, 125 μ l of mRNA, 40 μ g/ml creatine kinase, and 10 mg/ml asolectin liposomes) was prepared in a Thermo Scientific 10K MWCO Slide-A-Lyzer dialysis device, and the dialysis cup was immersed in 3.5 ml of dialysis solution (SUB-AMIX SGC, Cell-Free Sciences). The reaction was performed for 24 hrs at 15 °C. The NAK α 3/liposome complex was purified by centrifugation and resuspension in HBS as described above. The purified pellet was suspended in 300 μ l of a solubilization solution (200 mM HEPES-NaOH, pH 8.0, 750 mM NaCl, 4% (wt/vol) n-dodecyl- β -D-maltoside, 10% (vol/vol) glycerol, and 1 mM dithiothreitol). The suspension was sonicated using Branson Sonifier model 450 Advanced Cell Disrupters for 15 min at 20 °C, and centrifuged at 14,000 \times g for 15 min at 4 °C. The supernate containing solubilized NAK α 3 was collected and loaded on a Superdex 200 10/300 24-mL SEC column (GE Healthcare) for further purification (66). The concentration of the purified NAK α 3

micelles was determined from the absorbance at 280 nm using an extinction coefficient of 94770 (67).

SPR. SPR was performed on a ProteOn XPR36 Protein Interaction Array System (BIO-RAD) with standard system software. About 900 RU (resonance units) of C-terminally biotinylated PD peptide was immobilized on the GLC chip, on which NeutrAvidin had been pre-coupled, according to the manufacturer's instructions. Association and dissociation of ASPD with each PD peptide were measured at 25 °C as a function of time (sensorgram). To examine direct interaction between ASPD and NAK α 3, synthetic ASPD were diluted with a sodium acetate immobilization buffer (pH 4.5) and immobilized on the intermediate binding capacity-sensor chip GLM according to the manufacturer's instructions (~1600 RU of ASPD). Association and dissociation of ASPD with purified NAK α 3 micelles (see "Cell-free Protein Production of NAK α 3") were monitored as described above. Langmuir with Mass Transfer determined kinetic parameters in ProteOn Manager software (version 3.1).

Immunoprecipitation (IP). IP with haASD1 was performed using either an Immunocapturing Kit 100 MB-IAC Prot G (Bruker Daltonics 233794) or a Protein Capturing Kit MB-CovAC-Select (Bruker Daltonics 254733) as described previously using 3% (wt/vol) BSA (Sigma A7030) to suppress non-specific binding (19, 20). We confirmed that ASPD retained their structure and toxicity by means of TEM, dot blotting, and toxicity studies (19, 20). The ASPD amount was determined using dot blotting (19, 20). CoIP experiments were performed essentially as described above except that extracts were preincubated with ASPD (250 nM) for 30 min at r.t.

For Figs. 2 and 7, in order to remove other A β oligomers less than 100 kDa in mass, soluble extracts from AD or NCI brains were concentrated approximately 16 times using 100-kDa molecular mass cut off filters (Sartorius) (19, 20). IP was performed using N-hydroxysuccinimide (NHS)-Activated Magnetic Beads (Thermo Scientific), according to the manufacturer's instructions, except that BSA was used to suppress nonspecific binding. ASPD-specific haASD1 antibody or normal mouse IgG was coupled to NHS-magnetic beads. In order to remove nonspecific binding proteins to haASD1, pre-absorption of AD and NCI samples was performed with mouse IgG-coupled beads before antigen-antibody complex reaction. After a final washing step of the IP, the captured immunocomplex was separated by SDS-PAGE. The magnetic beads were resuspended in 20 μ l of SDS-loading buffer without reducing agent and the beads were heated at 70 °C for 10 minutes. The beads were then removed and the supernatant was collected for SDS-PAGE under denaturing conditions on non-reducing NuPAGE 12% Bis-Tris gels (Life Technologies) in MES buffer. Then, silver staining was performed using SilverQuest Silver Staining kit

(Life Technologies), according to the manufacturer's instructions.

Rat primary hippocampal neuronal cultures (21 DIV) were washed twice with PBS at 37 °C, incubated with 10 nM streptavidin in PBS for 15 min at 37 °C, washed twice with F12 without L-glutamine and phenol red at 37 °C, and incubated with 53 nM biotinylated ASPD in F12 without L-glutamine and phenol for 30 min at 37 °C. After three washes with F12 without L-glutamine and phenol at 37 °C, the cells were extracted using 1% (wt/vol) n-octyl- β -D-thioglucopyranoside for 30 min on ice, and the lysate was centrifuged for 30 min at 16,000 \times g at 4 °C. The biotinylated ASPD-ASPD binding protein complexes in the supernate were isolated using streptavidin magnetic beads (Promega) according to the manufacturer's protocol. The samples were separated on NuPAGE gels and silver-stained using SilverQuest Silver Staining kit (Invitrogen).

NAK activity. ATPase activities in membrane fractions were determined as described (40) with some modifications. Briefly, after vehicle or synthetic ASPD treatment, mature rat primary neuronal cultures (25-26 DIV) were extracted using 300 μ l of lysis solution (20 mM Tris-HCl pH 7.5, 0.6 mM EGTA, 0.1 mM PMSF). The lysate was homogenized once using a Potter-Elvehjem homogenizer, and the homogenate was centrifuged for 5 min at 1,500 \times g. The supernate was further centrifuged for 45 min at 120,000 \times g at 4 °C. The pellet was suspended on ice in a buffer (20 mM Tris-HCl pH 7.5 and 0.1 mM EGTA), and the suspension was used as the membrane fraction. ATPase activities were determined using the membrane fraction (20-30 μ g/assay) in 90 μ l of a buffer (18 mM Tris-HCl pH 7.5, 80 mM NaCl, 15 mM KCl, 3 mM MgCl₂ and 0.1 mM EGTA) with or without ouabain at different concentrations (40). The level of inorganic phosphate was quantified as ATPase activity using MicroMolar Phosphate Assay Reagent (Profoldin). The absorbance at 655 nm was measured and converted to the activity value by linear regression using a standard curve of phosphate ion standard solution (Wako). Since rodent NAK α 3 is extremely sensitive to ouabain (IC₅₀=1.6 nM) (26), the activity of the rodent NAK α 3 was determined by subtracting the ouabain-sensitive (100 nM) activity from the overall Mg²⁺-ATPase activity (40).

Ca²⁺ and Na⁺ imaging. Primary rat hippocampal cultures (19-24 DIV) on Celldesk were washed with HSS buffer (5.4 mM KCl, 130 mM NaCl, 1 mM CaCl₂, 0.6 mM MgCl₂, 5.5 mM glucose, and 20 mM HEPES, pH 7.3) and loaded with 4 μ M Fura-PE3AM (TEFLABS; a leakage-resistant Fura-2AM derivative that is retained in cells for hours) for 45 min at 37°C. The cells were mounted in the chamber where HSS buffer was perfused by a peristaltic pump at 37 °C. ASPD and other reagents were added to the perfusion line. Images were captured and processed using Metafluor software (Molecular Devices)

with a CCD camera (Hamamatsu Photonics ImagEM Model C9100-13) coupled to an Olympus IX70 microscope (Olympus). The cells were excited at 340 and 380 nm via a Lambda DG-5 light source (Sutter Instrument Co.) and emission at 500 nm was captured every 2 sec. The absorbance ratio at 340/380 nm is shown. In the case of sodium (Na^+) imaging, cultures were loaded with a Na^+ indicator, 2 μM Asante Na TRIUM Green TM-2AM (ANG-2AM) ($K_d = 20 \text{ mM}$; excitation 488-517 nm; emission 540 nm; TEFLABS) for 30 min at 37 °C. Na^+ imaging was performed in essentially the same way as Ca^{2+} imaging, except that the cells were excited at 470-495 nm and emission at 510-550 nm was captured using an Olympus NIBA filter set. In our system, as described above, HSS buffer was circulated. The treatment of neurons with ASPD or other reagents was initiated by changing the circulating HSS buffer to HSS buffer containing ASPD or the sample reagent. After the buffer change, it usually took ~ 110 sec for HSS buffer containing ASPD or the sample to reach the assay chamber, and by ~ 17.5 min the solution in the assay chamber was completely replaced with HSS buffer containing ASPD or the sample.

Time-lapse. Confocal images of the culture in a thermostatic chamber (37 °C, 5% CO_2) were taken with a 20x objective for 24 hrs (6 images/hr) using a confocal laser microscope system A1 and NIS-Elements C software (Nikon). The time of cell swelling or shrinkage was quantified based on the time of each shot.

Dot blotting. Dot blotting was performed as described (19, 20) using 40 ng/ml ASPD-specific rpASD1 antibody in 5% skim milk with membrane boiling, 1/5000 anti-pan-oligomer A11 antibody (StressMark Biosciences Inc.) in 5% skim milk without membrane boiling, and 12.5 ng/ml anti- $\text{A}\beta$ N-terminal 82E1 antibody in 10% skim milk without membrane boiling. Immunoreactions were detected with SuperSignal West Femto chemiluminescent substrates (Pierce) and quantified using a LAS-1000 Plus or LAS-4000 Mini (19, 20). In the case of synthetic ASPD, amounts were determined by quantitative amino acid analysis using a Waters AccQ-Tag system, according to the manufacturer's protocol, as in (19). In the case of patient ASPD, amounts of ASPD or $\text{A}\beta$ in samples were obtained from a standard curve generated from serial dilutions of synthetic ASPD, the concentration of which was pre-determined as described above (see 19). The linear range of the assay is 0.3 – 3000 nM in equivalents.

TEM. Samples were negatively stained with 4% (wt/vol) uranyl acetate solution on carbon or elastic carbon-coated Formvar grids and then immediately analyzed at 100 kV (19, 20). ASPD images were captured at a direct magnification of 200,000 and the size of each spherical assembly was determined at the equator (19).

SEM. Rat primary hippocampal neuronal cultures (20 DIV on Aclar films from Nisshin EM Co., Ltd) were treated for the indicated time with 35 nM synthetic ASPD, fixed with 4% (wt/vol) paraformaldehyde, 0.005% (vol/vol) glutaraldehyde, 15% (vol/vol) saturated picric acid, 0.1% (wt/vol) tannic acid, and 1% (w/v) sucrose in 0.1 M phosphate buffer pH 7.4 for 20 min at 37 °C, and dehydrated through a 50, 70, 80, 90, 95, and 100% (vol/vol) ethanol series. After replacement of ethanol with 100% (vol/vol) *tert*-butyl alcohol, the samples were dried using a Hitachi freeze dryer ES-2030. The dried specimens were sputter-coated with osmium. The surface and morphology of the cells were examined by scanning electron microscopy (SEM) using a Hitachi model S-4500 field emission electron microscope operating at 15 kV.

Human brain pathology and ISH. We examined 5 clinicopathologically confirmed AD cases (as in (19)) and 5 control cases, among which samples with well-preserved RNA were used for the study. The cases used for this purpose were as follows (patient number, age, gender, disease duration, post-mortem (p.m.) sampling time, brain weight): AD patients (AD1, 86 yr, female, 23 yr duration, 4 hr p.m., 995 g; AD2, 100 yr, female, 6 yr duration, 3 hr p.m., 935 g; AD3, 87 yr, female, 9 yr duration, 3 hr p.m., 1000 g) and NCI cases (NC1, 76 yr, male, no AD symptoms, 3 hr p.m., 1220 g; NC2, 82 yr, male, no AD symptoms, 2 hr p.m., 1170 g; NC3, 79 yr, female, no AD symptoms, 3 hr p.m., 1100 g). Paraffin-embedded 4- μ m sections of formalin-fixed brains were pretreated with microwaves for 30 min in 10 mM citrate buffer (pH 6.0), and treated with 0.3% (vol/vol) H₂O₂-methanol for 30 min followed by incubation with normal goat serum for 30 min at r.t. These sections were incubated overnight at 4 °C with primary antibodies against NAK α 1 (US Biological A4000-52, 1:300) and NAK α 3 (US Biological A4000-65c, 1:300), followed by incubation for 1 hr at r.t. with appropriate biotinylated secondary antibodies. Immunoreactivities were detected by the avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector). The chromogen reaction was developed with diaminobenzidine/H₂O₂ solution. Counterstaining was carried out with Mayer's hematoxylin. Sections were viewed by using a light microscope (Olympus AX80T) and images were captured with a digital camera (Olympus DP70). It is well known that the distribution patterns of neurofibrillary tangles and of senile plaques in AD brains develop in a predictable sequence (68). We assessed the overall pathology of the AD brains according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria. Since severity of neuronal degeneration usually appears to be associated with the cellular burden, we evaluated it according to the staging system of neurofibrillary pathology by using a phosphorylation-specific anti-tau antibody

(clone AT8)(68). We considered the cases with stages III/IV and V/VI neurofibrillary pathology as corresponding to “moderate” and “severe” degeneration, respectively.

ISH was performed on paraffin sections with some modifications. Briefly, 10- μ m sections were made from frontal cortex, cornu ammonis, and cerebellum. The digoxigenin-labelled riboprobes for *ATPIA1* (3189-3741 of human *ATPIA1* cDNA; accession number NM_000701.7) and *ATPIA3* (2882-3573 of human *ATPIA3* cDNA; accession number NM_001256213.1) were used and visualized with the Tyramide Signal Amplification (TSA) Biotin System (NEL700; Perkin-Elmer) according to the manufacturer’s protocol. After ISH staining, the sections were counterstained using Methyl Green (Vector Laboratory). Bright-field images were acquired using a Nikon Eclipse E600 microscope with a Nikon DS-Ri1 CCD camera and NIS-Elements Microscope Imaging Software (Nikon Instrument Inc.).

Glycerol gradient sedimentation. Patient ASPD (~16 pmol in a volume of 0.2 ml) was layered on top of a 4 ml 15-30% (vol/vol) linear glycerol gradient in a Beckman Coulter 344057 tube. The tube was centrifuged in a Beckman Coulter SW55Ti rotor at 86,000 \times g for 16 h at 4 °C. Fractions (0.35 ml each) were collected from the bottom of the tube. Fraction numbering begins with #1, which corresponds to the top 0.35 ml of fluid in the tube (the last fraction actually collected). Fractions were immediately dot-blotted to quantify A β content (see “Dot blotting”). Antibody 82E1 was used for A β and antibody rpASD1 was used for ASPD. Aliquots of selected fractions then were used in toxicity assays and for TEM. Standards (158 kDa, aldolase, 1 mg; 232 kDa, catalase, 0.3 mg; 440 kDa, ferritin, 4 mg; and 669 kDa, thyroglobulin, 0.5 mg) were centrifuged at the same time as were the ASPD, but in a separate tube, and used to determine the molecular mass of the ASPD fractions. Collected fractions of standard proteins were separated by SDS-PAGE using Novex 10-20 % Tricine gels and stained with a Colloidal Blue Staining Kit (Life Technologies). Densitometric analysis of each band was performed using an LAS-1000 Plus and the mobility shift of each standard protein thus determined was used to construct a standard curve of molecular mass versus relative mobility. Toxicity assays measured apoptotic cell death using propidium iodide and Hoechst 33258 (18). Briefly, after 24 h treatment, 19-22 DIV mature hippocampal neurons were gently rinsed and exposed for 30 min to 5 μ M propidium iodide in a buffer (130 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 20 mM HEPES, pH 7.3, 2 mM CaCl₂) at 37 °C. Dye uptake was measured using a fluorescence microscope (Axiovert 200M, Zeiss) with a computer-assisted cooled CCD camera unit (CoolSNAP HQ, Roper Scientific) using band-pass filters appropriate for each probe, and analyzed with MetaMorph imaging software (Universal Imaging Corp., West Chester, PA, USA). Cells with shrunken or fragmented nuclear staining were counted as dead cells. The total cell number was assessed

by 1 $\mu\text{g/ml}$ Hoechst 33258 nuclear staining after 10% formalin fixation. At least 400 cells were selected randomly from eight different fields. Data were expressed as the apoptotic ratio with respect to the total cell number. One unit is defined as the activity causing apoptosis in 1% of cells (determined by Hoechst 33258 staining)(18).

PD. A randomized 12-mer peptide library presented on M13 phages (Ph.D.TM-12 phage display library kit; New England Biolabs) was used for screening against ASPD immobilized on an 8-well strip (compatible with a 96-well plate) (Mitsubishi Chemical Medience Corp.) (19). BSA-only-immobilized 8-well strips were also used. Biopanning was performed according to the manufacturer's instructions. Briefly, the phages were incubated in the synthetic ASPD-immobilized wells for 1 hr at 25 °C. To remove phages with low affinity to ASPD, the wells were washed ten times with TBS-T. Bound phage was eluted in 0.2 M glycine-HCl buffer (pH 2.2) and immediately neutralized with Tris. The mixture was then incubated in the BSA-only-immobilized wells four times to subtract non-specific binders. Finally, the phage was transfected into *E. coli*, amplified, and recovered. After the third round of this biopanning processes, the phage was cloned and sequenced using an ABI PrismTM 310 genetic analyzer (Applied Biosystems).

NMR. ¹H-¹⁵N HSQC spectra of both monomeric ¹⁵N-A β ₁₋₄₀ and ¹⁵N-ASPD were recorded at 298 K on a 600 MHz Bruker *AVANCE*-III spectrometer equipped with a cryogenic TCI probe. Approximately 100 μM ¹⁵N-A β ₁₋₄₀ and 350 nM ¹⁵N-ASPD in 0.5 x PBS (supplemented with 5% D₂O) were used. 128 and 512 scans were accumulated, respectively. The data were processed and analyzed with NMRPipe (69) and Sparky. ¹⁵N-A β ₁₋₄₀ backbone signal assignments were taken from the literature (70).

Molecular modeling. A three-dimensional homology model of human NAK α 3 was constructed using the Prime program (Schrödinger). The structure of pig NAK α 1 (32) was selected as a template (PDB code 3B8E). To further refine the model, all the coordinates of the hydrogen atoms and heavy atoms of side chains and main chains except backbone C and N atoms were optimized with the Merck Molecular Force Field (MMFF) using the MacroModel program (Schrödinger). During the optimization, an implicit water solvent model was employed, and a van der Waals cut-off of 8 Å and an electrostatic cut-off of 20 Å were used. Finally, the model was appended with the β and γ subunits of pig NAK (PDB code 3KDP) (32) by superimposing the α subunits on these subunits. The homology model was visualized by the program PyMOL (Schrödinger).

SI Discussion of definition of ASPD

In Hoshi *et al.* (18), two fractions of soluble A β aggregates were obtained from the slowly rotated A β solutions after glycerol gradient sedimentation: (1) non-toxic aggregates of mass ~60 kDa and diameter <10 nm by TEM (termed “non-toxic ASPD”); and (2) toxic aggregates of mass ~346 kDa and diameter 10-25 nm in TEM (termed “toxic ASPD”). Solution AFM studies have shown that these aggregates both have a height/width ratio of 1 (Fig. 2C of (18)), suggesting they are spheres. We thus refer to them as such. Structure-activity studies demonstrated that the 10-15-nm spheres were the toxic entities within the population of 10-25 nm structures (18). However, all spheres were considered ASPD at that time because we did not know whether any difference, other than size, existed between non-toxic and toxic spheres. In subsequent studies by Noguchi *et al.* (19), toxic ASPD were used as immunogens to produce ASPD-specific antibodies, which reacted robustly with 10-15 nm spheres but very weakly with A β monomers, dimers, or fibrils (Fig. 1B in (19)). These antibodies detected the toxic 10-15-nm ASPD, but not the non-toxic, smaller spheres or the non-toxic, larger spheres >15 nm in diameter (19). Furthermore, these antibodies selectively immunisolated only the neurotoxic 10-15-nm spheres (Fig. 2B and C of Matsumura *et al.* (20)). Therefore, on the basis of neurotoxic activity and immunoreactivity, as well as size, we conclude that the 10-15-nm spheres are distinct from the spheres of diameter <10 nm and those of diameter >15 nm. These new data enable us to make our definition of ASPD more specific: namely ASPD are **“neurotoxic, spherical A β oligomers of 10-15-nm diameter (measured by TEM) that are recognized by ASPD-specific antibodies.”** Additional details follow.

By using ASPD-specific antibodies, we found that ASPD were recovered in soluble fractions of patients’ brains, but not from insoluble fractions, after treatment with 2% SDS or 70% formic acid [Figure S3 in Noguchi *et al.*, (19)]. We selectively isolated 10-15-nm ASPD by a combination of 100-kDa retention and immunoprecipitation using ASPD-specific antibodies (purity >95%) [Figure 2G, H, I in Noguchi *et al.*, (19)] and showed that the purified AD patient-derived ASPD were composed predominantly of A β ₁₋₄₀ and A β ₁₋₄₂ (by means of mass spectrometry) and were directly toxic to human mature neurons [Figure 2J, K in Noguchi *et al.*, (19)]. We have thus proved that ASPD are present in AD patients’ brains and we have defined the diameter, mass, and neurotoxicity of ASPD (Table S1).

To monitor ASPD formation *in vitro*, as well as to determine ASPD mass more accurately than with the glycerol gradient sedimentation analysis, we have developed a highly sensitive monitoring method using fluorescence correlation spectroscopy (FCS) combined with TEM and toxicity assays (20). With this method, we showed that the mass of 10-25 nm A β ₁₋₄₂ aggregates containing predominantly

ASPD-sized, 10-15-nm spheres as major species is 330 ± 58 kDa (Fig. 1B 16.5 h in (20)), in good agreement with the result (~ 346 kDa) of the sedimentation assay in (18). As described above, from these ASPD-containing aggregates, we selectively immunisolated pure (97%) 10-15-nm ASPD using the ASPD-specific antibody haASD1. This isolation procedure did not affect ASPD structure or neurotoxicity as described in (19). The 10-15-nm spheres were found to have a molecular mass of 128 ± 44 kDa (Fig. 2C in (20)).

To identify the receptor mediating toxicity of ASPD, in this work we used ASPD immunisolated with the haASD1 antibody 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. 2A. Importantly, these two patients were the same patients from whom we previously purified patient ASPD and characterized them in comparison with synthetic ASPD (19). To confirm the mass of patient ASPD used in this work, we performed glycerol gradient sedimentation because we cannot apply our FCS method to patient ASPD. In our previous work (18), thyroglobulin (669 kDa) and ferritin (440 kDa), two marker proteins, both were recovered in the bottom-most fraction of the gradient. To enable resolution of assemblies in this mass size range, in the current work, we centrifuged the sample in a Beckman Coulter SW55Ti rotor, instead of the TLS55 rotor we originally used, which allowed us to resolve thyroglobulin and ferritin within two distinct fractions. Note that our ASPD preparation method involves initial filtration through a 100-kDa MWCO filter, which eliminates the smaller (<10 nm) non-toxic aggregates, which pass through filters. Our higher resolution glycerol gradient system yields 10-15 nm spheres (i.e., synthetic ASPD) in fractions 1-3, centered at ~ 120 kDa in mass (as described in the text in (19)), while larger spheres were recovered in fractions 4-5. Patient ASPD were similarly recovered in fractions 1-3, centered at fraction 2, and of ~ 120 kDa in mass (Fig. 2E). In Parthasarathy *et al.* (31), we compared the size of synthetic ASPD and that of patient ASPD in TEM images using Image J software. The analysis indicated identical diameters, within experimental error, for synthetic ASPD (11.0 ± 2.1 nm; $n = 65$) and patient ASPD (10.9 ± 1.7 nm; $n = 34$). These data collectively indicated that synthetic ASPD and patient ASPD are essentially identical in size and in mass.

With respect to the methods of preparation of ASPD, in the case of $A\beta_{1-40}$, ASPD are prepared from $350 \mu\text{M}$ $A\beta_{1-40}$ solution in 0.5x PBS (-) by slowly rotating the solution for 5-7 days at 37°C (as described in (18)) or from $50 \mu\text{M}$ $A\beta_{1-40}$ solution in 0.5x PBS (-) by slowly rotating the solution for 5-7 days at 4°C (used in (20)). In either protocol, the level of $A\beta_{1-40}$ -ASPD is maximally $\sim 8\%$. In the case of $A\beta_{1-42}$, ASPD are formed in $50 \mu\text{M}$ $A\beta_{1-42}$ solution in F12 buffer without L-glutamine and phenol red by slowly rotating the solution for ~ 16 hrs at 4°C as shown in (19, 20) and the current study, except for the

fluorescence-based study using FCS. Since FCS is highly sensitive to fluorescent materials, we used F12 buffer without riboflavin, L-glutamine, and phenol red in order to eliminate the fluorescent background. The level of A β_{1-42} -ASPD in the A β_{1-42} solution after slow rotation is usually ~30 %. In addition to immunoisolation using ASPD-specific haASD1 antibody as described in (19, 20), synthetic ASPD are obtained by means of two-step filtration, in the fraction that passed through 0.22- μ m filters, but was retained on 100-kDa MWCO filters (Sartorius), as described in (19). Synthetic ASPD were prepared every week and the quality was confirmed by dot blotting, TEM, amino acid analysis, and toxicity assays using 19-27 DIV hippocampal neurons. In this work, we also confirmed the *K_d* of ASPD-specific rpASD1 antibody for synthetic ASPD each week using SPR. Patient ASPD were isolated by a combination of 100-kDa retention and immunoprecipitation using ASPD-specific antibodies (see “Immunoprecipitation (IP)” in *SI Materials and Methods* and “Human Brain Extracts” in Additional Experimental Procedures in Supplemental Data of Noguchi *et al.* (19). Patient ASPD are very stable, and our isolation procedures are highly reproducible, as evidenced by our ability to obtain essentially the same amounts of ASPD in multiple independent extractions and fractionations over time from the same patient's brain.

Discussion for a Plausible Mechanistic Explanation for Two Types of Ca²⁺-Responses Induced by ASPD. Mitochondria have been considered to act as a spatial Ca²⁺ buffer by: (i) taking up excess cytoplasmic Ca²⁺ mainly through the rapid mode of calcium uptake (the RaM) and partly through the uniporter; or (ii) releasing Ca²⁺ mainly through the Na⁺-dependent mechanism in neurons, to maintain cytoplasmic Ca²⁺ levels (71). At high ASPD concentrations (more than 40 nM), severe NAK impairment causes high levels of Ca²⁺ influx from VGCC, which will result in inactivation of the RaM. As a result, only the uniporter remains to sequester excess cytoplasmic Ca²⁺. The capacity of the uniporter to do so is limited, which allows the initial sustained increase of cytoplasmic Ca²⁺ levels observed at high ASPD concentrations (Fig. 4C2). At low ASPD concentrations, because of moderate levels of Ca²⁺ influx from VGCC, both the RaM and the uniporter can take excess cytoplasmic Ca²⁺ into mitochondria and as a result cytoplasmic Ca²⁺ levels will increase transiently and then decrease rapidly, as observed. In either case (high or low ASPD), in spite of the presence of the mitochondrial Na⁺/Ca²⁺ exchanger that releases excessive mitochondrial Ca²⁺, owing to continuous Ca²⁺ influx from outside, mitochondrial Ca²⁺ levels will continue to increase and sooner or later will reach abnormal levels. Since levels of Ca²⁺ influx from outside will correlate with levels of VGCC activation, i.e. levels of NAK impairment, higher concentrations of ASPD will cause mitochondria to reach the abnormal Ca²⁺ level earlier. That would

explain why cytoplasmic Ca^{2+} levels did not plateau within two hrs in the case of 18 nM ASPD treatment. In either case (high or low ASPD), eventual prolonged Ca^{2+} overload in mitochondria has been reported to cause the mitochondrial permeability transition pore (MPT) to open (72). This mitochondrial calcium-induced calcium release through MPT opening as well as the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger both cause the sharp Ca^{2+} increase after the initial sustained increase (Fig. S3E), leading to neuronal death induced by ASPD.

SI Discussion for the first and second categories shown in Fig. 8

The first system involves regulating the CNS concentration of $\text{A}\beta$ monomer available for assembly. Whereas RAGE transports $\text{A}\beta_{1-40}$ or $\text{A}\beta_{1-42}$ into the CNS and increases brain $\text{A}\beta$ levels (73), APOE stabilizes $\text{A}\beta_{1-40}$ or $\text{A}\beta_{1-42}$ through its binding, and as a result reduces brain $\text{A}\beta$ levels available for assembly (74). The binding of APOE4 to $\text{A}\beta_{1-42}$ is slightly weaker than that of APOE3 (75), which could be the reason why APOE4 increases the risk for AD onset.

The second system involves post-synaptic neurons. Here, $\text{A}\beta$ impairs synaptic connections by indirectly affecting NMDAR activity. $\text{A}\beta_{1-42}$ activates post-synaptic $\alpha 7\text{nAChR}$ leading to NMDAR internalization (76). Binding of dimers/trimers to EPHB2 induces degradation of EPHB2 by proteasomes. Loss of EPHB2 has been reported to reduce cell-surface NR1 subunit of NMDAR and impair NMDAR-mediated synaptic connections in *APP* transgenic mice (9). Binding of $\text{A}\beta$ oligomers larger than 500 kDa to PrP^{C} has been shown to activate FYN kinase, induce phosphorylation of the NR2B subunit of NMDAR, and facilitate a transient overexpression of NMDAR on the cell surface, leading to spine loss (77). Although ADDLs have been reported to co-immunoprecipitate with NMDR-containing synaptosomes (16), at present NMDAR is unlikely to be a direct target for $\text{A}\beta$. Recently, post-synaptic Sigma-2/PGRMC1 has been reported to serve as a receptor for 50~75 kDa $\text{A}\beta_{1-42}$ oligomers (43). It would be interesting in the future to examine whether $\text{A}\beta_{1-42}$ oligomers directly bind to Sigma-2/PGRMC1, and if so, how the $\text{A}\beta_{1-42}$ oligomers-Sigma-2/PGRMC1 interaction affects neuronal function.

AD is a progressive disease with risk highly correlated with ageing and with pathology that may vary due to micro-environmental and genetic differences among patients. Different receptor/ligand systems, as discussed above, thus may act synergistically or in a stage-dependent manner to contribute to AD pathogenesis.

SI FIGURE LEGENDS

Fig. S1. Effects of antagonists on ASPD neurotoxicity (DNA fragmentation) towards mature rat primary neuronal cultures (19 Day-In-Vitro (DIV)). Toxicity of synthetic ASPD (176 nM) towards mature neurons was blocked specifically by 2-hr pretreatment with 0.1 mg/ml ASPD-specific antibody mASD3, but not with the same concentration of another ASPD-specific antibody, haASD1, that detects a different epitope in ASPD, as we have shown previously (19). The antibody remained present during the overnight incubation with ASPD. This ASPD toxicity (176 nM) was unchanged by treatments with 1 μ M TTX (a potent sodium channel blocker), or glutamate receptor antagonists (10 μ M APV or MK801 for NMDA receptors, 100 μ M DNQX for non-NMDA kainate or α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, 500 μ M MCPG for metabotropic glutamate receptors (I/II), and 50 μ M LY341495 for metabotropic glutamate receptors (II)). Mean \pm SD; *, $p < 0.001$ Scheffé's *post-hoc* test, $n = 3$.

Fig. S2. Developmental NAK α 3 expression and its binding to synthetic ASPD. (A) Representative TEM of negatively stained synthetic ASPD is shown on top. Representative Far-western blotting using 3.5 nM synthetic ASPD (left) and a silver-stained gel used for the Far-western blotting (right) are shown. Binding was detected using anti-ASPD antibody haASD1. RIPA extracts (15 μ g/lane) of 2 DIV immature rat neurons (lane I) or 21 DIV mature rat neurons (lane M), or HEK293 cells (lane H) were used. Red arrows indicate a 105-kDa mature neuron-specific ASPD binding protein, NAK α 3. MS/MS analysis of the other ASPD-detected bands, derived from immature neurons or HEK293, showed that these bands contained intracellular proteins such as α -spectrin2 (pink* at ~238 kDa), an uncharacterized protein (black* at ~171 kDa), heat shock protein gp96 (green* at ~110 kDa), and eukaryotic translational elongation factor 2 (blue* at ~110 kDa) (Fig. 3A and Fig. S2A). Postsynaptic A β receptors such as 35 kDa PrP^c, 40.4 kDa RAGE, 54.2 kDa α 7nAChR, NMDAR (~100 kDa NR1 and 165 kDa NR2 subunits), 108 kDa EPHB2, were not detected among the ASPD-binding bands using MS/MS analysis. (B) Representative Far-western blotting using 3.5 nM synthetic ASPD detected by haASD1 antibody (left), 3.5 nM synthetic ASPD presaturated with 100 nM (equimolar in terms of A β ₁₋₄₂ monomer concentration) haASD1 (middle), and haASD1 alone (right). RIPA extracts (15 μ g/lane) were used. Red arrows indicate a 105-kDa mature neuron-specific ASPD binding protein, NAK α 3. Asterisks have the same meaning as in A. (C) Developmental changes in the levels of NAK α 3 and MAP2 were analyzed by Western blotting (representative blotting on the right). Data were normalized to actin and shown relative to each protein amount at 2 DIV. Mean \pm SD; *, $p < 0.008$ compared with data at time 0 (Scheffé's *post-hoc* test, $n = 3$).

(D) Using biotin-labelled ASPD as a ligand, NAK α 3 (red arrows) was co-immunoprecipitated from living mature neurons (see text). Far-western blotting using biotin-labelled ASPD is shown on the right. (E) Representative Z-stack images of 27 DIV hippocampal or 22 DIV cerebellar neurons, treated with 13 nM synthetic ASPD for 15 min were obtained as in Fig. 3E. The images showed anti-MAP2 (red) and anti-NAK α 3 (green) staining.

Fig. S3. Representative Na⁺ and Ca²⁺ imaging data. (A) Cytoplasmic Na⁺ levels of mature rat hippocampal neuronal cultures (20-24 DIV), treated with vehicle (*A*₁), 300 nM ouabain (*A*₂), synthetic ASPD (38 nM in *A*₃, 59 nM in *A*₄), or 100 μ M NMDA (*A*₅), were measured using a Na⁺ indicator, ANG-2AM (TEFLABS) (see “Ca²⁺ and Na⁺ imaging” in *SI* Materials and Methods). The treatment of neurons with ASPD or other reagents was initiated by changing the circulating HSS buffer to HSS buffer containing ASPD or the sample reagent. After the buffer change, it usually took ~110 sec for HSS buffer containing ASPD or the sample to reach the assay chamber (at the orange arrow) and at ~17.5 min the solution in the assay chamber was completely replaced with HSS buffer containing ASPD or the sample (at the yellow arrow). Treatment of neurons with 300 nM ouabain, which corresponds to 52 nM synthetic ASPD based on the average K_d values of ASPD (7.8 nM) and ouabain (45 nM (61-63)) for rat NAK α 3, elicited a Na⁺ increase similar to that induced by ASPD.

(B) Intracellular free Ca²⁺ level ([Ca²⁺]_i) of cell bodies of mature rat hippocampal neurons (19-20 DIV), treated with vehicle, 18 nM, 42 nM, or 88 nM synthetic ASPD (at the orange arrow; see text in *A*), was measured using a leakage-resistant Ca²⁺ indicator, Fura-PE3AM (TEFLABS) (see “Ca²⁺ and Na⁺ imaging” in *SI* Materials and Methods). The absorbance ratio at 340/380 nm is shown. The ratio of responsive cells was obtained as in Fig. 4D. Ca²⁺ levels were stable during vehicle treatment over 2 hrs, but increased transiently upon brief exposure to 100 μ M glutamate, proving that the neurons remained responsive. With 18 nM ASPD, neurons showed repetitive Ca²⁺ spikes starting at the time of ASPD treatment. Higher concentrations of ASPD persistently increased [Ca²⁺]_i, which showed a sudden rise to plateau levels. Higher concentrations of ASPD appeared to accelerate the process: with 35 nM ASPD, the initial Ca²⁺ response was transient, but a sustained increase was seen after 2-hr treatment (Fig S3C₁), whereas with 88 nM ASPD, [Ca²⁺]_i usually reached a plateau within 1 hr. Note that the data of 42 nM ASPD is also shown in Fig. 4C2.

(C) C₁, 35 nM synthetic ASPD caused repeated transient increases in [Ca²⁺]_i of neurons, leading to a sustained increase after 2-hr treatment. C₂, EGTA inhibited ASPD-induced increase in [Ca²⁺]_i of mature neurons (see text).

(D) $[Ca^{2+}]_i$ was monitored as described in *B* in mature rat hippocampal neurons (19 DIV) treated with 16 nM synthetic ASPD, with or without preincubation with 4 μ M ω -conotoxin (N-type VGCC specific inhibitor) or 20 μ M peptides derived from the 4th extracellular loop (Ex4) of NAK α 3 (see Fig. 7 C-G). The inhibitor was present while neurons were labelled with Fura-PE3AM for 40 min and was also present during the ASPD treatment. The peptides were preincubated with ASPD for 60 min on ice and were also present during the ASPD treatment.

(E) $[Ca^{2+}]_i$ changes of rat hippocampal neurons (24 DIV), treated with 50~80 nM synthetic ASPD, with or without 40-min preincubation with 20 μ M cyclosporin A (an inhibitor of MPT pore opening) and/or 3 μ M CGP37157 (a mitochondrial NCX inhibitor), were monitored as in *B*. The inhibitors were present while neurons were labelled with Fura-PE3AM and were also present during the ASPD treatment. Representative data are shown in *E*₁. Quantification of the data from three independent experiments (Mean \pm SD) is shown in *E*₂. In the case of ASPD-treated neurons, the second sharp Ca^{2+} rise was seen almost exclusively after the initial sustained $[Ca^{2+}]_i$ increase. As shown in Fig. S3E₂, treatment of neurons with cyclosporin A, CGP37157, or both did not affect this initial sustained $[Ca^{2+}]_i$ increase in terms of the responsive cell number and the response level ($= [Ca^{2+}]_i \Delta[\text{first peak height-baseline}]$). In contrast, treatment of neurons with these inhibitors markedly impaired the ASPD-induced second sharp $[Ca^{2+}]_i$ rise in terms of both the responsive cell number and the response level ($= [Ca^{2+}]_i \Delta[\text{second peak height-first peak height}]$) (*, $p < 0.001$; **, $p < 0.0001$ compared with ASPD alone using the Scheffé's *post-hoc* test, $n = 3$). In ASPD-treated neurons, the second sharp $[Ca^{2+}]_i$ rise, once it occurred, was saturated without decline, but in the presence of MPT pore opening inhibitor (cyclosporin A), the number of neurons showing this sharp $[Ca^{2+}]_i$ rise was markedly decreased, or, if the sharp $[Ca^{2+}]_i$ rise occurred, it gradually declined (not saturated) to the initial sustained level after it had reached the peak (Fig. S3E₁). The mitochondrial NCX inhibitor strongly inhibited occurrence of the sharp $[Ca^{2+}]_i$ rise, and a gradual increase of $[Ca^{2+}]_i$ was seen, instead of the sharp $[Ca^{2+}]_i$ rise, after the initial sustained $[Ca^{2+}]_i$ increase (Fig. S3E₁). Treatment with both cyclosporin A and CGP37157 appeared to inhibit the ASPD-induced second sharp $[Ca^{2+}]_i$ rise more potently than the single treatments. These results suggest that mitochondrial NCX and MPT pore openings are both involved in ASPD neurotoxicity.

(F) Effects of 100, 300, and 500 nM ouabain, which correspond to 17, 52, and 87 nM ASPD, respectively, based on the average K_d values of ASPD (7.8 nM) and ouabain (45 nM (61-63)) for rat NAK α 3. Ouabain elicited the same phenomena triggered by ASPD, including increases in $[Ca^{2+}]_i$, activation of CDK5, increase in the Tyr²¹⁶ phosphorylated active form of TPKI/GSK3 β , tau phosphorylation/destabilization, and MAP2 destabilization, except for decrease in the Ser⁹

phosphorylated inactive form of TPKI/GSK3 β (see Figs. S3B and 5C-F). The reason for the difference in the case of Ser⁹ is not clear at present. These data further support the conclusion that ASPD-induced NAK α 3 impairment is the main cause of these phenomena. Mean \pm SD; *, $p < 0.02$ Scheffé's *post-hoc* test, $n = 3$.

Fig. S4. Time lapse images of ASPD-treated neurons and human studies. (A) Representative time-lapse images of mature rat hippocampal neurons treated with each reagent (see Videos S1-4). (B) Immunohistochemical studies of non-clinically demented individual (NCI) and Alzheimer's disease (AD) brains using anti-NAK α 1 (see text). Bar = 50 μ m (C) ISH analyses using probes for *ATPIA3* (NAK α 3) and *ATPIA1* (NAK α 1) on 10- μ m paraffin-embedded adjacent sections.

Fig. S5. ASPD-binding peptides inhibit both ASPD binding to NAK α 3 and other ASPD-induced downstream phenomena. (A) SPR analyses were performed as in Fig. 3F. Ex4 of NAK α 3 bound to synthetic ASPD, but Ex1 did not. Representative data are shown. (B) Inhibition of synthetic ASPD (50 nM) binding to mature hippocampal neurons by the peptides (20 μ M). ASPD-binding sites (red) and anti-NAK α 3 staining (green) were detected as in Fig. 3E. (C) Activation of TPKII/CDK5 and TPKI/GSK3 β was determined by Western blotting as shown in Fig. 5C and 5D, respectively. Ex4 peptides were treated as in Fig. S3D. ASPD-binding Ex4 peptides (final concentration 20 μ M) significantly inhibited activation of these tau-phosphorylating kinases induced by 6-hr treatment with 35 nM synthetic ASPD. Mean \pm SD; *, $p = 0.011$ for TPKII/CDK5; 0.050 for pTyr216; 0.028 for pSer9 by Scheffé's *post-hoc* test, $n = 3$.

Fig. S6. Interference with aberrant ASPD-NAK α 3 interaction by masking the A β oligomer surface with specific masking peptides or peptidomimetics. ASPD (7.2 \pm 2.6 nm in diameter) bind and cause death of mature neurons. Molecular modeling suggests that the mature heterotetrameric NAK pump forms a 9.9 nm-wide opening. This diameter is similar to that of *in situ* ASPD (7.2 \pm 2.6 nm in diameter) and the Ex4 region, which is essential for the ASPD-NAK α 3 interaction, is on the edge of the opening. Based on these data, we present a possible model of ASPD-NAK α 3 interaction (left). Given the essential functions of the NAK pump in neurons, direct modulation of the NAK pump would be a risky approach. We propose a new strategy to interfere with the aberrant ASPD-NAK α 3 interaction by masking the A β oligomer surface with specific masking peptides or peptidomimetics (right).

Video Legends

Video S1. Primary rat hippocampal mature neurons (19-25 DIV) were imaged at intervals of 10 min for 11 h (see “Time-lapse” in *SI* Materials and Methods) after the administration of 141 nM ASPD at 20x magnification. The time-lapse videos were shortened (1 sec = 20 msec).

Video S2. Primary rat hippocampal mature neurons (19-25 DIV), treated with 100 nM ouabain, were imaged as in Video S1 at 20x magnification.

Video S3. Primary rat hippocampal mature neurons (19-25 DIV), treated with 1 μ M staurosporine, were imaged as in Video S1 at 20x magnification.

Video S4. Primary rat hippocampal mature neurons (19-25 DIV), treated with vehicle, were imaged as in Video S1 at 20x magnification.

Table S1 Summary of the Characteristics of ASPD Reported Previously (18-20) And Found in this Work

	Synthetic ASPD	AD patient-derived native ASPD
Source	<ul style="list-style-type: none"> ▪ Synthetic Aβ_{1-40} or Aβ_{1-42} ▪ Identified as the most toxic entities either in Aβ_{1-40} solutions (50 μM in 0.5x PBS(-), slowly rotated for 5-7 d at 4°C) or in Aβ_{1-42} solutions (50 μM in 0.5x PBS(-) or F12 media without L-glutamine and phenol red, slowly rotated for 14-16 h at 4°C) ▪ Obtained by two-step filtrations as the 100-kDa retentates of the 0.22-μm filtrates ▪ Purified (~97%) by immunoprecipitation using ASPD-specific haASD1 or mASD3 antibody 	<ul style="list-style-type: none"> ▪ Hippocampus or cerebral cortex of AD patients (not cerebellum cortex of AD patients) ▪ Increase with the disease severity and duration, along with the increase in neurodegeneration[†] ▪ Ultracentrifuge supernatant of the extracts of AD brains, homogenized in F12 media without L-glutamine and phenol red (no detergents) ▪ Purified (>95%) by immunoprecipitation using ASPD-specific haASD1 or mASD3 antibody from the 100-kDa retentates of the above supernatant ▪ Aβ_{1-40} and Aβ_{1-42} are detected as major components by MS analysis and by silver staining[†]
Size of active components	<ul style="list-style-type: none"> ▪ 10- to 15-nm spheres in TEM (11.8 \pm 2.1 nm; n = 94)[*] ▪ Complete spheres y/x = 0.982 (n = 100; in TEM) height/diameter = 1.0 (n = 100; solution AFM) ▪ ASPD mass peak is ~120 kDa in 15-30% glycerol gradient sedimentation assays (slightly smaller than 158-kDa aldolase). ▪ ASPD (purified (97%) using ASPD-specific haASD1 antibody) is 128 \pm 44 kDa in mass (FCS) ~30 \pm 10 mers (using 4.5 kDa for Aβ_{1-42}) 7.2 \pm 2.6 nm in height (solution AFM) 	<ul style="list-style-type: none"> ▪ 10- to 15-nm spheres in TEM (11.9 \pm 1.7 nm; n = 108)[*] ▪ ASPD mass peak is 123 \pm 20 kDa (n = 3)(slightly smaller than 158-kDa aldolase) in 15-30% glycerol gradient sedimentation assays[†]. ▪ The size of ASPD is estimated to be 128 \pm 44 kDa in mass 28~30 \pm 10 mers (using 4.3 kDa for Aβ_{1-40} and 4.5 kDa for Aβ_{1-42}) based on FCS analysis of synthetic ASPD
Structural characteristics	<ul style="list-style-type: none"> ▪ Originate from trimer ▪ A11-negative in dot blotting ▪ ASPD-specific antibodies (rpASD1, mASD3, haASD1, etc.)-positive in dot blotting ▪ Immunoprecipitated by haASD1 or mASD3 antibody, but not by 6E10 	<ul style="list-style-type: none"> ▪ A11-negative in dot blotting[†] ▪ ASPD-specific antibodies (rpASD1, mASD3, haASD1, etc.)-positive in dot blotting ▪ Immunoprecipitated by haASD1 or mASD3 antibody, but not by 6E10 ▪ Bind to a 105-kDa band in mature

	<ul style="list-style-type: none"> ▪ Solution NMR analysis indicated the presence of one defined structure in the ASPD sample and determined the amino acid sequences exposed on the ASPD surface[†], which are consistent with the previous epitope map of ASPD obtained by ASPD-specific antibodies. ▪ Bind to a 105-kDa band in mature neurons in Far-western ligand binding assay[†] 	<p>neurons in Far-Western ligand binding assay[†]</p>
<p>Biological effects</p>	<ul style="list-style-type: none"> ▪ Activate both TPKI/GSK-3β and TPXII/CDK5 and increase tau phosphorylations[†] ▪ NMDAR independent neuronal cell death of mature neurons ▪ Nontoxic against non-neuronal cells or immature neurons (human, monkey, and rat origins) ▪ Toxicity is neutralized by ASPD-specific antibodies (rpASD1 and mASD3) but not by 6E10 or 82E1 antibody. ▪ Impair NAKα3 activity in mature neurons[†] ▪ Activate N-type voltage gated calcium channels and cause mitochondrial calcium dyshomeostasis[†] ▪ Induce loss of tau and MAP2[†] 	<ul style="list-style-type: none"> ▪ Cause neuronal cell death of mature neurons ▪ Nontoxic to non-neuronal cells or immature neurons (human) ▪ Toxicity is neutralized by ASPD-specific mASD3 antibody. ▪ Colocalize with NAKα3 and this binding is inhibited by ASPD-binding peptides[†]

* Calculated from the data in Noguchi et al. JBC 2009

[†]Data from the current work

Table S2 Summary of the MS/MS analysis of ~105 kDa bands in Fig. S2A

Sample	MS hit	MS hit peak	MS/MS peak	MS/MS protein	Mass	pI	
2 DIV	-	-	1081	heat shock protein gp96 [†]	92713	4.72	
	-	-	2030	heat shock protein gp96 [†]	92713	4.72	
21 DIV	NAK α 3	776					
		1030					
		1158					
		1268					
		1284					
		1296					
		1055					
		1619	1619	NAK α 1, -2, -3	111664	5.26	
		1659	1659	NAK α 3	111664	5.26	
		1158					
		1431					
		1490					
		1766					
		1829	1829	NAK α 1, -2, -3	111664	5.26	
		1867					
		1236					
		2505					
		2464					
		2480					
		2505					
2866							
2882							
1442							
-	-	1501	adaptor-related protein complex 2 β 1 subunit [†]	105625	5.19		
-	-	1709	dynamain I*	95867	6.32		
-	-	1400	hexokinase I*	102342	6.29		
-	-	1081	heat shock protein gp96 [†]	92713	4.72		
HEK	-	-	1799	eukaryotic translation elongation factor 2 [†]	95277	6.41	
	-	-	2220	eukaryotic translation elongation factor 2 [†]	95277	6.41	
	-	-	1081	heat shock protein gp96 [†]	92713	4.76	

The MALDI-TOF/MS analyses of three independent experiments are shown. In each sample, the results are shown in high hit score order. NAK α 3 is the only protein that was detected by MS analysis, and this was confirmed by further MS/MS analyses. Proteins that were not detected by MS are considered insignificant.

*; detected only in one run only by MS/MS (without MS hit)

[†]; detected only by MS/MS (without MS hit)

Table S3 Summary of the representative A β ligand/target systems

Target	Target character	Ligand	Binding mode	Mechanism
Sigma-2/ PGRMC1 (43)	<ul style="list-style-type: none"> 21.6 kDa* Heme-binding protein, co-purified with progesterone binding proteins in liver Possible roles in cell survival and apoptosis High expression in liver and kidney, lower expression in lung, brain, in humans (. expression in liver, lung, e.g., kidney, and brain in rodents) Post-synaptic localization in neurons 	A β 1-42 oligomers (50-75 kDa mixtures)	<ul style="list-style-type: none"> $K_d = 518$ nM for neuronal cultures (using Aβ1-42 oligomers) PGRMC1 siRNA reduced oligomer binding to neurons. 	<ul style="list-style-type: none"> PGRMC1 levels increased ~30% after 48 h oligomer treatment. Aβ1-42 oligomers facilitate membrane trafficking (MTT assay), which is inhibited by small compounds that inhibit oligomer binding to neurons.
APOE (78)	<ul style="list-style-type: none"> 34.2 kDa glycoprotein* A major component of very low-density lipoproteins (VLDLs). VLDLs maintain normal levels of cholesterol and triglyceride in systemic tissues by carrying these lipids from the liver. E2, E3, E4 isoforms (E4 as a risk factor for AD) High expression in liver and in astrocytes in brains 	A β 1-40 A β 1-42	<ul style="list-style-type: none"> Direct binding of purified APOE to Aβ on the microplate. $K_d = 48.1$ nM for E2; 63.7 nM for E3; 75.9 nM for E4 (using Aβ1-42) 1:1 binding Lys16 of Aβ binds to Lys72, 75, 95, 143, 157 and 242 of APOE. 	<ul style="list-style-type: none"> Binding of APOE to Aβ prevents formation of Aβ fibrils. APOE stabilizes extracellular Aβ monomers and may reduce the extracellular Aβ concentration of Aβ monomers available for fibril formation.
PrP ^C (10, 79)	<ul style="list-style-type: none"> 35-36 kDa* GPI-anchored protein PrP^C's physiological function is a complex issue that requires further studies. Ubiquitous expression. Greater expression in CNS, spinal cord, and thymus, less in the other tissues. Localization in synaptic connections and in astrocyte in brains. 	A β 1-42 oligomers (~500 kDa in SEC)	<ul style="list-style-type: none"> Direct binding of purified PrP^C and Aβ1-42 oligomers using SPR $K_d = 50\text{--}100$ nM for neuron cultures (using Aβ1-42 oligomers); $K_d = 71$ nM with EPR spectroscopy PrP^C 23-27, 92-110 are both critical for binding to Aβ1-42 oligomers. 	<ul style="list-style-type: none"> Post-synaptic PrP^C mediates activity of certain type of Aβ oligomers (mitochondria dysfunction, reactive oxygen species production, Ca²⁺ influx and LTP suppression). Binding of Aβ oligomers activates FYN kinase, induce phosphorylation of NR2B subunit of NMDAR, and facilitate transient overexpression of NMDAR on the surface, leading to spine loss.
RAGE sRAGE (80)	<ul style="list-style-type: none"> 40.6 kDa* Multi-ligand receptor of the IgG superfamily (several ligands known including Aβ) Expression in the blood-brain barrier (BBB), glia, microglia, and neurons Post-synaptic localization in neurons 	A β 1-40 A β 1-42	<ul style="list-style-type: none"> Direct binding of ¹²⁵I-Aβ to purified RAGE on the microplate $K_d = 55$ nM for rat neuronal cultures (using Aβ1-40) 	<ul style="list-style-type: none"> RAGE mediates Aβ transport across BBB into CNS. RAGE inhibitors markedly reduced Aβ40 and Aβ42 levels in brain and normalized cognitive performance in aged <i>APP^{swE}</i> mice. RAGE mediates Aβ-induced oxidant stress in neurons and activation of microglia.
α 7nAChR (81)	<ul style="list-style-type: none"> Homopentamer (54.1 kDa)* Neuronal nicotinic AChR Abundant pre- and post-synaptic distributions 	A β 1-42	<ul style="list-style-type: none"> Direct binding of Aβ1-42 to purified α7nAChR by co-immunoprecipitation $K_d \sim$ pM and nM ranges for α7nAChR-expressing N-MC cell membrane (using Aβ1-42 monomers) N-terminal domain of α7nAChR is involved in binding. 	<p><u>Neuroprotective Effects:</u></p> <ul style="list-style-type: none"> Aβ1-42 monomers activate pre- and post-synaptic α7nAChR, promote calcium influx, enhance neurotransmitter release, and trigger protective signals such as ERK. <p><u>Pathologic Effects:</u></p> <ul style="list-style-type: none"> Aβ1-42 activates post-synaptic α7nAChR, promotes NMDAR internalization. ADDLs inactivate α7nAChR, impair LTP probably through

<p>EPHB2 (9)</p>	<ul style="list-style-type: none"> ▪ 108.1 kDa* ▪ EPH B2-type receptor tyrosine kinase ▪ Pre- and post-synaptic distributions 	<p>Aβ1-42 oligomers Dimers/trimers</p>	<ul style="list-style-type: none"> ▪ Direct binding of Aβ1-42 oligomers to purified EPHB2-Fc chimera by co-immunoprecipitation ▪ K_d undetermined ▪ Fibronectin type III repeats domain of EPHB2 is involved in binding. 	<p>NMDAR.</p> <ul style="list-style-type: none"> ▪ Dimers/trimers induce degradation of EPHB2 by the proteasome in neuronal cultures. ▪ Loss of EPHB2 reduce surface NR1 of NMDAR and impairs NMDAR-mediated synaptic connections in <i>hAPP</i> mice.
<p>NMDAR (12, 16, 82)</p>	<ul style="list-style-type: none"> ▪ Heterodimer (97.3 kDa for NR1; 132.3-165.2 kDa for NR2A, B, C, D)* ▪ Post-synaptic localization 	<p>ADDLs (3~24-mers mixtures)</p>	<ul style="list-style-type: none"> ▪ Synaptosomes (containing PSD95, NR1 and NR2A/2B) and ADDLs were co-immunoprecipitated by anti-ADDL antibody. ▪ Knockdown of NR1 with its anti-sense reduced ADDL binding to neurons. 	<ul style="list-style-type: none"> ▪ Morphology change and loss of spines are induced by ADDLs and dimers through NMDAR.
<p>NAKα3 (current work)</p>	<ul style="list-style-type: none"> ▪ 111.7 kDa* ▪ Neuron-specific catalytic subunit of NAK pump ▪ Mainly pre-synaptic localization 	<p>ASPD (128 kDa on average)</p>	<ul style="list-style-type: none"> ▪ Direct binding of ASPD to recombinant human NAKα3 by SPR ▪ $K_d = 28.6$ nM (using NAKα3 by SPR) ▪ 1:1 binding of ASPD to (NAKα3)₂(NAKβ1)₂ heterotetramer ▪ RLNW motif in extracellular loop 4 is involved in binding. 	<ul style="list-style-type: none"> ▪ ASPD directly bind to NAKα3, impair its activity, which activate N-type VGCC and induce calcium dyshomeostasis in cytoplasm and mitochondria, induce tau phosphorylation and loss, and degenerate mature neurons. ▪ Tetrapeptides inhibit ASPD binding and protect neurons from ASPD-induced death.

*Human protein's mass value is shown.

