ANESTHESIOLOGY

Dantrolene Ameliorates Impaired Neurogenesis and Synaptogenesis in Induced Pluripotent Stem Cell Lines Derived from Patients with Alzheimer's Disease

Yong Wang, M.D., Ph.D., Ge Liang, M.D., Shuqing Liang, M.D., Rachel Mund, Yun Shi, M.D., Huafeng Wei, M.D., Ph.D.

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EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Overactivation of ryanodine receptors in the endoplasmic reticulum and the resulting dysregulation of calcium homeostasis contribute to Alzheimer's disease–related pathophysiology
- Dantrolene is an antagonist of ryanodine receptors, and its chronic use has been suggested to improve memory function in experimental models of Alzheimer's disease

What This Article Tells Us That Is New

- Survival, proliferation, and differentiation of neuronal progenitors derived from patients with Alzheimer's disease are impaired when compared with healthy counterparts
- Chronic exposure of induced pluripotent stem cells, derived from patients with Alzheimer's disease, to dantrolene improves the survival, proliferation, and differentiation of these cells

ABSTRACT

Background: Overactivation of ryanodine receptors and the resulting impaired calcium homeostasis contribute to Alzheimer's disease–related pathophysiology. This study hypothesized that exposing neuronal progenitors derived from induced pluripotent stems cells of patients with Alzheimer's disease to dantrolene will increase survival, proliferation, neurogenesis, and synaptogenesis.

Methods: Induced pluripotent stem cells obtained from skin fibroblast of healthy subjects and patients with familial and sporadic Alzheimer's disease were used. Biochemical and immunohistochemical methods were applied to determine the effects of dantrolene on the viability, proliferation, differentiation, and calcium dynamics of these cells.

Results: Dantrolene promoted cell viability and proliferation in these two cell lines. Compared with the control, differentiation into basal forebrain cholinergic neurons significantly decreased by 10.7% (32.9 ± 3.6% *vs.* 22.2 ± 2.6%, N = 5, $P = 0.004$) and 9.2% (32.9 ± 3.6% *vs.* 23.7 ± 3.1%, N = 5, $P = 0.017$) in cell lines from sporadic and familial Alzheimer's patients, respectively, which were abolished by dantrolene. Synapse density was significantly decreased in cortical neurons generated from stem cells of sporadic Alzheimer's disease by 58.2% (237.0 ± 28.4 *vs.* 99.0 ± 16.6 arbitrary units, N = 4, $P = 0.001$) or familial Alzheimer's disease by 52.3% (237.0 ± 28.4 *vs.*113.0 ± 34.9 *vs.* arbitrary units, N = 5, $P = 0.001$), which was inhibited by dantrolene in the familial cell line. Compared with the control, adenosine triphosphate (30 μM) significantly increased higher peak elevation of cytosolic calcium concentrations in the cell line from sporadic Alzheimer's patients (84.1 ± 27.0% *vs.* 140.4 ± 40.2%, N = 5, $P = 0.049$), which was abolished by the pretreatment of dantrolene. Dantrolene inhibited the decrease of lysosomal vacuolar-type H^+-ATPase and the impairment of autophagy activity in these two cell lines from Alzheimer's disease patients.

Conclusions: Dantrolene ameliorated the impairment of neurogenesis and synaptogenesis, in association with restoring intracellular Ca^{2+} homeostasis and physiologic autophagy, cell survival, and proliferation in induced pluripotent stem cells and their derived neurons from sporadic and familial Alzheimer's disease patients.

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Alzheimer's disease is a devastating neurodegenerative disease.[1] The deficit in the development of new drugs targeting the amyloid pathology over the past several decades[2] warrants exploration of alternative pathways that could be the primary cause of Alzheimer's disease cognitive dysfunction. Ca^{2+} dysregulation *via* overactivation of the endoplasmic reticulum ryanodine receptor is thought to play a central, upstream role in the neuropathology and cognitive dysfunction in Alzheimer's disease.[3–7] Some familial Alzheimer's disease gene mutations cause ryanodine receptor overexpression, resulting in excessive endoplasmic reticulum Ca^{2+} release *via* activated

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ryanodine receptor,[8,9] which in turn worsen amyloid and Tau pathology, neurodegeneration, and synapse dysfunction. Moreover, adult neurogenesis, critical for maintaining synaptic and cognitive function during aging, is also impaired.[10,11] Considering the important role of Ca^{2+} in the regulation of neurogenesis,[12] it is important to understand the mechanisms of impairment of neurogenesis in Alzheimer's disease.

Sporadic Alzheimer's disease accounts for more than 95% of Alzheimer's disease patients, but its pathology is largely unknown. Lack of understanding of the mechanisms and inadequate cell or animal models of sporadic Alzheimer's disease limit the development of new effective drugs for treatment of Alzheimer's disease. Although the pathology and mechanisms of familial Alzheimer's disease have been relatively well studied, they are primarily in cell and animal models, not in patients. Recent advancement in the development of induced pluripotent stem cells from skin fibroblasts of Alzheimer's disease patients allows for a new cell model to study pathology in sporadic Alzheimer's disease and test the therapeutic efficacy of new drugs, especially for the process of neurogenesis.[13]

Dantrolene, which reduced mortality of malignant hyperthermia from 85% to less than 5%,[14] is the only U.S. Food and Drug Administration–approved clinically available drug to treat malignant hyperthermia. Chronic use of oral dantrolene is also utilized to treat muscle spasm, with relatively tolerable side effects.[15] Although not fully consistent,[3] the majority of recent studies,[5,6,16,17] indicate that chronic use of dantrolene significantly ameliorated memory loss and amyloid pathology in various familial Alzheimer's disease animal models, with acceptable adverse reactions. The mechanisms of dantrolene neuroprotection in Alzheimer's disease were also investigated *in vitro*, including the correction of Ca^{2+} disruption, inhibition of neurodegeneration and synapse dysfunction, etc.[15,18] However, significant gaps of knowledge need to be filled before dantrolene can be studied as a treatment for Alzheimer's disease patients: (1) mechanisms by which dantrolene ameliorates cognitive dysfunction in Alzheimer's disease; (2) efficacy of dantrolene neuroprotection in sporadic Alzheimer's disease cells or animal model, especially in tissues from sporadic Alzheimer's disease patients; and (3) effects of dantrolene on neurogenesis in human sporadic Alzheimer's disease models. We hypothesized that dantrolene inhibits impaired neurogenesis and synaptogenesis by correction of Ca^{2+} dysregulation caused by overactivation of ryanodine receptor and associated impairment of lysosome and autophagy function. In this study and with the use of induced pluripotent stem cells from both sporadic Alzheimer's disease and familial Alzheimer's disease patients and their derived neuroprogenitor cells and basal forebrain cholinergic neurons, we studied the effects and mechanisms of dantrolene on neurogenesis and synaptogenesis.

Materials and Methods

Cell Culture

Human control cells (AG02261) and sporadic Alzheimer's disease–induced pluripotent stem cells (AG11414) were obtained from John A. Kessler's lab.[19] Familial Alzheimer's disease– induced pluripotent stem cells (GM24675) were purchased from Coriell Institute (Camden, New Jersey). Each type of induced pluripotent stem cells was generated from skin fibroblasts of one heathy human subject or one patient diagnosed of either sporadic Alzheimer's disease or familial Alzheimer's disease. The AG02261 cell line was derived from a 61-year-old male healthy patient. Another AG11414 cell line came from a 39-year-old male patient with early onset Alzheimer's disease who displayed an APOE3/E4 genotype. The GM24675 cell line was derived from a 60-year-old familial Alzheimer's disease patient with APOE genotype 3/3.[20] The human pluripotent stem cells were maintained on Matrigel (BD Biosciences, USA)–coated plates in mTeSR™1 medium (catalog No. 05850, Stem Cell Technologies, Canada) and were cultured in a 5% CO_2 humidified atmosphere at 37°C. The culture medium was changed every day. We routinely checked the cells before the experiments for healthiness, and the unhealthy cells (*e.g.*, nonadherent cells) were removed. We randomly assigned the cells into two experimental groups. During the experiments, if the cultured cells were visibly infected, the data were removed and not used. This happened two times in the cell viability experiments.

Cell Viability

The cell viability on different wells in 96-well plates was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich, USA) reduction assay at 24 h as we previously described.[21,22] After being washed with phosphate-buffered saline, the samples were incubated with fresh culture medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/ml in the medium) at 37°C for 4 h in the dark. The medium was then removed, and formazan was solubilized with dimethyl sulfoxide. The absorbance was measured at 540 nm with plate reader (Synergy H1 microplate reader, BioTek, USA).

Cell Proliferation Assays

The induced pluripotent stem cells were plated onto cover glasses coated with Matrigel in mTeSR™1 medium. 5-Bromodeoxyuridine (Invitrogen, USA) was added to the mTeSR™1 medium 4 h before the end of treatment with a final concentration of 30 μM. The cells were then fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. For 5-bromodeoxyuridine detection, acid treatment (1 N HCl 10 min on ice followed by 2 N HCl 10 min at room temperature) separated DNA into single strands so that the primary antibody could access the incorporated

5-bromodeoxyuridine (the antibodies used are listed in Supplemental Digital Content, table 1, http://links.lww.com/ALN/C273). After being incubated with blocking solution (5% normal goat serum in phosphate-buffered saline containing 0.1% Triton X-100), the cells were incubated with rat monoclonal anti-5-bromodeoxyuridine primary antibody (1:100; Santa Cruz Biotechnology, USA) overnight at 4°C. After subsequent wash with phosphate-buffered saline containing 0.1% Triton X-100, the cells were incubated with fluorescently labeled secondary antibody conjugated with anti-rat IgG (1:1,000; Invitrogen, USA) for 2 h at room temperature. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen) for 5 min at room temperature. The immunostained cells were covered and then mounted on an Olympus BX41TF fluorescence microscope (200×; Olympus, USA). The images were acquired using iVision 10.10.5 software (Biovision Technologies, USA). Five sets of images were acquired at random locations on the cover glass and were subsequently merged using Image J 1.49v software (National Institutes of Health, USA). The percentage of 5-bromodeoxyuridine--positive cells over the total number of cells was calculated and compared across different groups from at least three different cultures.

Differentiation of Induced Pluripotent Stem Cells

The protocol for differentiation into cortical neurons and basal forebrain cholinergic neurons from induced pluripotent stem cells was adapted from previously described protocol.[23,24] Briefly, feeder-free culture was induced to neural progenitors *via* dual-SMAD inhibition. The cells were cultured in chemical defined condition with 2 μM SB431542 and 2 μM DMH1 (both from Tocris, USA) for 7 days.

For cortical neurons, change the medium to neural maintenance medium (this is a 1:1 mixture of N-2 and B-27-containing media; N-2 medium consists of Dulbecco's modified Eagle's medium/F-12 GlutaMAX, 1× N-2[2], 5 μg ml^{-1} insulin, 1 mM L-glutamine, 100 μM nonessential amino acids, 100 μM 2-mercaptoethanol, 50 units ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin; B-27 medium consists of Neurobasal, 1× B-27, 200 mM L-glutamine, 50 U ml^{-1} penicillins, and 50 mg ml^{-1} streptomycin) from day 12. Neural rosette structures should be obvious when cultures are viewed with an inverted microscope around days 12–17. From this point, medium was changed every other day.

For basal forebrain cholinergic neurons differentiation, the induced pluripotent stem cell-derived primitive neural stem cells were developed under recombinant human sonic hedgehog (500 ng/ml; 1845-SH) and then treated with nerve growth factor (1156-NG-100, 50-100 ng/ml; both from R&D Systems, USA) from day 24. At day 28 the neural progenitors were plated on the poly-L-ornithon/laminin–coated plates at a density of 5,000 cells/cm^2 and then cultured in neuronal differentiation medium consisting of neurobasal medium, N_2 supplement (Invitrogen) in the presence of nerve growth factor (50-100 ng/ml), cAMP (1 μM; Sigma), brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor (10 ng/ml; R&D), and recombinant human sonic hedgehog (50 ng/ml; R&D).[25]

Ca^{2+} Measurements

The changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) of induced pluripotent stem cells after adenosine triphosphate (ATP) exposure were measured using jellyfish photoprotein aequorin–based probe; 7.5–12 × 10^4 cells were plated on 12-mm coverslips on a 24-well plate, grown to 50-60% confluence, and then transfected with the cyt-Aeq plasmid using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The next day, the transfected cells were incubated with 5 μM coelenterazine for 1 h in modified Krebs–Ringer buffer (140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 11 mM glucoses, pH 7.4) supplemented with 1 mM $CaCl_2$ and then were transferred to the perfusion chamber. All aequorin measurements were carried out in Krebs–Ringer buffer, and anesthetics were added to the same medium as specified in the text. The experiments were performed in a custom-built aequorin recording system. For the extracellular Ca^{2+}-free experiment, the Ca^{2+}-free buffer was used (Krebs–Ringer buffer without Ca^{2+} with 5 mM EGTA). The experiments were terminated by lysing the cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The light signal was collected and calibrated into $[Ca^{2+}]_c$ values by an algorithm based on the Ca^{2+}-response curve of aequorin at physiologic conditions of pH, $[Mg^{2+}]$, and ionic strength, as previously described.[26,27]

The changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) of induced pluripotent stem cells after exposure to *N*-methyl-D-aspartate (NMDA) was measured by Fura-2/AM fluorescence (Molecular Probes, USA) using methods described before. Assays were carried out on an Olympus IX70 inverted microscope (Olympus America Inc., USA) and IPLab v3.71 software (Scanalytics, USA). In brief, the induced pluripotent stem cells were plated onto a 35-mm culture dish. After the cells were washed three times in Ca^{2+}-free Dulbecco's modified Eagle's medium (Gibco, USA) and loaded with 2.5 μm Fura-2/AM in the same buffer for 30 min at 37°C, the cells were then washed twice and incubated with Ca^{2+}-free Dulbecco's modified Eagle's medium for another 30 min at 37°C. Fura-2 AM was measured by recording alternate at 340- and 380-nm excitation, and emission at 510 nm was detected for up to 10 min for each treatment. The evoked changes were recorded in response to treatment of 500 μM NMDA with or without 30 μM dantrolene. The results were presented as a ratio of F340/F380 nm and averaged from at least three separate experiments.

Western Blotting

Western blotting was performed according to the standard procedure. Total protein extracts from induced pluripotent

stem cells were obtained by lysing the cells in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Triton X-100) in the presence of a cocktail of protease inhibitors.[28] After centrifugation, the supernatant was collected, and the total protein was quantified using a bicinchoninic acid protein assay kit (Thermo Scientific, USA). Equal amounts of protein for each lane were loaded and separated on 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% fat-free milk dissolved in phosphate-buffered saline-T for 1 h at room temperature and then stained with primary antibody at 4°C overnight. After the wash with phosphate-buffered saline-T, the membranes were incubated with secondary antibodies (horseradish peroxidase–conjugated anti-rabbit and anti-mouse IgG) at 1:1,000 dilutions, and β-actin served as a loading control. Signals were detected with an enhanced chemiluminescence detection system (Millipore, USA) and quantified by scanning densitometry.

Immunocytochemistry

The cells were fixed in 4% paraformaldehyde for 15 min followed by three 1× phosphate-buffered saline washes. They were then blocked by 5% normal goat serum in phosphate-buffered saline containing 0.1% Triton X-100 at room temperature for 1 h. Primary antibodies were applied for overnight at 4°C in 1× phosphate-buffered saline containing 1% bovine serum albumin and 0.3% Triton X-100. After three washes with phosphate-buffered saline, Alexa Fluor–conjugated secondary antibodies (1:1,000) together with 4′,6-diamidino-2-phenylindole (1:2,000) were added for 1 h. After three more washes, coverslips were mounted with Prolong Gold antifade reagent (both from Invitrogen) and imaged. Primary antibodies used were listed in the Supplemental Digital Content (http://links.lww.com/ALN/C273). Image acquisition and analysis are performed by people blinded to experiment treatment. Five sets of images were acquired at random locations on the cover glass and were subsequently merged using Image J 1.49v software (National Institutes of Health). The percentage of positive cells over the total number of cells was calculated and compared across different groups from at least three different cultures.

Lysosome Acidity Measurements

As we described before,[22] LysoTracker[regs] Red DND-99 (Molecular Probes) probe stock solution was diluted to a working concentration of 50 nM in Hanks' balanced salt solution. Induced pluripotent stem cells were plated on coverslips coated with Matrigel in mTeSR1. After being washed three times with Hanks' balanced salt solution, the cells were loaded with prewarmed (37°C) probe containing Hanks' balanced salt solution and incubated for 1 h at 37°C. Fresh medium was added to replace the labeling solution. The cells were observed by a fluorescent microscope fitted with the correct filter set for the probe used, to determine whether the cells were sufficiently fluorescent. LysoTracker Red used an emission maximum of ~590 nm and an excitation maximum of ~577 nm.

Data Analysis and Statistics

No statistical power calculation was conducted before the study. The sample size was based on our previous experience with this design. All data were tested for normal distribution by the Kolmogorov-Smirnov normality test and the Brown–Forsythe test to determine whether parametric or nonparametric tests are used for statistical analysis. Variables that satisfied the assumptions for parametric analysis were expressed as means ± SD and analyzed using one-way or two-way analysis of variance followed by Sidak's *post hoc* analysis. The nature of factors (*e.g.*, repeated measures) and groping of the factors were adequately addressed for analysis of variance. Variables that satisfied the assumptions for nonparametric analysis were analyzed using Kruskal–Wallis test followed by Dunn's multiple comparisons test. GraphPad Prism software (GraphPad Software, Inc., USA) was used for statistical analyses and graphs creation. A P value less than 0.05 was considered statistically significant.

Results

Dantrolene Promoted Cell Viability and Inhibited Impairment of Cell Proliferation in Induced Pluripotent Stem Cells from Alzheimer's Disease Patients

Induced pluripotent stem cells, neuroprogenitor cells, and neurons from the healthy human subject or sporadic Alzheimer's disease/familial Alzheimer's disease patients were cultured and characterized by specific antibodies targeting types of cells. There was no significant difference in cell viability determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay of induced pluripotent stem cells among healthy human subjects or sporadic Alzheimer's disease/familial Alzheimer's disease patients. However, dantrolene treatment resulted in a significantly greater 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (percentage of control) in sporadic Alzheimer's disease cells by 15.1% (103.8 ± 4.7% *vs.* 118.9 ± 10.3%, N = 8, $P = 0.006$) and familial Alzheimer's disease cells by 67.6% (96.4 ± 7.3% *vs.* 163.9 ± 10.1%, N = 7 replicates, $P < 0.0001$; fig. 1A) than control cells. Compared with healthy human subjects, induced pluripotent stem cells from sporadic Alzheimer's disease/familial Alzheimer's disease patients tended to have impaired proliferation ability as determined by 5-bromodeoxyuridine incorporation, more significantly in familial Alzheimer's disease induced pluripotent stem cells, which was inhibited by dantrolene (fig. 1B). Compared with control, dantrolene had no significant

effects on induced pluripotent stem cells differentiation into neuroprogenitor cells (data not shown).

Dantrolene Ameliorated the Impairment of Neuroprogenitor Cells Differentiation into Immature Neurons, Cortical Neurons, and Basal Forebrain Cholinergic Neurons in Both Sporadic and Familial Alzheimer's Disease Cells

Based on our pilot study to exert adequate dantrolene neuroprotection on neurogenesis, we treated induced pluripotent stem cells with dantrolene (30 μM) for 3 continuous days, beginning at the induction of induced pluripotent stem cells differentiation into neuroprogenitor cells (figs. 2 and 3). Differentiation of neuroprogenitor cell derived from sporadic Alzheimer's disease/familial Alzheimer's disease induced pluripotent stem cells into immature neurons at differentiation day 23 in the percentage of doublecortin-positive cells was less by 9.1% (13.7 ± 3.2% *vs.* 22.8 ± 3.2%, N = 6 replicates, $P = 0.004$) and 8.2% (14.6 ± 1.6% *vs.* 22.8 ± 3.2%, N = 6 replicates, $P = 0.011$), respectively, than the control, which was abolished by dantrolene (fig. 2). Compared with the control, mature cortical neurons (fig. 3) from sporadic Alzheimer's disease and familial Alzheimer's disease induced pluripotent stem cells decreased the percentage of Trb1-positive cells by 35.2% (23.5 ± 2.0% *vs.* 58.8 ± 12.3%, N = 5 replicates, $P < 0.0001$) and 15.8% (43.1 ± 6.9% *vs.* 58.8 ± 12.2%, N = 5 replicates, $P = 0.022$), respectively, compared with control, an effect that was abolished by dantrolene (fig. 3C). Using sonic hedgehog (fig. 3D), we further examined the generation of basal forebrain cholinergic neurons (choline acetyltransferase positive cells [green] from induced pluripotent stem cells), because the deficiency of basal forebrain cholinergic neurons has been considered a primary cause of memory loss and the basis of traditional treatments.[19,29] Compared with the control, differentiation into particular basal forebrain cholinergic neurons (fig. 3E) in the percentage of ChAT and MAP2 positive cells was less by 10.7% (22.2 ± 2.6% *vs.* 32.9 ± 3.7%, N = 5 replicates, $P = 0.004$) and 9.2% (23.7 ± 3.1% *vs.* 32.9 ± 3.6%, N = 5 replicates, $P = 0.017$) in sporadic Alzheimer's disease/familial Alzheimer's disease–induced pluripotent stem cells, respectively (fig. 3F), which was also abolished by dantrolene.

Dantrolene Rescued the Synaptogenesis Impairment of Neurons Generated from the Induced Pluripotent Stem Cells of Sporadic and Familial Alzheimer's Disease Patients

To determine the effects of dantrolene applied during the first 3 days of the induced pluripotent stem cell induction period on synaptogenesis of induced pluripotent stem cells originated neurons, we quantified the numbers of intersections between dendrites and concentric circles of the cortical neurons, shown as the distance (μm) of the circles from the soma (fig. 4A). Compared with the control neurons, the number of intersections (equivalent to synaptogenesis) was significantly lower in cortical neurons generated from

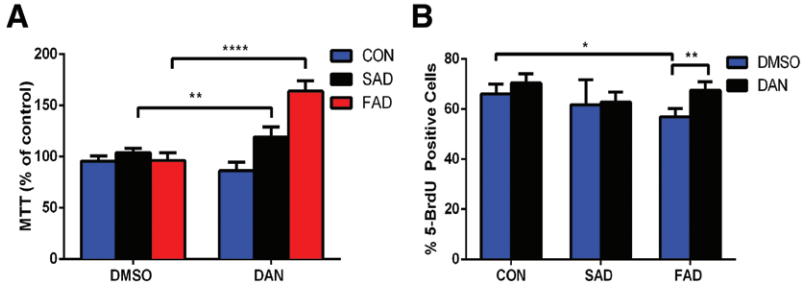

Fig. 1. Dantrolene promoted cell viability and inhibited impairment of cell proliferation in induced pluripotent stem cells from Alzheimer's disease patients. (*A*) Treatment of induced pluripotent stem cells with dantrolene (DAN; 30 μM) for 24 h did not affect induced pluripotent stem cells from healthy human subjects (CON) but resulted in a significantly greater cell viability of induced pluripotent stem cells from sporadic Alzheimer's disease (SAD; $P = 0.006$) and familial Alzheimer disease (FAD; $P < 0.0001$) patients. For cell viability, interaction, treatment, and cell type were all significant sources of variation ($F[2,40] = 92.56$, $P < 0.0001$; $F[1,40] = 110.40$, $P < 0.0001$; and $F[2,40] = 92.81$, $P < 0.0001$, respectively). (*B*) Cell proliferation, measured by the percentage of bromodeoxyuridine (BrdU)-positive cells, was significantly impaired in familial Alzheimer's disease cells compared with control healthy subject cells ($P = 0.022$). Compared with vehicle control, dimethyl sulfoxide (DMSO), dantrolene resulted in a greater proliferation in familial Alzheimer's disease cells ($P = 0.008$, familial Alzheimer's disease dantrolene to dimethyl sulfoxide). For proliferation, dantrolene treatment and cell type were significant sources of variation ($F[2,30] = 5.44$, $P = 0.009$; and $F[1,30] = 9.81$, $P < 0.039$, respectively). All data are expressed as the means ± SD from five to eight independent experiments (in *A*, familial Alzheimer's disease, n = 7; control, n = 8; sporadic Alzheimer's disease, n = 8; and in *B*, control treated with dimethyl sulfoxide, n = 7; with dantrolene, n = 5; sporadic Alzheimer's disease both DMSO and dantrolene group, n = 5; familial Alzheimer's disease dimethyl sulfoxide, n = 8; dantrolene, n = 6). $^{**}P < 0.01$; $^{***}P < 0.001$. Statistical significance was determined using two-way analysis of variance with Sidak's multiple comparison tests. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

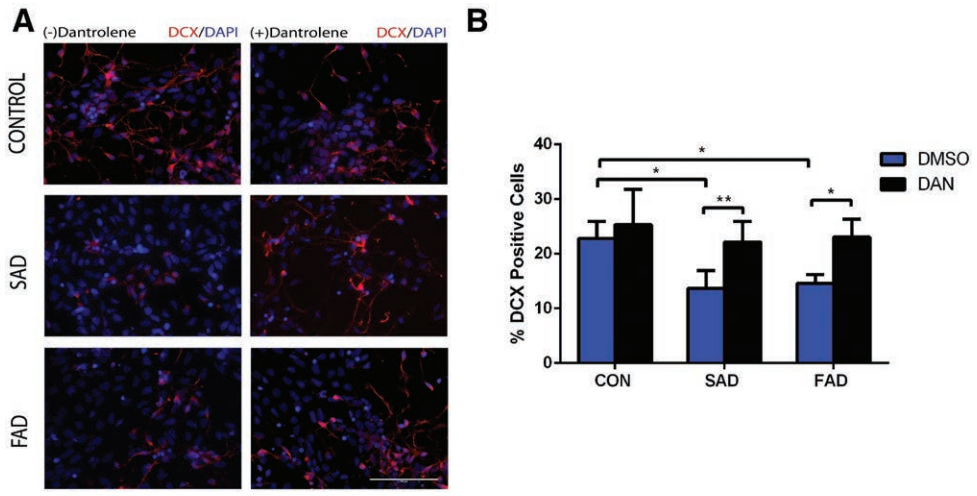

Fig. 2. Dantrolene ameliorated impairment of neuroprogenitor cells differentiation into immature neurons in Alzheimer's disease cells. Differentiation of neural progenitor cells into immature neurons (differentiation day 23) was significantly impaired in both sporadic Alzheimer's disease (SAD) and familial Alzheimer's disease (FAD), which was inhibited by dantrolene (DAN). (*A*) Representative immunofluorescence images of stained immature neurons by doublecortin (DCX; red), treated with or without dantrolene for 3 days, starting on induction day 0 from induced pluripotent stem cells. *Scale bar*, 100 μm. (*B*) Differentiations of both sporadic Alzheimer's disease cells ($P = 0.004$) and familial Alzheimer's disease cells ($P = 0.011$) were impaired compared with controls (CON). However, the differentiations of both sporadic Alzheimer's disease ($P = 0.008$) and familial Alzheimer's disease ($P = 0.008$) cells were enhanced after treatment with dantrolene. Cell type and treatment were significant sources of variation ($F[2,30] = 8.749$, $P = 0.001$; and $F[1,30] = 25.08$, $P < 0.0001$, respectively) using two-way analysis of variance with Sidak's multiple comparison tests. The data are represented by the means ± SD from six independent experiments (n = 6 for all groups). *$P < 0.05$; **$P < 0.01$. DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide.

both sporadic Alzheimer's disease and familial Alzheimer's disease patient induced pluripotent stem cells and most dramatically by 52.5% (19.7 ± 6.2 *vs.* 41.4 ± 19.3, N = 3 replicates, $P < 0.0001$) and 57.2% respectively (17.7 ± 10.2 *vs.* 41.4 ± 19.3 N = 3 replicates, $P < 0.0001$) at the distance around 150 μM from soma (fig. 4A), which was inhibited by dantrolene, especially in sporadic Alzheimer's disease cells (fig. 4B). We further examined the effects of dantrolene on synaptic density by determining presynaptic marker synapsin-1 (green) and postsynaptic marker PSD95 (red), using a double immunostaining technique (fig. 4C). Synapse density determined by either PSD95 (fig. 4D) or synapsin-1 (fig. 4E) was significantly less in cortical neurons generated from either sporadic Alzheimer's disease induced pluripotent stem cells by 58.2% (99.0 ± 16.6 *vs.* 237.0 ± 28.4 arbitrary units, N = 4 replicates, $P = 0.001$) or familial Alzheimer's disease induced pluripotent stem cells by 52.3% in PSD95 (113.0 ± 34.9 *vs.* 237.0 ± 28.4 arbitrary units, N = 5 replicates, $P = 0.001$) and sporadic Alzheimer's disease induced pluripotent stem cells by 59.1% (194.0 ± 52.3 *vs.* 474.5 ± 136.9 arbitrary units, N = 4 replicates, $P = 0.001$) or familial Alzheimer's disease induced pluripotent stem cells by 89.8% in synapsin-1 (48.5 ± 9.1 *vs.* 474.5 ± 136.9 arbitrary units, N = 5 replicates, $P < 0.0001$), and both were inhibited by dantrolene in familial Alzheimer's disease induced pluripotent stem cells.

Type 2 Ryanodine Receptor Was Significantly Greater in Human Alzheimer's Disease than in Control Induced Pluripotent Stem Cells

For mechanism studies, we first determined the expression of ryanodine receptor-2 using both immunoblotting (fig. 5, A and B) and immunostaining (fig. 5, C and D). Previous studies have demonstrated that ryanodine receptor-2 levels are abnormally elevated in Alzheimer's disease patients[8] and mice,[9] contributing to Alzheimer's disease pathology and cognitive dysfunction.[5,6,17] Similarly, compared with that of healthy human subjects, ryanodine receptor-2/β-actin levels were greater by 60.4% (0.8 ± 0.2 *vs.* 0.5 ± 0.1, N = 3, $P = 0.158$) in familial Alzheimer's disease (fig. 5B) and mean rank different by 11.1 (250.8 ± 75.7 *vs.* 397.5 ± 33.6 arbitrary units, N = 7 replicates, $P = 0.002$) in sporadic Alzheimer's disease (fig. 5D).

Dantrolene Significantly Inhibited ATP-mediated Abnormal Elevation of Cytosolic Ca^{2+} Concentrations ($[Ca^{2+}]_c$) in Induced Pluripotent Stem Cells from Both Sporadic and Familial Alzheimer's Disease Patients

We further investigated the possible mechanisms by which neurogenesis and synaptogenesis were impaired in sporadic Alzheimer's disease/familial Alzheimer's disease induced pluripotent stem cells and were ameliorated by dantrolene. Consistent with this elevated ryanodine

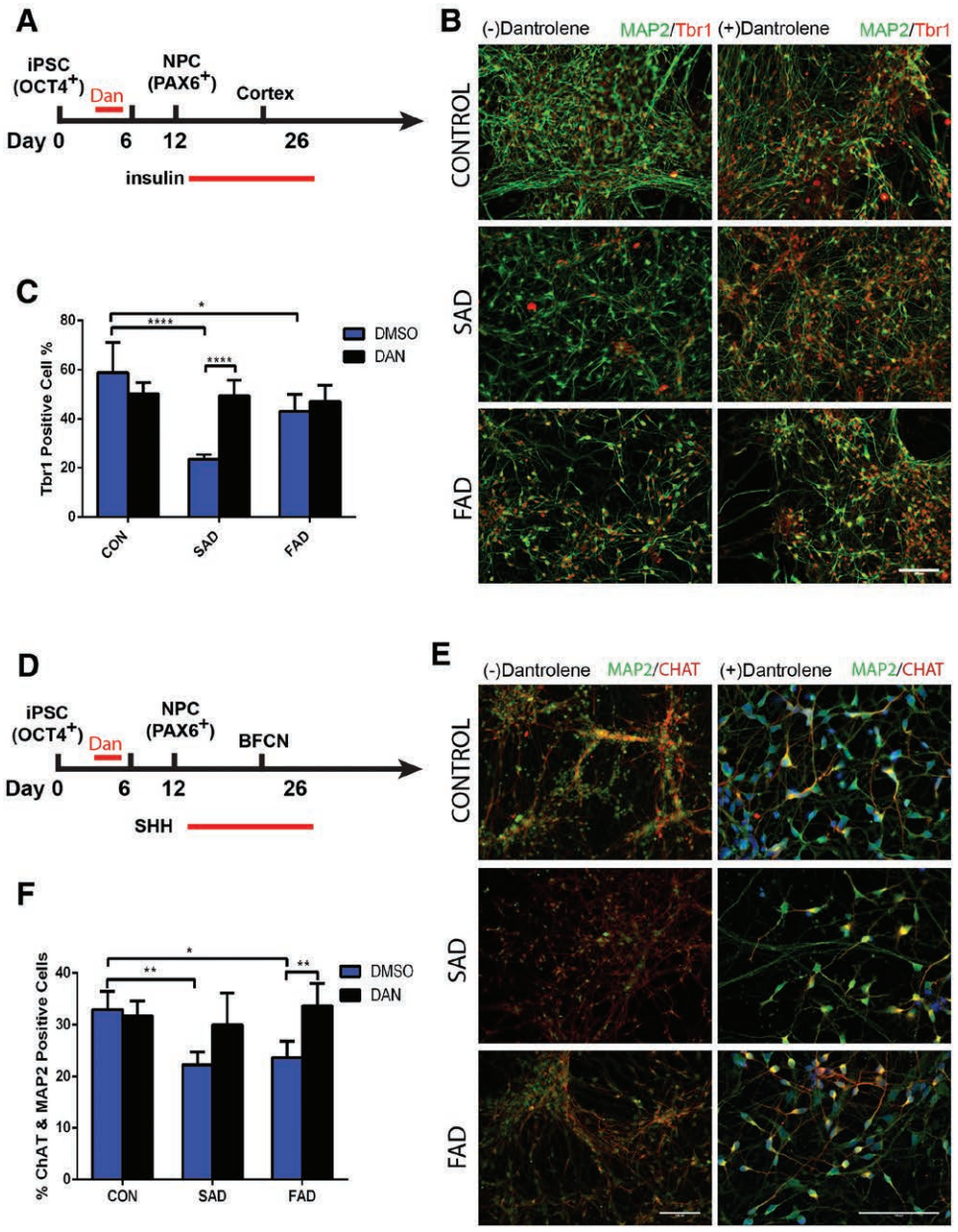

Fig. 3. Dantrolene inhibited differentiation of neural progenitor cells into cortical neurons and basal forebrain cholinergic neurons in Alzheimer's disease patient cells. (*A*) Differentiation timeline of neural progenitor cells (NPC) into mature cortical neurons. (*B*) Representative immunofluorescence images of double-stained neurons with thyroid hormone receptor-b (Trb1, red) and microtubule-associated protein-2 (MAP2, green). *Scale bar*, 100 μm. (*C*) The percentage of Trb1 positive cells was significantly less in both human sporadic Alzheimer disease (SAD; $P < 0.0001$) and familial Alzheimer disease (FAD) cells ($P = 0.022$) compared with control healthy subjects (CON) cells, but the sporadic Alzheimer's disease cells had significantly greater percentage of TrB1 positive cells after treatment with dantrolene (DAN or Dan; $P < 0.0001$). Interaction, cell type, and treatment were significant sources of variation ($F[2,24] = 14.84$, $P < 0.0001$; $F[2,24] = 15.94$, $P < 0.0001$; and $F[1,24] = 7.53$, $P = 0.011$, respectively). (*D*) Timeline for differentiation neural progenitor cells into mature basal forebrain cholinergic neurons (BFCN). (*E*) Representative immunofluorescence images of double-stained mature neurons by MAP2 (red) and choline acetyltransferase (CHAT or ChAT)–positive cells (green), with or without dantrolene treatment for 3 days starting from the induction of induced pluripotent stem cells (iPSC) differentiation into neurons. *Scale bars*, 100 μM. (*F*) The percentage of CHAT positive cells (basal forebrain cholinergic neurons) significantly decreased in both sporadic Alzheimer's disease ($P = 0.004$) and familial Alzheimer's disease ($P = 0.017$) cells, which was ameliorated by dantrolene treatment for familial Alzheimer's disease cells ($P = 0.008$) but not sporadic Alzheimer's disease cells ($P = 0.067$). Interaction, cell type, and treatment were significant sources of variation ($F[2,24] = 5.61$, $P = 0.010$; $F[2,24] = 6.27$, $P = 0.006$; and $F[1,24] = 14.78$, $P = 0.001$, respectively). Statistical significance was determined using two-way analysis of variance followed by Sidak's multiple comparison test. All data are represented as the means ± SD from five independent experiments (n = 5 for all groups). *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$. DMSO, dimethyl sulfoxide; SHH, Recombinant Human Sonic Hedgehog.

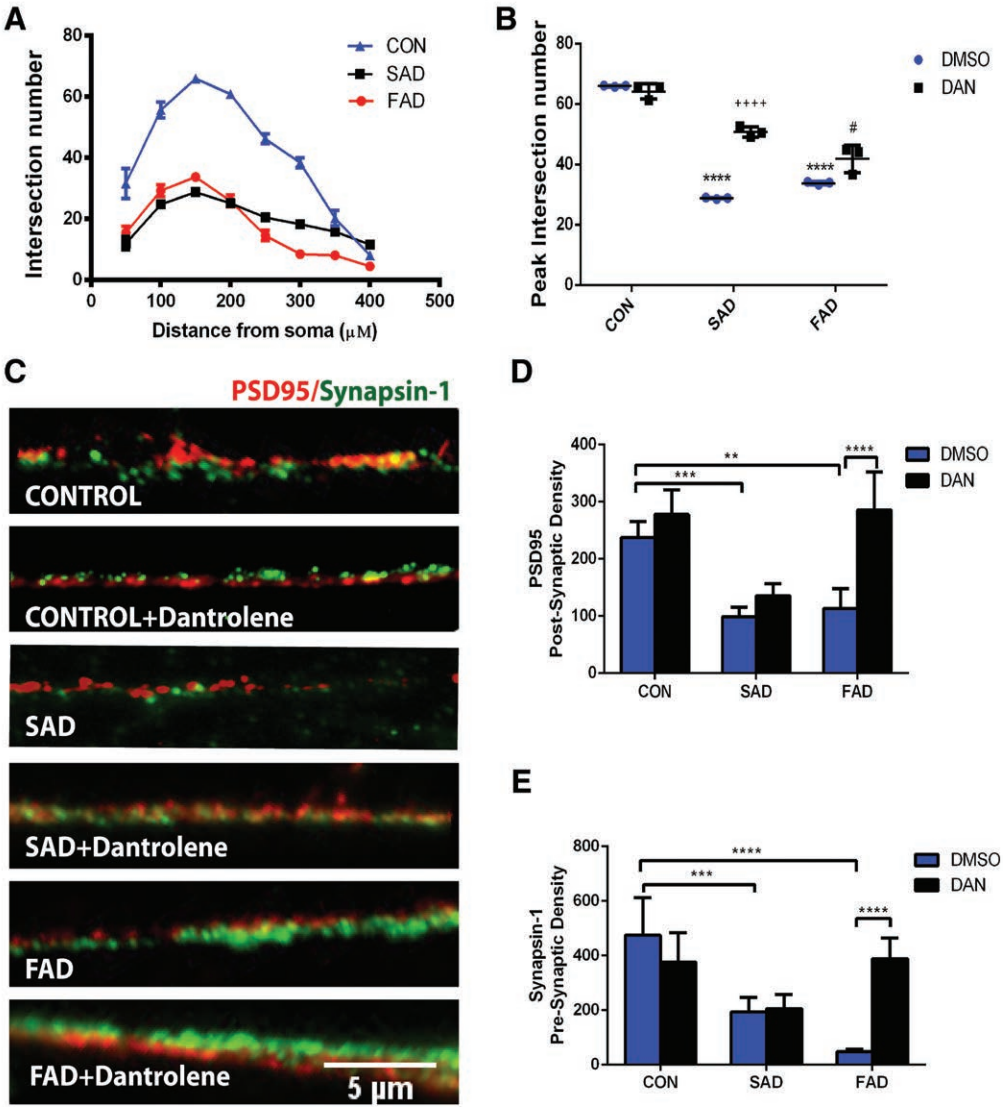

Fig. 4. Dantrolene inhibited impairment of dendrite intersection and synaptic density of neurons in Alzheimer's disease cells. Neural progenitor cells were differentiated into mature cortical neurons with insulin, and dantrolene (DAN) treatment was for 3 days starting from the induction of differentiation. The mean number of intersections between dendrites and concentric circles around the cortical neurons are shown as a function of the circle distance (μm) from the soma. (*A*) The number of intersections was significantly less in both sporadic Alzheimer's disease (SAD) and familial Alzheimer's disease (FAD) cells, which was inhibited by dantrolene in sporadic Alzheimer's disease cells. (*B*) The mean numbers of intersections at the distance around 150 μM from soma were less in sporadic Alzheimer's disease ($P < 0.0001$) and familial Alzheimer's disease cells ($P < 0.0001$) compared with controls (CON) but were significantly greater in both sporadic Alzheimer's disease ($P < 0.0001$) and familial Alzheimer's disease cells ($P = 0.014$) with dantrolene treatment. Interaction ($F[2,12] = 42.18$, $P < 0.0001$), cell type ($F[2,12] = 273.30$, $P < 0.0001$), and dantrolene treatment ($F[1,12] = 78.48$, $P < 0.0001$) were significant sources of variation. Statistical significance was determined by two-way analysis of variance and Sidak's multiple comparison test. (*C*) Synaptic density was determined by postsynaptic marker postsynaptic density protein 95 (PSD95; red) and presynaptic marker synapsin-1 (green) double immunostaining. *Scale bar*, 100 μm. (*D*) PSD95 density was significantly less in both sporadic Alzheimer's disease ($P = 0.001$) and familial Alzheimer's disease cells ($P = 0.001$) compared with controls but was significantly greater in familial Alzheimer's disease cells ($P < 0.0001$) with dantrolene treatment. Interaction ($F[2,23] = 8.78$, $P = 0.002$), cell type ($F[2,23] = 25.36$, $P < 0.0001$), and dantrolene treatment ($F[1,23] = 28.60$, $P < 0.0001$) were significant sources of variation. (*E*) Synapsin-1 was also significantly less in sporadic Alzheimer's disease ($P = 0.001$) and familial Alzheimer's disease $P < 0.0001$) cells and was significantly greater in familial Alzheimer's disease cells treated with dantrolene ($P < 0.0001$). Interaction ($F[2,23] = 18.12$, $P < 0.0001$), cell type ($F[2,23] = 21.46$, $P < 0.0001$), and dantrolene treatment ($F[1,23] = 7.18$, $P = 0.013$) were significant sources of variation. The data are represented by the means ± SD from at least four independent experiments: controls and familial Alzheimer's disease cells (N = 5) and sporadic Alzheimer's disease cells (N = 4). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Statistical significance was determined by two-way analysis of variance and Sidak's multiple comparison test. DMSO, dimethyl sulfoxide.

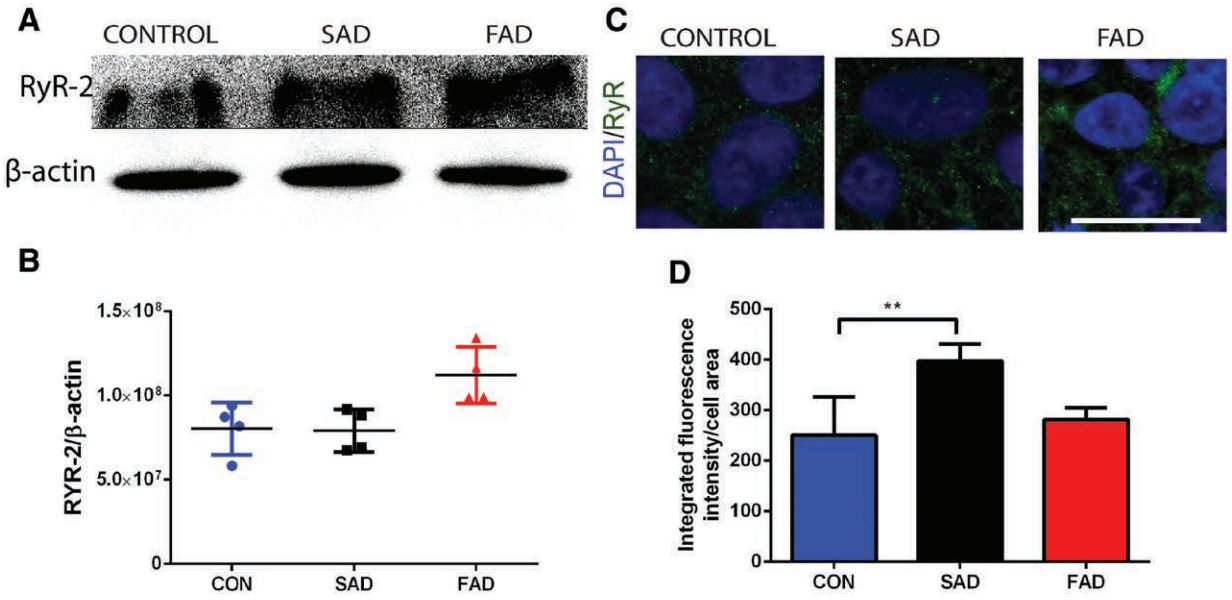

Fig. 5. Increased type 2 ryanodine receptors in human Alzheimer's disease cells. Type 2 ryanodine receptors (RyR, RyR-2, or RYR-2) were more in both sporadic Alzheimer's disease (SAD) and familial Alzheimer disease (FAD) cells and dramatically more in cells from familial Alzheimer's disease patients (*A* and *B*), determined by immunoblotting (Western Blot). Similarly, type 2 ryanodine receptors protein was significantly greater in sporadic Alzheimer's disease cells determined by immunofluorescence staining (*C* and *D*). All data are means ± SD from four independent experiments (N = 4 replicates, *B*) or 7 independent experiments (N = 7 replicates, *D*). The data in *B* was nonparametric (D'Agostino-Pearson omnibus normality test) and analyzed by the Kruskal–Wallis test ($P = 0.132$) followed by Dunn's multiple comparison tests ($P = 0.158$), compared to control healthy subjects (CON) cells. The data in *D* were also nonparametric and were analyzed by the Kruskal–Wallis test ($P = 0.002$) followed by Dunn's multiple comparison tests. *$P = 0.020$; **$P = 0.002$. *Scale bar*, 25 μm (*C*). DAPI, 4′,6-diamidino-2-phenylindole.

receptor-2 in Alzheimer's disease induced pluripotent stem cells, the NMDA-mediated elevation of integrated exposure (fig. 6, A and D) was higher in familial Alzheimer's disease and sporadic Alzheimer's disease induced pluripotent stem cells than in the normal control, which could be ameliorated by dantrolene (fig. 6, B–D). When three types of cells are examined of the Ca^{2+} release from the intracellular Ca^{2+} store by treating them with ATP (30 μM), as shown in figure 7, A through G, sporadic Alzheimer's disease/familial Alzheimer's disease induced pluripotent stem cells showed significantly higher peak elevation of $[Ca^{2+}]_c$ (percentage over baseline), (140.4 ± 40.2% *vs.* 84.1 ± 27.0%, N = 5 replicates, $P = 0.049$; 128.3 ± 40.6% *vs.* 84.1 ± 27.0%, N = 5 replicates, $P = 0.206$,) which was abolished by removal of extracellular Ca^{2+} and associated Ca^{2+} influx form extracellular space (fig. 7, A, B, and E) and pretreatment of dantrolene (30 μM) for 1 h (fig. 7, C and F). Without Ca^{2+} influx from extracellular space, ATP caused significantly lower overall elevation of $[Ca^{2+}]_c$ in all three types of cells (fig. 7E). In the absence of extracellular Ca^{2+} influx, dantrolene did not significantly inhibit ATP-mediated peak or overall elevation of $[Ca^{2+}]_c$ in sporadic Alzheimer's disease/familial Alzheimer's disease cells (fig. 7, D and G).

Dantrolene Ameliorated the Decrease of Lysosomal Vacuolar-type H^+-ATPase and Acidity in Induced Pluripotent Stem Cells from Alzheimer's Disease Patients

Decreased endoplasmic reticulum Ca^{2+} concentrations in Alzheimer's disease presenilin 1 mutation caused by overactivation of ryanodine receptor impaired synthesis and secretion of vacuolar-type H^+-ATPase from the endoplasmic reticulum into the lysosome, and subsequently decreased lysosome acidity and function.[30] We have determined the changes of lysosome *versus* endoplasmic reticulum vacuolar-type H^+-ATPase, as well as the lysosome acidity in various types of induced pluripotent stem cells. Location of vacuolar-type H^+-ATPase was determined by double immunostaining and colocalization targeting lysosome (LAMP-2), endoplasmic reticulum (calnexin), and endosome (fig. 8A), and the cellular acidity vehicles were determined by the Lysotracker (fig. 8B). The amount of lysosome vacuolar-type H^+-ATPase was significantly less in induced pluripotent stem cells from sporadic Alzheimer's disease by 39.3% (0.3 ± 0.02 *vs.* 0.6 ± 0.04 arbitrary units, N = 4 replicates, $P = 0.001$) and familial Alzheimer's disease by 30.4% (0.4 ± 0.07 *vs.* 0.6 ± 0.04 arbitrary units, N = 4 replicates, $P = 0.010$; fig. 8C; *compared with the

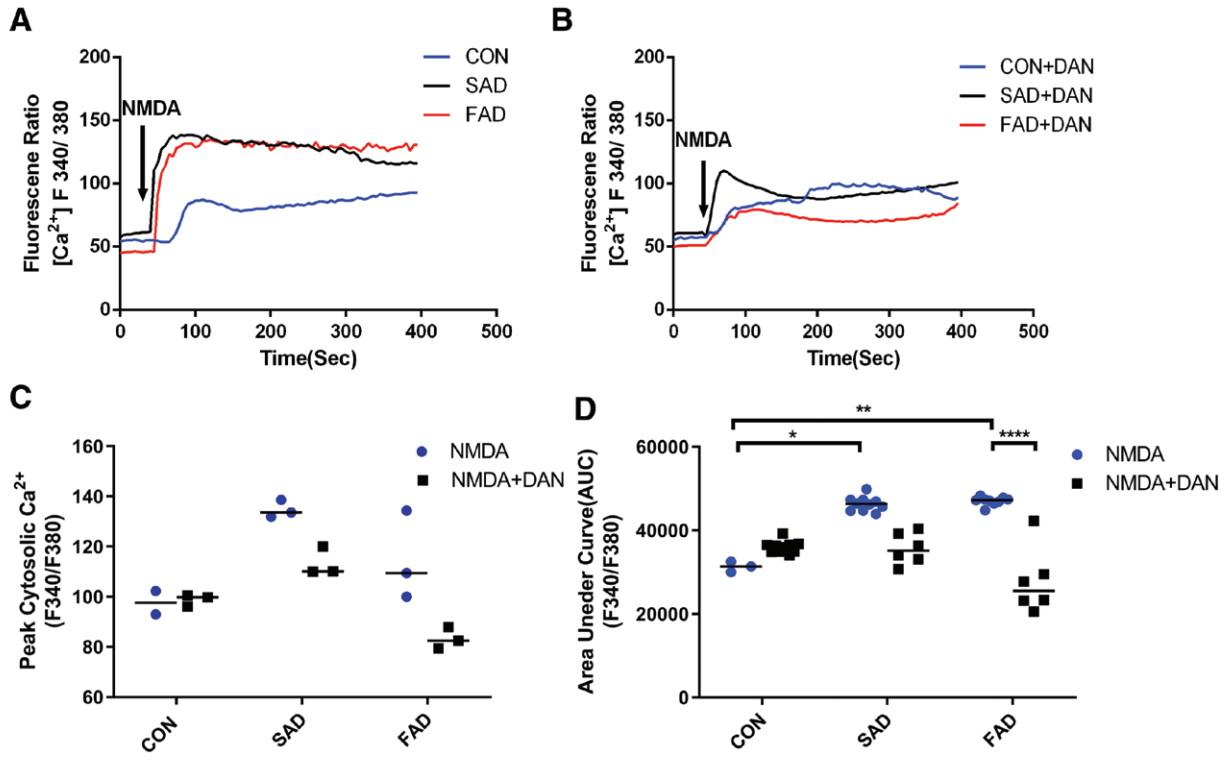

Fig. 6. Dantrolene significantly inhibited *N*-methyl-d-aspartate (NMDA) mediated elevation of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) in induced pluripotent stem cells from Alzheimer's disease patients. NMDA (500 μM) induced greater overall exposure of the integrated cytosolic Ca^{2+} represented by the area under curve (AUC; *A–D*) in sporadic (SAD) and familial (FAD) Alzheimer disease cells ($P = 0.041$ for sporadic Alzheimer's disease, $P = 0.008$ for familial Alzheimer's disease respectively) compared with normal human subjects (CON). Dantrolene (DAN, 30 μM) ameliorated the NMDA-mediated elevation of $[Ca^{2+}]_c$ and AUC in familial Alzheimer's disease cells ($P = 0.436$ for peak, $P < 0.0001$ for AUC, respectively; *B* and *D*). All data are expressed as medians [25th, 75th] from three independent experiments (N = 3). The data in *C* were nonparametric and analyzed by the Kruskall–Wallis test ($P = 0.020$) followed by Dunn's multiple comparison tests. The data for *D* were also nonparametric and analyzed using the Kruskal–Wallis test ($P < 0.001$) followed by Dunn's multiple comparison tests. *$P = 0.041$, **$P = 0.008$, ****$P < 0.0001$.

control), which could be inhibited by dantrolene, especially in familial Alzheimer's disease induced pluripotent stem cells (fig. 8D; *compared with the control). Consistently, the cellular acidity vehicle was significantly decreased by 49.3% (0.3 ± 0.1 *vs.* 0.7 ± 0.02 arbitrary units, N = 4 replicates, $P < 0.0001$) and 34.3% (0.4 ± 0.1 *vs.* 0.7 ± 0.02 arbitrary units, N = 4 replicates, $P = 0.004$), respectively, in both sporadic Alzheimer's disease and familial Alzheimer's disease induced pluripotent stem cells compared with that of the normal control, which were also significantly inhibited by dantrolene (fig. 8E; *compared with the control; $^{+}$compared with sporadic Alzheimer's disease; $^{\#}$compared with familial Alzheimer's disease).

Dantrolene Promoted Autophagy Activity in Induced Pluripotent Stem Cells from Alzheimer's Disease Patients

We further determined the effects of dantrolene on autophagy, considering the important role of lysosome in autophagy flux. The overall activity indicated by overall cellular level of autophagy biomarker LC3II was not significantly different among the three types of induced pluripotent stem cells (fig. 9, A–C). However, dantrolene treatment induced higher LC3II level by 42.9% (0.2 ± 0.01 *vs.* 0.1 ± 0.02 arbitrary units, N = 5, $P = 0.348$) in sporadic Alzheimer's disease (fig. 9B) and by 27.3% (0.1 ± 0.02 *vs.* 0.1 ± 0.02, ratio of LC3 II/β-actin, N = 3 replicates, $P = 0.0004$) in familial Alzheimer's disease (fig. 9C; $^{\#}$compared with familial Alzheimer's disease) induced pluripotent stem cells, respectively, which could be further elevated by the cotreatment with bafilomycin, an agent that impaired autophagy flux (fig. 9, B and C). This suggests that dantrolene promoted autophagy induction rather than impairing autophagy flux. The impaired autophagy flux in sporadic Alzheimer's disease/familial Alzheimer's disease induced pluripotent stem cells was further supported by the significantly elevated ratio of p62/β-actin mean rank different by 3 (1.6 ± 0.1 *vs.* 1.6 ±

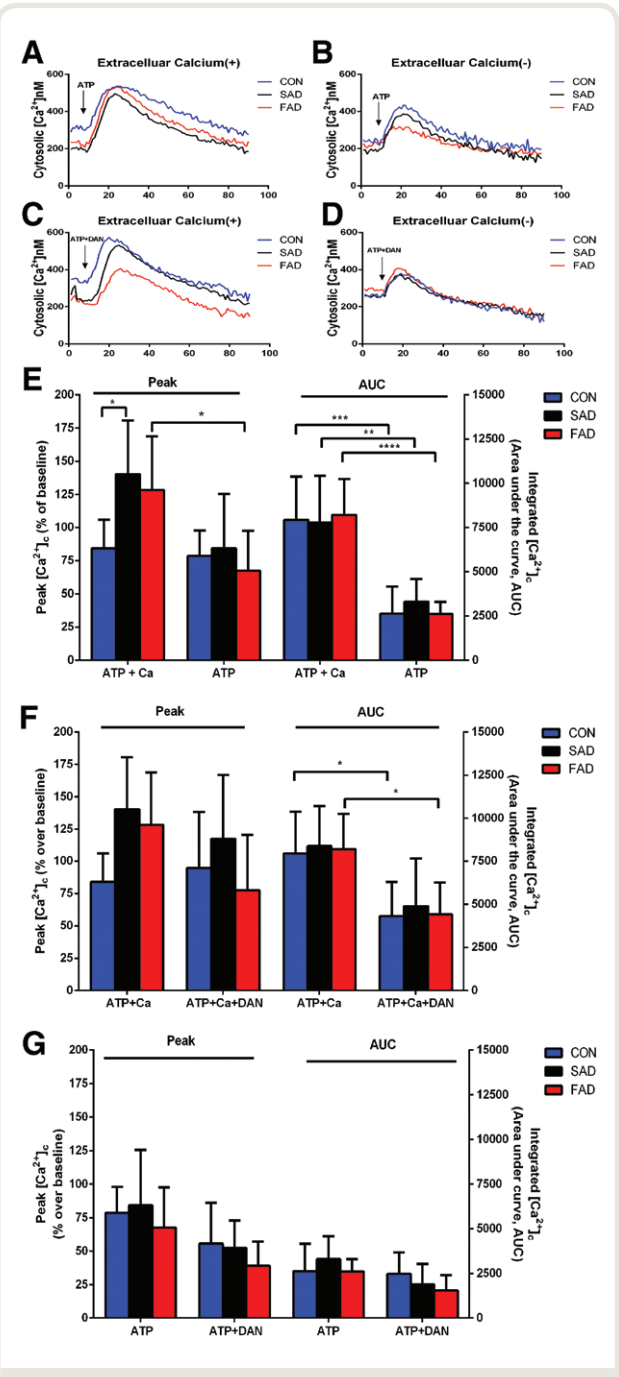

Fig. 7. The effect of dantrolene on the adenosine triphosphate (ATP)-mediated elevation of cytosolic calcium (Ca^{2+}) concentrations ($[Ca^{2+}]_c$) in basal forebrain cholinergic neurons from Alzheimer's disease patients. Both representing trace changes of cytosolic Ca^{2+} concentrations (*A–D*) and corresponding statistical analysis (*E–G*) are provided. Having examined the data carefully *via* the use of numerical and graphical summaries, a two-way analysis of variance was conducted to exam ATP (ATP + Ca^{2+}) and cell type: control (CON), sporadic Alzheimer's disease (SAD), familial Alzheimer's disease (FAD). ATP (30 μM), in the presence of 1 mM extracellular calcium (ATP + Ca^{2+}; *A*, *E*), was a significant source of variation (F[1,35] = 14.90, $P = 0.0005$) for the peak

0.3, N = 3 replicates, $P = 0.359$) in sporadic Alzheimer's disease and by 6 (1.5 ± 0.1 *vs.* 1.6 ± 0.3 arbitrary units, N = 3 replicates, $P = 0.015$) in familial Alzheimer's disease induced pluripotent stem cells, respectively (fig. 9D; *compared with the control).

Discussion

The primary finding of this study is that neurogenesis from neuroprogenitor cells to common cortical and Alzheimer's disease–specific deficient basal forebrain cholinergic neurons was significantly impaired in sporadic Alzheimer's disease/familial Alzheimer's disease patients compared with in healthy human subjects, which could be inhibited by dantrolene. In addition, dantrolene significantly inhibited synaptogenesis impairment in cortical neurons derived from induced pluripotent stem cells of sporadic Alzheimer's disease/familial Alzheimer's disease patients. In terms of mechanisms study, the ryanodine receptor-2 numbers in sporadic Alzheimer's disease/familial Alzheimer's disease

Fig. 7. (*Continued*) cytosolic Ca^{2+} concentrations $[Ca^{2+}]_c$ which were significantly higher in sporadic Alzheimer disease cells ($P = 0.049$) compared with control cells. ATP, in the absence of extracellular Ca^{2+} influx (ATP), caused significantly higher the ATP-induced peak $[Ca^{2+}]_c$ in familial Alzheimer's disease cells ($P = 0.031$) compared with familial Alzheimer's disease cells in the presence of extracellular Ca^{2+} influx (ATP+Ca^{2+}). (*B*, *E*) Furthermore, ATP with extracellular calcium (ATP + Ca^{2+}) was a significant source of variation (F[1,35] = 71.87, $P < 0.0001$) for the integrated cytosolic Ca^{2+} (area under the curve [AUC]), which was significantly less for control ($P = 0.0002$), sporadic Alzheimer's disease ($P = 0.005$), and familial Alzheimer's disease ($P < 0.0001$) cells compared with the same cells with ATP alone (ATP; *E*). Dantrolene (DAN, 30 μM) pretreatment of cells with ATP plus extracellular Ca^{2+} (ATP + Ca^{2+} + DAN) was a significant source of variation for Alzheimer's disease cell type (F[2,42] = 3.65, $P = 0.035$) for the peak cytosolic $[Ca^{2+}]_c$, although no significant differences were detected between the groups (*C*, *F*). The addition of dantrolene (ATP + Ca^{2+} + DAN) was also a significant source of variation (F[1,40] = 30.60, $P < 0.0001$) for Alzheimer's disease cell type for the AUC, which was significantly reduced for the controls ($P = 0.033$) and familial Alzheimer's disease cells ($P = 0.015$) compared with cells with just ATP + Ca^{2+} (*C*, *F*). Dantrolene (30 μM) pretreatment of the cells with ATP in the absence of extracellular Ca^{2+} (ATP + DAN; *D*) was a significant source of variation (F[1,33] = 10.01, $P = 0.003$) on the peak cytosolic $[Ca^{2+}]_c$, though no significant differences were found between the groups (*G*), and the absence of Ca^{2+} was a significant source of variation (F[1,33] = 5.95, $P = 0.020$) on the AUC with no differences detected between groups (*G*). Peak and integrated Ca^{2+} concentrations are shown as percentages of baseline from CON cells from normal human subjects. All data (*E–G*) are expressed as the means ± SD from at least five independent experiments (CON, n = 6 replicates; sporadic Alzheimer's disease, n = 5 replicates; familial Alzheimer's disease, n = 8–9 replicates). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Significance was determined by two-way analysis of variance followed by Sidak's multiple comparison tests.

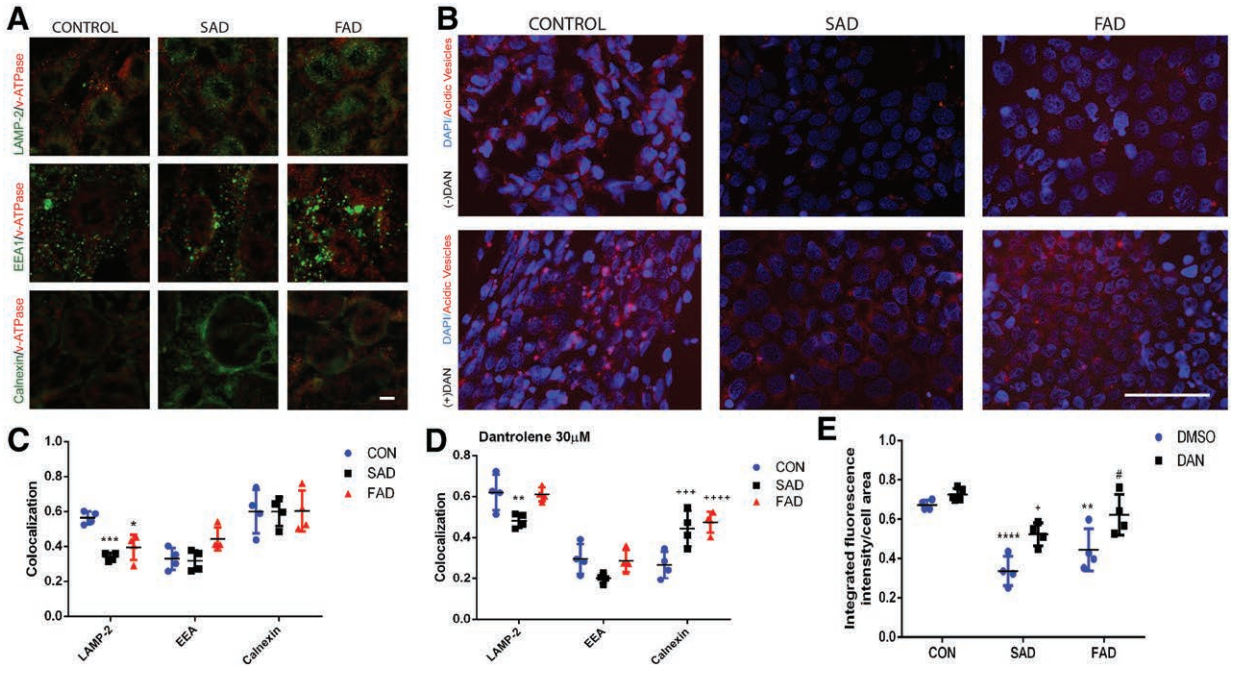

Fig. 8. Lysosomal ATPase and acidity in neurons derived from Alzheimer's disease patients were less than in control cells. (*A*) Colocalization of vacuolar-type H^+-ATPase (V-ATPase; *red*) was measured using immunostaining with specific markers targeting lysosomes (LAMP-2, *green*), endosomes (EEA, *green*), and endoplasmic reticulum (calnexin, *green*), in induced pluripotent stem cells of healthy human subjects (CON), sporadic Alzheimer's disease (SAD), or familial Alzheimer's disease (FAD) patients. (*B*) Cell acidity was measured by lysotracker-positive acidic vehicles (*red*) in control, sporadic Alzheimer's disease, and familial Alzheimer's disease cells (4′,6-diamidino-2-phenylindole [DAPI], *blue*). (*C*) Vacuolar-type H^+-ATPase in lysosomes (LAMP-2) was significantly lower in sporadic Alzheimer's disease ($P = 0.001$) and familial Alzheimer's disease cells ($P = 0.010$) than controls. There was significant source of variation for interaction ($F[4,23] = 4.35$, $P = 0.008$) and organelle type ($F[2,23] = 29.15$, $P < 0.0001$). (*D*) With the addition of dantrolene (DAN, 30 μM), V-ATPase in lysosomes (LAMP-2) in familial Alzheimer's disease cells was no longer significantly reduced ($P = 0.965$) but remained significantly lower in sporadic Alzheimer's disease cells ($P = 0.007$) compared with controls. In addition, vacuolar-type H^+-ATPase in the endoplasmic reticulum (calnexin) of the controls was significantly reduced compared with sporadic Alzheimer's disease ($P = 0.001$) and familial Alzheimer's disease ($P < 0.0001$) cells. There was significant source of variation for interaction ($F[4,27] = 8.66$, $P = 0.0001$), organelle type ($F[2,27] = 79.49$, $P < 0.0001$), and cell type ($F[2,27] = 5.96$, $P = 0.007$). (*E*) Lysotracker-positive acidic vesicles were significantly lower in sporadic Alzheimer's disease ($P < 0.0001$) and familial Alzheimer's disease ($P = 0.0004$) compared with control cells. Dantrolene also significantly increased tracker-positive acidic vesicles in both sporadic Alzheimer's disease ($P = 0.025$) and familial Alzheimer's disease ($P = 0.036$) cells compared with dimethyl sulfoxide (DMSO). Cell type and dantrolene were significant sources of variation ($F[2,19] = 29.88$, $P < 0.0001$; and $F[1,19] = 23.16$, $P = 0.0001$, respectively). All data are expressed as the means ± SD from four independents (n = 4 replicates for all groups) and were analyzed by two-way analysis of variance followed by Sidak's multiple comparison test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

induced pluripotent stem cells were abnormally increased, which contributed to the significant abnormal elevation of $[Ca^{2+}]_c$ by Ca^{2+} influx from extracellular space.[31–35] On the other hand, the abnormal Ca^{2+} release from the endoplasmic reticulum aggravated Ca^{2+} influx *via* capacitive calcium entry,[36] forming a vicious cycle to increase $[Ca^{2+}]_c$ and mitochondria Ca^{2+} concentration ($[Ca^{2+}]_m$), with simultaneous abnormal decrease of endoplasmic reticulum Ca^{2+} concentration ($[Ca^{2+}]_{er}$). All above pathologic Ca^{2+} dysregulation in cell lines derived from sporadic Alzheimer's disease/familial Alzheimer's disease patients contributed to the impairment of autophagy function, decrease of cell survival, and impairment of neurogenesis and synaptogenesis. Consistently, dantrolene significantly ameliorated the above pathologic Ca^{2+} dysregulation and therefore was neuroprotective in the cell lines from sporadic Alzheimer's disease/familial Alzheimer's disease patients.[5,6,17,18]

Recent studies indicate the important role of neurogenesis and synaptogenesis in cognitive function.[37] This is especially true in Alzheimer's disease because of an existing deficiency of memory formation–related neurons, such as basal forebrain cholinergic neurons.[19] Although the mechanisms remain unclear, neurogenesis and synaptogenesis in Alzheimer's disease are significantly impaired, contributing

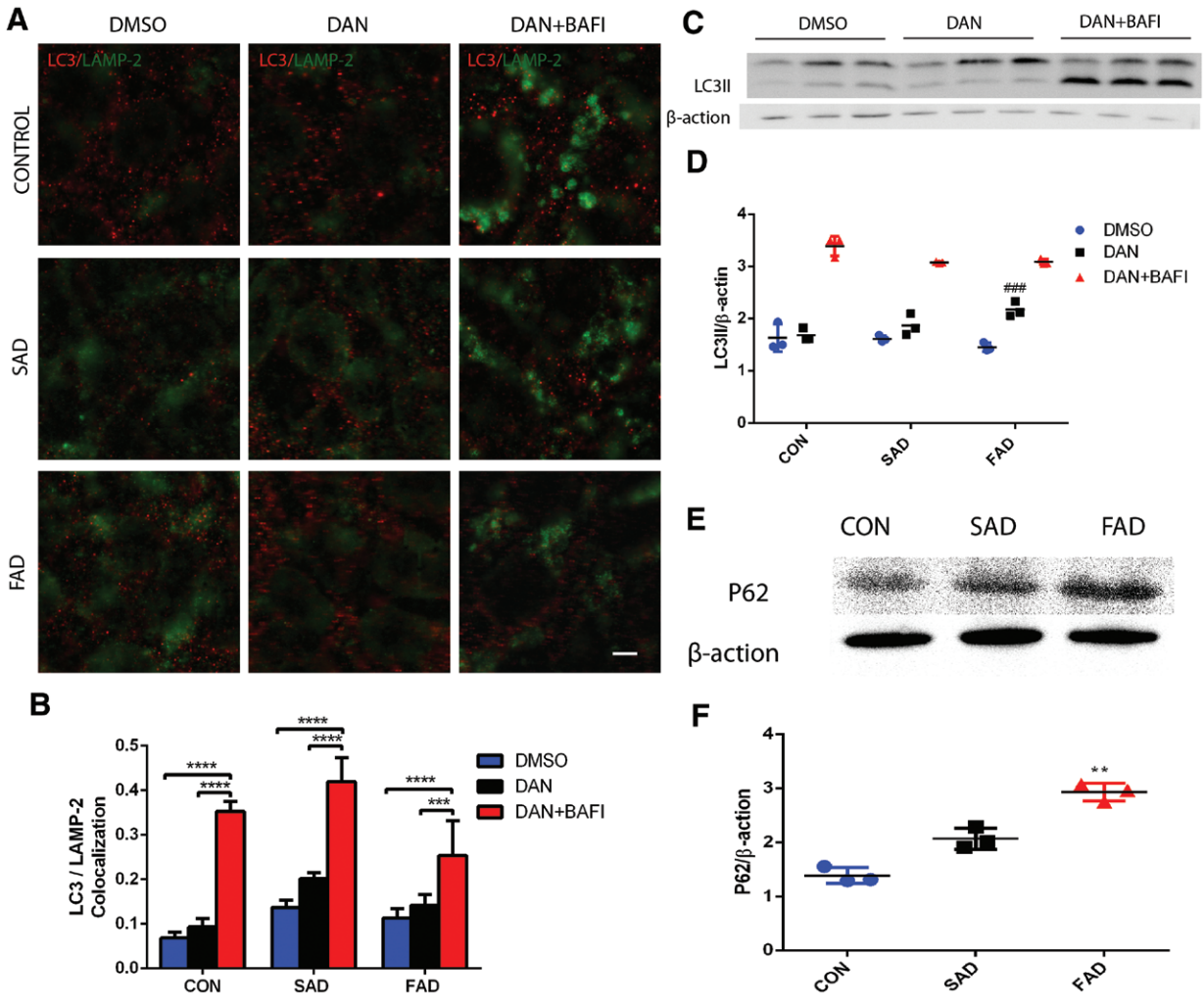

Fig. 9. Dantrolene increased LC3II levels in induced pluripotent stem cells from Alzheimer's disease patients. (*A*, *C*) Representative immunohistochemical images (*A*) and representative Western blots (*C*) of LC3II (*red*) in lysosomes (LAMP-2, *green*) in induced pluripotent stem cells from sporadic Alzheimer's disease (SAD), familial Alzheimer disease (FAD), and healthy human controls (CON) with dimethyl sulfoxide (DMSO), dantrolene (DAN), or dantrolene plus bafilomycins (BAFI). (*B*) Quantitation of the double-labeled immunostained cells showed that dantrolene with bafilomycins resulted in significantly greater LC3II in lysosomes (LAMP-2) in sporadic Alzheimer's disease ($P < 0.0001$), familial Alzheimer's disease ($P < 0.0001$), and CON ($P < 0.0001$) cells compared with dimethyl sulfoxide or dantrolene, respectively. There were significant sources of variation in interaction ($F[4,35] = 8.18$, $P < 0.0001$), cell type ($F[2,35] = 24.08$, $P < 0.0001$), and treatment ($F[2,35] = 177.00$, $P < 0.0001$) using two-way analysis of variance and Sidak's multiple comparison test. (*D*) Quantitation of Western blots similarly showed that dantrolene with bafilomycins resulted in significantly greater LC3II in lysosomes (LAMP-2) in sporadic Alzheimer's disease ($P < 0.0001$), familial Alzheimer's disease ($P < 0.0001$), and control ($P < 0.0001$) cells compared with dimethyl sulfoxide or dantrolene alone, respectively. Familial Alzheimer's disease cells treated with dantrolene were also significantly increased ($P = 0.0004$) compared with familial Alzheimer's disease treated with dimethyl sulfoxide cells. Interaction ($F[4,18] = 6.92$, $P = 0.002$) and treatment ($F[2,18] = 303.40$, $P < 0.001$) were significant sources of variation using two-way analysis of variance and Sidak's multiple comparison test. (*E*) Representative Western blot of P62 levels in control, sporadic Alzheimer's disease, and familial Alzheimer's disease cells. (*F*) Quantitation of P62 Western blots found that this marker of cellular stress is significantly greater in familial Alzheimer's disease cells ($P = 0.015$) compared with control using the Kruskal–Wallis test ($P = 0.004$) followed by Dunn's multiple correct tests. All data are expressed as the means ± SD from at least three independent experiments (n = 3 replicates for all groups). *$P < 0.05$; **$P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$.

to cognitive dysfunction.[11,38] A physiologic intracellular Ca^{2+} homeostasis plays an important role in neurogenesis/synaptogenesis and synapse function, whereas excessive Ca^{2+} release from the endoplasmic reticulum through overactivation of ryanodine receptor in Alzheimer's disease disrupts neurogenesis/synaptogenesis, which in turn, impairs synapse

and cognitive function. Theoretically, a method/approach to correct the disrupted intracellular Ca^{2+} homeostasis would inhibit impaired neurogenesis/synaptogenesis, synapse, and cognitive dysfunction. By targeting the upstream pathologic overactivation of ryanodine receptor in Alzheimer's disease, dantrolene is theoretically a good candidate to correct the disrupted intracellular Ca^{2+} homeostasis and associated neuropathology and could be therapeutic. In fact, dantrolene did inhibit the NMDA receptor activation–mediated Ca^{2+} dysregulation, impaired neurogenesis/synaptogenesis, and ameliorated lysosome dysfunction in sporadic Alzheimer's disease/familial Alzheimer's disease induced pluripotent stem cells/neuroprogenitor cell /neurons in this study. With its potency to inhibit impaired neurogenesis/synaptogenesis and to ameliorate the neurodegeneration in different types of neurodegenerative diseases[18,21] and its relatively tolerable side effects even after chronic administration,[39] dantrolene is expected to be therapeutic on both neuropathology and cognitive dysfunction in Alzheimer's disease patients, and further clinical trials are warranted.

Presenilin 1 mutation in familial Alzheimer's disease causes ryanodine receptor overactivation and Ca^{2+} dysregulation, resulting in worsened amyloid pathology, inflammation, neurodegeneration, and synapse dysfunction.[4,40] Comparatively, much less is known about the Ca^{2+} dysregulation and mechanisms in sporadic Alzheimer's disease cells, especially its role in the neurogenesis. The results from this study suggest that similar Ca^{2+} dysregulation caused by overactivation of ryanodine receptor and excessive Ca^{2+} release from the endoplasmic reticulum may also exist in sporadic Alzheimer's disease cells from patients, which can be corrected by dantrolene. Previous studies implicated the role of glutamate excitotoxicity, especially the NMDA overactivation and associated Ca^{2+} dysregulation, on neurodegeneration, amyloid pathology, and other neuropathology in Alzheimer's disease.[9,41] This has been the fundamental basis for the U.S. Food and Drug Administration approval of the last Alzheimer's disease treatment drug, memantine.[42] Dantrolene has been demonstrated to ameliorate the majority of NMDA-mediated elevation of $[Ca^{2+}]_c$ (up to 70%) in primary cortical neurons.[43] Because previous studies have also suggested that dantrolene could inhibit NMDA receptor–mediated Ca^{2+} influx,[44] it is possible that the inhibition of the NMDA receptor by dantrolene contributes to its amelioration on the elevation of $[Ca^{2+}]_c$. In the comparison of similar effects of removal extracellular Ca^{2+} influx and use of dantrolene, it is plausible to assume that dantrolene ameliorated ATP-mediated $[Ca^{2+}]_c$ elevation by primary inhibition of Ca^{2+} influx from extracellular space, although the inhibition of ryanodine receptor–mediated capacitive calcium entry may play a role on Ca^{2+} influx. However, additional experiments examining the exact Ca^{2+} channels on plasma membrane (*e.g.*, different types of voltage-dependent calcium channels, glutamate receptors, Orail 1 receptors etc.) involved for ATP-mediated elevation of $[Ca^{2+}]_c$ and the effects of dantrolene is beyond the scope of current study and should be investigated further in the future. Regardless of the detailed mechanisms, dantrolene can inhibit NMDA overactivation on both sporadic Alzheimer's disease and familial Alzheimer's disease cells, which contributes to its beneficial effects on ameliorating the impaired neurogenesis/synaptogenesis in induced pluripotent stem cells/neuroprogenitor cell /neurons from sporadic Alzheimer's disease/familial Alzheimer's disease patients. It is important to note that dantrolene is also neuroprotective in sporadic Alzheimer's disease cells because sporadic Alzheimer's disease accounts for most Alzheimer's disease patients. In a further step, these results need continuous confirmation in multiple cell lines from induced pluripotent stem cells of different sporadic Alzheimer's disease/familial Alzheimer's disease patients. Although the genetic background may not be the same in sporadic Alzheimer's disease and familial Alzheimer's disease, they seem to share a common mechanism on the disruption of intracellular Ca^{2+} homeostasis and associated impairment of neurogenesis/synaptogenesis, in addition to the Ca^{2+} dysregulation–mediated amyloid, Tau pathology, and neurodegeneration in previous studies.[45]

Normal and physiologic autophagy function play important roles in many cell functions, including neurogenesis and synaptogenesis,[46] whereas impaired physiologic autophagy may promote apoptosis, and pathologic autophagy may cause autophagic cell death directly.[47] Disruption of intracellular Ca^{2+} homeostasis, especially abnormal Ca^{2+} release from the endoplasmic reticulum *via* ryanodine receptor or inositol triphosphate receptor, contributed to both impaired physiologic autophagy and pathologic autophagic cell death.[47] Altered and/or impaired autophagy function have been demonstrated in familial Alzheimer's disease.[30] Although the mechanisms are not clear, Ca^{2+} dysregulation contributes to impaired autophagy function in Alzheimer's disease, because Ca^{2+} is an important messenger in the regulation of autophagy.[45] Activation of inositol triphosphate receptor and Ca^{2+} release from the endoplasmic reticulum can induce autophagy in a cytosolic Ca^{2+}-dependent way[48] or inhibit autophagy *via* mitochondrial pathway to increase production of ATP.[49] Ryanodine receptor overactivation, especially ryanodine receptor-3, impairs autophagy flux at the lysosome level[50] and promotes autophagy cell death.[51] The results from this study are consistent with the previous finding that abnormally elevated ryanodine receptor-2 (fig. 5) and resultant Ca^{2+} dysregulation (figs. 6 and 7) in Alzheimer's disease cells were associated with the impaired lysosome acidity and function (fig. 8). Autophagy flux was consistently impaired in Alzheimer's disease cells, but dantrolene seemed to primarily promote autophagy activity, although it also ameliorated impaired lysosome acidity and function. (figs. 8 and 9). It is reasonable to associate dantrolene-mediated inhibition of impaired neurogenesis/synaptogenesis in Alzheimer's disease

cells with its ability to promote overall autophagy activity and to ameliorate the impaired lysosome function.

Because of the lack of well recognized cell or animal models of sporadic Alzheimer's disease, the autophagy function and its relationship with intracellular Ca^{2+} regulation is much less defined in sporadic Alzheimer's disease. We have demonstrated in this study that, like in familial Alzheimer's disease cells,[30] the lysosome acidity and function was also impaired in sporadic Alzheimer's disease cells, which was rescued by dantrolene, suggesting a role of calcium dysregulation in disrupting the autophagy function in sporadic Alzheimer's disease cells. Additionally, dantrolene can promote autophagy induction and relieve impaired lysosome function in sporadic Alzheimer's disease cells. In combination with its beneficial effects in inhibiting impaired neurogenesis/synaptogenesis in this study, it is reasonable to assume that the ability of dantrolene to correct disruption of intracellular Ca^{2+} homeostasis contributes to its beneficial effects on autophagy function and neurogenesis/synaptogenesis in sporadic Alzheimer's disease cells.

Cholinergic cortical neurons in the prefrontal cortex play important roles in the formation of memory and have been considered major deficient neurons in Alzheimer's disease.[19] The majority of current symptom-relieving drugs for Alzheimer's disease are the cholinesterase inhibitors, aiming to raise level of presynaptic acetylcholine, antagonizing the effects of deficiency of cholinergic cortical neurons.[29] The results in this study indicated a reduced neurogenesis on cholinergic neurons, which can be inhibited by dantrolene. The beneficial effects of dantrolene to restore cholinergic cortical neurons theoretically make the drug a potential treatment of cognitive dysfunction in Alzheimer's disease, especially in sporadic Alzheimer's disease patients. Furthermore, the inhibition of impaired synaptogenesis on cholinergic neurons also helps to improve synapse and cognitive function in Alzheimer's disease. More translational studies are needed in sporadic Alzheimer's disease animal model or those models more translational to human Alzheimer's disease to promote dantrolene to be a potential future drug treatment for Alzheimer's disease patients.[52] Because neuroprotection of dantrolene is dose-dependent, a method or approach to improve dantrolene penetration into the central nervous system will be very helpful to promote dantrolene use for neuroprotection of various neurodegenerative diseases, including Alzheimer's disease and stroke.[53]

This study has the following limitations: (1) each sporadic Alzheimer's disease/familial Alzheimer's disease cell line is only from one patient, and more cell lines from different patients are needed to strengthen the finding and confirm the conclusion, especially on data with less consistency between sporadic Alzheimer's disease and familial Alzheimer's disease cells; (2) the mechanisms of dantrolene neuroprotection is primarily based on its inhibition of ryanodine receptor, but other possible effects, such as on NMDA receptors, need to be clarified in future studies; and (3) the lack of studies on synapse function by electrophysiology studies, which contribute to the cognitive dysfunction in Alzheimer's disease.

In conclusion, dantrolene significantly ameliorated impaired neurogenesis and synaptogenesis in both sporadic Alzheimer's disease and familial Alzheimer's disease cells from patients, which was associated with its effects to restore the intracellular Ca^{2+} homeostasis and to ameliorate lysosomal dysfunction and promote autophagy activity, calling for further investigation of using dantrolene to treat Alzheimer's disease patients in the future.

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Competing Interests

Dr. Wei was once a member of the Advisory Board of Eagle Pharmaceutical Company (Woodcliff Lake, New Jersey) for a 1-day meeting in 2017; the contract has expired. Eagle Pharmaceutical Company produces and sells Ryanodex, a new formula of dantrolene. Partial results of this article have been included in a U.S. provisional patent application titled "Intranasal Administration of Dantrolene for Treatment of Alzheimer's Disease" filed on June 28, 2019 (serial No. 62/868,820) by the University of Pennsylvania Trustee. Dr. Wei and Dr. Liang are listed as inventors on the provisional patent application. The patent application is also part of the research collaboration agreement between the University of Pennsylvania and Eagle Pharmaceutical Company. The dantrolene used in this study is purchased from the Sigma Company (Kawasaki, Japan). The other authors declare no competing interests.

Correspondence

Address correspondence to Dr. Wei: University of Pennsylvania, 305 John Morgan Building, 3610 Hamilton

Walk, Philadelphia, Pennsylvania 19104. weih@uphs.upenn.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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George S. Bause, M.D., M.P.H., Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio.