

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

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

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

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

We previously isolated neurotoxic A β oligomers, termed amylospheroids (ASPD), from the brains of AD patient (18–20). ASPD appear in transmission electron microscopic (TEM) images as

spheres of diameter $\sim 11.9 \pm 1.7$ nm (19). ASPD appear to be unique A β assemblies, as determined immunochemically. These structures are recognized strongly by ASPD-specific antibodies ($K_d \sim$ pM range), but not with the oligomer-specific polyclonal antiserum A11 (19). ASPD are distinct from A β dimers, ADDLs, dodecamers, and other A11-reactive entities (19).

ASPD cause severe degeneration of mature human neurons (19). ASPD levels in the cortices of AD patients correlate well

Significance

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

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with disease severity (19). In contrast, ASPD-like oligomers were minimally detectable in the brains of transgenic mice expressing human amyloid precursor protein (*APP*), in which no significant neuronal loss is observed (19). These findings suggest that ASPD are an important effector of neuronal death in AD patients. We sought to elucidate mechanisms of ASPD-induced neurotoxicity. We report here that ASPD interact with the α -subunit of neuron-specific Na^+/K^+ -ATPase (*NAK\alpha3*), resulting in presynaptic calcium overload and neuronal death.

Results

ASPD Bind To *NAK\alpha3* In Mature Neurons. ASPD caused degeneration of mature rat hippocampal neurons, but not immature neurons or nonneuronal HEK293 (Fig. 1*A*). ASPD toxicity required binding to mature neurons, because ASPD-specific mouse monoclonal amylospheroid (*mASD*)3 antibody blocked binding and toxicity of ASPD (Fig. 1*A* and *B*). [The K_d for ASPD binding = 5.43 ± 0.27 nM ($n = 3$) (Fig. 1*C*). ASPD concentration was determined using an average ASPD molecular weight of 128 kDa (20). See summary of the characteristics of patient and synthetic ASPD in Table S1. See also *SI Discussion* for an updated definition of ASPD.] Blockers of known $\text{A}\beta$ receptors (21), including glutamate receptors (NMDA, non-NMDA, and metabotropic types) and voltage-gated sodium channels, did not affect ASPD neurotoxicity (Fig. S1). These findings suggested ASPD exert their toxicity through binding to novel cell-surface molecules specific to mature neurons.

To identify ASPD-binding proteins on mature neurons, Far-Western ligand-binding assays were performed in a physiological medium. We used ASPD isolated (19) from the soluble brain extracts of the two AD patients displaying the most severe neurodegeneration and the highest ASPD concentrations among those shown in Fig. 2*A* (Fig. 2*B*). These ASPD were *A11*-negative (Fig. 2*C*), composed predominantly of $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$

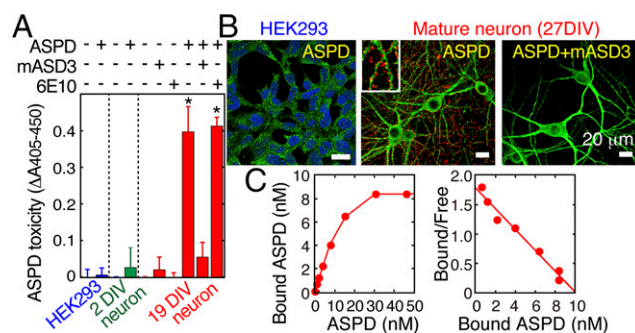


Fig. 1. Mature neuron-specific binding and toxicity of ASPD. (*A*) ASPD neurotoxicity was determined by measuring apoptotic DNA fragmentation in HEK293 cells, 2 DIV immature or 19 DIV mature rat hippocampal neurons after overnight treatment with 140 nM synthetic ASPD, with or without 2-h pretreatment of ASPD with each antibody (0.1 mg/mL for ASPD-specific *mASD*3 antibody; 0.4 mg/mL for $\text{A}\beta_{3-8}$ antibody 6E10) (mean \pm SD; * $P < 0.001$ Games-Howell post hoc test, $n = 6$). The antibody remained during overnight incubation with ASPD. As shown previously (19), *mASD*3 inhibited ASPD-induced neuronal death, but 6E10, targeting $\text{A}\beta_{3-8}$, did not. ASPD concentration is expressed in terms of the average ASPD mass 128 kDa (20). (*B*) Cells were treated for 30 min with 140 nM synthetic ASPD as in *A*. Images are representative of ASPD binding detected by *mASD*3 (red). Green represents anti-actin for HEK293 or anti-MAP2 for neurons. Neuronal 2D images were made from the z stack to show neurites (19). Neurons (19–27 DIV) gave essentially the same results as to binding and toxicity of ASPD. (*C*) Binding of synthetic ASPD was performed as in *B*, quantified (10), and shown as ASPD concentrations in 400 μL per well. Scatchard analysis gave $K_d = 5.43 \pm 0.27$ nM ($n = 3$). B_{max} of ASPD binding was 8.00 ± 1.0 nM for 30 min, from which the maximum level of ASPD binding was calculated to be 14 ± 0.7 pmol of ASPD/mg membrane protein.

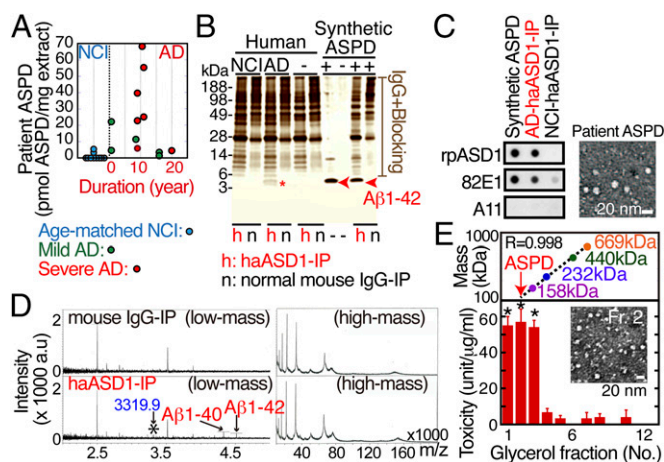


Fig. 2. Characterization of AD patient-derived ASPD. (*A*) Levels of ASPD in soluble extracts of the cerebral cortex are shown according to disease duration and the level of neurodegeneration (see *SI Materials and Methods, Human Brain Pathology and ISH*; reanalysis of the samples used as in ref. 19). ASPD are undetectable in most of the age-matched NCI cases, but are present even in the very early AD cases. ASPD levels in patients increase markedly in parallel with the severity and progression of the disease. The 100-kDa retentates of the extracts from the two patients containing the highest level of ASPD were used for isolation of patient ASPD in this study. In a severe AD patient 20 y after onset, ASPD levels were very low, likely because such widespread neuronal death already had occurred. (*B–D*) Synthetic ASPD and patient ASPD (see above in *A*) were purified by IP using ASPD-specific haASD1 antibody as in ref. 19. Soluble extracts from NCI brains and normal mouse IgG were used as controls for patient extracts and haASD1 antibody, respectively. Silver staining in *B*, dot blotting (*SI Materials and Methods*) along with a TEM image of negatively stained patient ASPD (see *SI Materials and Methods, TEM* for particle analysis) in *C*, and MALDI-TOF/MS analyses in *D* were performed as per ref. 19. Representative data are shown. In the silver-stained gels, a band corresponding to $\text{A}\beta_{1-42}$ or $\text{A}\beta_{1-40}$ (red asterisk) was detected only in haASD1-immunoprecipitated patient ASPD. Consistently, in MS of patient ASPD, significant peaks corresponding to $\text{A}\beta_{1-40}$ (4331.3 Da, centroid) and $\text{A}\beta_{1-42}$ (4515.5 Da, centroid) were reproducibly detected. Less-intense peaks at lower mass than $\text{A}\beta_{1-40}$ (e.g., see the asterisk in *D, Lower Left*) were occasionally, but not reproducibly, detected. The mass of the peak (3319.9 Da, centroid) is consistent with that of [pyroglutamylo- $\text{A}\beta_{11-42}$ + H^+]. Peaks at higher mass than $\text{A}\beta_{40}$ or $\text{A}\beta_{42}$ monomer were not detected (*Right*). These findings showed that $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ are the predominant components of patient ASPD. (*E*) Fractionation of patient ASPD (~ 16 pmol) in a 15–30% linear glycerol gradient (see *SI Materials and Methods, Glycerol Gradient Sedimentation*) (18). Protein standards (aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; and thyroglobulin, 669 kDa) were centrifuged at the same time as a reference and used for molecular mass determination (*Upper*). Fractions were collected and immediately assayed for neurotoxicity (*Lower*) (18). One unit of toxicity induces apoptosis in 1% of cells (see *SI Materials and Methods, Glycerol Gradient Sedimentation*). Data were obtained from three independent experiments and normalized to $\text{A}\beta$ concentration (mean \pm SD; Scheffé's post hoc test, * $P < 0.0001$ compared with vehicle alone). *Inset* shows a representative TEM image of the sample recovered in fraction 2 ($\sim 11.6 \pm 2.2$ -nm spheres; $n = 54$).

(Fig. 2*B* and *D*), had molecular masses 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*), consistent with previous data (18–20). We also used in vitro-reconstituted synthetic ASPD, which share essential characteristics with patient ASPD (19) (Table S1), as an analog.

Binding of ASPD was detected with ASPD-specific hamster monoclonal (*haASD*)1 antibody [K_d for ASPD ~ 0.5 pM (19)]. ASPD bound to a 105-kDa band in extracts from mature neurons cultured for 21 d in vitro (DIV) (red arrowhead in Fig. 3*A, Center*), but not immature 2 DIV neurons or HEK293 cells. This band was also recognized by synthetic ASPD (Fig. S2*A*, red arrow on the left), indicating that ASPD bind directly to this band, because purified synthetic ASPD contained no protein other than ASPD (19).

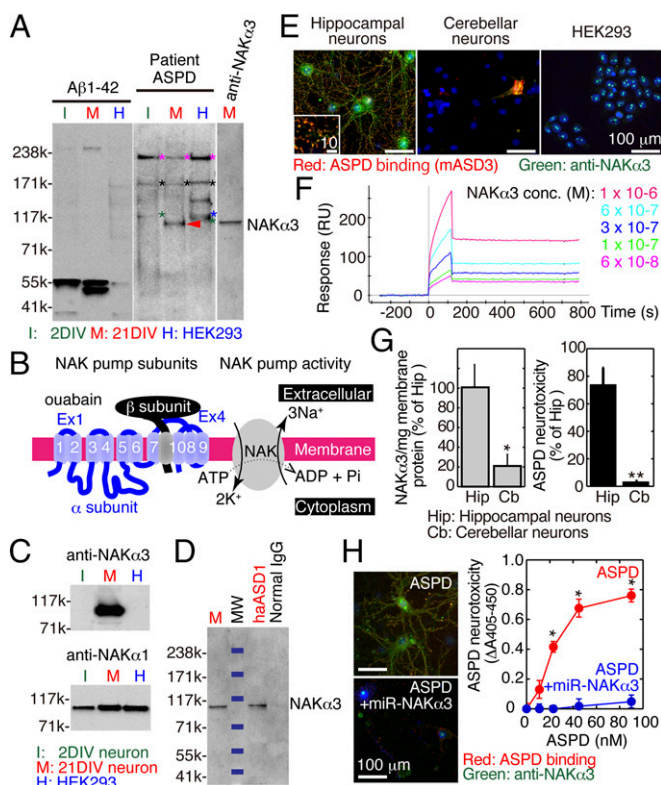


Fig. 3. Patient ASPD bind to NAK α 3. (A) Far-Western blotting of RIPA extracts (15 μ g per lane) was done using 6.6 nM patient ASPD (Center; detected with ASPD-specific haASD1 antibody) or 100 nM freshly dissolved A β _{1–42} containing monomers and dimers (Left; detected with anti-A β antibody 6E10). Bands marked with an asterisk represent intracellular proteins (Fig. S2A). Western blotting with anti-NAK α 3 antibody (excised from D) showed the mass of NAK α 3 (Right). Red arrow indicates a 105-kDa mature neuron-specific ASPD-binding protein, NAK α 3. (B) Schematic representation of NAK $\alpha\beta$ and its function. (C) Western blotting of RIPA extracts (used in A). (D) Coimmunoprecipitation (CoIP) of NAK α 3 with 250 nM synthetic ASPD. (E) Representative z-stack images of 27 DIV hippocampal or 22 DIV cerebellar neurons, or HEK293, treated with 13 nM synthetic ASPD for 15 min. ASPD-binding sites (red) on neuropil colocalize with anti-NAK α 3 staining (green) (high-power view in Inset; see SI Materials and Methods, Immunocytochemistry). (F) Direct interaction between ASPD on 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 tip without ASPD was subtracted as background. NAK β 1 did not interact with ASPD in the AlphaScreen system. (G) Rat hippocampal (19–20 DIV) or cerebellar (23 DIV) neurons were treated overnight with 110 nM ASPD. ASPD neurotoxicity was determined as in Fig. 1A and normalized to 0.2 μ M staurosporine toxicity. NAK α 3 levels in membrane fractions of these neurons before ASPD treatment were determined by Western blotting. Mean \pm SD; * $P = 0.002$; *** $P < 0.0001$ compared with hippocampal neurons, $n = 3$ –4 by Scheffé's post hoc test. (H) Representative images of 27 DIV hippocampal neurons, with or without selective knockout of NAK α 3 (see SI Materials and Methods, miR of ATP1A3), treated with 13 nM synthetic ASPD for 15 min. ASPD-binding sites (red) and anti-NAK α 3 staining (green) were detected as in E. ASPD neurotoxicity was assayed as in Fig. 1A (mean \pm SD; Scheffé's post hoc test, * $P < 0.0001$ compared with vehicle alone, $n = 3$).

This band was not detected when we used haASD1 alone or synthetic ASPD presaturated with haASD1 (Fig. S2B). Unlike ASPD, freshly dissolved A β _{1–42}, which consisted of monomers, dimers, and other low-molecular-weight (LMW) oligomers, did not bind to the 105-kDa band, but bound to 55-kDa and 45-kDa bands, likely corresponding to 54.2 kDa α 7nAChR and 40.4 kDa RAGE, respectively (Fig. 3A, Left). These findings showed that the 105-kDa band corresponds to ASPD-specific binding proteins present only in mature neurons.

MS and MS/MS analyses of the 105-kDa band (red arrow in Fig. S2A, Right) identified various NAK α -derived peptides (Table S2). We confirmed that NAK α appeared at 105 kDa (Fig. 3A, Right). ASPD-binding bands from immature neurons or HEK293 (asterisks in Fig. 3A, Center, and Fig. S2A, Left) were identified as intracellular proteins (see legend to Fig. S2A). Thus, NAK α , which has a molecular mass of 112–113 kDa, is likely to be the 105-kDa band entity.

NAK α is an essential catalytic subunit of the NAK pump (Fig. 3B), which is responsible for keeping the neuron resting membrane potential at about -70 mV (22). The functional NAK pump also requires a β -subunit (22) (Fig. 3B). In adult brains, NAK α 1 is ubiquitously expressed, whereas NAK α 3 is expressed exclusively in neurons, and NAK α 2 is found in astrocytes (22). We found that NAK α 3 was abundant in extracts of mature neurons only (Fig. 3C). Western blotting showed that NAK α 3 became detectable at 7 DIV and that its levels continued to increase until 21 DIV in our culture (Fig. S2C). This developmental increase in NAK α 3 level correlated well with that of the 105-kDa band in Far-Western blotting using synthetic ASPD (Fig. S2B). To establish whether NAK α 3 is the sole ASPD-binding isoform, we performed coimmunoprecipitation experiments. First, NAK α 3 in mature neuron extracts was coimmunoprecipitated directly with purified synthetic ASPD using haASD1 antibody (Fig. 3D). Second, biotin-labeled ASPD were incubated with mature hippocampal neurons, and MS/MS analysis confirmed that the NAK α 3 isoform was selectively coimmunoprecipitated as the 105-kDa band with biotin-labeled ASPD (red arrows in Fig. S2D). We then confirmed that ASPD and NAK α 3 were essentially colocalized within the neuropil of mature hippocampal neurons double-stained for synthetic ASPD and NAK α 3 (Fig. 3E, Left Inset). Furthermore, surface plasmon resonance spectroscopy (SPR) demonstrated that rat NAK α 3 directly bound to synthetic ASPD ($K_d = 7.8 \pm 2.5$ nM, $n = 3$) (Fig. 3F). The K_d value obtained from this SPR data was almost identical with that obtained from ASPD-binding to mature neurons (Fig. 1C) ($K_d = 5.43 \pm 0.27$ nM, as described above). We confirmed that human and mouse NAK α 3 bound directly to synthetic ASPD using SPR (human $K_d = 28.6 \pm 6.6$ nM, $n = 5$; mouse $K_d = 4.0 \pm 1.9$ nM, $n = 3$). Notably, most mature hippocampal neurons were stained for NAK α 3, whereas most mature cerebellar neurons were NAK α 3-immunonegative (Fig. S2E). This finding was consistent with Western blotting showing that the amount of membrane NAK α 3 in cerebellar neurons was only $\sim 20\%$ of that in hippocampal neurons (Fig. 3G, Left). Synthetic ASPD bound to NAK α 3-immunopositive neurons among hippocampal or cerebellar neurons (Fig. 3E) and induced death in those neurons at levels correlating with their membrane NAK α 3 amount (Fig. 3G). Stable knockout of NAK α 3 expression with microRNA (miR)-expressing virus vector decreased NAK α 3-expressing neurons to $19.8 \pm 14\%$ ($n = 3$; $P < 0.0001$ compared with the untreated neurons using Scheffé's post hoc test, $n = 3$) in hippocampal neurons and abolished ASPD-binding and ASPD-induced neurodegeneration (Fig. 3H). These data support the conclusion that NAK α 3 is the mature neuron-specific ASPD-binding protein that is linked to ASPD neurotoxicity.

ASPD Impair NAK α 3 Activity, Leading to Ca²⁺ Dyshomeostasis and Neuron Death. We next examined the effect of ASPD-binding on NAK α 3 activity. As shown in Fig. 4A, exposure of membrane preparations from mature hippocampal neurons to 110 nM synthetic ASPD rapidly ($\sim 70\%$ in 1 h) impaired NAK α 3-specific activity in those membrane preparations. Overnight treatment of intact mature hippocampal neurons with 140 nM synthetic ASPD caused a $>90\%$ decrease in NAK α 3-specific activity (Fig. 4B).

This NAK α 3 impairment should cause a failure in active transport of Na⁺ and K⁺ ions (Fig. 3B). Indeed, we found that cytoplasmic Na⁺ levels were increased immediately after exposure of neurons to synthetic ASPD (at the orange arrow in Fig. S3A), which reached a maximum after ~ 18 min (at the yellow arrow in

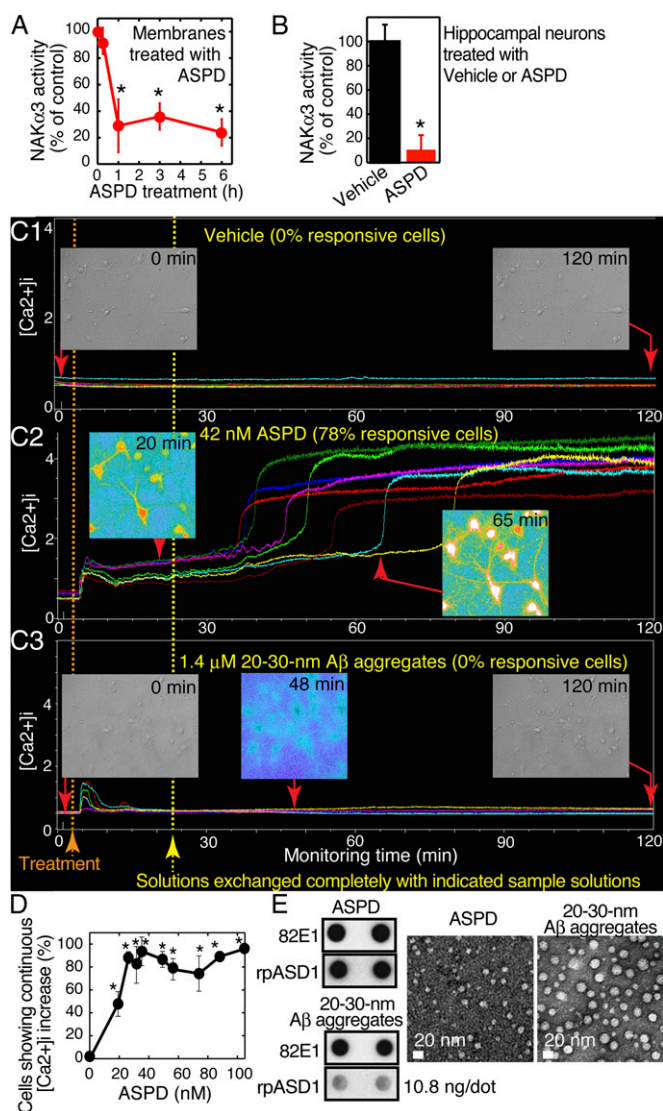


Fig. 4. ASPD impairs NAK activity and causes Ca^{2+} dyshomeostasis. (A) Membrane fraction of 25–26 DIV hippocampal neurons (30 μg per assay) was incubated with 110 nM synthetic ASPD and NAK α 3-specific ATPase activity [$K_d = 45$ nM for ouabain (61–63)] was determined by subtracting the 100-nM ouabain-sensitive activity from overall Mg^{2+} -ATPase activity (46). Mean \pm SD; * $P < 0.0001$ Scheffé's post hoc test compared with 0 h, $n = 4$. (B) NAK α 3-specific ATPase activity of 25–26 DIV hippocampal neurons treated overnight with 140 nM synthetic ASPD was determined as in A. Mean \pm SD; * $P < 0.0001$ Scheffé's post hoc test, $n = 3$. (C) Representative $[\text{Ca}^{2+}]_i$ changes of cell bodies of 19–20 DIV hippocampal neurons after treatment with each sample using Fura-PE3AM. The ratio of responsive cells was obtained as in D. The 20- to 30-nm $\text{A}\beta_{1-42}$ aggregates were obtained by concentration and redilution processes (see *SI Materials and Methods, Preparation of 20- to 30-nm $\text{A}\beta$ Aggregates*). For treatment with ASPD, the circulating buffer was replaced with the buffer containing ASPD. After the replacement, it took ~ 110 s until the ASPD-containing fluid reached the assay chamber (at the orange arrow) and it took ~ 17.5 min until all of the solution in the assay chamber was replaced by the ASPD solution (at the yellow arrow). Treatment with 20- to 30-nm $\text{A}\beta_{1-42}$ aggregates was done similarly. (D) The ratio of neurons that exhibited repetitive Ca^{2+} spikes or reached a saturated $[\text{Ca}^{2+}]_i$ within 2 h after the treatment was calculated from $[\text{Ca}^{2+}]_i$ data from three independent experiments. Mean \pm SD; * $P < 0.0001$ Scheffé's post hoc test. (E) TEM images and dot blotting of the samples used in C.

Fig. S3A₃ and A₄). ASPD-induced impairment of NAK α 3 activity thus increased cytoplasmic Na^+ levels and likely induced depolarization in the treated neurons.

This process would lead to an increase in intracellular Ca^{2+} level ($[\text{Ca}^{2+}]_i$) through the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) or voltage-gated Ca^{2+} channels (VGCC) activated by depolarization (22). We therefore monitored $[\text{Ca}^{2+}]_i$ using Fura-PE3AM staining. Vehicle treatments did not affect $[\text{Ca}^{2+}]_i$ for at least 2 h (Fig. 4C1 and Fig. S3B₁), but ASPD increased the number of Ca^{2+} -responsive cells dose-dependently (Fig. 4C2 and Fig. S3B₂–B₄), with a plateau at ~ 40 nM synthetic ASPD (Fig. 4D). The dose-dependent effect of ASPD on the number of Ca^{2+} -responsive cells correlated well with the level of ASPD-binding (Fig. 1C, Left), as well as that of ASPD neurotoxicity ($\text{EC}_{50} = 18 \pm 2.4$ nM, $n = 5$), suggesting that ASPD-binding to mature neurons caused increased $[\text{Ca}^{2+}]_i$, leading to eventual neuronal death.

Depending on the ASPD concentration, two types of Ca^{2+} -responses were observed (see *SI Discussion* for a plausible mechanistic explanation). With 18 nM ASPD, repetitive Ca^{2+} spikes were observed ~ 3 min after the treatment (Fig. S3B₂). In this case, $[\text{Ca}^{2+}]_i$ increased gradually and led to a sustained increase after 2 h (e.g., Fig. S3C₁). With higher concentrations of ASPD (e.g., 42 nM in Fig. 4C2), $[\text{Ca}^{2+}]_i$ increased ~ 2 min after the treatment, maintained a sustained increase during 0–40 min, and then rose precipitously before plateauing at ~ 40 –70 min. Much higher concentrations of ASPD accelerated the process, with $[\text{Ca}^{2+}]_i$ plateauing earlier (within 1 h; compare 88 nM synthetic ASPD in Fig. S3B₄ with 42 nM synthetic ASPD in Fig. S3B₃). Unlike ASPD, 1.4 μM 20–30-nm $\text{A}\beta_{1-42}$ aggregates (TEM in Fig. 4E), which were obtained by making a 20-fold concentration and then a 10-fold redilution of ASPD (*SI Materials and Methods, Preparation of 20- to 30-nm $\text{A}\beta$ Aggregates*), were rarely detected by ASPD-specific rabbit polyclonal (rpASD)1 antibody in dot blotting (Fig. 4E) and did not cause such a persistent increase in $[\text{Ca}^{2+}]_i$ (Fig. 4C3), and the neurons remained intact (inset bright-field images in Fig. 4C3).

This ASPD-induced increase in $[\text{Ca}^{2+}]_i$ required extracellular Ca^{2+} , because it was blocked by addition of EGTA to the medium (Fig. S3C₂). Furthermore, blockade of $[\text{Ca}^{2+}]_i$ overload by chelation with BAPTA-AM suppressed ASPD toxicity (Fig. 5A). We then asked which plasma membrane calcium conductors, VGCC or NCX, were involved in ASPD toxicity. Inhibitor effects on ASPD neurotoxicity showed that N-type VGCC were involved in ASPD-induced neuronal death, whereas the other VGCC subtypes and NCX were not involved (Fig. 5A). We confirmed that an inhibitor specific to N-type VGCC blocked ASPD-induced $[\text{Ca}^{2+}]_i$ increase (Fig. S3D₃). Notably, N-type VGCC are found primarily at presynaptic terminals of neurons (23), consistent with presynaptic binding of ASPD (19).

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

These findings indicate that impairment of NAK α 3 activity by ASPD binding increases cytoplasmic Na^+ levels (Fig. S3A₄), activates N-type VGCC, and causes continuous Ca^{2+} influx into cytoplasm. This in turn results in Ca^{2+} overload in mitochondria (observed as the sustained $[\text{Ca}^{2+}]_i$ increase) and subsequent Ca^{2+}

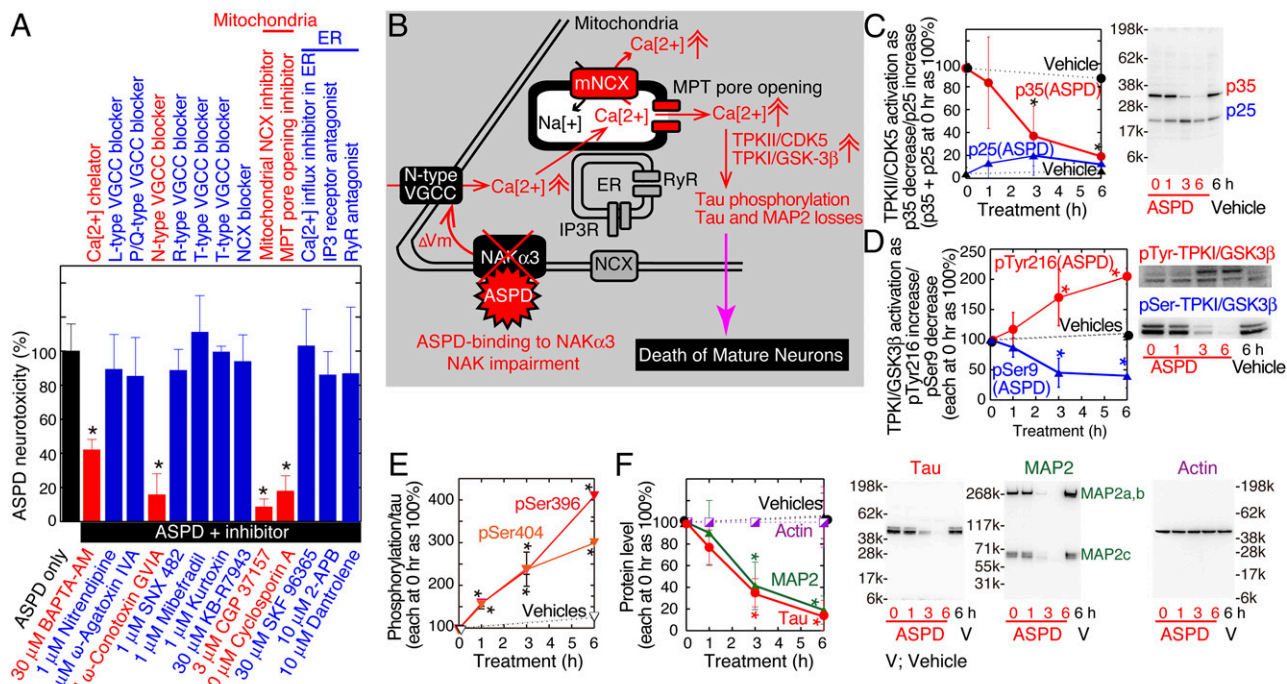


Fig. 5. Downstream signals leading to neuronal death after ASPD binding to NAK α 3. (A) Inhibitor effect on 140 nM synthetic ASPD neurotoxicity examined as in Fig. 1A. Mean \pm SD; * P < 0.001 compared with ASPD only (Scheffé's post hoc test, n = 3). (B) Schematic illustration of the mechanism of ASPD-induced neurodegeneration revealed in this study. (C) p25 generation from p35, a neuron-specific TPKII/CDK5 activator, after 110 nM synthetic ASPD treatment (Right; representative Western blot). Data were normalized to actin and presented relative to total p35 and p25 amount at time 0. Mean \pm SD; * P < 0.02 compared with data at time 0 (Scheffé's post hoc test, n = 4–6). (D) TPKI/GSK3 β activation as shown by phospho-Tyr²¹⁶ increase and phospho-Ser⁹ decrease after 110 nM synthetic ASPD treatment (Right; representative Western blot). Data were normalized to total TPKI/GSK3 β and presented relative to each phospho-protein amount at time 0. Mean \pm SD; * P < 0.002 compared with data at time 0 (Scheffé's post hoc test, n = 4–6). (E) Time-dependent increases in tau phosphorylation after 110 nM synthetic ASPD treatment. Data were normalized to total tau and presented relative to each phospho-protein amount at time 0. Vehicle controls are shown as inverted triangles in black for pSer³⁹⁶ and white for pSer⁴⁰⁴. Mean \pm SD; * P < 0.0001 compared with data at time 0 (Scheffé's post hoc test, n = 3–4). (F) Time-dependent decreases in MAP2 and tau after 110 nM synthetic ASPD treatment (Right; representative Western blot). Data were normalized to actin and presented relative to each protein amount at time 0. Mean \pm SD; * P < 0.002 compared with data at time 0 (Scheffé's post hoc test, n = 4–6).

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

Previously we have shown involvement of tau protein kinase I/glycogen synthase kinase-3 β (TPKI/GSK3 β) in ASPD neurotoxicity (18). We also found that tau protein kinase II/cyclin-dependent protein kinase 5 (TPKII/CDK5) was activated after ASPD treatment (Fig. 5C and D) and tau phosphorylation increased at Ser³⁹⁶ and Ser⁴⁰⁴, which are known to be phosphorylated in AD brains by TPKI/GSK3 β /TPKII/CDK5 and TPKII/CDK5, respectively (24) (Fig. 5E). Furthermore, tau and microtubule-associated protein 2 (MAP2) were rapidly lost after ASPD treatment (Fig. 5F). Tau phosphorylation and loss, together with MAP2 loss, could destabilize microtubules and promote neurodegeneration.

Next, time-lapse DIC images of mature neurons after ASPD treatment were taken. Because of osmotic imbalance, neurons started swelling at \sim 30 min after exposure to 140 nM synthetic ASPD, and cell shrinkage, a ubiquitous feature of apoptosis (25), took place in \sim 3–5 h (Fig. 6A, Fig. S4A, and Movie S1). Subsequently, DNA fragmentation became detectable after 5 h (Fig. 6B). Scanning electron microscopic (SEM) images clearly showed ASPD-induced neuronal swelling and shrinkage (Fig. 6C). The movie shows that the surface of the ASPD-treated neurons became rough within 30 min after the ASPD treatment (Movie S1) and holes appeared within 1 h (see arrows in Fig. 6C). Similar swelling and shrinkage of neurons were induced by 100 nM ouabain (Fig. 6A, Fig. S4A, and Movie S2), which selectively blocks rodent

NAK α 3 activity (26). Unlike ASPD and ouabain, staurosporine, an apoptosis inducer that inhibits multiple kinases, induced shrinkage but not swelling (Fig. 6A, Fig. S4A, and Movie S3). Vehicle treatment induced neither swelling nor shrinkage (Fig. 6A, Fig. S4A, and Movie S4). Ouabain also elicited the events triggered by ASPD (summarized in Fig. 5B; see also Fig. S3F). Our data are consistent with the conclusion that NAK α 3 is a death target for ASPD toxicity. We summarize a possible ASPD-induced sequence of events leading to neuronal death in Fig. 6A.

We next examined NAK α 3 localization in human brains. In nonclinically demented individual (NCI) cerebrum, punctate staining by anti-NAK α 3 antibody surrounded the cell body of pyramidal neurons (arrows in Fig. 6D, Upper Left) and diffuse staining was detected on axons and in neuropils. Intense staining was found in basket cells surrounding Purkinje cells in NCI cerebellum (Fig. 6D, arrowheads, Lower Left). NAK α 3 localization was consistent with that in adult mouse brain (27).

We found that NAK α 3 staining was essentially lost in AD cerebrum, whereas it was not decreased in AD cerebellum (Fig. 6D, Right). Quantitative dot blotting with an ASPD-specific antibody, rpASD1, showed patient ASPD levels in NCI cerebrum, AD cerebrum, and AD cerebellum of 0.7 ± 0.4 , 55.7 ± 7.0 , and 0.7 ± 0.7 pmol/mg soluble brain extracts, respectively (a representative blot in Fig. 6D), suggesting that decreased NAK α 3 staining correlated with patient ASPD levels in AD brains. Comparison of quantitative dot blotting results with rpASD1 and those with anti-A β antibody 82E1 indicated that patient ASPD

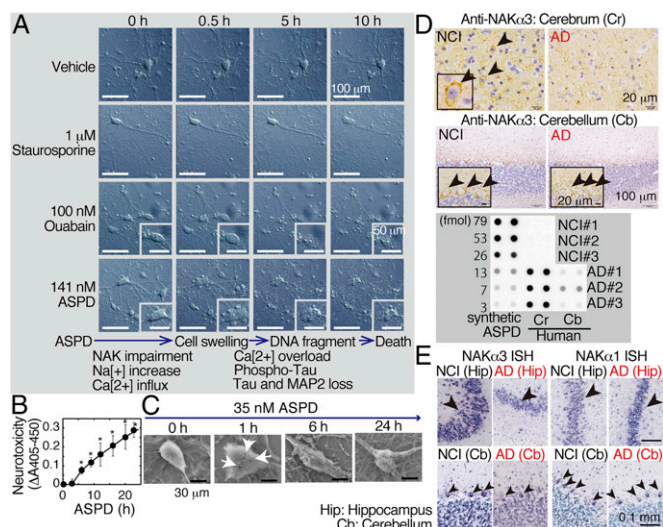


Fig. 6. ASPD effects on NAK α 3-expressing neurons in rat neurons and human brains. (A) Representative time-lapse images of mature rat hippocampal neurons treated with each reagent (Movies S1–S4). (B) Time-dependent increase in apoptotic DNA fragmentation (determined as in Fig. 1A) induced by 140-nM synthetic ASPD. (C) SEM showed the surface and morphology of mature rat hippocampal neurons treated with 35 nM synthetic ASPD. (D and E) Immunohistochemical studies using anti-NAK α 1 or anti-NAK α 3 antibody on 4- μ m paraffin-embedded sections in D and ISH analyses using probes for *ATP1A1* and *ATP1A3* on 10- μ m paraffin-embedded sections in E. Soluble extracts were obtained from these brains and amounts of patient ASPD (representative blots in D) and A β in the extracts were determined by dot blotting as 429 ± 50 and 695 ± 104 (μ g/g brain, $n = 3$), respectively.

accounted for $62 \pm 3\%$ ($n = 3$) of soluble A β . Unlike decreased NAK α 3 staining, anti-NAK α 1 antibody showed diffuse staining throughout NCI and AD cerebrum (Fig. S4B). Results of in situ hybridization (ISH) of adjacent sections of the same AD or NCI cases with NAK α 3 mRNA (*ATP1A3*) or NAK α 1 mRNA (*ATP1A1*) ($n = 3$ each) showed the same correlation with the histological data (compare Fig. 6E and Fig. S4C with Fig. 6D and Fig. S4B). With NAK α 3 ISH, the signal intensity of *ATP1A3* was clearly lower in layers of pyramidal neurons in the hippocampus in AD compared with that observed in NCI. No obvious differences were detected in the cerebellum between AD and NCI. With NAK α 1 ISH, the signal intensity of *ATP1A1* was not changed in both regions between AD and NCI cases. These results are consistent with a previous quantitative ISH study on AD patients (28) and suggest NAK α 3-expressing neurons are preferentially lost in AD-susceptible brain regions where ASPD concentrations are high.

Structural Characteristics of ASPD and Their Target, Ex4 of NAK α 3. To examine structural features of ASPD relevant to binding with NAK α 3, we acquired solution NMR spectra of 15 N-labeled ASPD. Although we detected 39 heteronuclear single quantum coherence (HSQC) signals in the case of freshly dissolved A β , we detected 14 of 39 HSQC signals representing peptide backbones of A β in ASPD, and these were nearly superimposable on the signals of the A β _{1–13}, A β _{15–16}, and A β ₂₃ regions of monomer (Fig. 7A). Remaining NMR-invisible amino acids presumably form a magnetically non-equivalent core. In other words, the NMR-visible amino acids, although derived from different A β regions, may exist in close proximity to form the ASPD surface (Fig. 7B), in accordance with our finding that specific binding of monoclonal mASD3 antibody to ASPD was blocked by pentapeptides covering A β _{2–8}, A β _{15–19}, or A β _{19–23} (19). Because mASD3 antibody neutralizes ASPD toxicity (19), the NMR-identified ASPD surface is likely involved in binding to NAK α 3. The HSQC spectra of ASPD were different from those of precursors of synaptotoxic dodecamers (termed

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

The above findings suggest that the unique ASPD surface has a key role in ASPD-NAK α 3 interaction. Because protein–protein interaction inhibitors can be designed based on the protein surface region essential for binding, we sought to determine which extracellular region of NAK α 3 serves as the ASPD target site. NAK α 1 and NAK α 3 share 96% sequence identity. Given ASPD's preferential binding to NAK α 3, the NAK α 3-specific region, either the first extracellular loop (Ex1) or the fourth extracellular loop (Ex4) (Figs. 3B and 7C), would be the key ASPD target. We found that a chemically synthesized octapeptide from Ex4 (RLNWDDRT) significantly blocked ASPD toxicity, whereas Ex1 peptides neither bound to ASPD, as determined by SPR (Fig. S5A), nor blocked ASPD toxicity (Fig. 7D). Scrambled Ex4 peptides failed to block ASPD toxicity (Fig. 7D). We confirmed that Ex4 interfered with binding of both patient ASPD and synthetic ASPD to mature hippocampal neurons (Fig. 7E and Fig. S5B), in addition to blocking the subsequent $[Ca^{2+}]_i$ increases (Fig. S3D₄) and activation of the kinases (Fig. S5C). Notably, with 15-min patient ASPD treatment, neurites began to degenerate (Fig. 7E, arrowheads). This degeneration was almost completely blocked by Ex4 (Fig. 7E). These findings suggest that Ex4 is the main ASPD target site (pink in Fig. 7C).

We then planned to develop ASPD-binding peptides with toxicity-neutralizing activity by using phage display (PD) analysis. Three independent screenings of a randomized 12-mer peptide library detected ~ 21 kinds of ASPD-binding peptides with high His and Trp contents. Among them, many showed similarity to Ex4 of NAK α 3. Chemically synthesized PD-identified dodecapeptides (PD2, -50, -68) bound to ASPD ($K_d = 5.8\text{--}8.9 \times 10^{-8}$ M by SPR). These dodecapeptides, which commonly contain a four-amino acid sequence (H*⁸⁷⁹NW) similar to Ex4 (RLNW; corresponding to positions 877–880), inhibited ASPD toxicity toward mature neurons as effectively as the Ex4-derived peptide, whereas other peptides containing H*⁸⁷⁹W (lacking NAK α 3-specific Asn) were ineffective (Fig. 7F). Notably, ASPD-binding tetrapeptide PD2-11 (HFNW) was enough to significantly block binding and neurotoxicity of ASPD (Fig. 7F and Fig. S5B). These findings suggest that the NAK α 3-specific region in Ex4 encompassing residues Asn⁸⁷⁹ and Trp⁸⁸⁰ is essential for ASPD-NAK α 3 interaction (Fig. 7C and G).

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

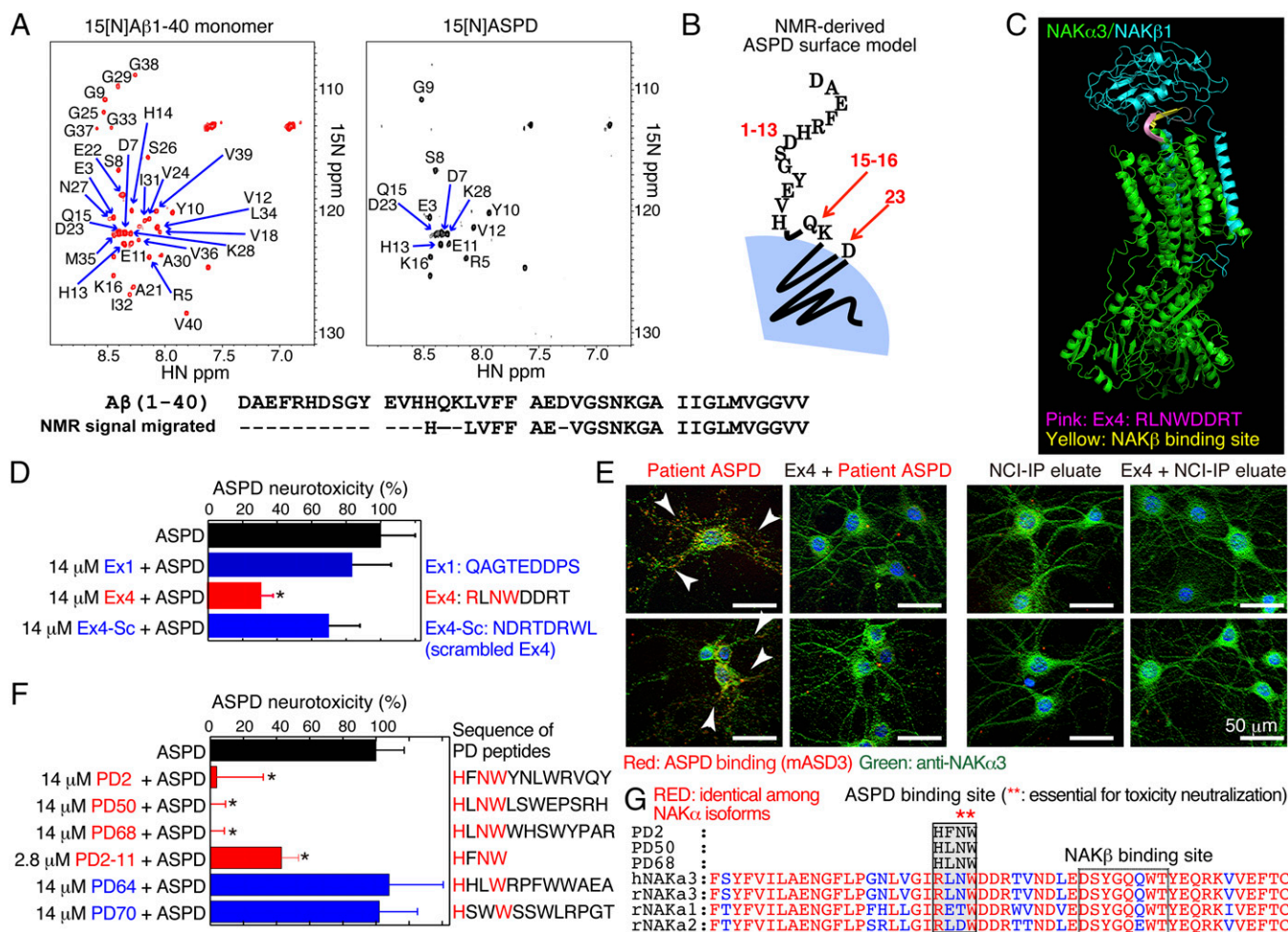


Fig. 7. Structural Basis for ASPD Binding To $\text{NAK}\alpha_3$. (A) ^1H - ^{15}N HSQC spectra of ~ 350 nM ^{15}N -ASP and ~ 100 μM ^{15}N - $\text{A}\beta_{1-40}$ monomers in $0.5\times$ PBS [supplemented with 5% (vol/vol) D_2O] were recorded at 298K on a 600-MHz Bruker AVANCE-III spectrometer equipped with a cryogenic TCI probe. (B) Schematic diagram of the NMR-derived ASPD surface. (C) A 3D homology model of human $\text{NAK}\alpha_3$ was constructed using the Prime program (Schrödinger) based on the structure of pig $\text{NAK}\alpha_1$ (32) as a template (PDB ID code 3B8E). (D) The effect of each $\text{NAK}\alpha_3$ -derived Ex1, Ex4, or scrambled Ex4 peptides on 140 nM synthetic ASPD neurotoxicity was examined as in Fig. 1A. Mean \pm SD; * $P < 0.001$ Scheffé's post hoc test, compared with vehicle, $n = 3$. (E) Representative z-stack images of 26 DIV hippocampal neurons, after 15-min treatment with 7 nM patient ASPD (immunoprecipitated from the patient extracts as in Fig. 2B), with or without 30-min pre-treatment with Ex4 (20 μM in final concentration). IP eluates of NCI brain extracts, in which ASPD were not detected by dot blotting, were used as a control. ASPD-binding sites (red) and anti- $\text{NAK}\alpha_3$ staining (green) were detected as in Fig. 3E. (F) The effect of each Ex4-mimicking ASPD-binding peptides on 140 nM synthetic ASPD neurotoxicity. All peptides used here contained a free N-terminal amino group and a carboxylic acid at the C terminus. Mean \pm SD; * $P < 0.001$ Scheffé's post hoc test, compared with vehicle, $n = 3$. (G) Alignment of Ex4 regions.

studies have shown that the NAK pump exists predominantly as $(\text{NAK}\alpha_3)_2:(\text{NAK}\beta_1)_2$ heterotetramer in membranes (35, 36) and ASPD appeared to bind almost all $\text{NAK}\alpha_3$ -containing NAK pumps in membranes (Fig. 3E), we infer, using Occam's razor (37), that the most likely ratio is 1:2 (i.e., 1:1 binding of ASPD with $\text{NAK}\alpha_3$ dimer). This inference is consistent with other ASPD binding data (Fig. S6). Because the tetrapeptide segment in Ex4 of $\text{NAK}\alpha_3$ is conserved in rodents and humans (Fig. 7G), this opens up possibilities for knowledge-based design of inhibitors of neuronal death in AD.

Discussion

Past studies have reported the loss of the NAK pump activity, and the protein itself, in the brains of AD patients (28, 38, 39). In particular, $\text{NAK}\alpha_3$ expression, but not $\text{NAK}\alpha_1$ expression, is reduced in the frontal cortices of AD patients and this reduction correlates inversely with levels of diffuse plaques in that region (28). No such reduction is observed in the unaffected cerebellum of the same patients (28). $\text{NAK}\alpha_3$ activity was reported to de-

crease in $\text{A}\beta_{1-40}$ -treated neuronal cultures (40) and the NAK pump activity was reported to decrease in 17- to 18-mo-old *APP* and *presenilin-1* transgenic mice (41). These previous observations suggest a potential link between $\text{A}\beta$ and $\text{NAK}\alpha_3$ impairment. However, until now, the question of whether $\text{NAK}\alpha_3$ impairment is the cause or the result of neuronal death has remained unanswered. We present here direct evidence that the neuron-specific α -subunit of the NAK pump, $\text{NAK}\alpha_3$, is the neuronal death-inducing target of ASPD.

SPR data demonstrated direct binding of ASPD to $\text{NAK}\alpha_3$ (Fig. 3F). ASPD-binding to $\text{NAK}\alpha_3$ impaired rapidly $\text{NAK}\alpha_3$ -specific NAK pump activity (Fig. 4A). Cytoplasmic Ca^{2+} overload was highly correlated with ASPD-binding (compare Figs. 1C and 4D), which eventually led to mitochondrial defects and neuronal death (Fig. 5A), as observed in AD (42). Stable knockout of $\text{NAK}\alpha_3$ expression with a microRNA-expressing virus vector decreased $\text{NAK}\alpha_3$ -expressing neurons and abolished ASPD-binding and ASPD-induced neurodegeneration (Fig. 3H). ASPD binding peptides inhibited the ASPD binding to $\text{NAK}\alpha_3$ and protected

mature neurons from ASPD neurotoxicity (Fig. 7 D–F, and Figs. S3D₄ and S5 B and C). In addition to these cell-based data, we found that NAK α 3-expressing neurons were lost in AD-susceptible regions, such as the cortex and the hippocampus in patients (where ASPD levels were high), but were not lost in the far less affected cerebellum of the same patients (where ASPD levels were low) (Fig. 6 D and E and Fig. S4 B and C). The explanation for these regional differences in ASPD concentrations must await further experimentation, but these observations further support our conclusion that NAK α 3 serves as the specific death-inducing target of ASPD.

Recent studies have shown that A β forms numerous structurally distinct oligomers that may contribute differently to disease pathogenesis (6, 19). A β receptor/ligand systems may be organized into three categories (Fig. 8 and Table S3). The first category involves regulating the CNS concentration of A β monomer available for assembly. The second category involves A β impairment of synaptic connections by indirectly affecting NMDAR activity (see *SI Discussion* for more details about the first two categories). Postsynaptic Sigma-2/PGRMC1 (progesterone receptor membrane component 1) has been reported to serve as a receptor for 50–75 kDa A β _{1–42} oligomers (43). We report here a third system involving presynaptic neurons. Presynaptic neuronal Ca²⁺ hyperactivation has been reported to occur near amyloid plaques in AD model mice (44). Interestingly, such hyperactivation of neurons in hippocampus has been associated with the cortical thinning in mild cognitive impairment patients and has been considered to be an early indicator of AD-related neurodegeneration (45). However, the underlying mechanisms were largely unknown. We have previously shown that ASPD colocalize with presynaptic Bassoon (19). Among different NAK α subunits, only NAK α 3 is present in the presynaptic side of neurons (27, 46–48). Electrophysiological studies have also shown the importance of NAK α 3 in the presynaptic function (49, 50). Our data are consistent with ASPD binding to presynaptic NAK α 3 of mature neurons, leading to activation of presynaptic N-type VGCC, and eventual death of NAK α 3-expressing neurons. Notably, NAK α 3 appears as punctate patterns restricted to layers III and V of the neocortex (27), where N-type VGCC are present (51). These layers are particularly vulnerable in AD brains (52). It is an intriguing speculation that the 100-kDa band sometimes observed by Gong et al. in ligand overlay assays with ADDLs was NAK α 3 (53).

The human NAK α 3 gene *ATPLA3* is located on chromosome 19q13.31, an AD linkage region, but at present there are no re-

ports that mutations in *ATPLA3* are linked to AD. The mutation rate in *ATPLA3* is very low in humans (54), suggesting that people with mutations in *ATPLA3* might be at high risk of neurodevelopmental/neuropsychiatric diseases and develop these diseases before they become old. Indeed, genetic studies have shown that mutations in the protein-coding region of *ATPLA3* cause rapid-onset dystonia parkinsonism, alternating hemiplegia of childhood, and cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss syndrome (54). Notably, although a direct linkage of *ATPLA3* mutations to AD is difficult to detect, AD and *ATPLA3*-mutated diseases share features such as convulsive seizures (derived from hyperactivity of neurons). A recent clinical study of AD patients has revealed that convulsive seizures occurred early in the course of AD, which often began around the same time when symptoms of neurodegeneration first appeared (55). Levetiracetam, which inhibits presynaptic calcium channels (56), suppresses neuronal hyperactivation, and reverses cognitive deficits in their AD model mice (57). These results are consistent with our findings (Fig. 8) and suggest that hyperactivation of presynaptic neurons occurs in AD patients contemporaneously with neurodegeneration.

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

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

Except for the NAK β subunit and agrin, all other NAK α -binding proteins have been reported to bind to the intracellular region of NAK α (59). Because this NAK β -binding region is located 10 amino acids more C-terminally than Trp⁸⁸⁰ (Fig. 7G), the Ex4-mimicking tetrapeptide is unlikely to interfere with the NAK α –NAK β interaction. In the case of the NAK α 2 mutation W887R, loss of NAK pump activity occurs without interfering with the NAK α –NAK β interaction because the NAK pump containing the NAK α and NAK β subunits is present at substantial levels in the plasma membrane (58). With respect to agrin, although its binding region in NAK α 3 has not been

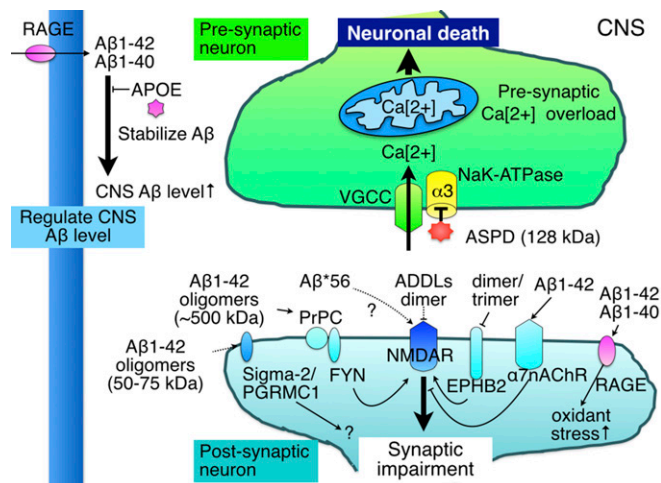


Fig. 8. Major A β ligand/receptor systems previously detected and the system identified in this work (Table S3).

determined, it has been suggested to take the place of NAK β , and accordingly, it is reasonable to consider that agrin and NAK β share the same binding region (59). Taken together, these observations suggest that peptidomimetics would not disrupt interactions between NAK α 3 and proteins other than ASPD. The Ex4-mimicking tetrapeptide (PD2–11, MW602.6) that inhibits ASPD neurotoxicity is sufficiently small that the molecule could serve as a lead compound for knowledge-based design of peptidomimetics. This class of compounds is expected to offer superior stability and pharmacokinetics compared with anti-A β antibodies. Given the essential functions of the NAK pump in neurons (22), direct modulation of the NAK pump would be a risky approach. We therefore propose a new strategy to treat AD by blocking ASPD–NAK α 3 interaction through masking the A β oligomer surface with specific peptidomimetics, as illustrated in Fig. S6. In summary, our data support the conclusion that ASPD–NAK α 3 interaction is a cellular basis for neuronal loss induced by ASPD and that blocking this interaction could be a useful strategy for AD treatment.

Materials and Methods

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

ASPD Preparation. ASPD are neurotoxic, spherical A β oligomers of 10- to 15-nm diameter (measured by TEM) that are recognized by ASPD-specific antibodies (19, 20). Patient ASPD were purified from the 100-kDa retentates of soluble AD brain extracts (devoid of LMW oligomers, prepared as in ref. 19) by immunoprecipitation (IP) using haASD1 (*SI Materials and Methods*). Synthetic ASPD were formed in 50 μ M A β _{1–42} solution (with or without 2 μ M biotinylated A β _{1–40}) using in-house-prepared highly soluble A β peptides (essential for obtaining ASPD) (*SI Materials and Methods, A β synthesis*) in F12 buffer without L-glutamine and phenol red by slowly rotating the solution for ~16 h at 4 °C (19). The level of A β _{1–42}-ASPD in this A β _{1–42} solution after slow rotation is usually ~30%. Synthetic ASPD were obtained in the fraction that passed through 0.22- μ m filters, but was retained on 100-kDa MWCO filters (Sartorius) (19). ¹⁵N-Labelled ASPD were prepared in the same

way using purified *Escherichia coli*-expressed ¹⁵N-labeled A β _{1–40} (60). ASPD quality was confirmed by dot blotting, TEM, amino acid analysis, and toxicity assays (20).

Statistical Analyses. Statistical analysis was performed using StatView5.0 (SAS Institute).

Other Methods. Methods for A β synthesis, preparation of 20- to 30-nm A β aggregates, primary neuronal cultures, ASPD toxicity, ASPD binding, immunocytochemistry, microRNA of *ATP1A3*, Western and Far-Western blotting and MS/MS, cell-free NAK α 3 production, SPR, IP, NAK activity, Ca²⁺ and Na⁺ imaging, time-lapse, dot blotting, TEM, SEM, Human brain pathology and ISH, glycerol gradient sedimentation, PD, NMR, and molecular modeling are described in *SI Materials and Methods*.

Supporting Information. Additional information can be found in *SI Materials and Methods, SI Discussion, Figs. S1–S6, Tables S1–S3, and Movies S1–S4*. Fig. S1 shows that glutamate receptor antagonists did not change ASPD neurotoxicity. Fig. S2 shows data on ASPD interactions with NAK α 3, developmental changes in NAK α 3 levels, coimmunoprecipitations, and immunostaining. Fig. S3 shows ASPD-induced Na⁺ increase, Ca²⁺ overload induced by different concentrations of ASPD, which were abolished by EGTA pretreatment, as well as ouabain-induced Ca²⁺ overload and tau phosphorylation/destabilization. Fig. S4 shows time-lapse shots and immunohistochemical studies of human brains using anti-NAK α 1 antibody and ISH. Fig. S5 shows ASPD-binding peptides inhibit ASPD-binding to NAK α 3 and other ASPD-induced downstream phenomena. Fig. S6 presents a model of ASPD–NAK α 3 interactions and how masking the ASPD surface with specific masking peptides or peptidomimetics could be a new therapeutic strategy.

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