I2020T mutant LRRK2 iPSC-derived neurons in the Sagamihara family exhibit increased Tau phosphorylation through the AKT/GSK-3β signaling pathway

Etsuro Ohta1,2,5, Tomoko Nihira3,5, Akiko Uchino4,7, Yoichi Imaizumi5, Yohei Okada5,10, Wado Akamatsu5,11, Kayako Takahashi12, Hideki Hayakawa3, Makiko Nagai13, Manabu Ohyama6, Masafumi Ryo13, Mieko Ogino13, Shigeo Murayama7,8,9, Akihiko Takashima14, Kazutoshi Nishiyama4,13, Yoshikuni Mizuno3, Hideki Mochizuki15, Fumiya Obata1,2 and Hideyuki Okano5,*

1R & D Center for Cell Design, Institute for Regenerative Medicine and Cell Design, Kitasato University School of Allied Health Sciences, Kanagawa, Japan, 2Division of Clinical Immunology, Graduate School of Medical Sciences, 3Department of Neuro-Regenerative Medicine, 4Department of Neurology, Graduate School of Medical Sciences, Kitasato University, Kanagawa, Japan, 5Department of Physiology, 6Department of Dermatology, Keio University School of Medicine, Tokyo, Japan, 7Department of the Brain Bank for Aging Research, 8Department of Neurology, 9Department of Bioresource Center (the Brain Bank for Aging Research), Tokyo Metropolitan Geriatriac Hospital and Institute of Gerontology, Tokyo, Japan, 10Department of Neurology, Aichi Medical University School of Medicine, Aichi, Japan, 11Center for Genomic and Regenerative Medicine, Juntendo University School of Medicine, Tokyo, Japan, 12Department of Medical Laboratory, Kitasato University Hospital, Kanagawa, Japan, 13Department of Neurology, Kitasato University School of Medicine, Kanagawa, Japan, 14Department of Neurobiology, National Center for Geriatrics and Gerontology, Obu, Japan and 15Department of Neurology, Osaka University Graduate School of Medicine, Osaka, Japan

*To whom correspondence should be addressed at: Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Tel: +81 353633747; Fax: +81 333575445; Email: hidokano@a2.keio.jp

Abstract

Leucine-rich repeat kinase 2 (LRRK2) is the causative molecule of the autosomal dominant hereditary form of Parkinson’s disease (PD), PARK8, which was originally defined in a study of a Japanese family (the Sagamihara family) harboring the I2020T mutation in the kinase domain. Although a number of reported studies have focused on cell death mediated by mutant LRRK2, details of the pathogenetic effect of LRRK2 still remain to be elucidated. In the present study, to elucidate the mechanism of neurodegeneration in PD caused by LRRK2, we generated induced pluripotent stem cells (iPSC) derived from fibroblasts of PD...
patients with I2020T LRRK2 in the Sagamihara family. We found that I2020T mutant LRRK2 iPSC-derived neurons released less dopamine than control-iPSC-derived neurons. Furthermore, we demonstrated that patient iPSC-derived neurons had a lower phospho-AKT level than control-iPSC-derived neurons, and that the former showed an increased incidence of apoptosis relative to the controls. Interestingly, patient iPSC-derived neurons exhibited activation of glycogen synthase kinase-3β (GSK-3β) and high Tau phosphorylation. In addition, the postmortem brain of the patient from whom the iPSC had been established exhibited deposition of neurofibrillary tangles as well as increased Tau phosphorylation in neurons. These results suggest that I2020T LRRK2-iPSC could be a promising new tool for reproducing the pathology of PD in the brain caused by the I2020T mutation, and applicable as a model in studies of targeted therapeutics.

Introduction
Parkinson’s disease (PD) is the second most common neurodegenerative disorder caused by loss of nigrostriatal dopaminergic neurons. Leucine-rich repeat kinase 2 (LRRK2) is the causative molecule of the autosomal dominant form of PD, PARK8, which was originally defined in a study of a Japanese family (Sagamihara family) harboring the I2020T mutation in the kinase domain (1–3). The G2019S mutation is that, which is detected most frequently, accounting for up to 5% of all cases of familial PD and 1% of cases of sporadic PD in the Caucasian population (4–6). Patients with LRRK2 mutations exhibit clinical features indistinguishable from those of patients with sporadic PD, and LRRK2 is postulated to be a key molecule in the etiology of the disease. However, the mechanism of neurodegeneration resulting from the mutation has not been conclusively clarified.

LRRK2 belongs to the receptor-interacting protein (RIP) family, characterized by leucine-rich repeat (LRR), Ras of complex (ROC), C-terminal ROC (COR), kinase and WD40 domains (7). It has been reported that LRRK2 phosphorylates itself (autophosphorylation), and that its potential substrates include Ezrin/Radixin/Moesin, 4E-BP1, β-tubulin, and MKK3, MKK6, MKK7, Tau and ribosomal protein s15 (8–12). Although hyper-kinase activity reported for mutant LRRK2 molecules, particularly G2019S LRRK2, may be one possible mechanism for the pathogenesis induced by this molecule (13–17), it has not been observed reproducibly in other LRRK2 mutations including I2020T (18). We previously reported that LRRK2 directly phosphorylates AKT1, a serine/threonine kinase involved in cell growth, proliferation and survival (19). The disease-associated mutations, R1441C, G2019S and I2020T, exhibited reduced interaction with, and phosphorylation of, AKT1. In addition, we recently reported that the kinase activity of wild-type (WT) LRRK2 for AKT phosphorylation was diminished by the presence of I2020T LRRK2 (20). Also, some studies have reported that LRRK2-containing inclusion bodies and autophagy are responsible for cytotoxicity (18,21).

Recently, it has been reported that neurons derived from iPSC from PD patients with G2019S LRRK2 show increased susceptibility to both oxidative and proteasomal stress (22–24). Furthermore, these G2019S mutant LRRK2 iPSC-derived neurons showed deficiency of the autophagic machinery, shorter neurites, a significantly increased incidence of apoptosis and passage-dependent deficiencies of nuclear-envelope organization (22,23,25,26). In addition, G2019S LRRK2 iPSC lines showed increased levels of Tau protein, α-synuclein protein and ERK phosphorylation (24). It has also been reported that R1441C and G2019S LRRK2 iPSC-derived neurons show mitochondrial DNA damage (27).

In the present study, to elucidate the pathogenetic effect of mutant LRRK2, we first generated and analyzed iPSC from fibroblasts of patients with familial PD harboring I2020T LRRK2. We found that I2020T mutant LRRK2 iPSC-derived neurons had a diminished degree of Ca2+-dependent dopamine release relative to control-iPSC-derived neurons. Furthermore, we demonstrated that patient iPSC-derived neurons showed an increased incidence of apoptosis, as well as activation of GSK-3β and high Tau phosphorylation through reduction of AKT phosphorylation. In addition, the postmortem brain of the patient from whom the iPSC had been established exhibited deposition of neurofibrillary tangles (NFTs) as well as increased Tau phosphorylation in neurons. These results indicate that LRRK2 iPSC-derived neurons replicate to some extent the pathologic phenotype evident in the brain of PARK8 patients.

Results
Generation, characterization and differentiation efficiency of iPSC
I2020T LRRK2-iPSC lines were first generated from dermal fibroblasts isolated from two PARK8 patients (LA and LB) harboring I2020T LRRK2 mutation using retroviruses carrying Oct4, Sox2, Klf4 and c-Myc genes, as previously described (28,29). To evaluate the iPSC lines established from the patient, we characterized their properties. Colonies from LA and LB iPSC lines (LAS, LA11, LB16 and LB21) demonstrated robust expression of the pluripotency markers OCT4, SSEA4 and TRA1–60 (Fig. 1A). Then, to choose the lowest expression levels of the transgene, mRNAs from the cell lines were examined using real-time reverse transcription (RT)-PCR. No significant differences between the LA and LB iPSC lines were observed (Fig. 1B). Patients-derived fibroblasts and iPSC lines showed the same heterozygous I2020T mutation (Fig. 1C). All of the patient iPSC lines demonstrated differentiation of all three germ layers spontaneously in vivo, and maintained a normal karyotype (Fig. 1D and E).

Next, to investigate the differentiation efficiency of iPSC lines established from the patient and the control (YA9 and WD39), iPSC-derived neurospheres were allowed to differentiate to neurons from all the iPSC clones. We found that >80% of the differentiated cells were positive for βIII-tubulin (a neuron-specific marker) and tyrosine hydroxylase (TH) (a dopaminergic neuronal marker). We found that Δ5% of the differentiated cells were positive for βIII-tubulin, and that Δ5% (among a total of 5200 cells) of neurons from all the iPSC clones were TH-positive (Fig. 1F and G). This differentiation tendency was similar among the iPSC clones. In addition, to investigate whether there were any differences in the level of TH expression in long-term culture (118 days), iPSC-derived neurons were analyzed by immunostaining and real-time RT-PCR. We found that Δ20% (among a total of 4000 cells) of neurons from all the iPSC clones were TH-positive (Fig. 1H and I). This differentiation tendency was similar among the iPSC clones. Furthermore, the levels of TH mRNA in all six long-term cultures of iPSC-derived neurons were increased Δ3.5-fold relative to those of short-term (14 days) cultured neurons (Fig. 1J).
Figure 1. Generation and characterization of LRRK2 iPSC. (A) Clones from LA and LB iPSC lines (LA5, LA11, LB16 and LB21) stained for the pluripotency markers Oct4 (green), SSEA4 (red) and TRA1-60 (red). SSEA1, a mouse pluripotency marker that is absent in undifferentiated human stem cells, was used as a non-pluripotency marker, and was co-stained using Hoechst33342 (blue). Scale bars: phase contrast, 500 μm. Immunocytochemical staining with Oct4, SSEA4 and TRA1-60, 200 μm. (B) Silencing of transgenes in LRRK2 iPSC clones using quantitative RT-PCR analysis. Expression levels were normalized to the positive control of LA transgene (TG) (=1). LA TG shows the transgene-infected fibroblasts. B7 iPSC was used as control-iPSC line. Data represent mean ± SEM of duplicate experiments. (C) Genomic DNA sequencing of exon 41 of the LRRK2 gene performed using DNA isolated from patient fibroblasts and iPSC lines. (D) Teratoma assay using iPSC subcutaneously injected into immunodeficient mice, and then stained with hematoxylin–eosin. Scale bars: 100 μm. (E) Karyotype analysis. (F) In 14 days in vitro, differentiation into neurons and midbrain dopaminergic neurons shown by immunostaining for TH and βIII-tubulin. Scale bars: 50 μm. (G) Differentiation efficiencies of all the cell lines in 14 days in vitro, expressed as a percentage of total cells, identified by nuclear positivity for the indicated marker. Data represent mean ± SEM of triplicate experiments. (H) In 118 days in vitro, differentiation into neurons and midbrain dopaminergic neurons shown by immunostaining for TH and βIII-tubulin. Ho, Hoechst33342. Scale bars: 100 μm. (I) Differentiation efficiencies of all the cell lines in 118 days in vitro, expressed as a percentage of total cells, identified by nuclear positivity for the indicated marker. Data represent mean ± SEM of triplicate experiments. (J) Quantitative RT-PCR analysis of TH expression level in differentiated cells for 14 days and 118 days from all six iPSC lines. The mRNA expression levels were normalized using β-actin. TH expression levels relative to WD39 are shown. Data represent mean ± SEM of triplicate experiments.
Figure 1. Continued.
The levels of LRRK2 protein and AKT phosphorylation of patient iPSC-derived neurons were lower than those of control-iPSC-derived neurons

We previously reported that the WT LRRK2/I2020T LRRK2-heterodimer has a short protein lifetime and reduced AKT1 phosphorylation at Ser473 (20). To examine the level of LRRK2 protein and AKT phosphorylation in I2020T-iPSC-derived neurons, we allowed neurons to differentiate from patient and control primary neurospheres. After 14 days in vitro, cell lysates were subjected to western blot analysis. We found that the levels of LRRK2 protein and AKT phosphorylation at Ser473 in LA- and LB-iPSC-derived neurons were lower than those in control-iPSC-derived neurons (Fig. 2A and C). Interestingly, Thr308 phosphorylation of AKT in I2020T-iPSC-derived neurons was markedly lower than that in control-iPSC-derived neurons (Fig. 2B and C). Similarly, the levels of LRRK2 protein and AKT phosphorylation were low in neurons derived from I2020T-iPSC tertiary neurospheres (Fig. 2D–F). In addition, using phospho-AKT substrate antibody, we found that the levels of phosphorylated AKT substrate proteins in patient iPSC-derived neurons were significantly lower than those in control-iPSC-derived neurons (Fig. 2G). These results suggested that AKT activity is suppressed in I2020T-iPSC-derived neurons.

Vulnerability to oxidative stress in I2020T-iPSC-derived neurons

To clarify the vulnerability of neurons derived from I2020T-iPSCs to oxidative stresses, we treated the cells with 200 μM hydrogen peroxide (H₂O₂) for 24 h. The cells were then fixed and stained for βIII-tubulin, TH and the activated form of Caspase-3 (C-CASP3). In short-term culture (14 days), ∼80% (among a total of 8000 cells) of differentiated cells were βIII-tubulin-positive and ∼5% were TH-positive. In all six cultures of iPSC-derived neurons, no significant differences between the percentages of βIII-tubulin-positive cells and TH-positive cells were observed. Also, the proportions of TH and C-CASP3 double-positive cells were similar among H₂O₂-untreated iPSC-derived neurons (Fig. 3A–C). In contrast, the proportions of TH and C-CASP3 double-positive cells among H₂O₂-treated neurons were significantly higher for...
LA- and LB-iPSC-derived neurons than for control-iPSC-derived neurons. Furthermore, in long-term culture (118 days), ~80% of differentiated cells (among a total of 3500 cells) were βIII-tubulin-positive and ~20% TH-positive. Interestingly, the proportions of βIII-tubulin and C-CASP3 double-positive cells and TH and C-CASP3 double-positive cells were markedly higher for LA- and LB-iPSC-derived neurons than for control-iPSC-derived neurons (Fig. 3D–F, Supplementary Material, Fig. S1A and B).

Although the numbers of C-CASP3-positive cells were increased among neurons derived from all six iPSC clones by H2O2 treatment, the number was markedly higher for LA lines and LB lines than for YA9 and WD39 lines. In addition, to confirm whether Caspase-3 showed any differences in activation, cell lysates of iPSC-derived neurons were subjected to western blot analysis. At 118 days in vitro, we found that C-CASP3 expression in H2O2-treated neurons from LA-iPSC lines and LB-iPSC lines was higher than that for control-iPSC-derived neurons (Fig. 3G).

On the other hand, to examine differences in cell death between iPSC-derived neurons from patients and controls, cell viability was assessed by trypan blue staining. The numbers of trypan blue-positive cells among patient iPSC-derived neurons were higher than those among control neurons (Supplementary Material, Fig. S1C). These results indicated that I2020T-iPSC-derived neurons might be more vulnerable to cell death via activation of C-CASP3 than control-iPSC-derived neurons.

**Dysfunction of dopamine release from I2020T-iPSC-derived neurons**

To examine functional properties of iPSC-derived neurons as dopaminergic neurons, both released and residual dopamine was measured in the conditioned medium and the lysate of iPSC-derived neurons using high performance liquid chromatography (HPLC). After long-term culture (86 days) in vitro, all six iPSC-derived neurons showed markedly enhanced release of dopamine evoked by 56 mM KCl relative to those of 4.7 mM KCl (Fig. 4A and B). The percentage of dopamine released by all cell lines was strongest in response to the first high-K⁺ solution employed to stimulate Ca²⁺ influx, and no difference in the peak of dopamine release was observed. Interestingly, the amount of dopamine released from iPSC-derived neurons of the LA and LB lines was markedly lower than that from control-iPSC-derived neurons.
neurons. Furthermore, the levels of dopamine and DOPAC were similar in control and patient iPSC-derived neurons (Fig. 4C and D). These results suggested that dopamine release from I2020T-iPSC-derived neurons might be impaired.

Next, to investigate possible differences in the SNARE complex associated with neurotransmitter release in iPSC-derived neurons, lysates of control and patient iPSC-derived neurons were analyzed by western blotting. Using blue-native-polyacrylamide gel
electrophoresis (PAGE), the level of oligomerized soluble NSF attachment protein receptor (SNARE) complex, which mediates vesicle fusion and exocytosis, was confirmed using an antibody against SNAP-25, which is one of the important components of the complex. In comparison with control-iPSC-derived neurons, the amount of oligomerized SNARE complex was decreased in patient iPSC-derived neurons (Fig. 4E). Furthermore, to elucidate whether SNARE protein show any differences in expression level, SNAP-25, Syntaxin-1A and VAMP2 protein in iPSC-derived neurons were analyzed by western blotting. These SNARE proteins in patient iPSC-derived neurons were lower than those of control-iPSC-derived neurons (Fig. 4F). Consistent with, the mRNA levels of SNAP-25, Syntaxin-1A and VAMP2 were decreased in patient iPSC-derived neurons (Fig. 4G). We also confirmed that cell lysate of all iPSC-derived neurons exhibited similar expression levels of TH (Supplementary Material, Fig. S2).

Furthermore, to examine whether AKT contributes to the dysregulation of SNARE proteins, control-iPSC-derived neurons were treated with the AKT inhibitor MK-2206 for 7 days. After long-term culture (86 days) of AKT inhibitor-treated or untreated iPSC-derived neurons in vitro, both released and residual dopamine were measured using HPLC, and the protein and mRNA levels of Syntaxin-1, VAMP2 and SNAP-25 were analyzed by western blotting and real-time RT-PCR. The amount of dopamine released from AKT inhibitor-treated control-iPSC-derived neurons was markedly lower than that from untreated control-iPSC-derived neurons (Supplementary Material, Fig. S3A). Also, control-iPSC-derived neurons under chronic AKT inhibition showed a significant decrease of SNARE proteins as well as mRNA (Supplementary Material, Fig. S3B and C). Similarly, AKT inhibitor-treated SH-SY5Y cells showed decreased cellular levels of protein and mRNA for Syntaxin-1, VAMP2 and SNAP-25 (Supplementary Material, Fig. S4A and B). These results suggested that the reduction in the amount of dopamine released from patient iPSC-derived neurons was related to a decrease in the levels of oligomerized SNARE complex and SNARE proteins, which are associated with synaptic exocytosis.

I2020T-iPSC-derived neurons show increased tendency for dysfunction of autophagy and protein oxidation

To evaluate the potential usefulness of this iPSC as a model of PD, lysates of control and patient iPSC-derived neurons were analyzed for autophagy function and accumulation of α-synuclein by western blotting. We found that the protein level of the autophagy marker LC3B-II in patient iPSC-derived neurons was
Figure 3. Increase of cleaved-Caspase-3 in patient iPSC-derived neurons. (A and B) All iPSC-derived neuronal lines from tertiary neurospheres cultured for 14 days untreated (A) or treated (B) with 200 μM H2O2. After 24 h, cells were co-stained with antibodies against cleaved-Caspase-3 (C-CASP3), TH and βIII-tubulin. Nuclei were stained with Hoechst 33342. Scale bars: 50 μm. (C) Graphical representation of the experiments shown in A and B, in which the percentages of C-CASP3-positive neurons (left panel) or C-CASP3-positive and TH-positive neurons (right panel) were determined. Data represent mean ± SEM (n = 3 wells per clone). Stars represent statistical comparisons by one-way ANOVA; *P < 0.05. (D and E) All iPSC-derived neuronal lines from tertiary neurospheres cultured for 117 days untreated (D) or treated (E) with 200 μM H2O2. After 24 h, long-term-cultured cells were co-stained with antibodies against C-CASP3, TH and βIII-tubulin. Nuclei were stained with Hoechst 33342. Scale bars: 50 μm. (F) Graphical representation of the experiments shown in D and E, in which the percentages of C-CASP3-positive neurons (left panel) or C-CASP3-positive and TH-positive neurons (right panel) were determined. Data represent mean ± SEM (n = 3 wells per clone). Stars represent statistical comparisons by one-way ANOVA; *P < 0.05, **P < 0.005. (G) Western blotting for Caspase-3 in H2O2-treated iPSC-derived neurons for 118 days. C-CASP3 protein levels were normalized using β-actin. Lower panels show graphical representations of western blot data. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.05.
higher than that in control-iPSC-derived neurons, and that the protein level of the autophagy adapter molecule p62 in patient iPSC-derived neurons tended to be higher than that in the controls (Fig. 5A). On the other hand, no difference in the level of α-synuclein expression was evident among the iPSC-derived neurons (Fig. 5B). Next, using OxyBlot analysis to detect protein carbonyl formation, a common marker of protein oxidation, lysates of control and patient iPSC-derived neurons were analyzed. This revealed that the levels of carbonylated proteins after dinitrophenylhydrazine (DNP) derivatization were higher in patient iPSC-derived neurons than in control neurons (Fig. 5C). These results suggested that I2020T LRRK2 iPSC-derived neurons exhibited autophagy dysfunction through LC3B and increased levels of protein oxidation.

Both I2020T LRRK2 iPSC-derived neurons and the postmortem brain of a PARK8 patient show increased Tau phosphorylation through activation of GSK-3β

Because the postmortem brains of PARK8 patients in the Sagamihara family were reported to show phosphorylated Tau and NFTs as pathologic features (30,31), we examined whether reduction of AKT phosphorylation would influence the activity of GSK-3β, also known as Tau kinase, and increase the phosphorylation of Tau. We differentiated neurons from both patient and control tertiary neurospheres. After 14 days in vitro, cell lysates were subjected to western blot analysis. In comparison with control-iPSC-derived neurons, I2020T LRRK2 iPSC-derived neurons of the LA and LB lines exhibited a reduction of phosphorylation at Ser9 of GSK-3β.
and an increase of phosphorylation at Tyr216 of GSK-3β (Fig. 6A). In addition, we found that Tau phosphorylation at Ser202/Thr205, Thr231/Ser235, Thr181 and Ser396 in patient iPSC-derived neurons was significantly higher than that in control-iPSC-derived neurons (Fig. 6B and C). Also, in an experiment using Phos-tag gel, I2020T LRRK2 was shown to comprehensively increase Tau phosphorylation (Fig. 6D). In addition, AKT inhibitor-treated control-iPSC-derived neurons exhibited Tau hyperphosphorylation relative to untreated control-iPSC-derived neurons (Supplementary Material, Fig. S5). These findings suggest that I2020T LRRK2 may affect Tau phosphorylation through a reduction of AKT.

Furthermore, to clarify whether I2020T LRRK2 affects Tau phosphorylation through activation of GSK-3β, control and patient iPSC-derived neurons after 13 days of in vitro culture were treated with 3 and 10 μM CHIR-99021 (a GSK-3 inhibitor) or 1 and 3 μM LRRK2-IN1 (a LRRK2 inhibitor). After 24 h, cell lysates of iPSC-derived neurons were subjected to western blot analysis. The degree of Tau phosphorylation in the presence of GSK-3 inhibitor was markedly lower than that in untreated iPSC-derived neurons (Fig. 6E and Supplementary Material, Fig. S6). Interestingly, Tau phosphorylation in I2020T-iPSC-derived neurons treated with LRRK2 inhibitor was lower than that in untreated I2020T-iPSC-derived neurons. Also, to examine whether Cdk5, which is involved in Tau phosphorylation, is activated, control and patient iPSC-derived neurons were analyzed by western blotting using a phospho-CDKs substrate antibody. We found that the levels of phosphorylated CDKs substrate proteins were
Figure 4. Reduction of dopamine release by patient iPSC-derived neurons. (A) Representative data of dopamine and DOPAC measurement using HPLC. (B) Dopamine release from iPSC-derived neurons was evoked with 4.7 and 56 mM KCl. After treatment, the KCl solutions including released catecholamine and the contents of iPSC-derived neurons including residual catecholamine were collected into tubes containing perchloric acid on ice. Secreted or remaining dopamine and DOPAC in each fraction were measured by HPLC. The amount of dopamine released into the medium was determined and expressed as a percentage of the total cellular content. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.001. (C) The concentration of intracellular level of dopamine in iPSC-derived neurons. (D) The concentration of intracellular level of DOPAC in iPSC-derived neurons. (E) Using blue-native PAGE, the level of oligomerized SNARE complex in control and patient iPSC-derived neurons was analyzed with antibody against SNAP-25. Middle panel shows β-actin as an internal control using SDS-PAGE. Lower panels show graphical representations of western blot data. The levels of oligomerized SNARE complex relative to WD39 is shown. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.005. (F) Western blotting for Syntaxin-1A, SNAP-25 and VAMP2 in patient and control-iPSC-derived neurons. The membrane was reprobed for detection of various antibodies. SNARE protein levels were normalized using GAPDH. Lower panels show graphical representations of western blot data. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.005. (G) Quantitative RT-PCR analysis for mRNA expression level in Syntaxin-1A, SNAP-25 and VAMP2 in patient and control-iPSC-derived neurons. The mRNA expression levels were normalized using β-actin. Each of mRNA expression levels relative to WD39 is shown. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.05.
similar in control and patient iPSC-derived neurons (Supplementary Material, Fig. S7). Taken together, these results suggested that I2020T LRRK2 increased Tau phosphorylation through activation of GSK-3β.

Next, to investigate whether I2020T LRRK2 affected Tau phosphorylation in a PD patient, we performed histopathological and biochemical analysis of postmortem brain tissue from patient LB. Gallyas-Braak staining, which specifically stain phosphorylated Tau, of the hippocampus demonstrated an increase of phosphorylated Tau relative to sporadic PD patient (Fig. 7A–C). Consistent with this observation, immunohistochemical staining with AT8 antibody exhibited a higher level of Tau phosphorylation at Ser202/Thr205 in neurons of the hippocampus, substantia nigra and locus ceruleus. The numbers of pretangles (PTs) revealed by AT8 staining were analyzed semiquantitatively and scored (−: none, +: sparse, ++: moderate, +++: severe) (Table 1). Similarly, the numbers of NFTs revealed by Gallyas-Braak staining were scored using a grading system (Supplementary Material, Table S1).

On the other hand, the level of Tau phosphorylation in neurons of the hippocampus, substantia nigra and locus ceruleus were similar in patients with LB and sporadic Alzheimer’s disease (AD) (Fig. 7A, B and D, and Table 1). Furthermore, biochemical analysis of a brain lysate from the patient LB revealed hyper-phosphorylation of Tau and activation of GSK-3β in comparison to the three controls (Fig. 7E). Similarly, the patient LC in the Sagamihara family who had been previously confirmed to have Tau pathology...
(30,31) exhibited hyper-phosphorylation of Tau and activation of GSK-3β relative to the three controls. Consequently, Tau phosphorylation in iPSC-derived neurons from patient LB showed a pattern consistent with the pathology evident in the brain. These results provide evidence that I2020T LRRK2 causes high phosphorylation of Tau through activation of GSK-3β.

Figure 6. Increased phosphorylation of Tau through the impaired AKT–GSK-3β pathway in patient iPSC-derived neurons. (A) Cell lysates of patient and control tertiary neurosphere-derived neurons differentiated for 14 days were analyzed by western blotting using antibodies against phospho-GSK-3β (Ser9), phospho-GSK-3β (Tyr216) and GSK-3β. Lower panels show graphical representations of western blot data. Phosphorylation levels of GSK-3β (Ser9) and GSK-3β (Tyr216) were normalized with total GSK-3β. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.005. (B) Western blots for phospho-Tau clones AT8 (Ser202/Thr205), AT180 (Thr231/Ser235), AT270 (Thr181), PHF1 (Ser396/Ser404) and Tau in patient and control neurons differentiated from tertiary neurospheres for 14 days. The membrane was probed for detection of various antibodies. (C) Graphical representation of Tau phosphorylation levels in the experiment shown in (B) after normalization with the amount of total Tau. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.005. (D) Western blot for the degree of Tau phosphorylation using 12.5% Phos-tag gel. The arrows indicate an increase in phosphorylated Tau in patient iPSC-derived neurons relative to those of control-iPSC-derived neurons. (E) Control (YA9) and patient (LA5) iPSC-derived neurons after 13 days of in vitro culture were treated with 3 and 10 μM CHIR-99021 (a GSK-3 inhibitor) or 1 and 3 μM LRRK2-IN1 (LRRK2 inhibitor). After 24 h, cell lysates of iPSC-derived neurons were analyzed by western blotting using antibodies against phospho-Tau clones AT270 (Thr181) and Tau. Phosphorylation levels of Tau (Thr181) were normalized against total Tau. The membrane was probed for detection of other antibodies. Lower panels show graphical representations of western blot data. Data represent mean ± SEM of triplicate experiments. Stars represent statistical comparisons by one-way ANOVA; *P < 0.005.
**Discussion**

Recently, several studies of dopaminergic neuron loss in familial PD using G2019S and R1441C mutant LRRK2-iPSC have been reported (22–27). In the present study, we first established LA and LB iPSC lines from two PARK8 patients harboring I2020T mutant LRRK2. We have previously reported that I2020T LRRK2 has a unique molecular feature, i.e. it is more susceptible to post-translational degradation than WT and G2019S LRRK2 (33,34). Impaired LRRK2 protein stability has also been reported for the pathogenic A1442P and R1441C ROC domain mutant (35). We have also reported that AKT1, a key molecule involved in promotion of cell survival through several mechanisms, including suppression of the Caspase cascade (36,37), is a convincing candidate for the physiological substrate of LRRK2 (19). In this study, we found that I2020T LRRK2 iPSC-derived neurons exhibited reduced levels of LRRK2 protein and AKT phosphorylation relative to control-iPSC-derived neurons. We also found that patient iPSC-derived neurons exhibited vulnerability to oxidative stress via elevated expression of C-CASP3. In our previous studies, LRRK2-knockdown HEK293 and SH-SY5Y cells exhibited vulnerability to oxidative stress via initiation of apoptosis as well as low phosphorylation of AKT1 (19). We also demonstrated that the I2020T LRRK2-transfected SH-SY5Y clone overexpressing WT LRRK2, leading to low phosphorylation of AKT1, exhibited vulnerability to oxidative stress (20). In fact, LRRK2 deficiency has been reported to induce apoptosis of human mesencephalic neural progenitor cells, leading to loss of dopaminergic neurons (38). Our data provide evidence that I2020T LRRK2 induces activation of effector Caspase, consistent with data for G2019S iPSC-derived neurons (22–26).

Neurotransmitter release is critically dependent on the Ca²⁺ sensor synaptotagmin-1 and the SNARE proteins syntaxin-1, synaptobrevin (VAMP2) and SNAP-25, which mediate membrane fusion by forming tight SNARE complexes that bridge the synaptic vesicle. It is considered that LRRK2 regulates synaptic vesicle trafficking (39–42). Recently, it has been reported that LRRK2 phosphorylates Snapin that interacts with numerous proteins including synaptic vesicle-related proteins such as SNAP-25 (43). Snapin promotes the binding of SNAP-25 to synaptotagmin-1 and stabilizes the binding of synaptotagmin-1 to the SNARE complex (44). In the present, all six iPSC-derived neurons exhibited a similar level of dopamine. On the other hand, patient iPSC-derived neurons showed reduction of dopamine release relative to control-iPSC-derived neurons. Furthermore, the level of oligomerized SNARE complex in patient iPSC-derived neurons was decreased. Our present data support the notion that I2020T LRRK2 affects neurotransmitter release through synaptic vesicle-related proteins. Also, it has been reported that expression of SNARE proteins activates the PI3K/AKT signaling pathway in pancreatic β cells (45). Consistent with this observation, our findings demonstrated that the protein and mRNA levels of the SNARE proteins SNAP-25, Syntaxin-1A and VAMP2 in patient iPSC-derived neurons were decreased relative to control-iPSC-derived neurons. Similarly, the protein and mRNA levels of syntaxin-1, VAMP2 and SNAP-25 in AKT inhibitor-treated SH-SY5Y cells were decreased. Interestingly, AKT inhibitor-treated control-iPSC-derived neurons exhibited a reduction of dopamine release relative to untreated control-iPSC-derived neurons. These findings suggest that reduction of AKT phosphorylation in I2020T LRRK2 iPSC-derived neurons may affect transcriptional activation of the SNARE complex genes involved in dopamine release.

It is important to understand how phosphorylation of Tau is controlled by LRRK2. Tau phosphorylation is controlled by several kinases such as GSK-3β and Cdk5 (46,47). It also has been reported that GSK-3β activity is directly inhibited by phosphorylation at Ser9 by AKT (48). On the other hand, the active form of GSK-3 in the brain of AD patients phosphorylates Tau, resulting in increased hyper-phosphorylation of Tau (46–48). Our results suggest that reduction of AKT phosphorylation, leading to activation of GSK-3β, but not Cdk5, is involved in the pathogenesis of Tau pathology in PARK8 patients. In Drosophila dopaminergic neurons, G2019S LRRK2 enhances Tau phosphorylation and microtubule fragmentation through auto-activated GSK-3β at Tyr216 (49). It has been reported that I2020T LRRK2-transfected COS-1 cells show increased phosphorylation of Tau at Thr181 and Thr231 in comparison with WT LRRK2 (31). We have previously reported that G2019S and I2020T recombinant LRRK2 increases Tau...
Figure 7. Increased phosphorylation of Tau in postmortem brain tissue of patient LB from whom iPSC were established. (A) Histological analysis of postmortem brain tissue of patient LB, from whom iPSC were established. Left panel: Gallyas-Braak staining of a hippocampus section. Scale bars: 500 or 250 or 100 μm. (B) Immunohistochemical staining of hippocampus, substantia nigra, and locus ceruleus sections of patient LB with AT8 antibody. Scale bars: 500 or 250 or 100 μm. (C) Histological analysis of postmortem brain tissue of age-matched sporadic PD patient. Left panel: Gallyas-Braak staining of a hippocampus section. Middle and right panels: Immunohistochemical staining of hippocampus, substantia nigra and locus ceruleus sections with AT8 antibody. Scale bars: 500 or 200 μm. (D) Histological analysis of postmortem brain tissue of age-matched sporadic AD patient. Left panel: Gallyas-Braak staining of a hippocampus section. Middle and right panels: Immunohistochemical staining of hippocampus, substantia nigra and locus ceruleus sections with AT8 antibody. Scale bars: 500 or 200 μm. (E) Western blots for LRRK2, phospho-Tau (Ser396), Tau, phospho-GSK-3β (Ser9), phospho-GSK-3β (Tyr216) and GSK-3β in postmortem brain tissue from LB and LC patients of the Sagamihara family, and three control patients (Control 1, Control 2 and Control 3) who had been clinically and neuropathologically diagnosed as having sporadic ALS. The ALS patients were used as controls. The membrane was reprobed for detection of various antibodies.
phosphorylation relative to WT LRRK2 (11). Our previous studies have provided evidence that LRRK2 phosphorylates Tau at Thr181 and regulates the dissociation of Tau from tubulin using human neuroblastoma cells (11). Furthermore, we have recently demonstrated that GSK-3β directly interacts with LRRK2 and enhances its activity (50). In the present study, in I2020T-iPSC-derived neurons treated with GSK-3 or LRRK2 inhibitor, we found that I2020T LRRK2 increased Tau phosphorylation through activation of GSK-3β. These findings suggest that I2020T LRRK2 may affect Tau phosphorylation through the AKT/GSK-3β pathway.

There is some evidence that autopsied brain tissues from PD patients harboring the LRRK2 mutation show various pathologic features such as Lewy body-negativity, Lewy body-positivity,
ubiquitin-positive inclusions, α-synuclein pathology, PSP-like pathology, senile plaques and NFTs (51). Tau pathology is also often seen in sporadic PD or as a result of aging-related changes (32). Tau pathology including NFTs in the brain has been reported previously in four PARK8 patients from the original Japanese Sagamihara family (30). Interestingly, postmortem brain tissue of the patient from whom iPSC were established exhibited deposition of NFTs as well as increased Tau phosphorylation in neurons relative to sporadic PD patient. Similarly, we found that the postmortem brain tissue of patients LB and LC had an increased level of Tau phosphorylation through activated GSK-3β. The present study has provided an insight into the mechanism by which I2020T LRRK2 increases the phosphorylation of Tau through the AKT/GSK-3β pathway.

Although G2019S and R1441C LRRK2 iPSC from PD patients have been reported, our study is the first to demonstrate that the phenotype of I2020T LRRK2 iPSC-derived neurons replicates the in vivo phenotype evident in postmortem brain tissue from the corresponding cell donor. In addition, I2020T-iPSC-derived neurons showed an increased tendency for the autophagy marker LC3B-II and the autophagy adapter molecule p62, consistent with data for G2019S and R1441C iPSC-derived neurons (24,25). Also, I2020T-iPSC-derived neurons exhibited increased levels of protein oxidation, consistent with data for postmortem brain samples from sporadic PD patients or G2019S and R1441C iPSC-derived neurons (22,23,27,52). Intriguingly, it has been reported that PD patient with I2020T mutation in the Sagamihara family might not be related to the accumulation of α-synuclein (31). Correspondingly, the protein levels of α-synuclein were similar in control and patient iPSC-derived neuron. Therefore, further studies will be needed to clarify the mechanism responsible for the abnormal phenotype expressed through mutant LRRK2. We suggest that LRRK2 iPSC-derived neurons may serve as a useful model in studies aimed at the development of targeted therapeutics.

**Materials and Methods**

**Isolation of human skin fibroblasts and generation of iPSC**

For the patient iPSC lines, LA iPSC (LA5 and 11; Passage 12–20) and LB iPSC (LB16 and 21; Passages 15–21) were established from skin-punch biopsies from two female PD patients LA and LB (66 and 78 years) in the Sagamihara family harboring the I2020T LRRK2 mutation, respectively. YA9 (36 years; Passages 19–21) and WD39 (16 years; Passages 12–16) iPSC were used as the control-iPSC lines (28). The maintenance of human dermal fibroblasts (HDFs), retrovirus production, infection, stem cell culture and characterization and teratoma formation were performed as described previously (29,53). Recently, it has been reported that age- and gender-matched iPSC lines exhibited different genome expression in cluster analysis (24). Because the control-iPSC lines exhibited a phenotype similar to centenarian iPSC lines (28,54), the control-iPSC lines were matched to the patient-iPSC lines with regard to gender, the method of reprogramming, and approximate passage number, exclusive of age. All of the experimental procedures for skin biopsy and iPS production were approved by the Keio University School of Medicine Ethics committee (Approval Number: 20-16-15) and Kitasato University School of Medicine Ethics committee (Approval Number: B-09-46, G-09-06).

**In vitro differentiation of human iPSC**

Neural differentiation of iPSC was performed as described previously (28,55). Briefly, iPSC colonies were detached from feeder layers and cultured in suspension as EBs for ~30 days in bacteriological dishes. EBs were then enzymatically dissociated into single cells, and the dissociated cells were cultured in suspension in serum-free media (MHM) (28) for 10–14 days to allow the formation of neurospheres. The neurospheres were passaged repeatedly by dissociation into single cells followed by culture in the same manner. These neurospheres were expandable by repeated passage and stored as a frozen stock, which enabled us to perform each experiment easily and promptly (28,56). The neurospheres obtained using this protocol give rise mainly to neurons, and the differentiation tendencies were similar from primary through tertiary neurospheres (28,56,57). Therefore, neurospheres between passages 1 and 3 were used for analysis. For terminal differentiation, dissociated or undissociated neurospheres were allowed to adhere to poly-L-ornithine- and fibronectin-coated coverslips and cultured for 14, 86 and 118 days. For long-term cultures (86 and 118 days), the differentiated neurons from neurospheres were cultured on poly-L-ornithine- and fibronectin-coated. Subsequently, MHM medium of iPSC-derived neurons were changed every 4–5 days.

**PCR amplification of genomic DNA**

Genomic DNA was purified from HDFs and iPSC using a DNeasy kit (Qiagen). The PCR conditions used have been described previously (28,55,58).

**Quantitative reverse transcription-PCR**

RNA isolation and RT-PCR were performed as described previously (28,55,58). The amount of cDNA was normalized to β-actin mRNA. Real-time RT-PCR was performed on an ABI PRISM...
Sequence Detection System 7900HT and ABI PRISM 7500 Real-Time PCR system (Applied BioSystems) using SYBR premix ExTaq (Takara). Primers for the detection of Oct4, LRRK2 and β, actin, the transgenes Oct4-tg, Sox2-tg, KI4-tg and c-Myc-tg, have been described previously (29,33). Primer sequences of TH, Syntaxin-1A, SNAP-25, VAMP2 and Synaptophysin, are listed in Supplementary Material, Table S2.

**Karyotype analysis**
To assess chromosomally aberrant iPSC, patient iPSC lines were karyotyped using standard protocols for high-resolution G-banding.

**Immunocytochemical analysis of iPSC and neurons**
For immunocytochemical analysis, cells were fixed with PBS containing 4% PFA for 30 min and permeabilized with 0.3% Triton X-100, 0.25 mM sucrose, 10 mM EGTA. SSEA-4 (Millipore), TRA-1-60 (Millipore), TH (Millipore) and α-actin, the transgenes Oct4-tg, Sox2-tg, Klf4-tg and c-Myc-tg, were cleaved-Caspase-3 (Cell Signaling). Cells were incubated with an Alexa Fluor 488-, Alexa Fluor 555- or Alexa Fluor 647-conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. Images were obtained using an LSM-710 confocal (Zeiss) microscope.

**Western blotting analysis**
Control and patient iPSC-derived neurons, and postmortem brain tissue from the patient LB from whom iPSC were established, a female PD patient (43 years) (the patient LC) in the Sagamihara family who had been previously confirmed to have Tau pathology by histopathological analysis (30,31), and three control patients who had been clinically and neuropathologically diagnosed as having sporadic amyotrophic lateral sclerosis (ALS) were suspended in cell lysis buffer containing 1% Triton X-100, 0.25 mM sucrose, 10 mM EGTA, 2 mM ethylenediaminetetraacetic acid, Complete Mini Protease Inhibitor Cocktail (Roche), and PhosSTOP (Roche), and rotated at 4°C for 1 h. Cell lysates were obtained by centrifugation at 10000g for 10 min at 4°C. Proteins separated by SDS-PAGE or blue-native PAGE were blotted onto polyvinylidene fluoride (PVDF) membranes, and analyzed with antibodies against LRRK2 (c41-2) (Epitomics), phospho-AKT (Ser473) (Cell Signaling), phospho-AKT (Thr308) (Cell Signaling), AKT (Cell Signaling), phospho-AKT substrate (RXXS/T) (Cell Signaling), phospho-CDK substrate (R/KRSPXK/R) (Cell Signaling), TH (Millipore), SNAP-25, Syntaxin-1A, VAMP2 (rabbit anti-SNAP-25ct, mouse anti-Syntaxin-1A and rabbit anti-VAMP2ct antibodies were a kind gift from Dr S. Yamamori (59,60), phospho-Tau clone AT8 (Ser202/Thr205) ( Pierce), phospho-Tau clone PHF1 (Ser396/Thr404) (PHF1 antibody was a kind gift from Dr F. Davies), phospho-Tau clone AT180 (Thr231/235) (Pierce), phospho-Tau clone AT270 (Thr181) (Pierce), phospho-Tau (Ser396) (SantaCruz), Tau (H-150) (SantaCruz), phospho-GSK 3β (Ser9) (SAB), phospho-GSK 3β (Thr216) (BD), GSK-3β (H-76) (SantaCruz), Caspase-3 (Cell Signaling), p62 (LSBio), LC3B (Cell Signaling), α-synuclein (Santa Cruz), Synaptophysin (GeneTex) and βIII-tubulin (Sigma). β-actin (Abcam) and GAPDH (Abcam) were used as internal controls.

**Dopamine release assay**
All iPSC-derived neurons were subjected to long-term culture. After 86 days, cells were subjected to Ca2+-dependent release using low-concentration 4.7 mM KCl and high-concentration 56 mM KCl (59). The cells were then washed three times with low-K+ solution. After the wash, cells were incubated twice with low-K+ solution for 1 min at 37°C, and then the buffer was immediately changed three times every 1 min with high-K+ solution to stimulate Ca2+ influx. After treatment, the KCl solutions including released catecholamine and contents of iPSC-derived neurons including residual catecholamine were collected into tubes containing perchloric acid on ice. Secreted or remaining dopamine and DOPAC in each fraction were measured by HPLC (60-62).

**Histological and immunohistochemical analysis of postmortem brain tissue**
The ethics committee of Kitasato University School of Medicine reviewed and approved the protocol for analysis of postmortem brain tissue (Approval Number: G-09-06). The patient concerned was informed of the study and provided written informed consent. Brain tissue from patient LB, sporadic PD patient and sporadic AD patient were obtained following their death at age 78, 89 and 86. The tissue was fixed with 20% formalin and then embedded in paraffin. Sections of the hippocampus, substantia nigra and locus ceruleus (4 μm thick) were cut, deparaffinized with xylen, and then rehydrated in ethanol. Hippocampus section was examined with Gallyas-Braak staining for histological analysis. Immunohistochemical analysis was performed with a Ventana BenchMark GX autostainer (Ventana Medical Systems), an antibody against phospho-Tau clone AT8 (Innogenetics), and an I-View Universal DAB Detection Kit (Roche) in accordance with Images were obtained using an Eclipse 90i (Nikon) microscope.
Protein carbonyl assay
The derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine was performed using an OxyBlot™ Protein Oxidation Detection Kit (Millipore) in accordance with the manufacturer’s protocol. The derivatized lysates of control and patient iPSC-derived neurons separated by SDS-PAGE were blotted onto PVDF membranes, and analyzed with antibodies against DNP (Millipore).

Statistical analysis
The data were analyzed by one-way ANOVA followed by the Ryan–Einot-Gabriel–Welsch multiple-range test.

Supplementary Material
Supplementary Material is available at HMG online.

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