Inhibition of p25/Cdk5 Attenuates Tauopathy in Mouse and iPSC Models of Frontotemporal Dementia

Jinsoo Seo,1,2,* Oleg Kritskiy,1,3* L. Ashley Watson,1,2 Scarlett J. Barker,1,2 Dilip Dey,1 Waseem K. Raja,1,2 Yuan-Ta Lin,1,2 Tak Ko,1 Sukhee Cho,1 Jay Penney,1,2 C. M. Catarina Silva,1 Steven D. Sheridan,1 Diane Lucente,1 James F. Gusella,1 Bradford C. Dickerson,1,2 Stephen J. Haggarty,1 and Li-Huei Tsai1,2,6

1Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, 2Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, 3Center for Genomic Medicine, Chemical Neurobiology Laboratory, Departments of Neurology and Psychiatry, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, 4Molecular Neurogenetics Unit, Center for Genomic Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, 5Massachusetts General Hospital Frontotemporal Disorders Unit, Gerontology Research Unit, and Alzheimer’s Disease Research Center, Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129, and 6Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

Received March 6, 2017; revised Aug. 15, 2017; accepted Sept. 4, 2017.

Key words: Alzheimer’s disease; cerebral organoids; cyclin-dependent kinase 5; iPSCs; isogenic; tauopathy

Inhibition of p25/Cdk5 Attenuates Tauopathy in Mouse and iPSC Models of Frontotemporal Dementia

Increased p25, a proteolytic fragment of the regulatory subunit p35, is known to induce aberrant activity of cyclin-dependent kinase 5 (Cdk5), which is associated with neurodegenerative disorders, including Alzheimer’s disease. Previously, we showed that replacing endogenous p35 with the noncleavable mutant p35 (Δp35) attenuated amyloidosis and improved cognitive function in a familial Alzheimer’s disease mouse model. Here, to address the role of p25/Cdk5 in tauopathy, we generated double-transgenic mice by crossing mice overexpressing mutant human tau (P301S) with mice endogenous p35 with the noncleavable mutant p35 (Δp35). We observed significant reduction of phosphorylated tau and its seeding activity in the brain of double transgenic mice compared with the P301S mice. Furthermore, synaptic loss and impaired LTP at hippocampal CA3 region of P301S mice were attenuated by blocking p25 generation. To further validate the role of p25/Cdk5 in tauopathy, we used frontotemporal dementia patient-derived induced pluripotent stem cells (iPSCs) carrying the Tau P301L mutation and generated P301L:Δp35KI isogenic iPSC lines using CRISPR/Cas9 genome editing. We created cerebral organoids from the isogenic iPSCs and found that blockade of p25 generation reduced levels of phosphorylated tau and increased expression of synaptophysin. Together, these data demonstrate a crucial role for p25/Cdk5 in mediating tau-associated pathology and suggest that inhibition of this kinase can remedy neurodegenerative processes in the presence of pathogenic tau mutation.

Significance Statement

Accumulation of p25 results in aberrant Cdk5 activation and induction of numerous pathological phenotypes, such as neuroinflammation, synaptic loss, Aβ accumulation, and tau hyperphosphorylation. However, it was not clear whether p25/Cdk5 activity is necessary for the progression of these pathological changes. We recently developed the Δp35KI transgenic mouse that is deficient in p25 generation and Cdk5 hyperactivation. In this study, we used this mouse model to elucidate the role of p25/Cdk5 in FTD mutant tau-mediated pathology. We also used a frontotemporal dementia patient-derived induced pluripotent stem cell carrying the Tau P301L mutation and generated isogenic lines in which p35 is replaced with noncleavable mutant Δp35. Our data suggest that p25/Cdk5 plays an important role in tauopathy in both mouse and human model systems.

Introduction

Taur is a microtubule-binding protein, which stabilizes and promotes assembly of microtubules. Hyperphosphorylation, insolubilization, and accumulation of tau are observed in various neurodegenerative diseases, including Alzheimer’s disease.

This work was supported by National Institutes of Health Grant R37NS051874, Robert A. and Renee E. Belfer Family Foundation, and Belfer Neuropathogenesis Consortium to L.-H.T., National Institutes of Health/National Institute of Neurological Disorders and Stroke Grant R21NS085487 to S.J.H. and B.C.D., Association for Frontotemporal Degeneration to M.C.S., and Tau Consortium to S.J.H. We thank all members of L.-H.T. laboratory for advice and discussion; and Ting Fu for technical assistance with iPSC reprogramming.

(AD) and frontotemporal dementia (FTD). Tau hyperphosphorylation can lead to a conformational change of the protein that triggers its dissociation from microtubules and reduced microtubule integrity. The breakdown of this tubular system disrupts intracellular organelle transport, such as the movement of mitochondria or other cargos to peripheral regions, which eventually results in the degeneration of axons (Mazanetz and Fischer, 2007; Kolarova et al., 2012; Kondati et al., 2014). Abnormal phosphorylation of tau also leads to its mislocalization to dendritic spines, resulting in synaptotoxicity through the abnormal recruitment of tau-binding proteins, such as a Fyn kinase into the synapse (Ittner et al., 2010).

Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase whose activity is necessary for neuronal migration, synapse development, and synaptic plasticity. Cdk5 is not catalytically active unless it is associated with a regulatory activator, such as p35. The abundance of p35 is regulated by two alternate pathways consisting of the rapid proteasomal degradation of p35, or the direct truncation of p35 to a soluble 25 kDa form (p25) by calpain, a Ca\(^{2+}\)-dependent cysteine protease. Whereas the former is common under physiological conditions, the latter is primarily associated with the function of Cdk5 under pathological conditions (Patrick et al., 1999; Ahlijianian et al., 2000; Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000).

To date, a large body of literature supports the role of Cdk5 in numerous pathological phenotypes in neurodegenerative disorders, including AD. For example, work in various neurodegenerative disease model systems or animal models of AD showed that pharmacological inhibition or targeted knockdown of Cdk5 relieved neurotoxicity and tauopathy (Piedrahita et al., 2010; Zhang et al., 2013a; Miller et al., 2015). The ability of Cdk5 to phosphorylate tau (pTau) was shown to be enhanced in the presence of p25 compared with p35 (Van den Haute et al., 2001; Hashiguchi et al., 2002; Noble et al., 2003). Consistent with these findings, several p25-overexpressing transgenic mouse models exhibit tau hyperphosphorylation and aggregation (Cruz et al., 2003; Noble et al., 2003). Together, these studies show that p25/Cdk5 induces tauopathy.

A recent study reported that p25 expression is increased in the brain of JNPL3 mice carrying a human mutant transgene harboring a P301L mutation. Inhibition of calpain reduced p25 levels and attenuated tauopathy in these mice (Rao et al., 2014). It suggests that, in addition to p25/Cdk5 inducing tauopathy, p25 production can itself be regulated by pathogenic tau. The novel question that we have not yet answered is whether or not p25 generation is a key factor in developing pathogenic tau mutation-induced pathology. Furthermore, it remains unclear whether p25/Cdk5 mediates tauopathy in patient-derived cell models. Recently, we developed the knock-in mouse (Δp35KI) incapable of generating p25 (Seo et al., 2014). In this work, we thoroughly characterized the Δp35KI mouse through biochemical, electrical, and behavioral assays. We did not observe any difference in Cdk5 activity between WT and Δp35KI mice, which is consistent with the fact that expression of p25 under basal conditions is low. These mice exhibit impaired LTD in hippocampal Schaffer collateral-CA1 synapses and a deficit in memory extinction, suggesting the role of activity-induced p25 in memory process. However, overall, they display normal brain development, synapse density, locomotion, and learning behavior. And no obvious pathological phenotype was observed in Δp35KI mice. In the current study, we use this mouse line to inhibit p25 generation in a mouse model of FTD.

Previous studies using isogenic human induced pluripotent stem cells (iPSCs) derived from AD, FTD or Down syndrome individuals have shown that these cells display a number of readily observable disease phenotypes (Israel et al., 2012; Mou et al., 2012; Fong et al., 2013; Zhang et al., 2013b; Silva et al., 2016). The iPSC model system provides a critically needed means by which to conduct mechanistic studies in living human cells. Moreover, the advent of the clustered regularly interspaced short palindromic repeats (CRISPR) system, using the Cas9 nuclease to induce guided DNA breaks, provides a major advance in our ability to manipulate the human genome (Komor et al., 2017). Last, 3D human neural culture systems, also known as cerebral organoids, have been recently developed to better recapitulate some specific features of the human brain, such as architectural complexity and cortical layer formation. We recently found that cerebral organoids derived from familial AD patient iPSCs endogenously develop Ab and tau aggregation, which has not been observed in conventional 2D culture systems (Raja et al., 2016). In the current study, we generated Δp35KI iPSCs from fibroblasts of an FTD patient by reprogramming along with genome editing techniques, which enabled us to address the role of p25/Cdk5 in a human tauopathy model.

Materials and Methods

Animals

All animal experiments were performed with approval from the Massachusetts Institute of Technology Committee on Animal Care. P301S Tg mice (PS19) (Yoshiyama et al., 2007) were obtained from the The Jackson Laboratory (https://www.jax.org/strain/008169) and crossed to the Δp35KI mouse to generate P301S/Δp35KI mice. Four-month-old littermates were used for all the experiments, if not otherwise indicated. Male mice were used for electrophysiology experiments, and female mice were used for all biochemistry experiments.

Immunoblot analysis

Hippocampal or cortical tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1% SDS) containing protease and phosphatase inhibitors. For organoids, 3 or 4 organoids were pooled, homogenized, and sonicated in RIPA buffer. Lysates were incubated on ice for 15 min and spun at 12,000 rpm for 15 min. Then, supernatants were transferred to new tubes and analyzed for protein concentration (Bio-Rad Protein Assay). SDS buffer was added to equal amounts of protein and subjected to SDS-PAGE and immunoblotting analysis.

Antibodies

Antibodies are as follows: p35 (laboratory of L.-H.T.), Cdk5, HA, GAPDH (Santa Cruz Biotechnology), pTau T181, pTau S202 (Cell Signaling Technology), synaptophysin (Sigma-Aldrich), NeuN (Synaptic Systems), and Tau5 (Thermo Fisher Scientific).

Immunoprecipitation-linked kinase assay

Hippocampal lysates were incubated with an anti-Cdk5 antibody overnight at 4°C, and the immunocomplex was subjected to a Cdk5 kinase assay as described previously (Seo et al., 2014).

Immunohistochemistry

Mice were transcardially perfused with 4% PFA in PBS under anesthesia (2% of ketamine/xylazine), and the brains were sectioned at 40 μm thickness with a VT1000S vibratome (Leica). Slices were permeabilized with blocking solution containing 0.1% Triton X-100, 1% glycine, 10% don-
key serum, and 2% BSA in PBS for 1 h at room temperature, and
incubated at 4°C for overnight with blocking solution containing primary
antibody. Slices were then incubated at room temperature for 1 h with
fluorescently conjugated secondary antibodies (Invitrogen), and nuclei
were stained with Hoechst 33342 (Invitrogen).

Microscopy
All images were captured using a Zeiss LSM 880 confocal microscope and
the ZEN software, and analyzed using the ImageJ software (National

Tau seeding activity assay
Brains were homogenized in 1× TBS supplemented with protease inhib-
itors using a probe sonicator (30% power; 15 pulses). After sonication,
the lysates were centrifuged at 16,000×g for 15 min to eliminate large,
insoluble material. The supernatant was stored at −80°C and used for all
future experiments. Protein concentration was determined using a Bio-
Rad Protein Assay Kit. Fluorescence resonance energy transfer (FRET)
biosensor cell lines described previously (Holmes et al., 2014) were pro-
vided by Marc J. Diamond. Cells were grown in DMEM (Invitrogen)
supplemented with 10% FBS and 1× penicillin/streptomycin and main-
tained at 37°C and 5% CO2 in a humidified incubator. For the assay, cells
were plated in a 96-well plate at a density of 40,000 cells/well. Sixteen
hours later, at 50% confluence, brain homogenate samples were trans-
duced into cells using 1 μl Lipofectamine/well. After a 48 h incubation
at 37°C, cells were harvested with 0.25% trypsin and fixed in 4% PFA (Elec-
tron Microscopy Services) for 15 min, and then resuspended in PBS. An
LSR II HST-2 flow cytometer was used to measure the FRET signal within
each cell. FRET quantification was accomplished using FlowJo version 10
software (TreeStar). Integrated FRET density was derived by multiplying
the percentage of FRET-positive cells in each sample by the median FRET
intensity of those cells.

Electrophysiology
Hippocampal slices (transverse, 400-μm-thick) were prepared in ice-
cold dissection buffer (in mM as follows: 211 NaCl, 1.3 NaH2PO4, 0.5 CaCl2,
10 MgCl2, 26 NaHCO3, and 11 glucose) using a VT1000s vibratome (Leica). Slices were then moved to the submerged chamber with 95% O2/5% CO2-saturated ACSF consisting of (mM) as
follows: 124 NaCl, 3.3 KCl, 1.3 NaH2PO4, 2.5 CaCl2, 1.5 MgCl2, 26 NaHCO3,
and 11 glucose at 30°C for at least 1 h before the recording. Extracellular recording at mossy fiber-CA3 synapses was performed as previously described
(Siegert et al., 2015). In brief, a tungsten bipolar electrode was placed in the
dentate granule cell layer to stimulate mossy fibers, and extracellular recordings were made in the stratum lucidum of CA3 using a glass microelectrode filled with ACSF (resistance of 2–3 MΩ).
LTP was induced by three trains of high-frequency stimulation (100 Hz
for 1 s) with 10 s intervals after observation of baseline stability. To verify
mossy fiber inputs, 1 μl (2S,2R,3R)-2-(2’-3’ dicycloxy cyclopropyl)gly-
lycine (Tocris Bioscience), a Group II metabotropic glutamate receptor agonist that selectively blocks mossy fiber responses, was applied at the
end of each recording. The amplitude of field EPSPs (fEPSPs) was mea-
sured to quantify the strength of synaptic transmission. A MultiClamp
700B amplifier and a Digidata 1440A A-D converter (Molecular Devices)
were used for data acquisition and data were analyzed with pClamp10
(Molecular Devices).

iPSC cultures
FTD patient (Tau P301L carrier) and related healthy individual dermal
fibroblasts were generated from a skin biopsy from subjects within the
Massachusetts General Hospital Frontotemporal Dementia Clinic as part of
the Massachusetts General Hospital Neurodegeneration Repository.
Approval for human subject work was obtained under a Partners/
Massachusetts General Hospital-approved Institutional Review Board
Protocol (#2010P001611/MGH). iPSCs (Tau-P301L MGH-2046
and nonmutant control MGH-2069) were generated using a synthetic mod-
ified mRNA-based reprogramming method (StemGent mRNA Modified
Reprogramming Kit). These iPSC lines have been fully characterized, and
presence of the P301L mutation in MAP7 was confirmed by sequencing
(data not shown). iPSCs were cultured on irradiated mouse embryonic
fibroblasts (MEFs, MTT-GlobalStem) in DMEM/F12, HEPEs media (Invit-
rogen) supplemented with 20% knock-out serum replacement (Invit-
rogen), 1× nonessential amino acids, 1× Glutamax (Invitrogen)
β-fibroblast growth factor (PeproTech), and 0.1 mM 2-mercaptoethanol
(Sigma-Aldrich) and maintained at 37°C and 5% CO2 in a humidified
incubator.

Generation of Δp35KI isogenic lines
Preparation of the CRISPR/Cas9-p35-sgRNA plasmid. A sgRNA for
targeting CDK5R1 gene (GGGGCGTGGGAAATGTTGAG, reverse)
was designed by http://crispr.mit.edu. Both sgRNA and repair template
(ssODNs: TGAGGGGCTTTCCACTGAGGAGGACCCCGGTC
TGGAGAACGAAGAGATTGTTGCCGGGGCGGCTGG
GCCGTTGGGGCTGGAGGAGCAGCAAGCAGACTCTTTCTCAGGTG
TCATTGTAGGGTGTAGTGGTGTGTCGTGTGTCGTGGGTGG
CTCACACCTCTGGATTCCTCTCTTCTTCTTGG)
were synthesized by In-
tegrated DNA Technologies. The CRISPR/Cas9 plasmid (pSpCas9-2A-
GFP, PX458) was purchased from Addgene, and the p35-sgRNA was
cloned into the plasmid as described previously (Ran et al., 2013).

Electroporation. iPSCs on MEF plates were dissociated with Accutase
(Thermo Fisher Scientific) and collected to a 15 ml Falcon tube. Cells
were washed with hES media once and then resuspended with hES media
to count the number of cells. A total of 5 million cells were transferred
to a new tube, and media was removed. Then, the CRISPR/Cas9-p35-
sgRNA and ssODNs were added to cells, mixed, and transferred to a
cuvette. The Nucleasefector (Amazix) and the Human Stem Cell Nucleo-
factor Kit 1 (Lonza) were used for the electroporation, and the cells were
resuspended in hES media with 10 μM Rho-associated protein kinase
inhibitor and transferred to new MEF plates.

Fluorescence-activated cell sorting (FACS). Two days after the electropo-
ration, cells were dissociated with Accutase and transferred to a 15 ml
Falcon tube. Cells were washed with hES media once, resuspended with
DPBS, and filtered using Falcon polystyrene test tube (#2235). Filtered
cells were collected and sorted by a BD Biosciences Aria II based on GFP
signal. Sorted cells were then collected in hES media with 1× penicillin/
streptomycin and 10 μM Rho-associated protein kinase inhibitor (Y-
27632, Millipore) and plated at a density of 80,000 cells/well.

Colonies inspection. Once colonies formed, single colonies were trans-
ferred to each well of 12-well MEF plates. After second transfer, cells
in the original plates were dissociated and genomic DNAs were extracted
as previously described (Ran et al., 2013). PCR was performed with the
primer set to target the CDK5R1 gene (p35F-CTGTCCCTGTCTCCCA
GCTA, p35R-GGCAGAGAACTCACCCAG), and the PCR product
was submitted to Geneviz for the sequencing.

Organoid culture
Organoids were created from iPSCs carrying the Tau P301L mutation,
the Δp35KI isogenic, or healthy control lines using the protocol de-
scribed previously (Raja et al., 2016). In brief, embryoid bodies were
formed by loading 12,000 iPSCs per well into 96-well plates precoated
with pluronic acid (1%, F-127, Sigma-Aldrich). Embryoid bodies main-
tained in the Media 1 consist of Glasgow-MEM supplement with 20%
nonessential amino acids, 1× sodium pyruvate, 10% FBS, and 1% Matrigel
(Tocris Bioscience), and 3 μM Wnt-inhibitor (IWR1, Tocris Bioscience)
for 20 d. Dorosmorphin, a BMP inhibitor (2 μM Tocris Bioscience),
was added for the first 3 d. Organoids were then transferred to nonadherent
Petri dishes and cultured in Media 2, consisting of DMEM/F12 supple-
mented with 1× Chemically Defined Lipid Concentrate and 1× N2-
supplement with 40% O2/5% CO2 to promote neuroepithelial formation.
From day 35, 5 μM heparin (Sigma-Aldrich), 10% FBS, and 1% Matrigel
(Invitrogen) were added to the medium.

Experimental design and statistical analysis
Data are mean ± SEM and were analyzed by Prism 6 software (GraphPad).
Student’s t-test was used to compare the means of two groups. One-way
ANOVA followed by Tukey’s post hoc analysis was used for multiple
comparison. p < 0.05 was considered significant.
Results

Abnormal p25 expression induces hyperactivation of Cdk5 in the brain of P301S mice

To first test whether p25 levels are affected by pathogenic tau, we used a C-terminal specific p35 antibody to detect both p35 and p25 species in the brain of P301S transgenic mice. Using hippocampal lysates from 4-month-old mice, we observed a twofold increase in p25/p35 ratio in P301S mice compared with that of WT (Fig. 1A, B; \( p = 0.030 \)). To measure Cdk5 activity in these samples, we immunoprecipitated Cdk5, then performed a kinase assay using radiolabeled ATP and histone H1 protein, as a substrate of Cdk5. We found that basal Cdk5 activity in WT mouse hippocampus is significantly increased compared with that of WT (Fig. 1C). These data indicate that hyperactivation of Cdk5 in P301S mouse brains is mediated by abnormally high p25 generation.

Blockade of p25 generation attenuates tauopathy in P301S mice brain

Overexpression of human P301S mutant tau was shown to induce tau pathology, including hyperphosphorylation of tau, increased levels of insoluble tau, and the formation of neurofibrillary tangles (Yoshiyama et al., 2007). Although the precise mechanisms of mutant-driven tau hyperphosphorylation remain unclear, a conformational change induced by such mutations is proposed to trigger phosphorylation of other residues of tau by various kinases, such as Cdk5 or GSK-3b, or inhibit dephosphorylation mediated by phosphatases (Mazanetz and Fischer, 2007; Kolarova et al., 2012). To address the contribution of p25/Cdk5 to tau hyperphosphorylation in P301S mouse brains, we performed immunohistochemistry with hippocampal slices from P301S mice, P301S;\( \Delta p35KI \) mice, and their WT littermates. We observed significant reduction of pTau levels in the hippocampus from P301S;\( \Delta p35KI \) mice compared with that of P301S mice (Fig. 2A). With antibodies against two different pTau epitopes (pTau T181 and pTau S202), we performed Western blotting; consistent with our immunostaining data, levels of pTau in hippocampal lysates from P301S;\( \Delta p35KI \) mice were significantly reduced compared with those of P301S mice, without changes in total levels of tau (Fig. 2B; \( p = 0.0097 \) for pTau T181, \( p = 0.0396 \) for pTau S202, \( p = 0.283 \) for Tau5).

Recent studies suggest that pathogenic tau seeds can spread across the brain and trigger tauopathy in regions it spreads toward (Clavaguera et al., 2009; Frost et al., 2009; de Calignon et al., 2012). Thus, we asked whether a reduction of phosphorylated tau by p25/Cdk5 inhibition affects tau seeding activity in P301S mouse brains. We measured tau seeding activity, we used a FRET-based flow cytometry biosensor assay reported recently (Holmes et al., 2014). This study showed that tau seeding activity of lysates from P301S mice could be detected as early as 2 months of age. Therefore, we prepared biosensor HEK293T cells (expressing P301S mutant tau fused to either CFP or YFP) and treated them with brain lysates from 2-month-old P301S, P301S;\( \Delta p35KI \), or WT littermate mice. After 24 h, we measured FRET signals corresponding to the formation of tau aggregates and found that, while tau seeding remained significantly elevated relative to WT brain lysates, inhibition of p25 production significantly reduced the seeding activity of P301S brain lysates (Fig. 2C; \( p < 0.0001 \) by ANOVA). Together, these data suggest that aberrant Cdk5 activity by p25 generation elevates tau hyperphosphorylation and increases tau seeding activity in P301S mouse brain.

Inhibition of p25 restores synaptic function at mossy fiber–CA3 synapses in P301S mice

Hyperphosphorylation and aggregation of tau are associated with synapse loss and cognitive impairment, and a significant reduction of synaptic density was observed in the hippocampal CA3.
region of P301S mice by 3 months of age (Yoshiyama et al., 2007). To address the effect of p25/Cdk5 inhibition on pathogenic tau-mediated synaptic loss, we measured synaptic density using an anti-synaptophysin antibody in the CA3 region of 4-month-old P301S, P301S;Δ35KI, and WT littermate mice. Consistent with previous observations, P301S mice showed significantly reduced levels of synaptophysin in CA3 compared with their WT littermates (Fig. 3A). We also observed a trend toward a reduction of synaptophysin levels in CA1 of P301S mice compared with WT, although it was not statistically significant (data not shown). This synaptic loss in CA3 was completely reversed to the levels of WT by inhibiting p25 generation (Fig. 3A; p = 0.0005 by ANOVA).

To functionally analyze synapse integrity, we next performed extracellular field recordings to measure synaptic strength and LTP at mossy fiber-CA3 synapses. We observed that, while basal synaptic transmission in P301S mice was reduced compared with WT controls, inhibition of p25 in P301S mice significantly rescued this effect. Importantly, neither Δ35KI mice nor P301S;Δ35KI mice exhibited different basal synaptic transmission compared with controls. The reduced baseline transmission in P301S mice seems to be due to a reduction of synapse number rather than an alteration of presynaptic neurotransmitter release because paired-pulse facilitation ratios were not different between P301S and WT mice (Fig. 3B). Consistent with the loss of synaptic density, P301S mice did not show any potentiation of fEPSPs after high-frequency stimulations. However, similar to control and Δ35KI mice, P301S;Δ35KI mice showed ~150% potentiation of fEPSP by the stimulations (Fig. 3C; p = 0.0013 by ANOVA), indicating the restoration of synaptic plasticity by p25 inhibition in P301S mice.

**Inhibition of p25 generation reduces the levels of pTau in human cerebral organoids carrying a P301L mutation in tau**

To validate the effect of p25 generation on tau hyperphosphorylation in human model systems, we used iPSCs derived from fibroblasts from an FTD patient carrying the Tau P301L mutation. We then derived 3D human cerebral organoids from this iPSC line (MGH-2046) as well as from the nonmutant control line (MGH-2069) as reported previously (Raja et al., 2016). This system enabled us to address the role of p25 in tau hyperphosphorylation in the context of a different tau mutation associated with tauopathy, as both tau and p35 are highly expressed in neurons. Two-month-old organoids derived from iPSCs carrying the P301L mutation showed higher p25/p35 protein ratios compared with those derived from the nonmutant control (Fig. 4A; p = 0.038). To determine the role of p25/Cdk5 in tau hyperphosphorylation, we generated isogenic lines in which endogenous p35 is replaced with Δ35 protein by targeting Cas9 to the endogenous CDK5R1 locus (encoding p35) alongside a template donor oligonucleotide harboring the Δ35 DNA sequence (Fig. 4B). Sanger sequencing confirmed the incorporation of Δ35 sequence into the genome (Fig. 4C). The top three potential off-target sites predicted using CRISPR design tool (http://crispr.mit.edu) were inspected, and sequencing data showed that no off-target effect was present in these regions (data not shown). Two-month-old organoids from P301L iPSCs and P301L;Δ35KI isogenic lines were subjected to Western blotting experiments. We first measured the levels of p25 generation in both groups and observed a significant reduction of p25 abundance by this genetic manipulation in human cerebral organoids (Fig. 4D; p = 0.0092). We then examined the levels of pTau using two different antibodies (pTau S202 and pTau T181) as well as total tau. Consistent with the data from our mouse study, we observed a substantial reduction of pTau levels in P301L;Δ35KI organoids compared with P301L organoids (Fig. 4D; p = 0.002 for pTau S202, p < 0.0001 for pTau T181). We also observed a similar reduction in total tau levels (p = 0.0029). And there was a trend to pTau/total tau being reduced, although it was not statistically significant. These effects on total tau do not appear to result from a reduced number of neurons, as indicated by comparable levels of NeuN (p = 0.94), and could instead be due to the reduced aggregation of unphosphorylated versus phosphorylated tau. The expression of synaptophysin was higher in P301L;Δ35KI organoids, which is also consistent with the observation from Δ35KI mice (Fig. 4D; p =
Immunofluorescence analysis of the cerebral organoids also confirmed a reduction of pTau levels in P301L organoids by inhibition of p25 (Fig. 4E; \(p = 0.023\)). These data provide the first demonstration in a human neuronal culture system that disruption of p25/Cdk5 activity can ameliorate tauopathy phenotypes.

**Discussion**

Whereas the relationship between p25/Cdk5 and amyloid pathology has been an active area of interest, the impact of p25 inhibition upon another hallmark of AD, tauopathy, is less clear. Cdk5 hyperactivation by overexpressing p25 was shown to induce tauopathy in mouse brains (Cruz et al., 2003; Noble et al., 2003). However, the contribution of p25/Cdk5 complex to the development of tauopathy under pathological conditions by loss-of-function study has not been investigated. In this study, by genetically abolishing p25 generation, we have attempted to address whether p25 generation is necessary for aspects of tau-associated pathologies.

In the brain of P301S mice, a model of FTD, we found that p25/Cdk5 inhibition significantly reduces hyperphosphorylation of tau and its seeding activity. The levels of pTau and tau seeding activity in P301S;Δp35KI mice are still higher than those of WT mice because these mice significantly overexpress P301S tau. However, we found that inhibition of p25 generation is sufficient to restore synaptic integrity and function in this animal. To further determine the role of p25/Cdk5 in mutant tau-mediated pathology in human neurons, we turned to iPSC systems. Previous studies showed that human neurons differentiated from iPSCs of FTD patients carrying mutations in tau expressed higher levels of pTau and total tau compared with those from unaffected individuals (Fong et al., 2013; Silva et al., 2016). This effect does not appear to be due to increased number of neurons by early maturation because the levels of neuronal markers were not affected. These data showed that pathogenic mutant tau induced hyperphosphorylation of tau as well as accumulation of total tau, which could be caused by abnormal protein folding and impaired clearance of this pathogenic protein. Using the CRISPR/Cas9 genome-editing technique, we have created isogenic lines of human iPSCs derived from an FTD patient carrying a P301L mutation in the MAPT gene, with endogenous p35 replaced with Δp35. Cerebral organoids derived from these isogenic iPSC lines demonstrated that blockade of p25 production reduced tau phosphorylation on multiple epitopes as well as lowering total tau. As noted above, the effect on total tau levels could be due to enhanced clearance of unphosphorylated tau. It is important to note that we did not observe such a reduction of total tau in P301S;Δp35KI mice compared with P301S mice. This discrepancy could be from characteristics of two different model systems. Unlike cerebral organoids that express endogenous levels of mutant tau, P301S mice overexpress mutant tau at levels more than fivefold higher than endogenous tau. This could lower the...
efficiency to reduce expression of total tau, even though p25 inhibition attenuates hyperphosphorylation of tau. Interestingly, we observed that inhibition of p25 generation increases levels of synaptophysin in both the P301S mice brain and Tau P301L organoids. Tau is generally localized to the axons of neurons; however, hyperphosphorylated tau accumulates in the somatodendritic compartment. This leads to mislocalization of tau-interacting proteins, such as the Fyn kinase. Previously, it was shown that abnormal expression of Fyn followed by tau mislocalization at the synapse results in the phosphorylation and activation of NMDA receptors, leading to neurotoxicity (Ittner et al., 2010). We showed previously that NMDA receptor activation leads to p25 generation, which subsequently causes synaptic depression (Seo et al., 2014). Therefore, it is conceivable that p25 generation not only facilitates tau hyperphosphorylation by leading to aberrant Cdk5 activation, but also mediates neurotoxicity-induced synaptic depression and subsequent synaptic loss at the synapses of brains with tauopathy.

In the progression of AD, an increase of Aβ is apparent as much as a couple of decades before the onset of clinical symptoms (Jack et al., 2010). Aβ accumulation is followed by hyperphosphorylation of tau, subsequent neuronal loss, and cognitive impairment. Although this suggests that Aβ could cause abnormal phosphorylation of tau, the lack of tauopathy in mouse models of amyloidosis is perplexing. One potential explanation is the different nature of the tau species in mouse versus humans. Mouse does not express certain tau isoforms; thus, it cannot recapitulate the human four-repeat tau (4R-tau):three-repeat tau (3R-tau) ratio change in which have been associated with the formation of tauopathy (Adams et al., 2010; Schoch et al., 2016). Recent studies using iPSC-derived neurons from AD patients showed that this human model system nicely recapitulates both upregulation of Aβ and hyperphosphorylation of tau (Israel et al., 2012; Muratore et al., 2014). Cdk5 is a well-established tau kinase. Because we saw the beneficial effect of p25/Cdk5 inhibition on Aβ-induced pathology in the 5XFAD mouse model (Seo et al., 2014) and on tauopathy in human model systems, we speculate that p25/Cdk5 mediates Aβ-induced hyperphosphorylation of tau. And this further increases p25 generation and Cdk5 hyperactivation as forming a feedforward loop. p25/Cdk5 could also facilitate tau phosphorylation by other kinases, such as GSK-3β (Kimura et al., 2014). As such, inhibition of p25/Cdk5 would likely be beneficial in this system.

In conclusion, our study, using P301S;Δp35KI mice and P301L;Δp35KI iPSCs, suggests that inhibition of p25/Cdk5 is effective in ameliorating disease-causing mutant tau-mediated pathology. Therefore, further efforts to develop inhibitors of p25-mediated Cdk5 dysregulation are warranted and could benefit both AD and FTD patients.