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of Tau: A FUNCTIONAL LINK
BETWEEN DOWN SYNDROME AND
ALZHEIMER DISEASE**

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DYRK1A-mediated Hyperphosphorylation of Tau

A FUNCTIONAL LINK BETWEEN DOWN SYNDROME AND ALZHEIMER DISEASE*

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Most individuals with Down syndrome show early onset of Alzheimer disease (AD), resulting from the extra copy of chromosome 21. Located on this chromosome is a gene that encodes the dual specificity tyrosine phosphorylation-regulated kinase 1A (*DYRK1A*). One of the pathological hallmarks in AD is the presence of neurofibrillary tangles (NFTs), which are insoluble deposits that consist of abnormally hyperphosphorylated Tau. Previously it was reported that Tau at the Thr-212 residue was phosphorylated by Dyrk1A *in vitro*. To determine the physiological significance of this phosphorylation, an analysis was made of the amount of phospho-Thr-212-Tau (pT212) in the brains of transgenic mice that overexpress the human DYRK1A protein (DYRK1A TG mice) that we recently generated. A significant increase in the amount of pT212 was found in the brains of DYRK1A transgenic mice when compared with age-matched littermate controls. We further examined whether Dyrk1A phosphorylates other Tau residues that are implicated in NFTs. We found that Dyrk1A also phosphorylates Tau at Ser-202 and Ser-404 *in vitro*. Phosphorylation by Dyrk1A strongly inhibited the ability of Tau to promote microtubule assembly. Following this, using mammalian cells and DYRK1A TG mouse brains, it was demonstrated that the amounts of phospho-Ser-202-Tau and phospho-Ser-404-Tau are enhanced when DYRK1A amounts are high. These results provide the first *in vivo* evidence for a physiological role of DYRK1A in the hyperphosphorylation of Tau and suggest that the extra copy of the *DYRK1A* gene contributes to the early onset of AD.

Down syndrome (DS)³ is the most common genetic disorder with a frequency of 1 in 800 live births, and it is caused by the presence of an extra copy of whole or part of human chromosome 21 (1, 2). DS patients suffer various symptoms, including congenital heart defects, immune and endocrine system defects, mental retardation, and early onset of Alzheimer disease (AD) (3). Both DS and AD patients have pathological hallmarks, amyloid plaques and neurofibrillary tangles (NFTs) that are insoluble deposits made of proteins called β -amyloid ($A\beta$) and hyperphosphorylated Tau, respectively (4–6). Although an early onset AD in DS patients is not clearly understood, one potential mechanism is the presence of three chromosomal copies of β -amyloid precursor protein (*APP*) gene. However, the APP overexpression alone in mice does not show the endosome abnormalities observed in AD-like pathology (7), implying the necessity of additional genes on the chromosome 21 for a full spectrum of AD pathologies.

NFTs found in AD are composed of paired helical filaments (PHFs), which are mainly composed of hyperphosphorylated Tau protein (8). To date, more than 30 phosphorylation sites and 7–10 mol of phosphates per mol of Tau have been observed in PHF-Tau (9, 10). Although Tau protein is phosphorylated *in vitro* by numerous kinases, it is unclear how many kinases actually phosphorylate Tau *in vivo*. Currently, only glycogen synthase kinase 3 β (GSK3 β), cyclin-dependent kinase 5, cAMP-dependent protein kinase A, and microtubule affinity-regulating kinase are known to modulate Tau phosphorylation *in vivo* at some level, either directly or indirectly (11).

The *DYRK1A* (dual specificity tyrosine (Y)-phosphorylation-regulated kinase 1A) gene is isolated from human chromosome 21 and encodes a protein kinase (12–15). DYRK1A is ubiquitously expressed with strong expression in brain and heart (13, 15–18), and mRNA in DS fetal brains has been shown to be

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³ The abbreviations used are: DS, Down syndrome; AD, Alzheimer disease; DYRK1A, dual specificity tyrosine phosphorylation-regulated kinase 1A; DYRK1A TG mice, transgenic mice that overexpress the human DYRK1A protein; NFTs, neurofibrillary tangles; PHFs, paired helical filaments; pS202, phospho-Ser-202-Tau; pT212, phospho-Thr-212-Tau; pS404, phospho-Ser-404-Tau; protein nomenclature, Dyrk1A for mouse protein and DYRK1A for human protein; WT, wild type; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; siRNA, short interfering RNA; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; APP, β -amyloid precursor protein; $A\beta$, β -amyloid.

overexpressed (19). Dyrk1A has dual substrate specificities as follows: autophosphorylation on the tyrosine 321 residue for self-activation and phosphorylation of target proteins on serine/threonine residues with a preferred phosphorylation consensus motif of RPX(S/T)P (12, 20, 21). Dyrk1A potentially phosphorylates or interacts with several proteins, including transcription factors (NFATc, STAT3, Forkhead, CREB, and Gli1) and eukaryotic initiation factor 2B ϵ , Dynamin 1, cyclin L2, Hip-1, and α -synuclein, implying the multiple biological functions of DYRK1A (22). Dyrk1A knock-out mice show a general delay in fetal development and are embryonic lethal, indicating the vital and nonredundant biological functions of Dyrk1A (23). We recently generated transgenic mice that overexpress human DYRK1A (DYRK1A TG mice) by introducing a bacterial artificial chromosome that only carries the human DYRK1A genomic DNA, including the endogenous promoter (24). These DYRK1A TG mice show learning and memory deficits similar to what is seen in DS patients, a finding that is comparable with previous studies on the mouse Dyrk1A cDNA transgenic mice or the human genomic transgenic mice carrying a few chromosome 21 genes, including *DYRK1A* on a yeast artificial chromosome (24–26).

Previously, it was reported that Dyrk1A phosphorylates Tau at Thr-212 *in vitro*, a residue that is phosphorylated in fetal Tau and hyperphosphorylated in filamentous Tau from AD brains (27). In this study, we investigated the physiological significance of this phosphorylation event using transgenic mice that overexpress human DYRK1A (DYRK1A TG mice). It was found that that Tau phosphorylation at Thr-212 is significantly increased in DYRK1A TG mice. Furthermore, Tau protein was also phosphorylated at Ser-202 and Ser-404 residues by Dyrk1A *in vitro*, and the levels of the phosphorylation at those residues were increased in DYRK1A TG mice. These results indicate that the overexpression of DYRK1A in DS brains may contribute to early onset of AD pathology through the hyperphosphorylation of Tau.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies—The full-length mouse Dyrk1A cDNAs (wild-type and Y321F kinase-inactive mutant) were cloned into pET-25b (Novagen), followed by isopropyl 1-thio- β -D-galactopyranoside induction in *Escherichia coli* BL21(DE3) codon plus RIL (Stratagene). Using its endogenous 13-histidine repeat, the Dyrk1A protein was purified with nickel-nitrilotriacetic acid resin as described by the manufacturer (Qiagen). Human recombinant Tau, nonglycosylated and nonphosphorylated form of 441-amino acid Tau splice variant, was obtained from Calbiochem. Bovine tubulin was purchased from Cytoskeleton, and JNK2 was from Upstate. Antibodies for Tau (Tau-5) and phospho-Tau (pS202, pT212, pS404) were obtained from BIOSOURCE. Phosphopeptides and non-phosphopeptides were purchased to perform the peptide competition assay for all three phospho-Tau antibodies (BIOSOURCE). The anti-Dyrk1A antibody was custom-made as described previously (24).

Immunohistochemistry—Mice were anesthetized with isoflurane, and perfused transcardially with phosphate-buffered saline (PBS, pH 7.4) followed by fixative (4% paraformaldehyde in PBS, pH 7.4). The brains were fixed at 4 °C overnight and

were cryoprotected. Sagittal sections of mouse brains were prepared as described previously (24) and incubated first with phospho-Tau antibodies (pS202, pT212, and pS404; 1:100 dilution), then with biotinylated anti-rabbit IgG (1:200 dilution), and finally with avidin biotinylated horseradish peroxidase (Vector Laboratories). After visualization with an SG reagent set (Vector Laboratories), signals were examined with a Zeiss Axioplan microscope using conventional optics.

Preparation of Mouse Brain Lysates and Immunoblotting—DYRK1A TG mice that overexpressed the human *DYRK1A* gene, which was transcribed from the genomic *DYRK1A* DNA carried on a bacterial artificial chromosome, were produced and maintained as described previously (24). Experiments were performed in accordance with guidelines set forth by the Inje University Council Directive for the proper care and use of laboratory animals. Mouse brain lysates were prepared by homogenization in a tissue grinder (Fisher) in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 8.0, 0.5% deoxycholic acid) containing a protease inhibitor mixture (Calbiochem), 20 μ M sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was determined using the BCA method (Sigma). Proteins were separated by SDS-PAGE and analyzed by immunoblot with phospho-Tau and Tau antibodies. Densitometric quantification of Western blotting signal bands was carried out with a Science Lab 2001 Image Gauge (version 4.0, Fuji Photo Film Co., Ltd.).

In Vitro Tau Phosphorylation—Human recombinant Tau protein (1.1 μ g) was incubated for 90 min at 30 °C with the purified Dyrk1A (300 ng) in kinase buffer (20 mM MOPS, pH 7.0, 10 mM MgCl₂, 1 mM dithiothreitol, 20 μ M sodium orthovanadate, and 100 μ M ATP; 30- μ l reaction volume). The reaction mixtures were separated on SDS-PAGE and analyzed by immunoblot with Tau and phospho-Tau antibodies.

Microtubule Assembly—Tau protein (9.2 μ M) was phosphorylated by either no kinase, JNK2 (0.83 μ M), or Dyrk1A (0.90 μ M) for 2 h as described above. Phosphorylated Tau proteins were subjected to microtubule assembly assay as described previously (28). Briefly, nonphosphorylated or phosphorylated Tau (final concentration, 1.3 μ M) was mixed with tubulin (20 μ M) in polymerization buffer (80 mM Na-PIPES, pH 6.9, 10 mM MgCl₂, 1 mM EGTA and 1 mM GTP) at 37 °C. Microtubule assembly was initiated by addition of glycerol (final concentration, 5%) and was monitored by measuring the absorbance at 340 nm over 20 min.

Plasmids, siRNAs, and HEK293T Cell Transfection—A full-length wild-type *Dyrk1A* cDNA from a mouse brain and a full-length human *Tau* cDNA were cloned into pcDNA3.1 (Invitrogen). The Y321F kinase-inactive mutant *Dyrk1A* cDNA was generated by DpnI-mediated site-directed mutagenesis. For siRNA experiment, the mouse *Dyrk1A*-specific siRNA (5'-GGUAGAAAUCGACUCCUU) with the TT overhang was designed and synthesized by Samchully Pharm. Co. (South Korea). For siRNA delivery, Dyrk1A-specific duplex siRNA (60 pmol for 6-well plate) was co-transfected into human embryonic kidney (HEK293T) cells (8×10^5 cells/well) with Dyrk1A and Tau plasmids using Lipofectamine 2000 (Invitrogen). Fluorescein isothiocyanate-conjugated GFP-specific siRNA (5'-GUUCAGCGUGUCCGCGAGTT) was used as a nonsilenc-

Tau Hyperphosphorylation by DYRK1A

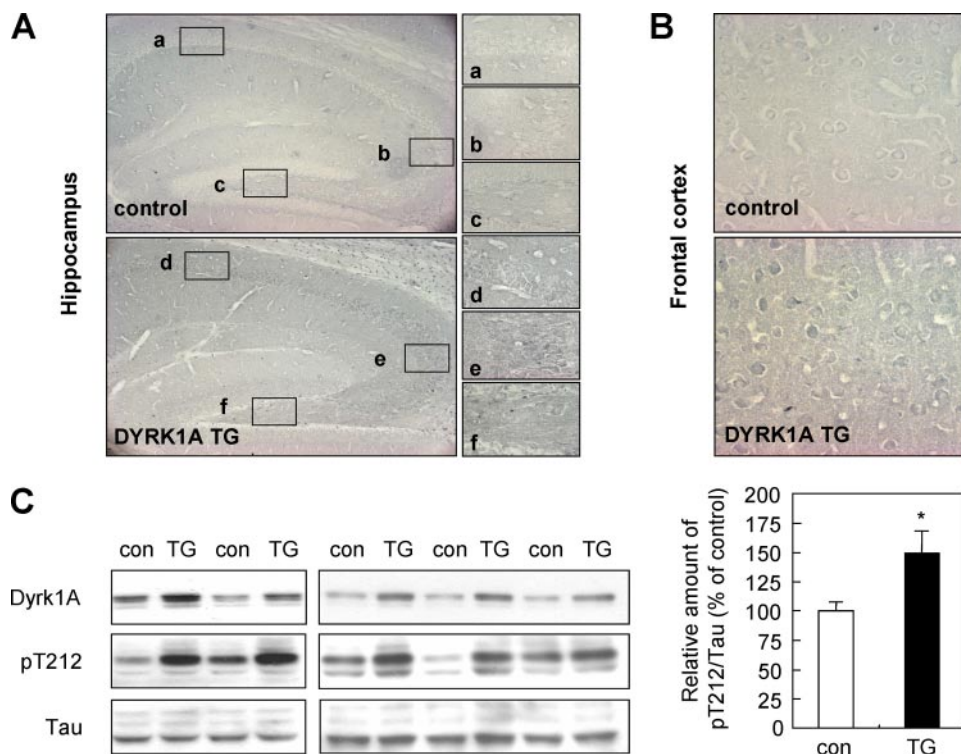


FIGURE 1. Increased phospho-Thr-212-Tau in the DYRK1A TG mouse brain. Immunohistochemistry of sagittal sections of hippocampus (*A*) and frontal cortex (*B*) from DYRK1A TG mice (8-week-old, *lower panel*) and control littermates (*upper panel*) performed with a phospho-Thr-212-Tau (pT212)-specific antibody. The figures shown are representative of results obtained with 3 pairs of DYRK1A TG and control littermate mice. *Panels a and d*, CA1; *panels b and e*, CA3; *panels c and f*, polymorphic layer of the dentate gyrus. *A*, $\times 50$; *panels a–f*, $\times 400$; *B*, $\times 400$. *C*, immunoblots of hippocampal lysates of 4–8-week old DYRK1A TG mice and control littermates carried out with Dyrk1A, pT212, and Tau antibodies (*left panel*). Densitometric analysis (*right panel*) of the immunoblots was normalized with respect to the Tau signal. The amount of pT212 in DYRK1A TG mice is plotted as the percent of that in wild-type littermate controls. Data are shown as means \pm S.E. *, $p < 0.05$ versus littermate control by Student's *t* test.

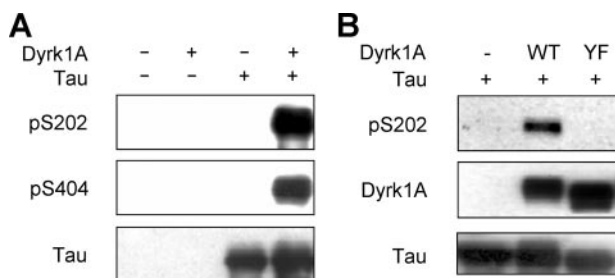


FIGURE 2. *In vitro* phosphorylation of Tau at Ser-202 and Ser-404 by Dyrk1A. *A*, human recombinant Tau protein was phosphorylated *in vitro* in the presence or absence of purified Dyrk1A. The reaction mixtures were subjected to SDS-PAGE, followed by immunoblot analyses with phospho-Ser-202-Tau (pS202), phospho-Ser-404-Tau (pS404), and Tau antibodies. *B*, Tau was phosphorylated by wild-type (WT) and Y321F kinase-inactive mutant (YF) Dyrk1A protein *in vitro* and was then subjected to immunoblot analysis for pS202, Tau, and Dyrk1A.

ing control. After 24 h of siRNA treatment, cell lysates were prepared for immunoblot analyses.

Immunocytochemistry—COS-7 cells on PDL-coated coverslip (6-well) were transfected with Dyrk1A and Tau plasmids. After 24 h, cells were fixed with 4% (w/v) paraformaldehyde in PBS, pH 7.4, permeabilized with 0.1% Triton X-100 in PBS, and blocked with PBS containing 1% normal goat serum for 2 h. Cells were incubated with the primary antibodies (Dyrk1A, 1:200; Tau, 1:500) at room temperature for 2 h, and then with

Alexa Fluor 488 goat anti-rabbit or Cy3 goat anti-mouse-coupled secondary antibodies (1:200) for 1 h. Technical support by examining the images using a confocal microscope (Carl Zeiss LSM 510) was provided by the Korea Basic Science Institute.

Co-immunoprecipitation—Brain lysate (1 mg) from the DYRK1A TG mice was incubated with control IgG (R & D Systems), Tau (BIO-SOURCE), or Dyrk1A antibody for overnight at 4 °C in RIPA buffer with protease inhibitors and 1 mM phenylmethylsulfonyl fluoride. The next day after a 1-h incubation with protein A beads (Pierce), the beads were gently washed with 1% Triton X-100 in RIPA buffer, and then the bound proteins were subjected to immunoblot analysis for the indicated antibodies.

RESULTS

Increased Phospho-Thr-212-Tau in DYRK1A TG Mice—To investigate the link between DYRK1A and Tau phosphorylation at the Thr-212 residue *in vivo*, transgenic mice that overexpress human DYRK1A (DYRK1A TG mice) were analyzed. These DYRK1A TG mice contained two copies of the mouse *Dyrk1A*

gene and one copy of the human *DYRK1A* genomic DNA, and the human DYRK1A protein expression was tightly controlled by the endogenous promoter. The overexpression of DYRK1A in these mice resulted in approximately a 70% increase in kinase activity.⁴ Immunohistochemical analyses revealed that phospho-Thr-212-Tau (pT212) expression was markedly increased in the hippocampus and frontal cortex of DYRK1A TG mice compared with control littermates (Fig. 1, *A* and *B*). The increase in pT212 was observed in the pyramidal cell layer of the hippocampus and the polymorphic layer of the dentate gyrus where DYRK1A overexpression was observed previously (24). The results were corroborated by Western analysis of brain lysates prepared from the hippocampus of DYRK1A TG mice and control littermates (Fig. 1*C*). After normalization with Tau, the amounts of pT212 in the DYRK1A TG mice were increased by $49 \pm 18\%$ ($n = 8$, $p < 0.05$), relative to control littermates. The fact that increased phosphorylation of Tau at Thr-212 was evident in both immunohistochemical and immunoblot experiments suggests that overexpression of DYRK1A is functionally linked to Tau phosphorylation *in vivo*.

⁴ Ryoo, S.-R., Cho, H.-J., Lee, H.-W., Jeong, H. K., Radnaabazar, C., Kim, Y.-S., Kim, M.-J., Son, M.-Y., Seo, H., Chung, S.-H., and Song, W.-J., *J. Neurochem.*, in press.

In Vitro Phosphorylation of Tau at Ser-202 and Ser-404 by Dyrk1A—Previously it was reported that Dyrk1A preferentially phosphorylates substrate proteins at serine and/or threonine residue(s) when proline is present at the +1 position, (S/T)P (20). An examination of the amino acid sequence of Tau reveals the presence of multiple serine/

threonine residues followed by proline. To further explore the role of Dyrk1A in Tau hyperphosphorylation, we examined whether this kinase phosphorylates Tau *in vitro* at other residues that are implicated in NFT formations in AD, such as Ser-202 and Ser-404 residues (29). The full-length mouse Dyrk1A proteins (wild-type (WT) and Y321F kinase-inactive mutant (YF)) were expressed in *E. coli* and were purified as ~90 kDa.⁴ The recombinant full-length human Tau was incubated in the presence or absence of Dyrk1A, and the reaction mixture was then subjected to immunoblotting analysis. Phosphorylation of Ser-202 and Ser-404 was detected with phospho-Ser-202-Tau (pS202)- and phospho-Ser-404-Tau (pS404)-specific antibodies, respectively. Only in the presence of both Tau and Dyrk1A were the pS202- and pS404-specific antibodies able to detect a protein band that corresponded to Tau on the immunoblotted membrane (Fig. 2A). This result indicates that Dyrk1A can phosphorylate the Ser-202 and Ser-404 residues of Tau directly *in vitro*. The phosphorylation of Tau by Dyrk1A did not result from any contaminating *E. coli* kinase activity during Dyrk1A purification, as the Tau protein was phosphorylated only by the WT Dyrk1A and not by the Y321F mutant (Fig. 2B). The Y321F mutant Dyrk1A showed slightly faster mobility on the SDS-PAGE, because of mutation of the Tyr-321 residue, the autophosphorylation site required for kinase activation (Fig. 2B and Fig. 4A). When the WT and Y321F proteins were treated with λ phosphatase for dephosphorylation, they showed the same mobility (data not shown).

Inhibition of Microtubule Assembly by Dyrk1A-mediated Tau Phosphorylation—It is known that hyperphosphorylated Tau inhibits microtubule assembly, whereas normal Tau stimulates assembly and stabilizes the microtubule. We examined the extent to which Tau phosphorylation by Dyrk1A affects the ability of Tau in promoting microtubule assembly. Phosphorylation of Tau by Dyrk1A strongly inhibited microtubule assembly (Fig. 3). After incubation for 20 min, assembly of the samples incubated with Tau phosphorylated by Dyrk1A was inhibited by $80 \pm 7\%$ ($n = 4, p < 0.01$) when compared with that of control (no kinase). The extent of Dyrk1A-mediated inhibition was comparable with that of JNK2, a known Tau kinase that phosphorylates several Tau residues (28). The strong inhibition of the microtubule assembly in the presence of Dyrk1A supports that Dyrk1A potentially phosphorylates multiple residues in Tau.

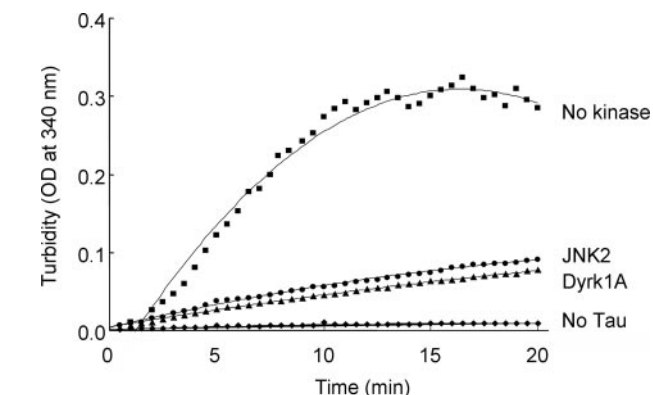


FIGURE 3. Inhibition of microtubule assembly by Dyrk1A-mediated phosphorylated Tau. Following the incubation of Tau with no kinase, JNK2 (0.83 μ M) or Dyrk1A (0.90 μ M) for 16 h at 30 °C, the reaction mixture was added to tubulin. Tubulin polymerization was initiated by addition of glycerol and was monitored by measuring turbidity at 340 nm for 20 min. There was no change in turbidity in the absence of Tau (No Tau).

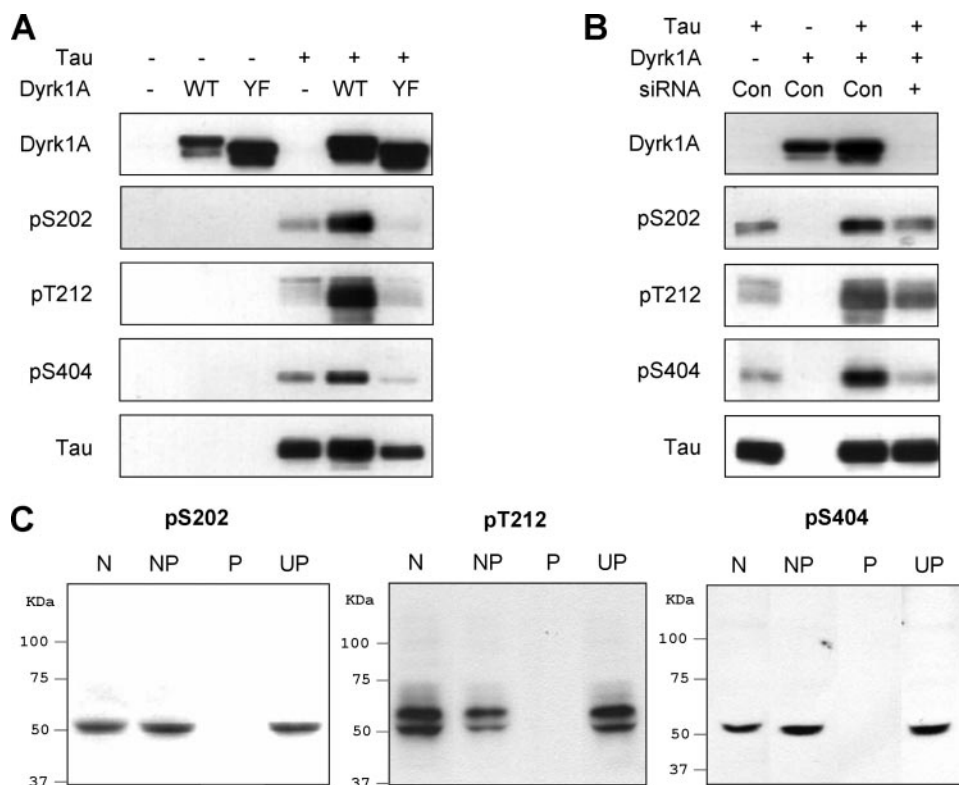


FIGURE 4. Tau phosphorylation by Dyrk1A in mammalian cells. A, HEK293T cells were transfected with indicated plasmid(s) or a control pcDNA3.1. After 24 h, cell lysates were subjected to SDS-PAGE, followed by immunoblot analyses for the indicated phospho-Tau, Tau, and Dyrk1A. B, HEK293T cells were co-transfected with human Tau, mouse Dyrk1A, and Dyrk1A-specific siRNAs. The level of Dyrk1A, phospho-Tau, and Tau were examined by immunoblot analyses. Fluorescein isothiocyanate-conjugated GFP-specific siRNA was used as a nonsilencing siRNA control (Con). C, peptide competition assay for phospho-Tau antibodies. Mouse hippocampal lysates were analyzed by Western blotting with indicated phospho-specific Tau antibodies preincubated in the absence (N) or presence of non-phosphopeptides (NP), phosphopeptides (P), or unrelated phosphopeptides (UP). The pS404 phosphopeptide was used as an unrelated phosphopeptide control for the pS202 antibody competition assay and vice versa. The pS202 phosphopeptide was used as an unrelated phosphopeptide control for the pT212 antibody.

Tau Hyperphosphorylation by DYRK1A

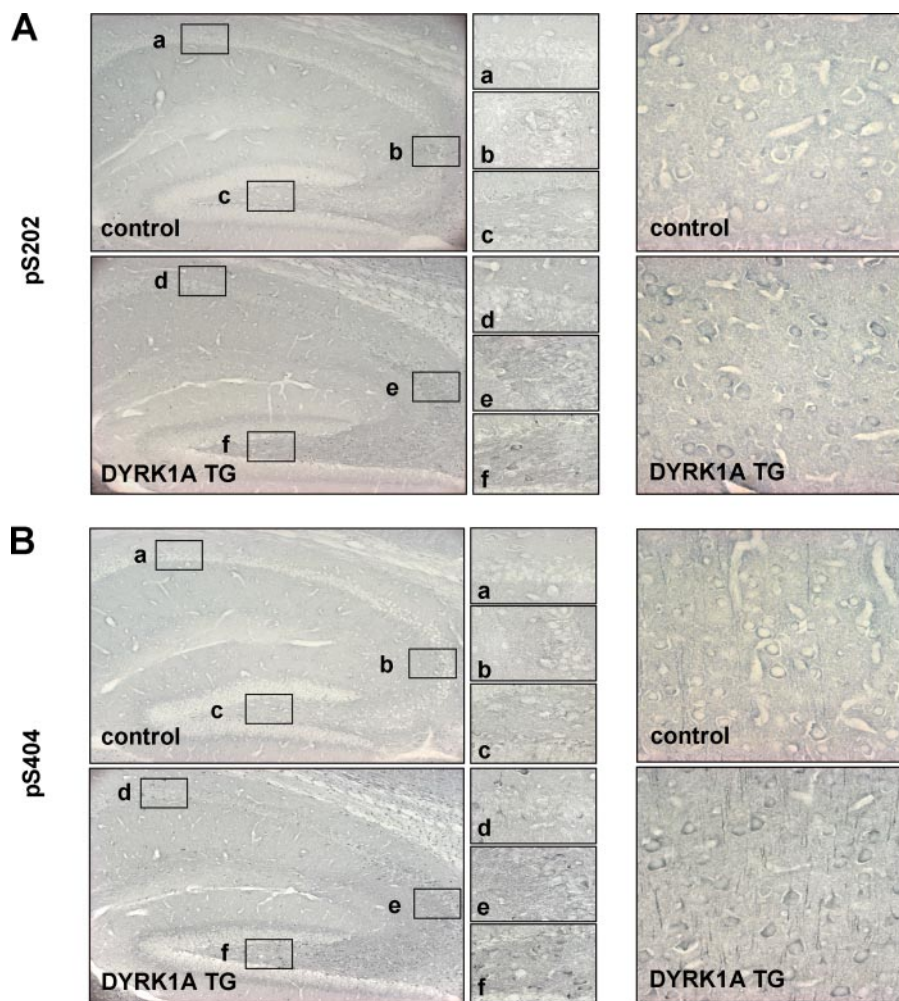


FIGURE 5. Immunohistochemical analysis of pS202 and pS404 levels in the DYRK1A TG mice. *A* and *B*, immunohistochemistry of sagittal sections of hippocampus (left panel) and frontal cortex (right panel) from DYRK1A TG (8-week-old, lower panels) and control littermates (upper panels) performed with pS202 (*A*) and pS404 (*B*) antibodies. Panels *a* and *d*, CA1; panels *b* and *e*, CA3; panels *c* and *f*, polymorphic layer of the dentate gyrus. *A*, $\times 50$; panels *a*–*f*, $\times 400$; *B*, $\times 400$. The figures shown are representative of results obtained with 3 pairs of DYRK1A TG and control littermate mice.

Tau Phosphorylation by the Dyrk1A in Mammalian Cells—To determine whether Dyrk1A phosphorylates full-length Tau on Ser-202, Ser-404, and Thr-212 *in vivo*, a Western analysis was performed with lysates of human embryonic kidney (HEK293T) cells that had been transiently transfected with a full-length Tau expression plasmid, either in the presence or absence of a plasmid encoding mouse Dyrk1A (WT or Y321F). Although the amounts of Tau were similar, the amounts of pS202, pT212, and pS404 were strongly increased in cells expressing Dyrk1A WT, but not Dyrk1A Y321F (Fig. 4A), indicating that full-length Tau is phosphorylated by Dyrk1A in cells. The Ser-202, Thr-212, and Ser-404 of Tau were weakly phosphorylated in the absence of exogenous mouse Dyrk1A, potentially on account of the presence of endogenous Dyrk1A and other Tau-phosphorylating kinases, including JNK (28). Furthermore, Y321F mutant, a dominant negative kinase-inactive mutant of Dyrk1A, may inhibit kinase activity of Dyrk1A by competing with endogenous Dyrk1A in HEK293T cells, resulting in further inhibition of Dyrk1A-mediated Tau phosphorylation, compared with that of Tau-transfected control (Fig. 4A).

To confirm the above finding, HEK293T cells were co-transfected with Dyrk1A siRNA along with plasmids encoding the full-length mouse Dyrk1A and human Tau. A nonsilencing fluorescein isothiocyanate-conjugated GFP-specific siRNA was used as a negative control. After 24 h of treatment, Dyrk1A siRNA strongly inhibited expression of Dyrk1A. Correspondingly, the levels of pS202, pT212, and pS404 were also reduced, whereas Dyrk1A siRNA did not affect Tau expression (Fig. 4B). This result supports that Dyrk1A phosphorylates Tau on Ser-202, Thr-212, and Ser-404 *in vivo*.

Specificity of each phospho-specific antibody was demonstrated by the peptide competition assay. Fig. 4C showed that the specific band signal in an immunoblot of mouse brain lysate was blocked only when the membrane was preincubated with the corresponding phosphopeptide, but not with the non-phosphopeptide or the unrelated phosphopeptide.

Increase in the Amounts of Phospho-Ser-202 and Phospho-Ser-404 in the Hippocampus and Frontal Cortex of DYRK1A TG Mice—To determine the physiological significance of Tau phosphorylation at the Ser-202 and Ser-404 residues, DYRK1A TG mice were analyzed with immunohistochemistry and immunoblotting. Immunohistochemical analyses revealed that pS202 and pS404 were increased in the hippocampus and frontal cortex of DYRK1A TG mice relative to control littermates (Fig. 5). Strong immunohistochemical signals were observed in the neuronal cell bodies of the hippocampus or apical dendrites of the frontal cortex in DYRK1A TG mice relative to those of control mice. Increases in pS202, pT212, and pS404 were also observed in scattered neurons in the hippocampus and axonal tract in the corpus callosum of DYRK1A TG mice (Fig. 1A and Fig. 5).

The results were corroborated by Western analysis of brain lysates prepared from the hippocampus or frontal cortex of DYRK1A TG mice and control littermates. Immunoblot analyses showed that after normalization with Tau, the amounts of pS202 and pS404 in the hippocampal lysates of DYRK1A TG mice were increased by $26 \pm 9\%$ ($n = 11, p < 0.01$) and $29 \pm 7\%$ ($n = 9, p < 0.05$) relative to that of control littermates, respectively (Fig. 6A). In addition, after normalization with Tau, the amounts of pS202 and pS404 in the frontal cortex of DYRK1A TG mice were also increased by $32 \pm 4\%$ ($n = 3, p < 0.05$) and

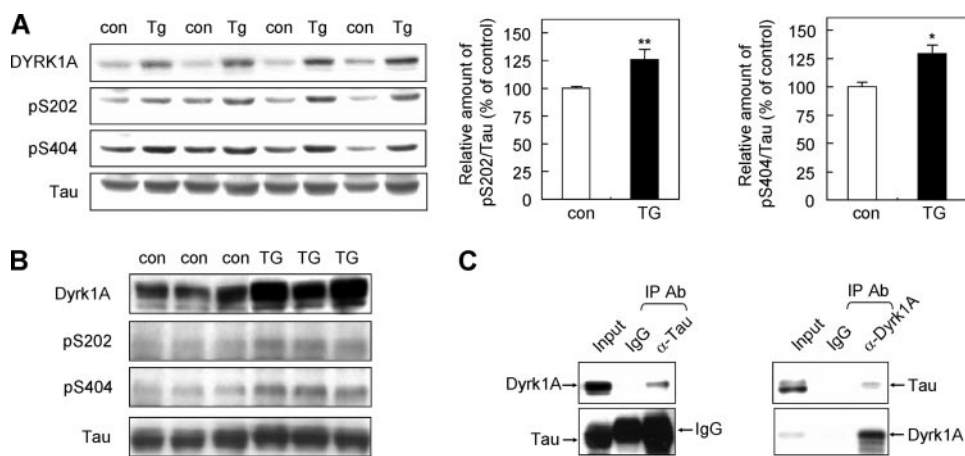


FIGURE 6. Immunoblotting analysis of pS202 and pS404 levels in the DYRK1A TG mice. *A*, immunoblots (left panel) of hippocampal lysates from 4- to 24-week-old DYRK1A TG mice (TG) and control littermates (con) carried out with Dyrk1A, pS202, pS404, and Tau antibodies. Densitometric analysis (right panel) of the immunoblots was normalized with respect to the Tau signal. The amount of pS202 and pS404 in the DYRK1A TG mice is plotted as the percent of that in wild-type littermate controls. Data are shown as means \pm S.E. *, $p < 0.05$; **, $p < 0.01$ versus littermate control by Student's *t* test. *B*, immunoblots of frontal cortex lysates from DYRK1A TG mice (TG) and control littermates (con) carried out with Dyrk1A, pS202, pS404, and Tau antibodies. *C*, co-immunoprecipitation (IP). Brain lysate (1 mg) from DYRK1A TG mice was immunoprecipitated with control IgG, anti-Tau, or anti-Dyrk1A antibody (Ab) and then was subject to immunoblot analysis for the indicated antibodies. The position of mouse immunoglobulin is marked IgG. Input, 10 μ g of brain lysate.

$74 \pm 6\%$ ($n = 3$, $p < 0.01$) relative to that of control littermates, respectively (Fig. 6B). Taken together, these results demonstrate that phosphorylation of Tau at Ser-202 and Ser-404 is enhanced when the amount of DYRK1A is increased suggesting that overexpression of DYRK1A is functionally linked to Tau hyperphosphorylation *in vivo*.

Interaction of Dyrk1A with Tau—To determine whether Tau is phosphorylated through the physical interaction with Dyrk1A, the brain lysates of DYRK1A TG mice were immunoprecipitated with anti-Tau-antibody. As shown in Fig. 6C, immunoprecipitation of Tau led to co-immunoprecipitation of Dyrk1A, indicating that Dyrk1A interacts with Tau in DYRK1A TG brains. Furthermore, reverse co-immunoprecipitation experiments with anti-Dyrk1A antibody also revealed an interaction between Dyrk1A and Tau as shown in Fig. 6C.

DISCUSSION

We show here that Tau residues at Ser-202, Thr-212, and Ser-404 are phosphorylated by Dyrk1A *in vitro* and *in vivo* and that their phospho-Tau amounts are increased in the brains of DYRK1A TG mice. This study provides the first *in vivo* evidence of a physiological role for DYRK1A in Tau hyperphosphorylation, a modification that is known to be necessary for the formation of NFTs in AD. Our transgenic mice show about 50% overexpression of Dyrk1A at the protein level (24) and $\sim 70\%$ increase in Dyrk1A kinase activity,⁴ compared with those of littermate controls. Despite a slight increase in the Dyrk1A kinase activity ($\sim 20\%$), these TG mice provide a good clinical model of Down syndrome for studying the roles of Dyrk1A *in vivo*. Expression of the DYRK1A protein in the frontal cortex of DS brains was increased by 45% when compared with age-matched normal controls.⁴ Therefore, overexpression of DYRK1A in DS brains, resulting from triplication of

the *DYRK1A* gene on human chromosome 21, may cause early onset of AD pathology through the hyperphosphorylation of Tau protein.

A recent study showed that DYRK1A immunoreactivity is also increased in the frontal cortex, entorhinal cortex, and hippocampus of AD patients (30), suggesting a role for DYRK1A in AD pathogenesis. Strong immunohistochemical signals of phospho-Tau in the neuronal cell bodies of the hippocampus and apical dendrites of the frontal cortex in DYRK1A TG mice are consistent with those from previous studies that showed that, in AD and other neurodegenerative diseases, hyperphosphorylated Tau is directed from the axons to the neural cell bodies and dendrites (31). The presence of NFTs in 12-month-old DYRK1A

TG mice brain was not detected using the silver method of Campbell (43) and Gallyas (44), possibly either because the mice were not old enough for development of NFTs or because DYRK1A-mediated Tau hyperphosphorylation alone is not sufficient for the formation of NFTs (data not shown). In future studies, it will be interesting to determine whether the elevations in Tau phosphorylation observed in DYRK1A TG mice enhance the formation of NFTs, either in aged DYRK1A mice or when they are crossed with mouse models that produce tangles.

Phosphorylation of proline-rich regions of Tau induces conformational changes (32), which may cause global structural and functional transformations of Tau from its less-phosphorylated, microtubule-bound form to its hyperphosphorylated, aggregated form. Consistent with this, Tau phosphorylation by Dyrk1A strongly inhibited the ability of Tau to promote microtubule assembly (Fig. 3). In the Tau hyperphosphorylation cascade, which culminates in NFT formation, the AT8 epitopes (pSer-202 and pThr-205) were visible prior to the generation of NFTs (31). It has been reported that phosphorylation at Ser-202 enhances Tau polymerization and that phosphorylation at both Ser-202 and Thr-205 not only induces polymerization but also facilitates filament formation (33). An analysis of pseudo-phosphorylation at the Thr-212 residue of Tau indicates that this phosphorylation event may act primarily to stabilize filaments by slowing the rates of dissociation (34). The PHF-1 antibody, which recognizes Tau phosphorylation at Ser-396/Ser-404, specifically stains AD brain sections (35), and phosphorylation at Ser-396/Ser-404 has been reported to enhance Tau polymerization and to attenuate the ability of Tau to stabilize microtubules (36). Therefore, the present findings, in which Tau is phosphorylated by Dyrk1A at Ser-202, Thr-212, and Ser-404, suggest important roles for DYRK1A in the loss of microtubule assembly and in the formation of NFTs.

Tau Hyperphosphorylation by DYRK1A

It has been reported that the reduction of the ability of Tau to promote microtubule assembly is proportional to the level of Tau phosphorylation (28). Phosphorylation by Dyrk1A strongly reduced the ability of Tau to promote microtubule assembly (Fig. 3). The effect was comparable with that of JNK2, which potentially phosphorylates ~10 Tau residues (27). Therefore, it is likely that in addition to the three phosphorylated residues, pS202, pT212, and pS404, examined here, additional Tau residues could be phosphorylated by Dyrk1A. Given that all potential phosphorylation sites of Tau in DYRK1A TG mice were not extensively examined, the link, if any, between DYRK1A and other Tau phosphorylation sites remains to be elucidated. In contrast to this study, a recent study concluded that DYRK1A phosphorylated only the Thr-212 residue in Tau (28). This discordant finding between that investigation and the present study is likely because of the preparation of Dyrk1A. Glutathione S-transferase-tagged truncated Dyrk1A (1–500 amino acids) was used to phosphorylate Tau in a previous study (28), whereas the full-length Dyrk1A (1–763 amino acids) was used in this study. Yoshida and Goedert (37) reported that the phosphorylated Tau by Dyrk1A inhibited microtubule assembly. However, they also used glutathione S-transferase-tagged truncated Dyrk1A (1–500 amino acids), and the inhibition was minimal (less than 15%), whereas we used the full-length Dyrk1A (1–763 amino acids) and the inhibition was much stronger ($80 \pm 7\%$, $p < 0.01$). It is possible that either a glutathione S-transferase tag at the N terminus potentially inhibits the interaction of Dyrk1A with Tau or that a C-terminal domain of Dyrk1A is necessary for efficient interaction with and for the phosphorylation of Tau.

A previous study showed that Tau is phosphorylated by Dyrk1A *in vitro* at the Thr-212 residue and that this phosphorylation primes Tau for phosphorylation by GSK3 (27). However, increased Tau phosphorylation at Thr-212 was not detected in Dyrk1A cDNA-containing transgenic mice in an immunohistochemical analysis (30). The discrepancy between the data obtained with Dyrk1A cDNA TG mice (30) and the DYRK1A TG mice used in this study (24) may have resulted from the difference in the promoters used for the production of the transgenic mice. The exogenous inducible sheep metallothionein-1a promoter was used to drive mouse Dyrk1A expression in cDNA transgenic mice (25), whereas the endogenous human promoter was used to drive expression of the human *DYRK1A* gene in the DYRK1A TG mice (24). It is also possible that the seven amino acid differences that exist between the human and mouse Dyrk1A proteins contribute to this conflicting result.

The following mechanism is proposed for the regulation of early onset of AD in DS brains. DYRK1A immunoreactivity is increased in the frontal cortex, entorhinal cortex, and hippocampus of AD patients (30), suggesting a role for DYRK1A in AD pathogenesis. It was recently found that Dyrk1A also phosphorylated APP at Thr-668 *in vitro* and *in vivo*, and it caused an increase in the amounts of β -amyloid.⁴ The increased amounts of DYRK1A in DS brains give rise to the hyperphosphorylation of Tau (this study) and elevated amounts of A β via an enhanced phosphorylation of APP.⁴ These processes possibly contribute to the early onset of AD pathogenesis in DS brains. Emerging evidence suggests that the plaques and NFTs are indications of disease but are

not the cause of AD, thereby therapeutic approaches aimed at reducing the levels of A β and abnormal Tau phosphorylation may be effective (38–42). From the results herein, we propose that DYRK1A can serve as a potential therapeutic target for the treatment of AD in both DS and AD patients.

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