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Modeling Alzheimer’s Disease with iPSCs Reveals Stress Phenotypes Associated with Intracellular Aβ and Differential Drug Responsiveness

（アルツハイマー病患者由来 iPS 細胞を用いた細胞内 Aβ 関連ストレスと薬剤応答性の解明）

近藤 孝之
Modeling Alzheimer’s Disease with iPSCs Reveals Stress Phenotypes Associated with Intracellular Aβ and Differential Drug Responsiveness


SUMMARY

Oligomeric forms of amyloid-β peptide (Aβ) are thought to play a pivotal role in the pathogenesis of Alzheimer’s disease (AD), but the mechanism involved is still unclear. Here, we generated induced pluripotent stem cells (iPSCs) from familial and sporadic AD patients and differentiated them into neural cells. Aβ oligomers accumulated in iPSC-derived neurons and astrocytes in cells from patients with a familial amyloid precursor protein (APP)-E693D mutation and sporadic AD, leading to endoplasmic reticulum (ER) and oxidative stress. The accumulated Aβ oligomers were not proteolytically resistant, and docosahexaenoic acid (DHA) treatment alleviated the stress responses in the AD neural cells. Differential manifestation of ER stress and DHA responsiveness may help explain variable clinical results obtained with the use of DHA treatment and suggests that DHA may in fact be effective for a subset of patients. It also illustrates how patient-specific iPSCs can be useful for analyzing AD pathogenesis and evaluating drugs.

INTRODUCTION

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disorder. One of the pathological features of AD is the oligomerization and aggregation and accumulation of amyloid-β peptide (Aβ), forming amyloid plaques in the brain. Cognitive impairment observed in clinical AD is inversely well correlated with the amount of Aβ oligomers in the soluble fraction rather than the amount of Aβ fibrils (amyloid plaques) constituting the oligomers (Haass and Selkoe, 2007; Krafft and Klein, 2010). Increasing evidence has shown that Aβ oligomers extracted from AD model mice or made from synthetic peptides cause...
Figure 1. Establishment of Control and AD Patient-Specific iPSCs, and Derivation of Cortical Neurons Producing Aβs from iPSCs

(A) Established iPSCs from both controls and AD patients showed embryonic stem cell-like morphology (Phase) and expressed pluripotent stem cell markers NANOG (red) and TRA1-60 (green). The scale bar represents 200 μm.

(B) Genomic DNA sequences showed the presence of the homozygous genotype for E693 deletion and the heterozygous genotype for V717L mutation on the APP gene only in AD iPSCs.

(C) Estimation of neuronal differentiation from control and AD-iPSCs. After 2 months of differentiation, neurons were immunostained with antibodies against the neuronal marker TUJ1 and the cortical neuron markers TBR1 and SATB2. The scale bar represents 30 μm.

(D) Proportions of TUJ1-, TBR1-, and SATB2-positive cells in control and AD-iPSCs. Data represent mean ± SD (n = 3 per clone).

(E) Aβ40 and Aβ42 secreted from iPSC-derived neural cells into the medium (extracellular Aβ) were measured at 48 hr after the last medium change. Data represent mean ± SD (n = 3 per clone). Levels of Aβ40 and Aβ42 in AD(APP-E693Δ) without β-secretase inhibitor IV (BSI, 1 μM) were significantly lower than those of the others (*, p < 0.006), and the level of Aβ42 and the ratio of Aβ42/Aβ40 in AD(APP-V717L) without BSI were significantly higher than those of the others.

(F) Intracellular levels of Aβ40 and Aβ42 expressed as fmol/mg protein.

(G) Extracellular levels of sAPPβ expressed as pg/ml.

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neurotoxicity and cognitive impairments in vitro and in vivo (Walsh et al., 2002; Gong et al., 2003; Lesné et al., 2006), and this was also true in humans (Kuo et al., 1996; Shankar et al., 2008; Noguchi et al., 2009). Therefore, the formation and accumulation of Aβ oligomers has been presumed to play a central role in the pathogenesis and clinical symptoms of AD. Aβs are composed of 38–43 amino acid residues and are generated from the amyloid precursor protein (APP) by β- and γ-secretase-mediated sequential cleavages. A number of mutations linked to familial AD in the APP gene have been identified. Recently, an atypical early-onset familial AD, caused by an E693A mutation of an APP-producing variant Aβ lacking 22nd Glu was discovered in Japan (Tomiyama et al., 2008). This APP-E693A mutation presents rare, autosomal-recessive mutations of the APP gene related to familial AD. Patients with the mutation show overt early-onset symptoms of AD but lack Aβ deposition, according to positron emission tomography (PET) scan analysis with a [11C] Pittsburgh compound-B (PIB) radiophore (Tomiyama et al., 2008; Shimada et al., 2011). The 22nd Glu within the Aβ sequence has a destabilizing effect on the formation of oligomeric structures because of the electrostatic repulsion between the adjacent side chain of 22nd Glu (Kassier et al., 2010), and the deletion of the amino acid residue leads to the ready formation of Aβ oligomers in vitro (Nishitsuji et al., 2009). APP-E693Δ transgenic mice show AD-like pathology, including intracellular oligomer accumulation, but lack extracellular amyloid plaque formation (Tomiyama et al., 2010). However, it remains unclear whether Aβ oligomers are accumulated in familial and sporadic AD patient neural cells and how intracellular Aβ oligomers play a pathological role. The compound and/or drugs that might rescue the Aβ oligomer-induced pathological phenotypes are also unclear. Recent developments in induced pluripotent stem cell (iPSC) technology have facilitated the investigation of phenotypes of patient neural cells in vitro and have helped to overcome the lack of success in modeling sporadic AD.

Here, we report the derivation and neuronal and astrogial differentiation of iPSCs from a familial AD patient with an APP-E693A mutation, a familial case with another APP mutation, as well as other sporadic cases. Using patient neurons and astrocytes, we addressed the accumulation and possible pathological roles of intracellular Aβ oligomers in familial and sporadic AD. We found that Aβ oligomers were not proteolytically resistant and that docosahexaenoic acid (DHA) treatment attenuated cellular phenotypes of AD neural cells with intracellular Aβ oligomers in both familial and sporadic AD patients.

RESULTS

iPSC Generation and Cortical-Neuronal Differentiation

Dermal fibroblasts were reprogrammed by episomal vectors (Okita et al., 2011). Control iPSC lines from three unrelated individuals, two and three familial AD iPSC lines from patients with E693Δ[AD(APP-E693Δ)] and V717L[AD(APP-V717L)] APP mutations, respectively, and two sporadic iPSC lines (AD3E211 and AD8K213) from two unrelated patients (Figure S1A available online) were generated (Figures 1A, 1B, and S1B–S1H). To characterize cortical neurons derived from the iPSC lines, we established differentiation methods for cortical neurons by modifying previous procedures (Morizane et al., 2011) (Figure S1I). The differentiated cells expressed the cortical neuron subtype markers SATB2 and TBR1 (Figure 1C), and the differentiated neurons were functionally active (Figures S1J and S1K). There was no prominent difference in the differentiation propensity between control and AD neurons (Figures 1D and S1L).

We analyzed the amounts of extracellular Aβ40 and Aβ42 (Figures 1E and 1F). As expected, both Aβ species were strongly decreased in all cloned AD(APP-E693Δ) neural cells in comparison to those in control neural cells. In familial AD(APP-V717L) neural cells, an increase in the extracellular Aβ42 level and a corresponding decrease in the intracellular Aβ42 level were observed, and the Aβ42/Aβ40 ratio in the culture medium was increased up to 1.5-fold, suggesting that the abnormality of APP metabolism in AD is dependent on the mutation sites in APP. Extracellular Aβ levels in sporadic AD neural cells were not changed in comparison to those in control neural cells, but intracellular Aβ in sporadic AD8K213 neural cells apparently decreased (that is, below the detection limit). APP expression levels in the AD(APP-E693Δ) neural cells were lower than in the others, but the levels of x- and β-secretase-mediated APP processing remained unaltered in all neural cells (Figures 1G, S1M, and S1N). Soluble APPβ production was strongly inhibited by treatment with β-secretase inhibitor IV (BSI) (Figure 1G). Aβ levels in the original fibroblasts and iPSC-derived astrocytes, in which APP expression levels were relatively higher than those in neural cells (data not shown), were lower than those of the corresponding neuronal cells (Figures S1O and S1P).

Intracellular Accumulation of Aβ Oligomers in AD(APP-E693Δ) and in One of the Sporadic AD Neural Cells

Using an immunocytochemical method with the Aβ-oligomer-specific antibody NU1 (Lambert et al., 2007), we investigated whether AD(APP-E693Δ) neural cells harbor Aβ oligomers or not. We found that Aβ oligomers were accumulated as puncta in the neurons of AD(APP-E693Δ) and in one of the sporadic AD cases (Figure 2A). The area of Aβ-oligomer-positive puncta was significantly increased in AD(APP-E693Δ) neuronal cells relative to control neuronal cells (Figure 2B). Dot blot analysis using cell lysates revealed that Aβ oligomers were markedly elevated in the AD(APP-E693Δ) and sporadic AD8K213 neural cells (Figures 2C and 2D), whereas Aβ oligomers were not detected in the culture medium (data not shown). Another antibody against Aβ1, 11A1, which detects low-molecular-weight oligomers rather than the Aβ monomer (Murakami et al., 2010), showed results similar to those observed with NU1 (Figures 2E and 2F).
**Figure 2. Familial AD(APP-E693Δ) and Sporadic AD iPSC-Derived Neurons Have Intracellular Ab Oligomers**

(A) Intracellular Ab oligomer accumulation in iPSC-derived neurons (red, MAP2-positive cells) was detected by the Ab-oligomer-specific monoclonal antibody NU1 (green) with a punctate pattern. Ab oligomer accumulation was massive in AD(APP-E693Δ) and sporadic AD(AD8K213) neurons but only faint in control neurons. Treatment with 1 μM BSI decreased Ab oligomer accumulation. DAPI, nuclear staining (blue). The scale bar represents 30 μm.

(B) Quantification of Ab oligomer accumulation in (A); the ratio of the NU1-positive area in the MAP2-positive area was analyzed. Data represent mean ± SD (n = 3 per clone). Ab oligomer levels in the AD(APP-E693Δ) and sporadic AD(AD8K213) neural cells without BSI were significantly different from those of other neural cells (*, p < 0.005) and from corresponding neural cells with BSI (#, p < 0.005).

(C) Dot blot analysis with the use of NU1 antibody. Control (N116213, N117322, 409B2), APP-E693Δ(APP1E111, APP1E211, APP1E211), APP-V717L(APP2E22, APP2E26), and sporadic AD (AD3E211, AD8K213) neural cells were dotted from the left. Blank is RIPA buffer only.

(D) Signals of blot in (C) were quantified. Data represent mean ± SD (n = 3 per clone). Ab oligomer levels in AD(APP-E693Δ) and sporadic AD(AD8K213) neurons without BSI were significantly different from those of other neurons (*, p < 0.001) and from corresponding neurons treated with 1 μM BSI (#, p < 0.001).

(E) Ab oligomer accumulation in AD astrocytes. The scale bar represents 30 μm.

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S2A–S2D). However, Aβ oligomers were not detected in cell lysates from the fibroblasts that generate iPSC lines (Figure S2E). To confirm whether Aβ oligomers were derived from mutant APP(E693Δ), we transduced a lentivector driven by an EF1α promoter to overexpress wild or mutant APP(E693Δ) in control iPSC-derived neural cells and found that Aβ oligomers emerged inside control neural cells overexpressing mutant APP(E693Δ) (Figure S2F).

To investigate the intracellular accumulation of Aβ oligomers in astrocytes derived from control and AD iPSCs, we established an astrocyte-enrichment culture by modifying the method previously reported (Krencik et al., 2011) (Figures S2G–S2J). Dot blot analysis using Aβ oligomer antibodies revealed that the astrocytes of AD(APP-E693Δ) and one of the sporadic AD iPSCs accumulated Aβ oligomers intracellularly (Figures 2E, S2K, and S2L), which was compatible with the results of neurons. On the other hand, we detected no difference in the uptake of extracellular glutamate between control and AD astrocytes (Figure S2M).

Aβ oligomers were also detected as a protein band with a molecular mass of 50–60 kDa by western blot analysis (Figures 2F and S2N). The accumulation of Aβ oligomers was inhibited by treatment with BSI (Figures 2A–2G, S2A–S2D, and S2N). To clarify whether the E693Δ mutation results in accelerated Aβ oligomerization and/or in a proteolytically resistant and stable form of Aβ oligomers, we analyzed the levels of Aβ oligomers over a course of time after BSI treatment. Intracellular Aβ oligomers started to disappear from 2 hr after the treatment with BSI, almost reaching the control level by 8 hr (Figures 2G and 2H). Secretion of Aβ40 from control neural cells was already inhibited at 2 hr after BSI treatment, but the secretion from AD neural cells was under the detection limit in both the presence and absence of BSI (Figure 2I).

**Cellular Stress Responses Caused By Intracellular Aβ Oligomers in AD iPSC-Derived Neural Cells**

Extracellular Aβ deposition in patient brains carrying APP with an E693Δ mutation is predicted to be extremely low, as amyloid PET imaging with a [11C] PIB probe revealed a far lower signal in the patients than those observed in sporadic AD brains (Tomiyama et al., 2008). Given that processing by β- and γ-secretases largely proceeds within vesicular endosomal compartments, it was possible that Aβ oligomers were associated with specific organelles. We characterized the Aβ oligomer-positive punctate structures in AD(APP-E693Δ) neural cells and astrocytes by coimmunostaining with antibodies for markers of vesicular compartments and subcellular organelles. Subpopulations of Aβ oligomer-positive puncta in the AD neurons showed positive immunostaining for an endoplasmic reticulum (ER) marker, binding immunoglobulin protein (BiP); an early endosomal marker, early endosome-associated antigen-1 (EEA1); and a lysosomal marker, lysosomal-associated marker protein 2 (LAMP2) (data not shown).

To uncover molecules that might be implicated in the dysfunction of AD(APP-E693Δ) neural cells, we analyzed gene expression profiles of control and AD neural cells (Figure 3A and Table S1). Gene ontology analysis revealed that oxidative-stress-related categories, including peroxiredoxin, oxidoreductase, and peroxidase activities, were upregulated in the AD, whereas glycosylation-related categories were downregulated (Figures 3B and 3C and Table S1), suggesting that ER and Golgi function might be perturbed in AD neural cells. Western blot analysis clarified that the amounts of both BiP and cleaved caspase-4 were elevated in the neurons and astrocytes of the AD(APP-E693Δ) case, and that of BiP in one of the sporadic AD cases, AD8K213, but not in fibroblasts (Figures 3D–3F and S3A–S3F). We also found that BSI treatment not only prevented the increase in Aβ oligomer-positive puncta area per cell in the context of AD(APP-E693Δ) lines but also decreased the amount of BiP and cleaved caspase-4 (Figures 3D–3F). PRDX4-coding antioxidant protein peroxiredoxin-4 was the most highly upregulated gene (Figure 3C). Western blot analysis confirmed that the amount of peroxiredoxin-4 was increased up to approximately 5- to 7-fold in lysates from AD(APP-E693Δ) and one of the sporadic AD cases, AD8K213 neural cells, but not in fibroblasts, and was decreased by the BSI treatment (Figures 3D, 3G, S3A, S3D, S3G, and S3H), indicating that the antioxidant stress response was provoked by Aβ oligomer formation in AD(APP-E693Δ) and sporadic AD8K213. To identify pathogenic species evoking oxidative stress in AD(APP-E693Δ), we visualized reactive oxygen species (ROS) and found that ROS was increased in both neurons and astrocytes in AD(APP-E693Δ) and AD8K213 (Figures 3H–3J and S3I–S3L). This increase was counteracted by the BSI treatment. These results indicated that intracellular Aβ oligomers provoked both ER and oxidative stress, and the increase in ROS most likely occurred via a vicious cycle between ER and oxidative stress (Malhotra and Kaufman, 2007).

**Allieviation of Intracellular Aβ Oligomer-Induced Cellular Stress by DHA**

We evaluated BSI and three additional drugs that had been reported to improve ER stress or to inhibit ROS generation: (1) DHA (Begum et al., 2012), (2) dibenzoylmethane (DBM14-26) (Takano et al., 2007), and (3) NSC23766 (Lee et al., 2002) (Figures 4 and S4). DHA treatment significantly decreased the protein level of BiP, cleaved caspase-4, and peroxiredoxin-4 in AD (APP-E693Δ) neural cells (Figures 4A, 4B, S4A, and S4B), and BiP and peroxiredoxin-4 in sporadic AD8K213 (Figures S4C and S4D). Furthermore, DHA treatment also decreased the generation of ROS in AD(APP-E693Δ) neural cells (Figures 4C and 4D), whereas the amount of Aβ oligomers in cell lysates

(F) Western blot analysis of control and AD neural cells in the presence or absence of BSI. BSI treatment (1 μM) disappeared 6E10-positive = 55 kDa protein bands in cell lysates of AD(APP-E693Δ) and sporadic AD(AD8K213) neural cells.

(G) Disappearance of Aβ oligomers after BSI treatment was analyzed by dot blot analysis with the use of the NU1 antibody. Intracellular Aβ oligomers started to disappear 2 hr after BSI treatment.

(H) Signals of blots in (G) were quantified. Data represent mean ± SD (n = 3 per clone). BSI treatment (1 μM) decreased intracellular Aβ in AD neural cells and was reduced to 16–23% of vehicle control by 8 hr. Post hoc analysis revealed that the amounts of Aβ oligomers at 2 hr after BSI treatment were significantly decreased in comparison to those of DMSO control oligomers (*, p < 0.005).

(I) Changes in extracellular Aβ40 levels were analyzed in the experimental condition of (G). Data represent mean ± SD (n = 3 per clone).

See also Figure S2.
Figure 3. Cellular Stress Responses Caused by Intracellular Aβ Oligomers in Familial AD(APP-E693D) and Sporadic AD(AD8K213) iPSC-Derived Neural Cells

(A) Hierarchical clustering analysis of differentiated neuronal cells and a heatmap of significantly up- and downregulated genes in AD neural cells. The statistically significant cutoff p value is < 0.05.

(B) GO term and p value
- Oxidative stress related gene ontology
  - Peroxidase activity: 0.0169007
  - Peroxidase activity: 0.0169007
- Glycosylation related gene ontology
  - Heme-lysyl oxidase: 1.54E-06
  - Beta-glucuronidase activity: 1.54E-06
  - Heme-lysyl oxidase, acting on glycoproteins: 8.89E-06
  - Glycosaminoglycan catabolic process: 1.65E-06
  - Polynucleotide catabolic process: 3.84E-06
  - Glycosaminoglycan metabolic process: 0.004289

(C) List of genes related to peroxiredoxin activity
- PRDX4
- FXN
- MGST3
- MEG31
- PRDX1
- PRDX5

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was not altered (Figures S4E–S4G). In contrast, the high concentration of DHA, DBM14-26, or NSC23766 treatment increased the protein level of BiP (Figure S4B). Finally, to confirm the protective effects of DHA in short-term screening, we analyzed the effect on the survival of AD(APP-E693Δ) neural cells. Neuronal cells were labeled with a lentiviral vector expressing synapsin I-promoter-driven EGF and cultivated in the medium depleted of neurotrophic factors and neural culture supplements mix. The real-time survival rate of AD(APP-E693Δ) neurons was lower than that of normal control neurons; however, DHA treatment for 16 days partially rescued AD(APP-E693Δ) cell viability (Figures 4E–4G). The real-time survival rate of sporadic AD(AD3E211, AD8K213) neurons for 16 days was unchanged (Figures 4E and 4F and Table S2). We confirmed these results through a lactate dehydrogenase (LDH) assay (Figure 4G). The AD(APP-E693Δ) neurons were also vulnerable to oxidative stress by hydrogen peroxide treatment (Figure S4H). Extracellular Aβ levels were not altered in the assay (Figure 4H).

**DISCUSSION**

The present study shows that neural cells derived from a patient carrying the pathogenic APP-E693Δ mutation and a sporadic AD patient produce intracellular Aβ oligomers, and the use of these neural cells provided an experimental system for addressing whether such oligomers would cause cellular stress and the killing of neurons and how such intracellular Aβ oligomers might contribute to the disease pathology, despite only one patient carrying the E693Δ mutation being available. Our findings also suggest that the possible heterogeneity of familial and sporadic AD stems from phenotypic differences of intracellular Aβ oligomers and suggests the possibility that DHA, a drug that failed in some clinical trials of AD treatment, might be effective in a portion of AD patients.

We demonstrated that Aβ oligomers were formed and accumulated inside AD(APP-E693Δ) and sporadic AD(AD8K213) neurons by immunostaining (Figures 2A and 2B), dot blot analysis (Figures 2C and 2D), and western blot analysis (Figures 2F and S2N). In addition, accumulation of Aβ oligomers, which has been supposed to be proteolytically resistant, disappeared after treatment with BSI in both AD neurons (Figures 2G and 2H), indicating that AD(APP-E693Δ) and sporadic AD(AD8K213) neurons still seemed to retain a degrading activity toward Aβ oligomers in which proteasomes, autophagosomes, and/or lysosomes may be involved and, thereby, that the pathological property of Aβ oligomers in a part of AD might be completely abrogated. The sporadic AD(AD8K213) neurons may retain a specific cellular environment that permits the formation of Aβ oligomers. Additional studies aimed at identifying the factors causing such an environment are needed.

We observed that the accumulation of Aβ oligomers induced ER and oxidative stress both in AD(APP-E693Δ) and in sporadic AD(AD8K213) neurons, although caspase-4 activation appeared not to accompany sporadic AD, probably because of the lesser extent of ER stress in comparison to AD(APP-E693Δ). Previously, Nishitsuji et al. (2009) reported that accumulated Aβ oligomers in ER provoke ER stress. This result suggests that oligomers represent a self-aggregating state of Aβ. During this process, Aβ generates ROS, which is supported by the fact that Aβ coordinates the metal ions zinc, iron, and copper, which induce the oligomerization of Aβ. Iron and copper then cause the generation of toxic ROS and calcium dysregulation (Barnham et al., 2004), leading to membrane lipid peroxidation and the impairment of the function of a range of membrane-associated proteins (Hensley et al., 1994; Butterfield, 2003), antioxidant factors being thought to protect ER-stress-induced cellular toxicities (Malhotra and Kaufman, 2007).

We found that intracellular Aβ oligomers were accumulated not only in a case of familial AD with APP-E693Δ mutation but also in a sporadic AD case, although only three clones derived from one familial AD patient carrying an APP-E693Δ mutation and two clones from two sporadic AD patients were analyzed in this study because of the limited number of patients. In contrast, in familial AD with the APP-V717L mutation, of which only one case was available, intracellular Aβ oligomers were not detected, but the extracellular Aβ/42/Aβ/40 ratio, which is increased in mutant presenilin-mediated familial AD, as reported previously (Yagi et al., 2011), was increased, lending support to the notion that AD could be classified into two categories: extracellular Aβ type and intracellular Aβ type. Although it has been supposed that environmental factors and/or the aging process contribute to neurodegenerative diseases, our findings support the idea that a genetic factor might play a role in a part of sporadic AD, a finding that is compatible with a previous report (Israel et al., 2012). However, identifying the genetic factor would require a larger sample size. The sporadic AD case with intracellular Aβ oligomers might correspond to the case without extracellular Aβ elevation of Israel et al. (2012). Analysis of neurons
Figure 4. DHA-Alleviated Cellular Stress Caused By Intracellular Aβ Oligomers

(A) Control and AD(APP-E693Δ) neural cells at day 72 were treated with DHA for 48 hr. Then, cells were lysed and subjected to immunoblot analysis (1 μM, 5 μM, and 15 μM of docosahexaenoic acid [DHA]).

(B) Densitometric analysis of (A) is shown. Measured values were normalized by that of β-actin. Data represent mean ± SD (n = 3 per clone). Two-way analysis of variance (ANOVA) showed significant main effects of DHA treatment (BIP, F[3,64] = 136.712, p < 0.001; cleaved caspase-4, F[3,64] = 50.855, p < 0.001) with a significant interaction between APP mutation and DHA treatment (BIP, F[3,64] = 99.658, p < 0.001; cleaved caspase-4, F[3,64] = 53.005, p < 0.001). Post hoc analysis revealed significant differences between DMSO (control) and DHA treatment (1, 5, and 15 μM) in AD(APP-E693Δ) neural cells (**, p < 0.001). Two-way ANOVA for peroxiredoxin-4 showed significant main effects of DHA treatment (F[3,64] = 16.995; p < 0.001) with a significant interaction between APP mutation and DHA treatment (F[3,64] = 32.093; p < 0.001). Post hoc analysis revealed significant differences between DMSO-control and DHA treatment (5 and 15 μM) in AD(APP-E693Δ) neural cells (**, p < 0.001). In control neural cells, the 5 μM DHA group was significantly different from the other groups (#, p < 0.005).

(C) Typical images of ROS-CellROX and Hoechst33342 signals after treatment with vehicle or 5 μM DHA. The scale bar represents 50 μm.

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and astrocytes, as we performed here, from larger numbers of patients might result in the classification of sporadic AD.

To date, the clinical effectiveness of DHA treatment is still controversial (Freund-Levi et al., 2006; Quinn et al., 2010). It is of particular interest that one of two sporadic AD neurons accumulated intracellular Aβ oligomers and showed cellular phenotypes that could respond to DHA but the other did not, and this result may explain why DHA treatment was effective for some AD patients, those with the intracellular Aβ oligomer-associated type of AD, although the timing (that is, the stage of disease development) for starting the treatment would be another critical factor. These results may suggest that patient-specific iPSCs provide a chance to re-evaluate the effect of a drug that failed in AD clinical trials, depending on the selection of the patient type. In the present study, the amount of Aβ oligomers in our culture was not affected by DHA, although it would be effective for reducing cellular stresses, and reducing the oligomerization of Aβ was also presumed to be a candidate mechanism of DHA treatment (Cole and Frautschy, 2006). These results indicate that therapy with DHA would alleviate symptoms. Furthermore, the data showing that BSI treatment leads to a reduction in ROS formation at a relatively similar level (Figure 2G) in both AD and control cells might indicate an Aβ oligomer-independent effect, in addition to an Aβ oligomer-dependent effect, of BSI.

In any event, patient-specific iPSCs would provide disease pathogenesis, irrespective of the disease being in a familial or sporadic form, as well as enable the evaluation of drug and patient classification of AD.

**EXPERIMENTAL PROCEDURES**

**Derivation of Patient-Specific Fibroblasts**

Control and AD-derived human dermal fibroblasts (HDFs) were generated from explants of 3 mm dermal biopsies. After 1–2 weeks, fibroblast outgrowths from the explants were passaged.

**iPSC Generation**

Human complementary DNAs for reprogramming factors were transduced in HDFs with episomal vectors (SOX2, KLF4, OCT4, L-MYC, LIN28, and small hairpin RNA for p53). Several days after transduction, fibroblasts were harvested and replated on an SNL feeder cell layer. On the following day, the medium was changed to a primacy embryonic stem cell medium (ReproCELL, Japan) supplemented with 4 ng/ml basic FGF (Wako Pure Chemical Industries, Japan). The medium was changed every other day. iPSC colonies were picked up 30 days after transduction.

**Statistical Analysis**

All data are shown as mean ± SD. For comparisons of the mean between two groups, statistical analysis was performed by applying Student’s t tests after confirming equality between the variances of the groups. When the variances were unequal, Mann-Whitney U tests were performed (SigmaPlot 11.2.0, Systat Software, USA). Comparisons of the mean among three groups or more were performed by one-way, two-way, or three-way analysis of variance followed by a post hoc test with the use of Student-Newman-Keuls Method (SigmaPlot 11.2.0). p values < 0.05 were considered significant.
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Supplemental Figures

Figure S1. Characterization of iPSCs and Neuronal Cells Derived From iPSCs, Related to Figure 1
Figure S2. Analysis of Intracellular Accumulation of Aβ Oligomers in AD Fibroblasts, iPSC-Derived Neurons, and iPSC-Derived Astrocytes, Related to Figure 2
Figure S3. Analysis of Cellular Stress Responses in AD Fibroblasts and Astrocytes, Related to Figure 3
Figure S4. Drug Evaluation in Relation to Improvement in ER Stress and Aβ Oligomer Accumulation, Related to Figure 4

Table S1. Changes in Gene Expression between Control and AD iPSC-Derived Neural Cells and Gene Ontology Analysis, Related to Figure 3
Table S2. Summary of iPSC Clones and Their Phenotypes, Related to Figure 4
Figure S1. Characterization of iPSCs and Neuronal Cells Derived from iPSCs, Related to Figure 1

(A) Summary of iPSC clones.

(B) Morphology and expression of human embryonic stem cell markers. iPSCs from both control and AD patients showed ES-like morphology (Phase image) and expressed pluripotent stem cell markers, NANOG and TRA1-60. Scale bar: 200 μm.

(C) iPSCs from both control and AD patients showed a normal karyotype.

(D) Bisulfite genomic sequencing of the promoter regions of NANOG and OCT4. Open and closed circles indicate unmethylated and methylated CpGs, respectively. CpGs of both NANOG and OCT4 promoters in all iPS clones were demethylated.

(E) In vitro differentiation of established iPSCs to representative three-germ layer: TUJ1 (ectoderm), αSMA (mesoderm), and SOX17 (endoderm). Scale bar: 50 μm.

(F) In vivo (teratoma) differentiation with evidence of all three germ layers: cartilage (mesoderm), gut-like epithelium (endoderm), and neural tube-like tissues (ectoderm). Scale bar: 50 μm.

(G) Hierarchical clustering showing that the global gene expression profiles of control and AD hiPSCs as similar to human embryonic stem cells (H9) and different from human fibroblasts.

(H) Pearson’s correlation coefficient of each global gene profile.

(I) Schematic diagram showing the differentiation protocol of cortical neurons.

(J) A typical recorded neuron identified using Synapsin::EGFP. Scale bar: 50 μm.

(K, left panel) Whole-cell voltage-clamp recordings of Na⁺ (rapid inward) and K⁺ (slow outward) currents. Cells were held at –60mV, and voltage was increased stepwise from –60 mV to +30 mV in 10-mV intervals.

(K, right panel) Current-clamp recordings of spontaneous action potentials. Na⁺/K⁺ currents and action potentials were measured to examine whether iPSC-derived neurons were functionally active. In 20 EGFP-positive neurons, 95% (n = 19) presented Na⁺/K⁺ currents, and 80% (n = 16) exhibited action potentials. Representative figures are shown. Inward currents, which were estimated to be spontaneous postsynaptic currents, were also recorded in 75% (n = 15) (data not shown).

(L) Both control and AD iPSC-derived neurons expressed neuronal marker (TUJ1, Green) and cortical transcriptional markers (Red, including TBR1/SATB2). Scale bar: 100 μm.

(M) Full-length APP levels in cell lysates from control and AD neural cells were measured by quantitative western blot analysis. FL-APP expression levels in AD(APP-E693Δ) were significantly different from those in the other neural cells (*p < 0.001). Data represent mean ± SD (n = 3 per group)

(N) Soluble APPα levels in culture media from control and AD neural cells were measured by MSD electrochemiluminescence assay. Data represent mean ± SD (n = 3 per group).

(O, P) Extracellular Aβ in control and AD fibroblasts (O), and in control and AD iPSC-derived astrocytes (P), were measured and the Aβ42/Aβ40 ratio was calculated. Levels of Aβ40 and Aβ42 in AD(APP-E693Δ) fibroblasts were significantly lower than those of the others (*p < 0.001), and levels of Aβ42 and the ratio of Aβ42/Aβ40 in AD(APP-V717L) were significantly higher than those of the others (*p < 0.006). Aβ40 and Aβ42 levels and the ratio of Aβ42/Aβ40 in AD(APP-E693Δ) astrocytes are significantly lower than those of the others (*p < 0.001). Data represent mean ± SD (n = 3 per group)
Figure S2. Analysis of Intracellular Accumulation of Aβ Oligomers in AD Fibroblasts, iPSC-Derived Neurons, and iPSC-Derived Astrocytes, Related to Figure 2

(A) Intracellular Aβ oligomer accumulation in iPSC-derived neurons (Red, MAP2-positive cells) was detected by anti-Aβ oligomer monoclonal antibody 11A1 (Green) with a punctate pattern. Aβ oligomer accumulation was massive in AD(APP-E693Δ) neurons, but only faint in control neurons. Treatment with 1 μM BSI decreased Aβ oligomer accumulation in AD neurons. DAPI staining (Blue). Scale bar: 30 μm.

(B) Quantification of Aβ oligomer accumulation in panel A; ratio of the 11A1-positive area in the MAP2-positive area was analyzed. Data represent mean ± SD (n = 3 per group). Levels of Aβ oligomers in AD(APP-E693Δ) and sporadic AD(AD8K213) neurons without BSI were significantly different from those of the other neurons (*p < 0.001).

(C) Dot-blot analysis using anti-Aβ oligomer antibody 11A1. Control (N116213, N117322, 409B2), APP-E693Δ(APP1E111, APP1E211, APP1E211), APP-V717L (APP2E22, APP2E26), sporadic AD (AD3E211, AD8K213) are dotted from the left. Blank is RIPA buffer only.

(D) Signals of blots in (C) were quantified. BSI treatment (1 μM) decreased Aβ oligomers in AD neural cells. Levels of Aβ oligomers in AD(APP-E693Δ) and sporadic AD(AD8K213) neural cells without BSI differed significantly from those of the other neural cells (*p < 0.005). Data represent mean ± SD (n = 3 per group).

(E) Aβ oligomers were not accumulated in control and AD fibroblasts.

(F) Control neurons, transduced APP with lentivirus expressing APP-E693Δ under control of EF1α promoter, presented intracellular Aβ oligomer formation. Intracellular Aβ oligomer accumulation in iPSC-derived neurons (Red, MAP2-positive cells) was detected by anti-Aβ oligomer antibody NU1 (Green, dot-like pattern). Aβ oligomer accumulation was massive in the mutant APP transduction, but only faint in wild-type APP. Scale bar: 30 μm.

(G) Schematic procedures for astroglial differentiation.

(H) Hierarchical clustering analysis of fibroblasts, iPSCs, primary astrocytes, and differentiated neural stem cells and astrocytes.

(I) Estimation of astroglial differentiation from control and AD-iPSCs. After 180 days of differentiation, astrocytes were immunostained with an antibody against GFAP. Scale bar: 100 μm.

(J) Proportions of GFAP-positive cells in control and AD-iPSCs. Data represent mean ± SD (n = 3 per clone).

(K) Dot-blot analysis using anti-Aβ oligomer antibody NU1. Cell lysates of control (N116213, N117322, 409B2), APP-E693Δ(APP1E111, APP1E211, APP1E211), APP-V717L (APP2E22, APP2E26), and sporadic AD (AD3E211, AD8K213) astrocytes were dotted from the left. Blank is RIPA buffer only.

(L) Signals of blots in panel F were quantified. Data represent mean ± SD (n = 3 per group). Levels of Aβ oligomers in AD(APP-E693Δ) and sporadic AD(AD8K213) astrocytes were significantly different from those of the other astrocytes (*p < 0.001).

(M) Assay of glutamate transport in control and AD astrocytes. Data represent mean ± SD (n = 3 per group).

(N) Full western blot of Aβ oligomers (dodecamers) in control and AD neural cells in the presence or absence of BSI.
Figure S3. Analysis of Cellular Stress Responses in AD Fibroblasts and Astrocytes, Related to Figure 3

(A) Western blot analysis of ER stress markers (BiP, caspase-4), peroxiredoxin-4, and reference protein (β-actin) in lysates from control and AD fibroblasts.

(B, C) Densitometric analysis of BiP protein (B) and cleaved caspase-4 (C) in (A). Their measured values were normalized by that of β-actin. Data represent mean ± SD (n = 3 per group).

(D) Western blot analysis of ER stress markers (BiP, caspase-4) and reference protein (β-actin) in lysates from control and AD astrocytes.

(E, F) Densitometric analysis of BiP protein (E) and cleaved caspase-4 (F) in panel D. Their measured values were normalized by that of β-actin. Data represent mean ± SD (n = 3 per group). Levels of ER stress markers in the AD(APP-E693Δ) astrocytes were significantly different from those of the others (*p < 0.001). Levels of BiP in the sporadic AD(AD8K213) astrocytes were significantly different from those of the others (*p < 0.001).

(G) Densitometric analysis of peroxiredoxin-4 in panel A. Their measured values were normalized by that of β-actin. Each column represents mean ± SD (n = 3 per group).

(H) Densitometric analysis of peroxiredoxin-4 in panel D. Their measured values were normalized by that of β-actin. Each column represents mean ± SD (n = 3 per group). Levels of peroxiredoxin-4 in the AD(APP-E693Δ) and the sporadic AD(AD8K213) astrocytes were significantly different from those of the others (*p < 0.001).

(I) Typical images of ROS detected by ROS-HFF and Hoechst33342 staining of control and AD fibroblasts with or without BSI treatment. Scale bar: 30 μm.

(J) Quantitative data of panel I. Each value is shown as a ratio of the ROS-stained area, adjusted with DAPI counts. Data represent mean ± SD (n = 3 per group).

(K) Typical images of ROS detected by ROS-CellROX and Hoechst33342 staining of control and AD fibroblasts with or without BSI treatment. Scale bar: 30 μm.

(L) Quantitative data of panel K. Each value indicates a ratio of the ROS-stained area, adjusted with DAPI counts. Data represent mean ± SD (n = 3 per group). Levels of ROS in AD(APP-E693Δ) and sporadic AD(AD8K213) astrocytes were significantly different from those of the others (*p < 0.001).
Figure S4. Drug Evaluation in Relation to Improvement in ER Stress and Aβ Oligomer Accumulation, Related to Figure 4

(A) After treatment with vehicle or DHA at indicated concentrations, cells were lysed and subjected to immunoblot analysis with antibodies of an ER stress marker (BiP) and a reference protein (β-actin) (DHA concentration: 4, 20 and 100 μM).

(B) Densitometric analysis of ER stress markers with or without drug treatment: 1) DHA, 2) DBM14-26, 3) NSC23766. Data represent mean ± SD (n = 3 per group). Measured values were normalized by that of β-actin. Data represent mean ± SD (n = 3 per group, per each concentration).

(C) Sporadic AD(AD8K213) neural cells at day 72 were treated with vehicle or DHA at indicated concentrations for 48 h. Then, cells were lysed and subjected to immunoblot analysis with antibodies of ER stress markers (BiP, caspase-4), peroxiredoxin-4 and a reference protein (β-actin) (DHA concentration: 5 and 15 μM).

(D) Densitometric analysis of ER stress markers and peroxiredoxin-4 in panel C. Measured values were normalized by that of β-actin. Each column represents mean ± SD (n = 3 per group) (*p < 0.005).

(E) Dot-blot analysis using NU1 antibody, after treatment with vehicle or DHA at indicated concentrations.

(F) Densitometric analysis of dot-blot analysis using NU1 antibody, with or without drug treatment. Data represent mean ± SD (n = 3 per group).

(G) Summary of effects of drugs.

(H) Treatment with 10 μM DHA (n = 3) for 48 h blocked hydrogen peroxide (200 μM)-induced death of AD neurons. Data represent mean ± SD (n = 3 per group). The control group of AD(APP-E693Δ) neural cells was significantly different from the other groups (*p < 0.005).
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>APP E693A (APP1E 111 211, 311)</th>
<th>APP V717L (APP2E 22, 26)</th>
<th>Sporadic AD 1 (AD3E 211)</th>
<th>Sporadic AD 2 (AD8K 213)</th>
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<tr>
<td>Intracellular Aβ oligomer</td>
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<td>BiP</td>
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<td>Survival rate</td>
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Intracellular Aβ oligomers, cellular stresses and survival rates of iPSC clones.
+
+, accumulated; -, not detected; ↑, increased; ↓, decreased; →, no significant change.
Supplemental Experimental Procedures

Karyotyping and Genotyping
Karyotyping was performed in our institute. Genotyping of APP single nucleotide mutation was performed by PCR amplification of genomic DNA and directly sequenced (3100 Genetic Analyzer; Applied Biosystems, Life Technologies). APOE gene was amplified by PCR (forward primer TCC AAG GAG CTG CAG GCG GCG CA; reverse primer ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC A). The PCR products were digested by HhaI at 37°C for 2 h and then subjected to electrophoresis to analyze band size.

In Vitro Differentiation
hiPSCs were harvested by treatment with CTK solution and used for embryoid body (EB) formation. Clumps of cells were transferred to petri dishes in DMEM/F12 containing 20% knockout serum replacement (KSR; Life Technologies), 2 mM L-glutamine, 0.1 M nonessential amino acids, 0.1 M 2-mercaptoethanol (Life Technologies), and 0.5% penicillin and streptomycin. The medium was changed every other day. For spontaneous differentiation, 8-day-old EBs were plated onto gelatin-coated coverslips and allowed to differentiate in DMEM + 10% fetal bovine serum for an additional 8 days.

Generation of hiPSC-Derived Teratomas
Undifferentiated hiPSCs were harvested by CTK solution and the pellets were suspended in DMEM/F12. The cells were subcutaneously transplanted into NOG mice (Central Institute for Experimental Animals, Japan). Eight weeks after injection, tumors were dissected and fixed with PBS containing 4% formaldehyde. Paraffin-embedded tissue was sliced and stained with hematoxylin/eosin.

Bisulfite Genomic Sequencing
Genomic DNA (1 μg) from iPS cells was processed for bisulfite modification using an EZ DNA methylation Gold Kit (Zymo Research Corporation). One conserved CpG-enriched region in the Oct-4 promoter and one conserved CpG-enriched region in the NANOG promoter were selected for amplification by PCR with ExTaq Hot start version (TaKaRa Bio, Japan). The PCR products were subcloned into pCR4 vector (Life Technologies). Ten clones of each sample were verified by sequencing with Sp6 universal primer. All primer sequences were as follows: Oct4-3F ATT TGT TTT TTG GGT AGT TAA AGG T; Oct4-3R CCA ACT ATC TTC ATC TTA ATA ACA TCC; NANOG_BS-F TGG TTA GGT TGG TTT TAA ATT TTT G; NANOG_BS-R AAC CCA CCC TTA TAA ATT CTC AAT TA.

Microarray Analysis for iPSCs
Total RNA from HDF and hiPSCs was labeled with Cy3. Samples were hybridized with Whole Human Genome Microarray 4 Å~44K (G4112F, Agilent Technologies). Each sample was hybridized once with the one-color protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent Technologies). Data were analyzed by GeneSpring GX7.3.1 software (Agilent Technologies).

Induction of Cortical Neuron Differentiation
hiPSCs were dissociated to single cells and quickly reaggregated in U-bottom 96-well plates for suspension culture (Greiner Bio-One), pre-coated with 2% Pluronic F-127 (Sigma-Aldrich) in 100% ethanol (Supplementary Figure S3A). Aggregations,
embryoid bodies (EB), were cultured in ‘DFK5% medium’ (DFK5%; DMEM/Ham’s F12 (Gibco, USA) supplemented with 5% KSR (Gibco), NEAA (Invitrogen), L-glutamine (Sigma-Aldrich), 0.1 M 2-mercaptopethanol (Invitrogen)) with or without 2 μM dorsomorphin (Sigma-Aldrich) and 10 μM SB431542 (Cayman Chemical) in a neural inductive stage (day 0 to 8). After neural induction, EB were transferred onto Matrigel (Becton Dickinson)-coated 6-well culture plates and cultured in DFK5% supplemented with 1x N2 supplement (Invitrogen), 2 μM dorsomorphin, and 10 μM SB431542 in the patterning stage (day 8 to 24). A large number of neural precursor cells (NESTIN-positive) were observed to migrate from the EB core. After the patterning stage, migrated neural precursor cells were separated from the plate bottom using Accutase (Innovative Cell Technologies, Inc.) and cultured in Neurobasal medium FULL, Neurobasal Medium (Invitrogen/Gibco) supplemented with 1x B27 without Vitamin A (Invitrogen/Gibco), 1x Glutamax (Invitrogen/Gibco), 10 ng/ml BDNF, 10 ng/ml GDNF and 10 ng/ml NT-3 on Matrigel-coated 24-well culture plates or cover-slip in the neural maturation stage (day 24 to 72).

Electrophysiological Recordings
Whole-cell patch-clamp recordings were performed from iPSC-derived neurons expressing Synapsin::EGFP by lentivirus vector under fluorescence microscopy in combination with differential interference contrast imaging. The recording micropipettes were filled with intracellular solution consisting of 140 mM KCl, 2 mM MgCl2, 10 mM HEPES, and 1 mM EGTA, adjusted to pH 7.4 with NaOH. Cells were maintained at 30°C during the experiment, and were continuously superfused with oxygenated Krebs-Ringer solution consisting of 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 1 mM MgCl2, 2 mM CaCl2, and 20 mM glucose. Voltage-clamp and current-clamp recordings were made using an EPC 9 amplifier (HEKA Elektronik, Lambrecht) and data were analyzed with Patchmaster software (HEKA).

Production of VSV-G-Pseudotyped Lentivirus
EF1α (kind gift from Dr. Akitsu Hotta) or Synapsin I promoter was inserted into pENTR1A vector (Invitrogen). The production of VSV-G-pseudotyped lentivirus was performed as reported previously (Hioki et al. 2007). Briefly, the viral plasmid carrying a gene was cotransfected with a mixture of packaging plasmids (pLP1, pLP2, and pLP/VSVG; Invitrogen) into the 293FT producer cell line (Invitrogen), using LipoFectamine 2000 (Invitrogen). Sixty hours after transfection, the viral particles in the culture supernatant were collected and filtered through 0.45-μm filters (Millipore, Corning).

Immunocytochemistry
Cells were fixed in 4% paraformaldehyde (pH 7.4) for 30 min at room temperature and rinsed with PBS. The cells were permeabilized in PBS containing 0.2% Triton X-100 for 10 min at room temperature, followed by rinsing with PBS. Nonspecific binding was blocked with PBS containing 10% donkey serum for 60 min at room temperature. Cells were incubated with primary antibodies overnight at 4°C, and then labeled with appropriate fluorescent-tagged secondary antibodies. DAPI (Life Technologies) was used to label nuclei. Fluorescence images were acquired on an FV1000 laser-scanning confocal microscope (OLYMPUS) or LSM710 microscope (Carl Zeiss). The following primary antibodies were used in immunocytochemistry: NANOG (1:10 dilution; R&D Systems), TRA1-60 (1:400; Cell Signaling Technology), αSMA (1:1,000; DAKO), SOX17 (1:200; Millipore), TUJ1 (1:4,000; Chemicon), CTIP2 (1:400; Abcam), SATB2 (1:800; Abcam), TBR1 (1:800; Abcam), NU1 (1:100), MAP2 (1:1,000; Millipore), BiP (1:400; Millipore), GFAP (1:2,000; DAKO) and 11A1 (1:400; IBL, Japan). 3’-(p-hydroxyphenyl) fluorescein (HPF) (Sekisui Medical, Japan) or CellROX deep red reagent (Invitrogen) was used to image intracellular ROS according to the manufacturer’s protocol. For evaluating the positive count ratio of immunocytochemistry, we imaged the cells using automated microscopy by IN CELL Analyzer 2000 (GE Healthcare) and then
counted the immunostained structures by using IN CELL Developer toolbox software 1.9 (GE Healthcare).

**Immunoblots**

Cells were lysed in RIPA buffer (50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktail (Roche Diagnostics)). Each 5-, 10- or 20-μg sample of protein was subjected to SDS-PAGE (5-20% gradient SDS-polyacrylamide gels, ATTO Co.), and separated proteins were transferred to polyvinylidene fluoride membrane (Hybond-P, GE Healthcare) or nitrocellulose membrane (PROTRAN; 0.2 µm pore-size, Whatman). The membranes were incubated with primary antibodies, followed by appropriate secondary antibodies, and then visualized using ECL plus or ECL prime (GE Healthcare). For dot-blot analysis, cell lysate samples (each 2 or 4 µg/spot) were loaded on a nitrocellulose membrane. The membranes were incubated with primary antibodies, followed by appropriate secondary antibodies, and then visualized using ECL prime (GE Healthcare). The images were acquired on LAS 4000 (GE Healthcare). The intensity of the protein band or spot was analyzed using Science Laboratory 2001 Image Gauge software (GE Healthcare). Each set of experiments was repeated at least two times to confirm the results. The following primary antibodies were used: NU1 (1:1,000), 6E10 (1:3,000), BiP (1:7,500; Abcam), caspase-4 (1:3,000, MBL), peroxiredoxin-4 (1:6,000, Abcam), and β-actin (1:20,000, MBL).

**Extracellular Aβ Measurement Using Electrochemiluminescence Assays**

Aβ species and sAPPβ in culture media were measured by Human (6E10) Aβ 3-Plex Kit and sAPPβ Kit (Meso Scale Discovery). For Aβ species, this assay uses 6E10 antibody to capture Aβ peptide and SULFO-TAG-labeled different N-terminus specific anti-Aβ antibodies for detection by electrochemiluminescence with Sector® Imager 6000 (Meso Scale Discovery). Each sample was measured in duplicate, and the mean was adopted.

**Intracellular Aβ Measurement Using Sandwich ELISA**

At day 72 from neuronal differentiation, two-day incubated conditioned media were collected from cultured neuronal cells (2 x 10^5 cells) and centrifuged at 4,000 g for 10 min. The resultant clear supernatants were added to 6 M guanidine solution containing 50 mM Tris-HCl, protease inhibitor cocktail Complete (Roche Diagnostics) and 0.7 µg/ml pepstatin A (Peptide Institute, Japan) (pH 7.6) to give 0.5 M (final concentration), and then sonicated before application to ELISA as an extracellular fraction. The cells were washed twice with ice-cold PBS, collected with a cell scraper, and sonicated in 6 M guanidine solution. The cell lysate was diluted 12 times to reduce the concentration of guanidine and was then used as intracellular or cell-associated fraction. The samples were subjected to sandwich ELISA (Immuno-Biological Laboratories Co.) with a combination of monoclonal antibodies specific to the N-terminal of Aβ and specific to the C-terminal of Aβ40 or Aβ42, to determine the amounts of secreted Aβ. We also examined the effect of BACE1 inhibitor IV (Merck) on Aβ oligomer levels. All media were replaced with new media containing 1 µM BACE1 inhibitor IV, and two-day conditioned media were analyzed as described above.

**Astroglial Differentiation**

At day 90 from neuronal differentiation, iPS-derived neuronal cells were plated at 40 x 10^5 cells per 6-cm dish without coating in Neurobasal medium FULL (described above). After passage, neurons could not attach to a non-coated polystyrene dish surface or they died by anoikis. Astroglia, on the other hand, could attach and proliferate. By repeated passage in the same manner at days 120, 150 and 180, astroglia increased their own abundance ratio and showed positive GFAP-immunostaining.
(more than 80%). After day 200 from neuronal differentiation, culture medium was changed to DMEM/F12 Glutamax (Invitrogen/Gibco) with 10% FBS (Invitrogen/Gibco) and astroglial cells were used for assay or stocked in a -80°C deep-freezer. Human primary astrocytes were purchased from Lonza as control cells for astrocyte differentiation.

**Glutamate Clearance Assay**
Differentiated astroglial cells were replated at 4 x10^4 cells per well in 48-well plates coated with 0.1% gelatin. Three days after replating, L-glutamate (final concentration 250 μM) (Nacalai, Japan) was added to assess glutamate uptake. To measure extracellular glutamate concentrations, Glutamate Assay Kit colorimetric assay II (Yamasa Corporation, Tokyo, Japan) was used. We added an equivalent amount of glutamate (250 μM concentration) to iPS-derived astrocyte culture medium and calculated glutamate uptake at 1-4-hour time points.

**Microarray Analysis for Differentiated Neural Cells**
Total RNA from differentiated neural cells was extracted by RNasey mini kit (QIAGEN) and altered into fragmented/BIOTINYLATED cDNA by Ovation Pico WTA System/ Encore Biotin Module kit (NuGENE). Fragmented cDNA samples were hybridized with GeneChip Human Gene 1.0 ST Array (Affymetrix). Each sample was hybridized once with the one-color protocol. Arrays were scanned with a GeneChip® Scanner 3000 7Go (Affymetrix). Data were analyzed by GeneSpring GX7.3.1 software (Agilent Technologies).

**Drug Treatment**
iPSC-derived neurons at day 72 were incubated in media containing BSI, DHA (Nacalai), DBM14-26 or Rac1 inhibitor (NSC23766, Calbiochem). All media were replaced with new media containing each drug, and iPSC-derived neurons were subjected to immunoblot analysis 48h later.

**Neuronal Survival Analysis**
At day 65 after neuronal differentiation, lentiviral vectors expressing synapsin I promoter-driven EGFP (Synapsin::EGFP) were transduced, and then plated at 2 x10^5 cells per well in 12-well plates coated with matrigel. EGFP-positive neurons were observed within five days after transduction. After confirming that the increasing number of EGFP-positive neurons had reached a stable plateau, iPSC-derived neural cells were replated at 4 x10^5 cells per well in 96-well plates coated with matrigel. Five days after replating, neurobasal medium FULL (described above) was changed to Neurobasal® Medium Minus Phenol Red (Invitrogen), without B27 supplement/Glutamax/neurotrophic factors, with or without 5 μM DHA. EGFP-positive neurons were imaged automatically at intervals of 48 h using IN CELL Analyzer 2000. The number of EGFP-positive neurons at each time point/well was automatically counted by IN CELL Developer toolbox software 1.9. Neuronal death was determined by cell membrane rupture, blebbing, or loss of EGFP fluorescence. Neuronal survival rate was calculated with the formula ‘counts at analysis/counts at start (%)’. Lactate dehydrogenase (LDH) assay was performed to assess the cytotoxic state during neuronal survival analysis. LDH, a cytoplasmic enzyme, is released into culture medium when the cell membrane is damaged. Released LDH in culture medium was measured by Cytotoxicity Detection Kit (LDH) (Roche Diagnostics). LDH levels were converted to a relative value (%), in comparison with the N116213 clone without DHA at day 16 of the survival assay.