

		福井次矢, 高木誠, 小室一成					
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雑誌

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長谷川一子, 石田篤子, 加藤睦子, 原輝文.	パーキンソン病患者を対象 としたロピニロール塩酸塩 錠（レキップ錠）の長期使 用に関する特定使用成績調 査	Progress in Medicine	33	929-955	2013
長谷川一子.	パーキンソン病の病態に合 わせた薬の選択と使い方	ファルマシア	49	844-848	2013
長谷川一子.	restless legs syndrome	Clinical neuroscience	31	1325-27	2013
長谷川一子.	進行期の患者さんに伝えたい パーキンソン病の治療と 自己管理の基本	マックス	41	1-5	2013
長谷川一子.	パーキンソン病の非運動症 状	難病と在宅ケア	19	33-36	2013
長谷川一子.	ジストニアの定義と分類	神経症候群 (第2版) —その他 の神経疾患を含め て—日本臨床 新 領域別症候群シリ ーズ	27	201-206	2014
長谷川一子.	Huntington 病と認知障害 「症候性 dementia」	神経内科	80	24-33	2014
長谷川一子.	Neurodegeneration with brain iron accumulation-1 NBIA1	神経症候群 (第2版) —その他 の神経疾患を含め て— 日本臨床 新領域別症候群シ リーズ	27	284-288	2014
長谷川一子.	新規ドパミンアゴニストと 既存薬の使い分け。「進化す るパーキンソン病診療」	Progress in Medicine	34	49-53	2014
長谷川一子.	ドパミンアゴニスト徐放性 製剤の使い方とその治療戦 略	Pharma Medica	32	80-85	2014

IV. 研究成果の刊行物・別刷

Modeling Alzheimer's Disease with iPSCs Reveals Stress Phenotypes Associated with Intracellular A β and Differential Drug Responsiveness

Takayuki Kondo,^{1,2,7} Masashi Asai,^{7,9,10} Kayoko Tsukita,^{1,7} Yumiko Kutoku,¹¹ Yutaka Ohsawa,¹¹ Yoshihide Sunada,¹¹ Keiko Imamura,¹ Naohiro Egawa,¹ Naoki Yahata,^{1,7} Keisuke Okita,¹ Kazutoshi Takahashi,¹ Isao Asaka,¹ Takashi Aoi,¹ Akira Watanabe,¹ Kaori Watanabe,^{7,10} Chie Kadoya,^{7,10} Rie Nakano,^{7,10} Dai Watanabe,³ Kei Maruyama,⁹ Osamu Hori,¹² Satoshi Hibino,¹³ Tominari Choshi,¹³ Tatsutoshi Nakahata,¹ Hiroyuki Hioki,⁴ Takeshi Kaneko,⁴ Motoko Naitoh,⁵ Katsuhiro Yoshikawa,⁵ Satoko Yamawaki,⁵ Shigehiko Suzuki,⁵ Ryuji Hata,¹⁴ Shu-ichi Ueno,¹⁵ Tsuneyoshi Seki,¹⁶ Kazuhiro Kobayashi,¹⁶ Tatsushi Toda,¹⁶ Kazuma Murakami,⁶ Kazuhiro Irie,⁶ William L. Klein,¹⁷ Hiroshi Mori,¹⁸ Takashi Asada,¹⁹ Ryosuke Takahashi,² Nobuhisa Iwata,^{7,10,*} Shinya Yamanaka,^{1,8} and Haruhisa Inoue^{1,7,8,*}

¹Center for iPS Cell Research and Application (CiRA)

²Department of Neurology, Graduate School of Medicine

³Department of Biological Sciences, Graduate School of Medicine and Department of Molecular and Systems Biology, Graduate School of Biostudies

⁴Department of Morphological Brain Science, Graduate School of Medicine

⁵Department of Plastic and Reconstructive Surgery, Graduate School of Medicine

⁶Organic Chemistry in Life Science, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8507, Japan

⁷Core Research for Evolutional Science and Technology (CREST)

⁸Yamanaka iPS Cell Special Project

Japan Science and Technology Agency (JST), Saitama 332-0012, Japan

⁹Department of Pharmacology, Faculty of Medicine, Saitama Medical University, Saitama 350-0495, Japan

¹⁰Laboratory of Molecular Biology and Biotechnology, Department of Molecular Medicinal Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8521, Japan

¹¹Department of Neurology, Kawasaki Medical School, Okayama 701-0192, Japan

¹²Department of Neuroanatomy (Biotargeting), Kanazawa University Graduate School of Medical Sciences, Ishikawa 920-8640, Japan

¹³Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Hiroshima 729-0292, Japan

¹⁴Department of Functional Histology

¹⁵Department of Psychiatry

Ehime University Graduate School of Medicine, Ehime 791-0295, Japan

¹⁶Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Hyogo 650-0017, Japan

¹⁷Department of Neurobiology, Northwestern University, Evanston, IL 60208, USA

¹⁸Department of Neuroscience, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan

¹⁹Department of Neuropsychiatry, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8577, Japan

*Correspondence: haruhisa@cira.kyoto-u.ac.jp (H.I.), iwata-n@nagasaki-u.ac.jp (N.I.)

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SUMMARY

Oligomeric forms of amyloid- β peptide (A β) 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. A β oligomers accumulated in iPSC-derived neurons and astrocytes in cells from patients with a familial amyloid precursor protein (APP)-E693 Δ mutation and sporadic AD, leading to endoplasmic reticulum (ER) and oxidative stress. The accumulated A β 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 (A β), forming amyloid plaques in the brain. Cognitive impairment observed in clinical AD is inversely well correlated with the amount of A β oligomers in the soluble fraction rather than the amount of A β fibrils (amyloid plaques) constituting the oligomers (Haass and Selkoe, 2007; Krafft and Klein, 2010). Increasing evidence has shown that A β oligomers extracted from AD model mice or made from synthetic peptides cause



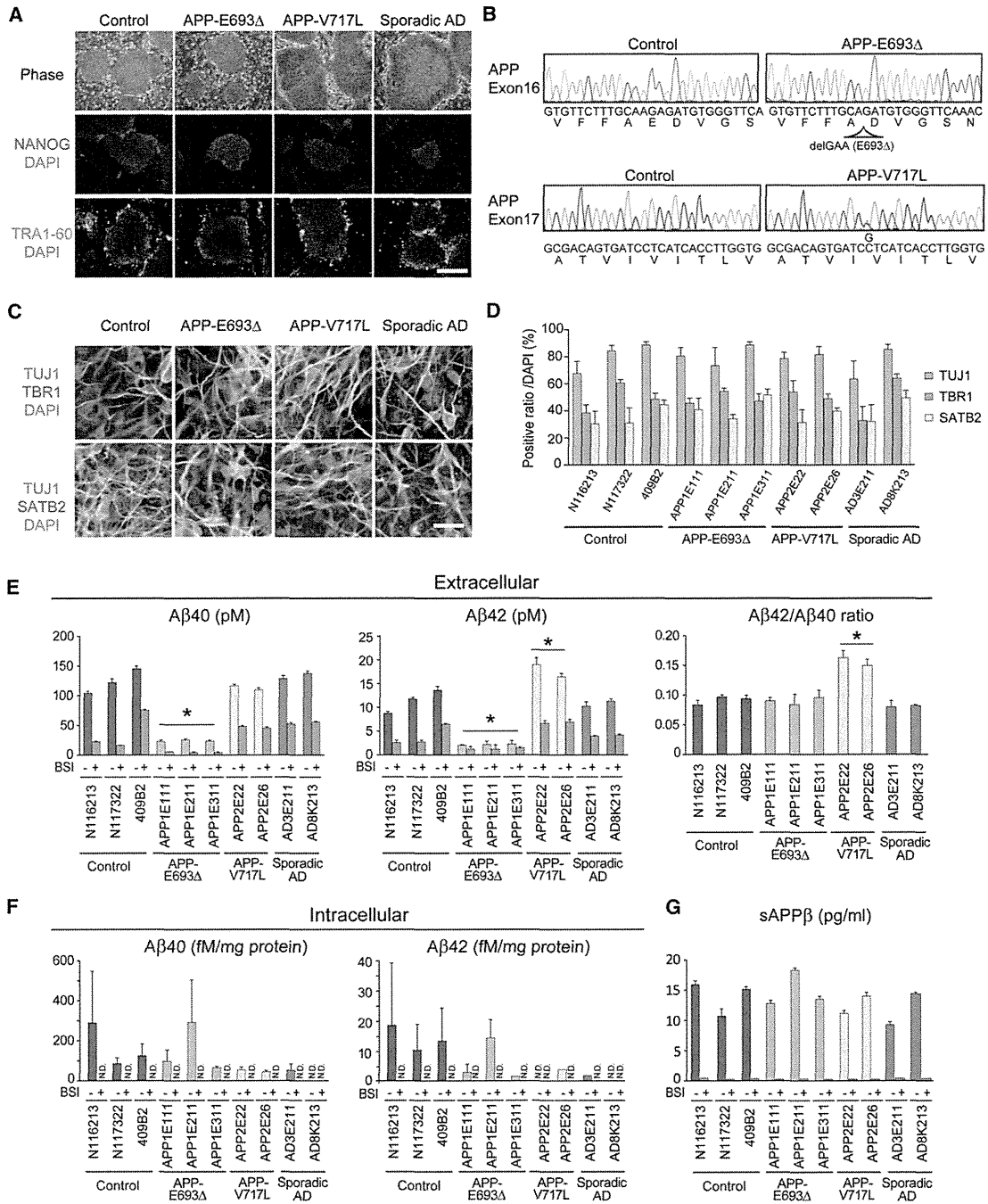


Figure 1. Establishment of Control and AD Patient-Specific iPSCs, and Derivation of Cortical Neurons Producing Aβs from iPSCs
 (A) Established iPSCs from both controls and AD patients showed embryonic stem cell-like morphology (Phase) and expressed pluripotent stem cell markers NANOG (red) and TRA1-60 (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 (legend continued on next page)

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 A β oligomers has been presumed to play a central role in the pathogenesis and clinical symptoms of AD. A β s 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 E693 Δ mutation of an APP-producing variant A β lacking 22nd Glu was discovered in Japan (Tomiya et al., 2008). This APP-E693 Δ 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 A β deposition, according to positron emission tomography (PET) scan analysis with a [¹¹C] Pittsburgh compound-B (PIB) radioprobe (Tomiya et al., 2008; Shimada et al., 2011). The 22nd Glu within the A β sequence has a destabilizing effect on the formation of oligomeric structures because of the electrostatic repulsion between the adjacent side chain of 22nd Glu (Kassler et al., 2010), and the deletion of the amino acid residue leads to the ready formation of A β oligomers in vitro (Nishitsuji et al., 2009). APP-E693 Δ transgenic mice show AD-like pathology, including intracellular oligomer accumulation, but lack extracellular amyloid plaque formation (Tomiya et al., 2010). However, it remains unclear whether A β oligomers are accumulated in familial and sporadic AD patient neural cells and how intracellular A β oligomers play a pathological role. The compound and/or drugs that might rescue the A β 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 astroglial differentiation of iPSCs from a familial AD patient with an APP-E693 Δ 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 A β oligomers in familial and sporadic AD. We found that A β oligomers were not proteolytically resistant and that docosahexaenoic acid (DHA) treatment attenuated cellular phenotypes of AD neural cells with intracellular A β 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 indi-

viduals, three and two familial AD iPSC lines from patients with E693 Δ [AD(APP-E693 Δ)] 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 extra- and intracellular A β 40 and A β 42 (Figures 1E and 1F). As expected, both A β species were strongly decreased in all cloned AD(APP-E693 Δ) neural cells in comparison to those in control neural cells. In familial AD(APP-V717L) neural cells, an increase in the extracellular A β 42 level and a corresponding decrease in the intracellular A β 42 level were observed, and the A β 42/A β 40 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 A β levels in sporadic AD neural cells were not changed in comparison to those in control neural cells, but intracellular A β in sporadic AD8K213 neural cells apparently decreased (that is, below the detection limit). APP expression levels in the AD(APP-E693 Δ) neural cells were lower than in the others, but the levels of α - and β -secretase-mediated APP processing remained unaltered in all neural cells (Figures 1G, S1M, and S1N). Soluble APP β production was strongly inhibited by treatment with β -secretase inhibitor IV (BSI) (Figure 1G). A β 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 neural cells (Figures S1O and S1P).

Intracellular Accumulation of A β Oligomers in AD(APP-E693 Δ) and in One of the Sporadic AD Neural Cells

Using an immunocytochemical method with the A β -oligomer-specific antibody NU1 (Lambert et al., 2007), we investigated whether AD(APP-E693 Δ) neural cells harbor A β oligomers or not. We found that A β oligomers were accumulated as puncta in the neurons of AD(APP-E693 Δ) and in one of the sporadic AD cases (Figure 2A). The area of A β -oligomer-positive puncta was significantly increased in AD(APP-E693 Δ) neuronal cells relative to control neuronal cells (Figure 2B). Dot blot analysis using cell lysates revealed that A β oligomers were markedly elevated in the AD(APP-E693 Δ) and sporadic AD8K213 neural cells (Figures 2C and 2D), whereas A β oligomers were not detected in the culture medium (data not shown). Another antibody against A β , 11A1, which detects low-molecular-weight oligomers rather than the A β monomer (Murakami et al., 2010), showed results similar to those observed with NU1 (Figures

(*, $p < 0.001$). There are significant differences between dimethyl sulfoxide (DMSO)-control and BSI treatment in each case (*, $p < 0.001$) except that of AD(APP-E693 Δ) for A β 42.

(F) A β 40 and A β 42 in cell lysates (intracellular A β). N.D., not detected. Data represent mean \pm SD ($n = 3$ per clone).

(G) The amount of soluble APP β was not altered in control and AD. Data represent mean \pm SD ($n = 3$ per clone).

See also Figure S1.

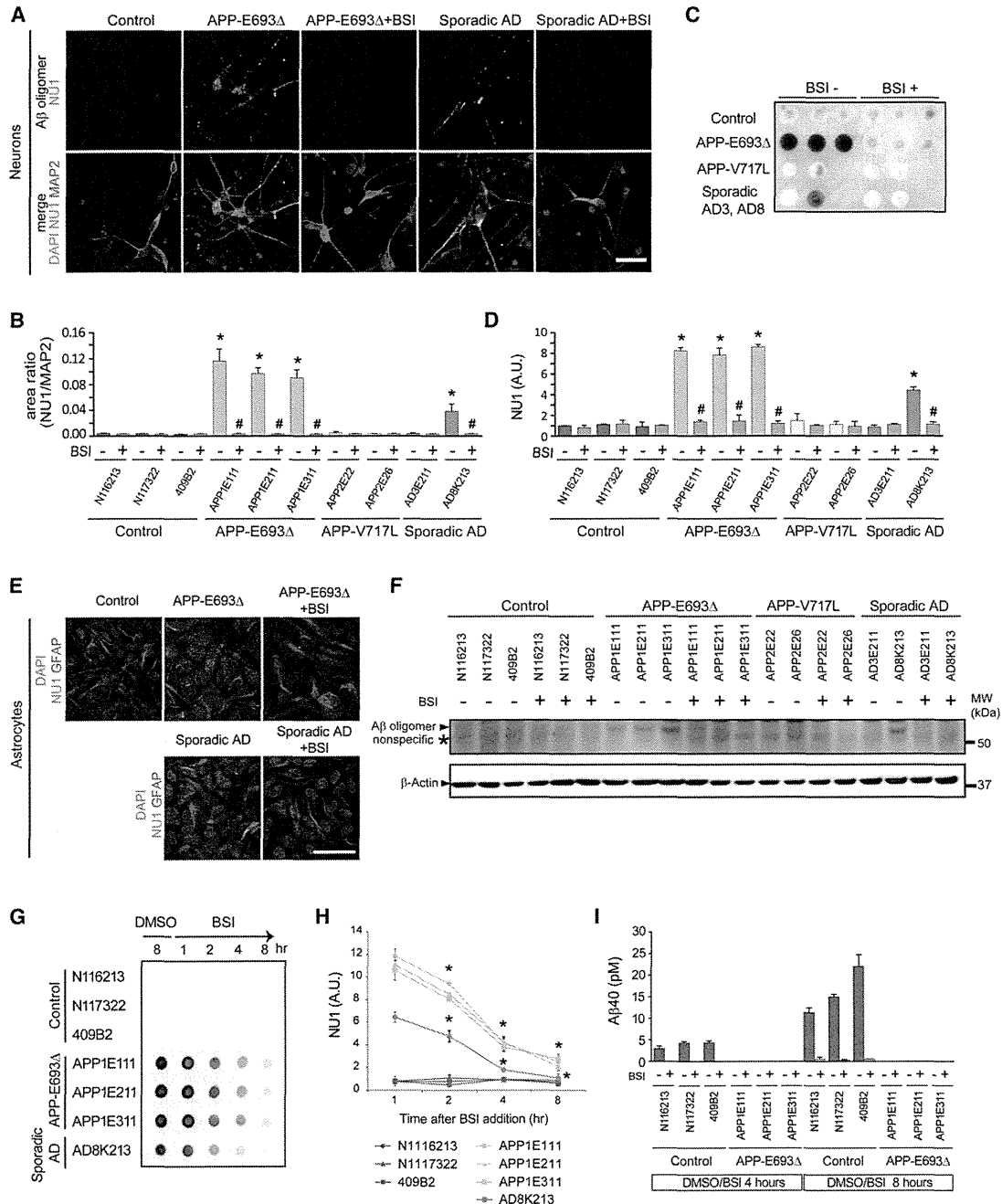


Figure 2. Familial AD (APP-E693Δ) and Sporadic AD iPSC-Derived Neurons Have Intracellular Aβ Oligomers

(A) Intracellular Aβ oligomer accumulation in iPSC-derived neurons (red, MAP2-positive cells) was detected by the Aβ-oligomer-specific monoclonal antibody NU1 (green) with a punctate pattern. Aβ 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 Aβ oligomer accumulation. DAPI, nuclear staining (blue). The scale bar represents 30 μm.

(B) Quantification of Aβ 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). Aβ 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). Aβ 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) Aβ oligomer accumulation in AD astrocytes. The scale bar represents 30 μm.

(legend continued on next page)

S2A–S2D). However, A β oligomers were not detected in cell lysates from the fibroblasts that generate iPSC lines (Figure S2E). To confirm whether A β oligomers were derived from mutant APP(E693 Δ), we transduced a lentiviral vector driven by an EF1 α promoter to overexpress wild or mutant APP(E693 Δ) in control iPSC-derived neural cells and found that A β oligomers emerged inside control neural cells overexpressing mutant APP(E693 Δ) (Figure S2F).

To investigate the intracellular accumulation of A β 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 A β oligomer antibodies revealed that the astrocytes of AD(APP-E693 Δ) and one of the sporadic AD iPSCs accumulated A β 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).

A β 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 A β oligomers was inhibited by treatment with BSI (Figures 2A–2G, S2A–S2D, and S2N). To clarify whether the E693 Δ mutation results in accelerated A β oligomerization and/or in a proteolytically resistant and stable form of A β oligomers, we analyzed the levels of A β oligomers over a course of time after BSI treatment. Intracellular A β 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 A β 40 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 A β Oligomers in AD iPSC-Derived Neural Cells

Extracellular A β deposition in patient brains carrying APP with an E693 Δ 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 A β oligomers were associated with specific organelles. We characterized the A β oligomer-positive punctate structures in AD(APP-E693 Δ) neural cells and astrocytes by coimmunostaining with antibodies for markers of vesicular compartments and subcellular organelles. Subpopulations of A β 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-E693 Δ) 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-E693 Δ) 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 A β oligomer-positive puncta area per cell in the context of AD(APP-E693 Δ) 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-E693 Δ) and in 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 A β oligomer formation in AD(APP-E693 Δ) and sporadic AD8K213. To identify pathogenic species evoking oxidative stress in AD(APP-E693 Δ), we visualized reactive oxygen species (ROS) and found that ROS was increased in both neurons and astrocytes in AD(APP-E693 Δ) and AD8K213 (Figures 3H–3J and S3I–S3L). This increase was counteracted by the BSI treatment. These results indicated that intracellular A β 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).

Alleviation of Intracellular A β 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-E693 Δ) 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-E693 Δ) neural cells (Figures 4C and 4D), whereas the amount of A β oligomers in cell lysates

(F) Western blot analysis of control and AD neural cells in the presence or absence of BSI. BSI treatment (1 μ M) disappeared 6E10-positive \approx 55 kDa protein bands in cell lysates of AD(APP-E693 Δ) and sporadic AD(AD8K213) neural cells.

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

(H) Signals of blots in (G) were quantified. Data represent mean \pm SD (n = 3 per clone). BSI treatment (1 μ M) decreased intracellular A β in AD neural cells and was reduced to 16–23% of vehicle control by 8 hr. Post hoc analysis revealed that the amounts of A β 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 A β 40 levels were analyzed in the experimental condition of (G). Data represent mean \pm SD (n = 3 per clone). See also Figure S2.

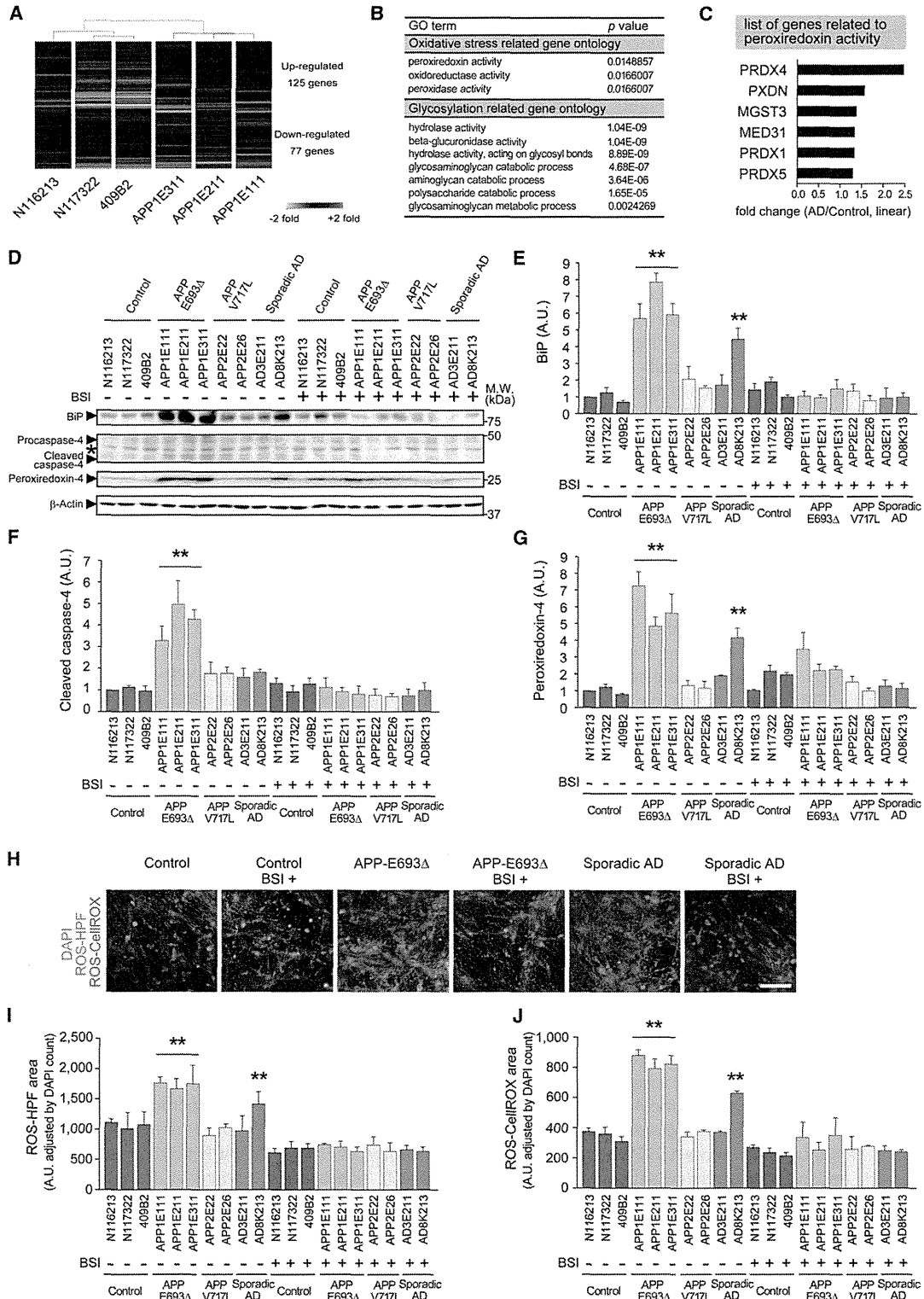


Figure 3. Cellular Stress Responses Caused by Intracellular A β Oligomers in Familial AD (APP-E693 Δ) and Sporadic AD (AD8K213) iPSC-Derived Neural Cells

(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 .

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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 EGFP 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 pathogenesis, 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, intracellular 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, auto-

phagosomes, 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 Aβ₄₂/Aβ₄₀ 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 Aβ₄₀ elevation of Israel et al. (2012). Analysis of neurons

(B) The gene ontology (GO) term list, calculated from the significantly altered gene expression patterns in the microarray analysis of AD versus control neural cells.

(C) Altered expression levels of genes related to peroxidation activity detected by GO analysis. All values were significantly different from that of the control ($p < 0.05$).

(D–G) Western blot analysis of ER stress markers (BiP and caspase-4), peroxiredoxin-4, and a reference protein (β-actin) in the presence or absence of BSI.

(E–G) Densitometric analysis of (D) are shown. Measured values of proteins were normalized by β-actin. Data represent mean ± SD ($n = 3$ per clone). Levels of BiP (E), cleaved caspase-4 (F), and peroxiredoxin-4 (G) in AD(APP-E693Δ) and sporadic AD(AD8K213) neural cells without BSI were significantly different from those of the other neural cells (**, $p < 0.005$).

(H) Typical images of reactive oxygen species (ROS) staining, detected by HPF or CellROX, in control and AD neural cells with or without BSI treatment. Scale bars represent 30 μm.

(I and J) Quantitative data of (H), ROS-HPF (I), and ROS-CellROX (J). Each value was shown as a ratio of the HPF-stained or CellROX area (average of random 25 fields per sample) adjusted with DAPI counts. Data represent mean ± SD ($n = 3$ per clone). ROS-generation levels in AD(APP-E693Δ) and sporadic AD(AD8K213) neural cells were significantly different from those of the others (**, $p < 0.001$). Data represent mean ± SD ($n = 3$ per clone).

See also Figure S3 and Table S1.

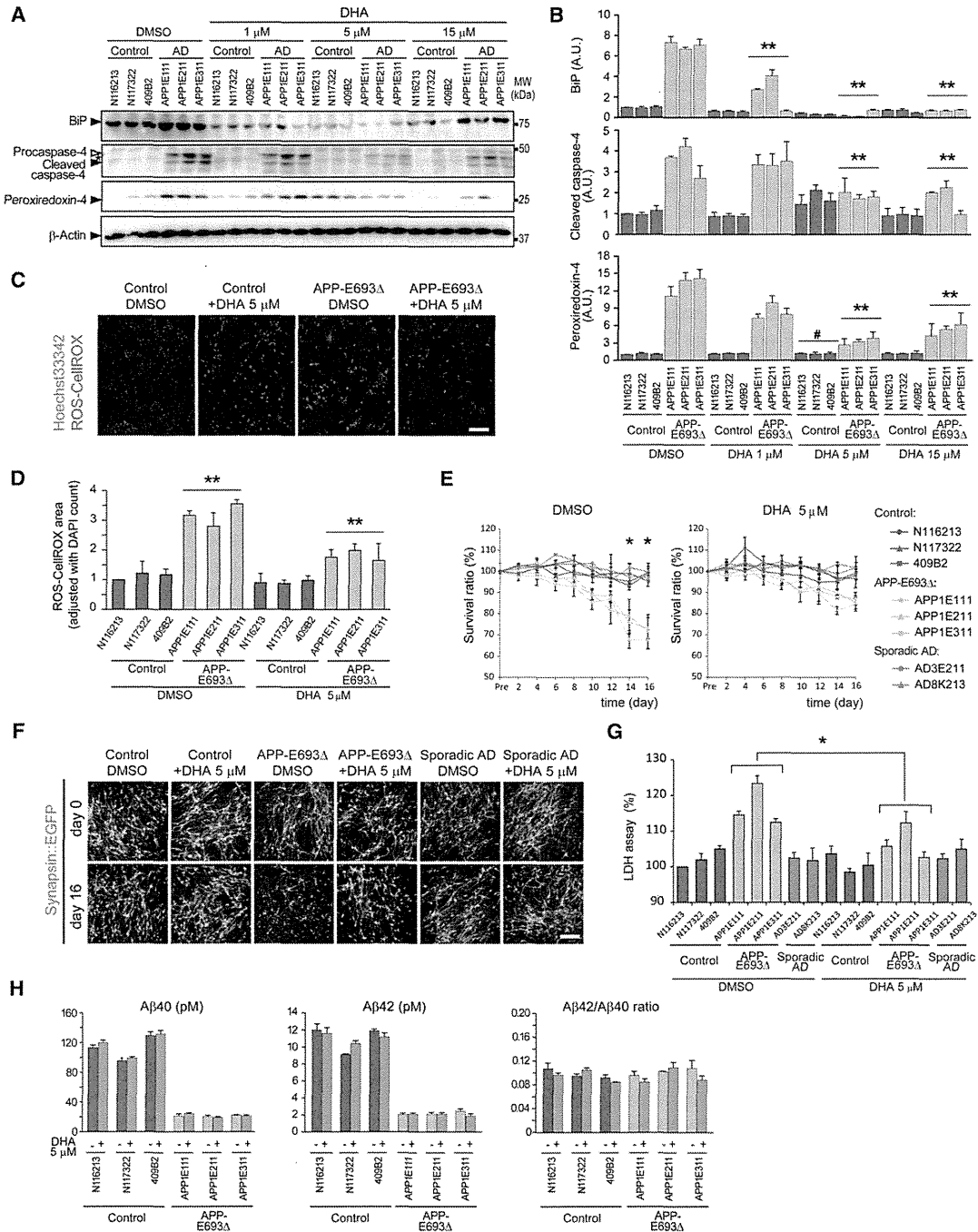


Figure 4. DHA-Alleviated Cellular Stress Caused by Intracellular Aβ Oligomers

(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-CelliROX and Hoechst33342 signals after treatment with vehicle or 5 μM DHA. The scale bar represents 50 μm.

(legend continued on next page)