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A human stem cell model of early Alzheimer's disease pathology in Down syndrome

Yichen Shi¹, Peter Kirwan¹, James Smith¹, Glenn MacLean², Stuart H. Orkin², and Frederick J. Livesey^{1,*}

¹Gurdon Institute and Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QN

²HHMI, Department of Pediatric Oncology, Dana-Farber Cancer Institute, Division of Hematology/Oncology, Children's Hospital Boston and Harvard Stem Cell Institute, Boston, Massachusetts

Abstract

Human cellular models of Alzheimer's disease (AD) pathogenesis would enable the investigation of candidate pathogenic mechanisms in AD and the testing and developing of new therapeutic strategies. We report the development of AD pathologies in cortical neurons generated from human induced pluripotent stem (iPS) cells derived from patients with Down syndrome. Adults with Down syndrome (caused by trisomy of chromosome 21) develop early-onset Alzheimer's disease, probably due to increased expression of the amyloid precursor protein (APP) encoded by a gene on chromosome 21. We found that cortical neurons generated from iPS cells and embryonic stem (ES) cells from Down syndrome patients developed Alzheimer's disease pathologies over months in culture, rather than years in vivo. The cortical neurons processed the transmembrane APP protein resulting in secretion of the pathogenic amyloid- β 42 (A β 42) peptide fragment. A β 42 peptides formed insoluble intracellular and extracellular amyloid aggregates. Production of A β peptides was blocked by a gamma-secretase inhibitor. Finally, hyperphosphorylated tau protein, a pathological hallmark of AD, was found to be localized to cell bodies and dendrites in iPS cell-derived cortical neurons from Down syndrome patients, recapitulating later stages of the AD pathogenic process.

INTRODUCTION

Alzheimer's disease is a major global health problem for which there are no disease-modifying treatments. A key challenge for developing effective treatments for Alzheimer's disease (AD) is that the aetiology and pathogenesis of the sporadic form of the disease are not well understood. The classic pathological hallmarks of AD are amyloid plaques

* Author for correspondence: Rick Livesey, Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QN. Telephone: +44-1223-763281 rick@gurdon.cam.ac.uk.

Contributions: YS, PK and FJL designed the experiments. YS generated cortical neurons from pluripotent stem cells and carried out immunohistochemistry and confocal microscopy studies. PK carried out electrophysiology and super-resolution microscopy studies. YS and JS carried out ELISAs. GM and SHO generated and characterized the DS ES cell line. YS, PK, JS and FJL analysed the data. YS, PK and FJL wrote the manuscript.

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composed of A β peptides, which are products of the transmembrane APP protein, and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated forms of the microtubule-associated protein tau (1). There is an ongoing debate as to the pathogenic process in AD and the relative contributions of A β peptides and tau to AD pathogenesis (2). Support for a causal role for A β peptides in AD pathogenesis comes from studies of autosomal dominant forms of familial AD. Mutations that cause the rare familial forms of AD are all found either in APP or in components of the enzyme complex that processes APP; all of these mutations result in an increase in A β peptide production and aggregation (3). In contrast, mutations in the tau protein lead to neurodegenerative disorders that are phenotypically distinct from AD including frontal temporal dementia (FTD) and progressive supranuclear palsy (PSP) (4).

Animal models, although useful for modelling aspects of APP processing in AD, have been of limited use in developing treatments for AD (5). Rodent models do not capture many key aspects of the disease process, and only triple transgenic mice that express mutant forms of human APP, presenilin and tau develop both plaque and tangle pathology in brain tissue (5). Therefore, it has been argued that these models are useful for studying the initiation of AD, but not the disease process itself (5). A human cellular model of AD would enable detailed functional studies of AD pathogenesis. An effective human cellular model would use the appropriate cell type, in this case glutamatergic projection neurons of the human cerebral cortex, would develop relevant molecular pathology (altered APP processing, A β aggregation and tau hyperphosphorylation), and would do so in a reproducible manner over a timescale short enough for practical use. A pressing question for the usefulness of this approach is whether neurological diseases that take decades to become manifest in humans can be successfully modeled over a reasonable timescale (6, 7).

Here, we report an *in vitro* human model for AD pathogenesis in Down syndrome. We applied a process that we developed for directed differentiation of human induced pluripotent stem (iPS) cells into cerebral cortex projection neurons (8). We generated cortical neurons from iPS cells derived from patients with Down syndrome caused by trisomy of chromosome 21. Down syndrome is the commonest genetic cause of mental retardation, occurring in approximately 1 in 700-800 live births (9). Individuals with Down syndrome also have a very high incidence of AD (10), attributed in part to the presence of three copies of the gene encoding the amyloid precursor protein (APP), which is located on chromosome 21 (11, 12). Duplication of the APP gene in humans results in autosomal dominant early-onset dementia (13), and mice with increased APP gene dosage develop amyloid plaques and the neuropathological hallmarks of Alzheimer-type dementia (14). A number of other genes on chromosome 21 may also contribute to the greatly increased risk of dementia in Down syndrome patients including the gene that encodes the Dyrk1A kinase that phosphorylates tau (15).

Increased production of A β peptides from processing of APP has been observed in children with Down syndrome, with plaques characteristic of AD pathology present in the nervous system as early as teen years (16). Given the early onset of amyloid pathology in Down syndrome patients, we hypothesized that cortical neurons generated from iPS cells derived from these patients could potentially develop phenotypes typical of AD quickly *in vitro*.

RESULTS

Cortical neurons from Down syndrome iPS cells

To investigate the potential for modeling AD pathology in Down syndrome, we differentiated human healthy control (17) and Down syndrome iPS cells (DS1-iPS4) (18) into cortical neural stem and progenitor cells (Fig. 1A-H). Control and Down syndrome iPS cells (referred to here as DS-iPS cells; Suppl Fig. 1) generate cortical neuronal stem and progenitor cells at high efficiency, defined by expression of Pax6, Vimentin and Otx1/2 (Fig. 1A-D). As described for human iPS cells and embryonic stem (ES) cells (8), control and Down syndrome iPS cell-derived cortical neuronal stem cells develop as polarized neuroepithelial rosettes (Fig. 1E, F), with a subpopulation of basal progenitor cells that express the basal progenitor cell-specific transcription factor Tbr2/Eomes (Fig. 1G, H).

Under neuronal differentiation conditions, control and DS iPS-cell derived cortical neuronal stem cells efficiently differentiate into glutamatergic projection neurons of each cortical layer (Fig. 1I-N, Q) and then generate astrocytes (Fig. 1O, P). As previously described (8), all neurons generated by control and DS-iPS cells using this process are glutamatergic (Suppl. Fig. 2). Mouse models of Down syndrome have suggested that DS cortical neuronal stem and progenitor cells produce proportionally fewer upper layer cortical neurons (19, 20), although it is not clear that this occurs in humans with trisomy 21. DS iPS cells did not demonstrate a significant neurogenic or neuronal differentiation phenotype. In particular, no difference was observed in the numbers of neurons produced or in the relative numbers of different classes of projection neurons generated by DS-iPS cells (Fig. 1Q).

Synapse Formation by cortical neurons derived from DS iPS cells

DS iPS cell--derived cortical neurons mature in culture, as shown by their acquisition of the ability to fire trains of action potentials upon current stimulation (Fig 2A, B). These neurons also form functional synapses, as evidenced by the presence of miniature excitatory post-synaptic currents in cultures 45 to 100 days old (Fig. 2C, D). The formation of physical synapses among cortical projection neurons derived from DS iPS cells was confirmed using super-resolution (structured illumination) microscopy to visualize the localization of pre- and post-synaptic proteins. The localization of post-synaptic proteins on or within a few 100 nm of the dendritic shaft, as well as closely juxtaposed pre- and post-synaptic proteins was used to define synapses (Fig. 2E-J). The glutamatergic post-synaptic density protein PSD95 was found to be localised close to MAP2 positive dendrites in neurons derived from DS-iPS and control iPS cells (Fig. 2E, F). In addition, both PSD95 and Homer1, another protein enriched in excitatory post-synaptic compartments, were frequently found in close association with the presynaptic proteins Synaptophysin and Munc13-1 (Fig 2E-J). No difference in the density of synapses per unit length of dendrite was found between DS iPS cell-derived neurons and neurons generated from four different control iPS cell lines (Fig. 2K).

Increased A β peptide generation in DS-iPS cell cortical neurons

A key stage in AD pathogenesis *in vivo* is the increased generation of short A β peptides (38-43 amino acids in length) from APP by glutamatergic neurons in the cerebral cortex.

These peptides form soluble and insoluble aggregates, or amyloid plaques (12) that are deposited in brain tissue. We monitored the time-course of the extracellular accumulation of A β 40 and A β 42 peptides by cortical neurons derived from stage- and cell density-matched control iPS and DS iPS cell cultures over a period of two weeks from the onset of neuronal differentiation (Fig. 3A). Healthy cortical neurons typically produce considerably more A β 40 than A β 42 *in vivo* (21). Extracellular concentrations of A β 40 peptides are low in both control and DS cortical neuronal cultures at the onset of neuronal differentiation (Fig. 3A). However, DS cortical neurons increased their production of A β 40 to high levels over the subsequent 10 days, reaching an average of 233pg/ml by day 28 of differentiation (n=3 for each 48 hour window of production and collection), whereas control neuron production of A β 40 remained consistently low at less than 100pg/ml (n=3 for each timepoint; Fig. 3A; $p<0.01$).

Extracellular accumulation of pathogenic A β 42 peptide from control or DS cortical neurons was not detected at this early stage of neuronal differentiation. However, analysis of the extracellular media from older (day 70) cultures of DS and control iPS cell-derived cortical neuron cultures confirmed that older DS neurons produce large amounts of both A β 40 (mean of 843pg/ml, n=3) and A β 42 (183pg/ml, mean of n=3) in a 48 hour period (Fig. 3B), whereas control cortical neurons continue to generate low concentrations of A β 40 (control iPS cell-derived neurons, mean of 124pg/ml, n=3) and A β 42 (control iPS cell-derived neurons mean of 32pg/ml, n=3; Fig. 3B; $p<0.01$). High-level production of A β peptides was specific to DS cortical neurons, as production of A β 40 and A β 42 peptides by fibroblasts from Down syndrome patients was low and undetectable, respectively (Fig. 3B).

To validate the DS-iPS cell-based model of the early stages of AD pathogenesis, we asked whether A β 40 and A β 42 peptide generation by DS-iPS cell-derived cortical neurons could be reduced by pharmacological inhibition of the gamma-secretase complex, one of the two essential protease complexes that process APP to generate A β peptides. Inhibition of gamma-secretase also inhibits Notch signaling in neural stem cells, resulting in cell cycle exit and differentiation (22). Therefore, the gamma-secretase inhibitor DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]- S-phenylglycine t-butyl ester) was administered at a stage (day 50; as we have shown in multiple iPS cell lines) when approximately 70% of cells in culture are post-mitotic neurons (8). DAPT inhibition of gamma-secretase over 4 days reduced A β 40 and A β 42 peptide production from DS cortical neurons by almost half, whereas longer-term treatment (21 days) reduced secretion of both A β peptides to below detectable levels (Fig. 3B).

DS-iPS cell-derived cortical neurons generate A β aggregates

Given the increased generation of A β 40 and A β 42 peptides by DS-iPS cell-derived cortical neurons over time, we assayed the development of amyloid plaques in these neurons in culture by live staining of intracellular and extracellular aggregates of amyloid with the thioflavin T analog, BTA1 (23) (Fig. 4A, B). Over a two-month period after the initiation of cortical differentiation, BTA1-positive neurons or extracellular aggregates were not observed in cultures of control iPS-cell derived cortical neurons (Fig. 4A). However, DS-iPS cell-derived cortical neurons generated both intracellular and extracellular aggregates of

BTA1-labelled amyloid over the same time period (Fig. 4B). Polyclonal and monoclonal antibody staining and confocal microscopy demonstrated the presence of numerous A β 42-containing aggregates both within and outside DS-iPS cell-derived cortical neurons (Fig. 4C-F; Suppl. Fig. 3), with extracellular A β 42-positive aggregates often found around neurites (Fig. 4F), confirming that A β 42 is produced by DS cortical neurons and aggregates over time. A β 42-positive aggregates were rarely found in cultures of control iPS cell-derived cortical neurons (Fig. 4C; Suppl. Fig. 3), or in cultures of DS fibroblasts (Suppl. Fig. 3).

AD pathogenesis is recapitulated by Down syndrome ES cells

Variation among iPS cell lines at the epigenetic level, together with the occurrence of genetic mutations during reprogramming from adult fibroblasts has been suggested to be an important factor in interpreting the reliability of disease-related phenotypes observed in iPS cell systems (24-26). To investigate the reproducibility of the AD phenotypes observed from DS iPS cells and their association with the iPS cell state, we also generated cortical neurons from Down syndrome and control (H9) human ES cells (Suppl. Fig. 4).

Down syndrome ES cells (DS-ES cells) differentiate efficiently into cortical projection neurons in numbers equivalent to those observed for iPS cells (Fig. 5A, B). Control and DS-ES cell-derived cortical neurons do not display marked differences in the expression or cellular localization of full length APP protein (Fig. 5C, D). However, extracellular and intracellular aggregates of A β 42 peptides are abundant in DS-ES cell cortical neuronal cultures (Fig. 5E, F, I), and have the same distribution as seen for DS-iPS cell-derived cortical neuronal cultures. DS-ES cell-derived and DS iPS cell-derived cortical neurons produce similar amounts of A β 40 and A β 42 peptides over a 48-hour collection period (Fig. 5G). These amounts are more than 4-fold higher than the amounts produced by control ES cell-derived and iPS cell-derived neurons (Fig. 5G). Notably, in addition to the increased secretion of both A β 40 and A β 42 peptides, there was also a marked reduction in the A β 40:A β 42 peptide ratio in both DS-ES cell-derived and DS-iPS cell-derived cortical neuronal cultures (from 6.6 and 6.9 in control ES and iPS cell cultures, to 4.7 and 4.4 in DS-ES cell and DS-iPS cell cultures). This demonstrates that DS cortical neurons do not simply increase their overall production of A β peptides, but also disproportionately increase their secretion of the pathogenic A β 42 peptide. The increased production of A β peptides is accompanied by an increase in release of the soluble APP β fragment of APP into the extracellular medium (Fig. 5H), as would be predicted for increased processing of APP proteins by the β -secretase protease (27).

Hyperphosphorylation and redistribution of tau in DS iPS cell-derived cortical neurons

Hyperphosphorylation of the microtubule-associated protein tau, dissociation of tau from axonal microtubules and redistribution of hyperphosphorylated tau to the neuronal soma and dendritic tree are well-described later stage pathologies in AD (28). Detection of tau phosphorylated at Ser202 and Thr205 using the AT8 antibody and immunofluorescence and confocal microscopy (29), in control and DS-iPS cell-derived and DS-ES cell-derived cortical neurons revealed abnormal localisation in the neurons derived from Down syndrome patients (Fig. 6A-L). Co-staining with axonally-localised Tuj1 and dendritically-localised

MAP2 was used to define the subcellular localization of tau staining positively with the AT8 antibody (AT8+ tau) in control and DS cultures. AT8+ tau was found in both DS and control neurons, but it was aberrantly localised into linear foci in the cell bodies and dendrites of DS-iPS and DS-ES cortical neurons (Fig. 6K, L) compared with a diffuse localization primarily to axons in control cultures (Fig. 6A-F).

Given that soluble phosphorylated tau is commonly found in the cerebrospinal fluid (CSF) of AD patients (30), potentially due to synaptic dysfunction or cell death, we performed ELISA assays to assess the concentrations of total and phosphorylated tau in the culture medium of DS-iPS cell-derived and DS-ES cell-derived compared to age-matched control cortical neurons (day 55). Total extracellular tau was 3-4 fold higher in media collected over a 48 hour window from DS-iPS and DS-ES cortical neurons compared to control cortical neurons ($p < 0.01$; Fig. 6M). In addition, two forms of phosphorylated tau, pSer396 and pThr231, were only detectable in the media from DS neurons, and not from matched control cultures (Fig. 6 N, O). In age-matched cultures, neuronal cell death was increased approximately two-fold in DS-iPS cell-derived and DS-ES cell-derived cortical neuronal cultures (Fig. 6P), suggesting that apoptosis and cell lysis of neurons is a major contributor to the appearance of tau in the extracellular medium.

DISCUSSION

We report here the reproducible development of AD pathology in cortical neurons generated from induced pluripotent stem cells derived from Down syndrome patients, including neuron-specific A β peptide production, A β 42 aggregate formation, altered Tau protein phosphorylation and localization, and cell death. These pathologies were developed by both DS iPS cell-derived and DS ES cell-derived cortical neurons, demonstrating that these pathologies are reproducible and are not influenced by the variations and mutations introduced by the cellular reprogramming strategy. The increased A β peptide production by DS iPS cell-derived cortical neurons can be reversed by gamma-secretase inhibition, demonstrating the usefulness of these systems for testing new disease intervention strategies and for drug screening.

A striking finding in this study is that high-level secretion of A β peptides and the formation of amyloid aggregates is specific to DS neurons; production by DS fibroblasts, DS iPS cells and DS cortical neuronal stem cells of A β 40 peptide is low and of A β 42 peptide is undetectable. This is particularly noteworthy given the ubiquitous expression of APP in all tissues. AD pathology only occurs in the brain, and the restriction of the development of AD pathology to pluripotent stem cell-derived neurons provides a point of entry for studying both the nervous system specificity of AD and the selective vulnerability of different parts of the brain to AD. Recent reports have shown that neurons generated from individuals with autosomal dominant, early onset AD also display increased A β peptide secretion at a relatively early stage (31-33), suggesting that this early aspect of AD pathogenesis is a general property of neurons.

Using iPS cells generated from two patients with familial Alzheimer's disease, heterogeneous cultures of neurons of a number of different types have recently been shown

to increase production of A β 40 peptides and alter Tau phosphorylation (32). Using Down syndrome pluripotent stem cells differentiated to cerebral cortex neurons, the major cell type affected in the disease, we report here increased production both of A β 40 and the more pathogenic A β 42 peptide. This was accompanied by a change in the A β 40:A β 42 ratio, demonstrating that DS cortical neurons disproportionately increase A β 42 production, as seen in Alzheimer's disease *in vivo*. Furthermore, Down syndrome iPS and ES cell-derived cortical neurons go on to form intracellular and extracellular aggregates of A β 42 peptides, a classic Alzheimer's disease neuropathology. Increased phosphorylation of Tau is a feature common to the familial AD model (32) and the Down syndrome model reported here. In addition, we observe relocalisation of phosphorylated Tau to the cell body and dendrites of Down syndrome cortical neurons, as occurs in the Alzheimer's disease brain. Finally, these pathologies are associated with a marked increase in neuronal cell death by Down syndrome cortical neurons. The development of these classical Alzheimer's disease phenotypes has not been reported to date in familial or sporadic AD models, perhaps due to the heterogeneity of the neuronal types generated, or to the relative functional immaturity of the neurons analysed, as noted by the authors (32).

It is noteworthy that the development of AD pathologies is accelerated in culture. For example, the formation of A β 42 aggregates occurs in this system over a period of months. Why this aspect of the disease process is accelerated in culture is a focus of ongoing work, but may be due to the increased production of reactive oxygen species by Down syndrome neurons in culture (34). In addition, children with Down syndrome already have significant increases in soluble A β 42 in early life (35), and amyloid plaques can be found in teenagers with Down syndrome (16). Therefore, individuals with Down syndrome show a predisposition to the early development of amyloid phenotypes compared with the age of onset of dementia, which typically occurs in middle age in these people (36). The combination of the lack of clearance mechanisms for A β peptides in this culture system, together with the rapid increase in A β peptide production from DS-PS cell-derived cortical neurons, may account for the relatively rapid appearance of A β aggregates in this system. Similarly, whereas it is possible that the changes in Tau phosphorylation and localization observed here are solely dependent on the increased A β peptide secretion by DS cortical neurons, increased expression of the Dyrk1A kinase may accelerate that process (15), underlining the complexity of AD pathogenesis and dementia development in Down syndrome.

A key question arising from this work is whether the development of A β peptide secretion and aggregation phenotypes that we have reported here are a general property of iPS cells from Down syndrome individuals. We demonstrate here that both DS iPS cell-derived and DS ES cell-derived cortical neurons can specifically, robustly and reproducibly develop the A β phenotype, compared with DS fibroblasts and control iPS cell- and hES cell-derived cortical neurons. In that respect, the DS iPS cell-derived cortical neurons reproduce the observed *in vivo* phenotypes (16). However, the incidence of overt dementia in the Down syndrome community is the subject of debate, as it has historically been challenging to diagnose dementia in severely learning disabled people. A range of incidence rates has been reported up to as high as 100% in Down syndrome patients over the age of 50 (36, 37). It

will be of interest in future work to investigate the penetrance of A β phenotypes in cortical neurons generated from iPS cells derived from cohorts of individuals with Down syndrome with and without confirmed diagnoses of dementia in later life.

In conclusion, we report here the use of iPS cell- and ES cell-derived cortical neurons to model AD pathogenesis in Down syndrome. This approach provides a potentially powerful *in vitro* system for functional analyses of pathways regulating A β 42 peptide production in human cortical neurons, cellular mechanisms regulating disease pathogenesis, and the identification and testing of candidate disease-modifying compounds.

METHODS

Culture of human induced pluripotent stem cells

Human ES cell research was approved by the Steering Committee for the UK Stem Cell Bank and for the Use of Stem Cell Lines, and carried out in accordance with the UK Code of Practice for the Use of Human Stem Cell Lines. Human DS ES cells (SC-321 cell line) were derived by the Reproductive Genetics Institute (Chicago) under their IRB approval for the Orkin laboratory and the use of cells approved by Children's Hospital Embryonic Stem Cell Research Oversight Committee (ESCRO; January 2007). Culture of hESCs (H9, WiCell Research Institute, and SC-321, Down syndrome ES cells) and hiPS cell lines (DS1-iPS4, Harvard Stem Cell Institute (18); BBHX and CRL healthy control iPS lines, kindly provided by Dr Ludovic Vallier, MRC Laboratory for Regenerative Medicine, Cambridge (17)) was carried out on mitomycin-treated mouse embryonic fibroblasts (MEFs) according to standard methods (38). Briefly, cells were maintained in hESC medium (all components Invitrogen unless otherwise stated): DMEM/F12 containing 20% KSR, 6 ng/ml FGF2 (PeproTech), 1mM L-Gln, 100 μ M non-essential amino acids, 100 μ M 2-mercaptoethanol, 50 U/ml Penicillin and 50 mg/ml Streptomycin.

Directed differentiation of human ES and iPS cells

Directed differentiation of hESCs and iPSCs to cerebral cortex was carried out as described (8). Briefly, dissociated pluripotent stem cells were plated on Matrigel (BD) coated 12-well plates in MEF-conditioned hESC medium with 10 ng/ml FGF2. Neural induction was initiated by changing the culture medium to a culture medium that supports neural induction, neurogenesis and neuronal differentiation, a 1:1 mixture of N2- and B27-containing media (8). Culture media were supplemented with 500 ng/ml mouse Noggin-CF chimera (R&D Systems) and 10 μ M SB431542 (Tocris) to inhibit TGF β signaling during neural induction (38). Neuroepithelial cells were harvested by dissociation with Dispase and replated in 3N medium including 20 ng/ml FGF2 on polyornithine and laminin-coated plastic plates. FGF2 was withdrawn to promote differentiation. Cultures were passaged once more with Accutase, replated at 50,000 cells/cm² on poly-ornithine and laminin-coated plastic plates and maintained for up to 100 days with a medium change every other day.

Immunocytochemistry and imaging

Cultures were fixed in 4% paraformaldehyde in PBS or in ice-cold methanol and processed for immunofluorescent staining and confocal microscopy. Antibodies used for this study:

Tbr1 (Abcam), Tbr2 (Millipore), CTIP2 (Abcam), Prominin/CD133 (Abcam), phosphorylated Histone H3 (Abcam), gamma-tubulin (Abcam), Pax6 (Chemicon), Oct4 (Abcam), Ki67 (BD), Vimentin (Abcam), Otx1/2 (Millipore), Doublecortin (Santa Cruz), β -Tubulin III (Chemicon), β -Tubulin III (Covance), vGlut1 (Synaptic Systems), Cux1 (Santa Cruz), Brn2 (Santa Cruz), Satb2 (Abcam), cleaved Caspase 3 (Cell Signaling), C-terminus of A β 42 polyclonal (Chemicon) and monoclonal (Covance), phosphorylated Tau (AT8, Pierce), Homer1 (Synaptic Systems 160 011), Munc13 (Synaptic Systems 126 103), PSD-95 (Abcam ab2723), synaptophysin (Abcam ab68851), MAP2 (Abcam ab10588). Secondary antibodies used for primary antibody detection were species-specific, Alexa-dye conjugates (Invitrogen).

For quantifying numbers of cells expressing specific cortical neuronal markers, cell cultures were dissociated into single cells with Accutase and resuspended at a density of 100,000 cells/ml. 20,000 cells were plated onto each poly-lysine coated glass slide with a Cytospin Centrifuge (Thermo Scientific), and fixed and stained for confocal microscopy. For live staining of amyloid in neuronal cultures, BTA-1 (Sigma) in DMSO was added to a final concentration of 100 nM for 20 minutes before washing and imaging. Super-resolution microscopy imaging of synaptic proteins was carried out using standard fixation and staining techniques, visualized with a Deltavision OMX system (Applied Precision).

Quantifications of A β 40, A β 42, sAPP β , total Tau, pT231-Tau and pS396-Tau were carried out with commercial sandwich ELISAs (Millipore, Covance and Invitrogen) using 50 μ l of cell culture supernatant from cultures of DS-iPS, DS-ES, iPS control and H9 hES cortical neurons. Inhibition of gamma-secretase was carried out in DS cortical cultures by addition of 10 μ M DAPT (Calbiochem) every 48 hours from day 50 of differentiation onwards.

Electrophysiology

Whole cell current clamp recordings were performed at room temperature in artificial cerebral spinal fluid containing (in mM): 125 NaCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 3 KCl, 2 CaCl₂, 1 MgCl₂, 25 Glucose, 3 Pyruvic Acid, bubbled with 95% O₂, 5% CO₂. Borosilicate glass electrodes (resistance 6-10 M Ω) were filled with an intracellular solution containing (in mM): 135 K-Gluconate, 7 NaCl, 10 Hepes, 2 Na₂ATP, 0.3 Na₂GTP, 2 MgCl₂. Cells were viewed using a BW50WI microscope (Olympus) with infrared DIC optics. Recordings were made with a Multiclamp 700A amplifier (Molecular Devices). Signals were filtered at 6kHz, sampled at 20kHz with 16-bit resolution, and analysed using Matlab (MathWorks).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Accessible Summary

Alzheimer's disease is a major global health problem for which there are no disease-modifying treatments. A human model of Alzheimer's disease would enable detailed functional studies of Alzheimer's disease pathogenesis and the development of novel therapies. An effective cellular model would use the appropriate cell type, in this case glutamatergic projection neurons of the human cerebral cortex, would develop relevant pathology and would do so in a reproducible manner over a timescale short enough for practical use. A pressing question for the usefulness of this approach is whether neurological diseases that take decades to become manifest in humans can be successfully modeled over a reasonable timescale. To address this problem, we used technologies we have developed to make cerebral cortex neurons from people with a genetic predisposition to developing Alzheimer's disease (AD). For this, we used human induced pluripotent stem cells reprogrammed from skin fibroblasts and human embryonic stem cells from people with Down syndrome. People with Down syndrome/Trisomy 21 have a very high risk of developing AD because they carry an extra copy of a major AD-associated gene, APP. Making cortical neurons from Down syndrome stem cells, we saw reproducible development of AD pathology within months, rather than the years that this takes in vivo. These pathologies were developed by both DS iPS cell-derived and DS ES cell-derived cortical neurons, demonstrating that these pathologies are reproducible and are not influenced by the variations and mutations introduced by the cellular reprogramming strategy. The development of this model enables functional studies of the initiation and progression of AD in human neurons. The first pathology we observe in Down syndrome cortical neurons can be reversed by drugs that are known to target this step, demonstrating the usefulness of these systems for testing new disease intervention strategies and for drug screening.

Figure 1. Directed differentiation of iPS cells into cortical neurons

Directed differentiation of healthy control and Down syndrome iPS cells into cerebral cortex projection neurons. (A-F) Induced pluripotent stem (iPS) cells from healthy individuals (control) and Down syndrome (DS) patients were induced to differentiate into cortical neural stem and progenitor cells expressing Pax6 over 15 days. The stem and progenitor cells formed polarized neuroepithelial rosettes expressing Pax6 (green; A, B), Vimentin (red; A, B), Otx1/2 (green; C, D) and CD133 (A-F). No difference in differentiation efficiency was noted between the control and DS iPS cell lines. Scale bars: A-F, 50 μ m. (G, H) Within the neuroepithelial rosettes generated from control (G) and DS iPS cells (H), was a subpopulation of proliferating basal progenitor cells (Ki67 positive; turquoise) expressing the transcription factor Tbr2 (red), in addition to a population of newly-born neurons that expressed Tbr2 and Doublecortin (Dcx, green). Scale bar, 50 μ m.

(I, J) Differentiation of early-born, layer 6 glutamatergic cortical projection neurons expressing Tbr1 (red) from healthy control (I) and DS (J) iPS cell-derived cortical neuronal stem cells. Scale bar, 50 μ m.

(K-N) Control (K, M) and DS (L, N) iPS cells generate later-born neurons of upper cortical layers defined by expression of the layer-specific transcription factors Cux1 (turquoise), Brn2 (red), and Satb2 (green). Scale bar, 50 μ m.

(O, P) Astrocytes expressing S100 (red) were generated last in both control- (O) and DS (P) iPS cell-derived cortical neuronal cultures. Scale bar, 50 μ m.

Q. Approximately equal numbers of deep and upper layer cortical neurons were generated in both control and DS-iPS cell derived cortical neuronal cultures. Deep layer transcription factors: Tbr1, Satb2; upper layer transcription factors: Brn2 and Satb2. For each cell specific marker, a minimum of n=3 experiments were counted; error bars, s.e.m.

Figure 2. Synapse formation by DS iPS cell-derived cortical neurons

(A, B) Control and DS iPS-cell derived cortical neurons become functionally mature *in vitro*, firing trains of action potentials upon current stimulation (control, n=15 neurons; Down syndrome, n=23 neurons). A representative example of a single neuron's responses to step current stimulation is shown. Voltage and time scales are as shown.

(C, D) Detection of miniature excitatory post-synaptic currents by whole cell recordings from control (C) or DS (D) iPS cell-derived cortical neurons (average of n=4 neurons). Representative recordings from single neurons are shown. Scales are as shown.

(E, F). Super-resolution microscope images of dendrites (MAP2, green) of iPS cell-derived cortical neurons showing localization of foci of PSD95 (red), an excitatory synapse-specific protein. (G-J). Physical synapses were identified as containing juxtaposed pre- and post-synaptic protein complexes of >100nm in width containing either synaptophysin (red) and PSD95 (green) (G, H) or Munc13 (red) and Homer (green) (I, J). Scale bar, 1μm. (K)

Synapse density was measured by the number of PSD95+ foci per unit dendrite length. No difference was found between cortical neurons derived from DS iPS cells and four different control iPS cell lines (control1-4).

differences (Student's t-test) in production of both A β 40 and A β 42 between control and DS-iPS cell-derived cortical neurons. Inhibition of the gamma-secretase protease with DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) for 4 days reduced secretion of both A β 40 and A β 42 peptides by approximately half in cortical neurons derived from DS iPS cells. Inhibition of gamma-secretase for 21 days reduced production of both A β peptides to undetectable levels ($p < 0.01$, Student's t-test).

Figure 4. Formation of amyloid aggregates by DS iPS-cell derived cortical neurons

(A, B). Live staining of amyloid aggregates in control iPS (A) and DS-iPS (B)-cell derived cortical neurons after 90 days in culture using the thioflavin analogue BTA-1. White arrows in panel B indicate BTA-1-positive aggregates in the DS-iPS cell-derived cortical neuronal cultures. Scale bar, 100 μ m.

(C, D). A β 42-positive staining (green) is infrequently observed in cultured cortical neurons derived from control iPS cells (C). In contrast, large numbers of intracellular and extracellular deposits of A β 42 (green) were found in cultures of DS-iPS cell-derived cortical neurons between 60 and 90 days of culture (D). Scale bars C, D 50 μ m.

(E). 90-day old cultures of DS-iPS cell-derived cortical neurons showed extracellular amyloid aggregates (green) indicated by white arrows. Some amyloid aggregates were found outside neurons in regions lacking DAPI-positive nuclei and Tuj1-positive neurites, as can be seen in the X-Z and Y-Z projections shown on the top and left sides of the main image. Scale bar, 20 μ m.

(F) A 3D rendering of a series of confocal images of 90-day cultures of DS-iPS cell derived cortical neurons showing extracellular amyloid plaques (green) indicated by white arrows around cortical neurites. Scale bar, 20 μ m.

Figure 5. DS ES cell-derived cortical neurons recapitulate AD phenotypes

(A, B). ES cells from Down syndrome patients differentiate into deep layer (A) cortical projection neurons expressing Tbr1 (red), and CTIP2 (green) and (B) upper layer cortical projection neurons expressing Brn2 (red) (a few also express Satb2, green). Representative images of day 55 cultures are shown. Scale bar, 50 μ m.

(C, D). Similar expression and localization patterns for APP protein (green) were seen in control (H9) and DS ES cell-derived cortical neurons. Scale bar, 50 μ m.

(E, F). Intracellular and extracellular deposits of A β 42 (green; white arrows) are found in cultures of DS ES cell-derived cortical neurons (F) but not control ES-cell derived cortical neurons (E) at day 55. Scale bar, 50 μ m.

(G). Both DS ES and iPS cell-derived neurons produced approximately 4-fold more A β 40 and A β 42 peptides (measured by sandwich ELISA) over a 48 hour period in culture, compared to age-matched control ES and iPS cell-derived neurons. Differences between matched ES and iPS cell-derived cortical neuronal cultures were highly significant ($p < 0.01$, Student's t-test). A β 40 and A β 42 concentrations are expressed relative to the total amount of cellular protein in each culture in order to control for cell number. Numbers above each set

of samples denote the A β 40:A β 42 ratio for each cell type (calculated from n=3 cultures for each line).

(H). Beta-secretase cleavage of APP was measured by release of soluble APP β (sAPP β) into the extracellular medium. The amount of sAPP β produced was significantly higher in DS ES and iPS cell-derived cortical neurons ($p < 0.01$, Student's t-test).

(I). A β 42 aggregates in control and DS ES and iPS cell-derived cortical neuron cultures was calculated as a function of cell number (day 62; n=3 for each line). The number of aggregates was greater ($p < 0.05$, Student's t-test) in both DS ES and iPS cell-derived neuronal cultures compared to their respective controls.

Figure 6. Phosphorylation and redistribution of tau in DS iPS cell-derived cortical neurons (A-L). Confocal microscopic images for Tau phosphorylated at Ser202 and Thr205 detected using AT8 antibody (green) (29) with counterstaining for the dendritic protein MAP2 (red) in age-matched control and DS ES and iPS cell-derived cortical neuron cultures. Dual color (A, D, G, J) and single channel images (B, C, E, F, H, I, K, L) are shown for clarity. Linear foci of phosphorylated Tau (Ser202 and Thr205; AT8+) were observed in dendrites (MAP2 positive) in DS iPS cell-derived-cortical neurons (white arrow in H), but not in control neurons (B, E). Phosphorylated Tau (AT8+) is also found in the cell body, axon and dendrites of neurons in DS iPS cell-derived neuronal cultures (white arrowheads, panels K, L) but is found at much lower levels in control neurons, and not in the cell body and dendrites. Scale bar, 50 μ m.

(M-O). Soluble tau protein is found in the extracellular medium of DS iPS cell-derived neurons at higher levels than in control cultures (I, p < 0.01, Student's t-test). Two different phosphorylated tau epitopes, pSer396 and pThr231, were detected in the extracellular

medium of DS iPS cell-derived cortical neurons (J, K). Neither form of tau was found in the culture medium of control iPS cell-derived cortical neurons ($p < 0.01$, Student's t-test). In each case, the total amount of each form of Tau is expressed relative to the total amount of cellular protein in each culture in order to control for cell number. (P) Quantification of cell death in control and DS ES and iPS cell-derived cortical neuron cultures (day 62; $n = 3$ for each line). Apoptotic cells with pyknotic condensed nuclei associated with cleaved Caspase-3 (aCasp3+) were counted as a percentage of all cells. The proportion of apoptotic cells was increased ($p < 0.05$, Student's t-test) in both DS ES and iPS cell-derived cortical neuronal cultures compared to their respective controls.