Molecules and Cells

cAMP Response Element-Binding Protein- and Phosphorylation-Dependent Regulation of Tyrosine Hydroxylase by PAK4: Implications for Dopamine Replacement Therapy

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https://doi.org/10.14348/molcells.2021.2250
www.molcells.org

Parkinson's disease (PD) is characterized by a progressive loss of dopamine-producing neurons in the midbrain, which results in decreased dopamine levels accompanied by movement symptoms. Oral administration of l-3,4-dihydroxyphenylalanine (L-dopa), the precursor of dopamine, provides initial symptomatic relief, but abnormal involuntary movements develop later. A deeper understanding of the regulatory mechanisms underlying dopamine homeostasis is thus critically needed for the development of a successful treatment. Here, we show that p21-activated kinase 4 (PAK4) controls dopamine levels. Constitutively active PAK4 (caPAK4) stimulated transcription of tyrosine hydroxylase (TH) via the cAMP response element-binding protein (CREB) transcription factor. Moreover, caPAK4 increased the catalytic activity of TH through its phosphorylation of S^{40}, which is essential for TH activation. Consistent with this result, in human midbrain tissues, we observed a strong correlation between phosphorylated $PAK4^{S474}$, which represents PAK4 activity, and phosphorylated TH^{S40}, which reflects their enzymatic activity. Our findings suggest that targeting the PAK4 signaling pathways to restore dopamine levels may provide a new therapeutic approach in PD.

Keywords: dopamine, PAK4, Parkinson's disease, post-treatment, tyrosine hydroxylase

INTRODUCTION

Parkinson's disease (PD) is an age-dependent disorder characterized by progressive degeneration of dopamine (DA) neurons in the substantia nigra (SN) pars compacta, leading to a decrease in DA levels in the striatum (Dauer and Przedborski, 2003). Most patients appear to experience motor symptoms when dopamine levels drop by 60% to 80% in the brain (Marsden, 1990). The resultant major symptoms include tremor, akinesia, postural instability and bradykinesia (Marsden, 1990). Currently, initial treatment starts with oral administration of l-3,4-dihydroxyphenylalanine (L-dopa), which alleviates motor symptoms, but L-dopa-induced dyskinesia subsequently develops (Mones et al., 1970). Therefore, there is an urgent need for a better method of DA replacement in

Received 20 December, 2020; revised 29 March, 2021; accepted 13 May, 2021; published online 7 July, 2021

eISSN: 0219-1032

patients with PD.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of DA synthesis (Tekin et al., 2014; Zhu et al., 2012); thus, regulation of TH protein levels and activity represent a central means for controlling DA synthesis. The TH gene contains a cAMP response element (CRE) and an activating protein-1 (AP-1)-binding site in its promoter (Ghee et al., 1998). CRE is involved in the basal and inducible transcription of the TH gene. TH activity is modulated by multiple protein kinases that phosphorylate TH at S^{19}, S^{31} and S^{40}; of these, S^{40} represents a key residue for TH activation (Haycock and Haycock, 1991; Ramsey and Fitzpatrick, 1998). A reduction in TH activity results in diminished DA synthesis and contributes to motor impairment in PD; thus, dysregulation of TH activity is an essential component of the pathogenesis of PD. Therefore, a therapeutic strategy aimed at improving TH expression and activity in PD is of widespread interest.

p21-activated kinase 4 (PAK4) stimulates cAMP response element-binding protein (CREB) transcriptional activity (Won et al., 2016), which promotes transcription of the TH gene; thus, PAK4 may regulate TH expression via CREB. Moreover, S^{40} of TH is compatible with the consensus motif for PAK4-mediated phosphorylation (Rennefahrt et al., 2007). We therefore sought to investigate the role of PAK4 as an upstream regulator of TH and examined its implications in PD.

MATERIALS AND METHODS

Immunohistochemistry of human brain tissue

Human tissue was obtained from the Victoria Brain Bank Network (Australia) (Supplementary Table S1). Experiments were performed in accordance with a protocol approved by the Ethics Review Committee of the Institutional Review Board of Chungbuk National University (approval No. CBNU-IRB-2011-T01). Paraffin-embedded human brain tissue slices were deparaffinized in xylene and subjected to citrate antigen retrieval prior to immunohistochemical analysis. For light microscopy, brain tissues were incubated with a biotin-conjugated secondary antibody followed by streptavidin-conjugated HRP (VECTASTAIN ABC Kits; Vector Laboratories, USA). Immunostaining was visualized by incubating samples in a 0.1 M-PB solution containing 0.05% diaminobenzidine-HCl (DAB) and 0.003% hydrogen peroxide. To coimmunostain pPAK4 with pTH^{S40}, brain tissues were incubated with alkaline phosphatase-conjugated secondary antibodies (VECTASTAIN ABC Kits). Immunostaining was visualized using either Alkaline Phosphatase Substrate Kit I (red) or Alkaline Phosphatase Substrate Kit III (blue) (VECTASTAIN ABC Kits).

Cell cultures

Primary rat midbrain neuron cultures were produced as previously described (Nam et al., 2015). PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics in a humidified 5% CO_2 incubator at 37°C.

Plasmid construction

The generation of wild type PAK4 (WT-PAK4) and caPAK4 ($PAK4^{S445N/S474E}$) tagged with N-terminal 3×-Myc was previously described (Park et al., 2013). TH-alanine (A) mutants for serine (S) 19, 31 and 40 were produced by site-specific mutagenesis using a Quick Change Site-Directed Mutagenesis Kit (Stratagene California, USA).

siRNAs

siRNAs for rat CREB #1, UCAAGGAGGCCUUCCUACA; #2, UCUAGUGCCCAGCAACCAA and scrambled siRNA were purchased from Bioneer (Korea). CREB siRNAs (100 nM) were transfected using Lipofectamine LTX (Invitrogen, USA) for 48 h.

Transfection

For transient transfection, a mixture of DNA or siRNA and Lipofectamine 2000 reagent (Invitrogen) was prepared according to the manufacturer's instructions and incubated with cells for the indicated times.

CRE-reporter assay

To assess the effects of PAK4, PC12 cells were cotransfected for 24 h with WT-PAK4 or caPAK4 plus the TH-luciferase reporter (30 ng), kindly provided by professor. Dr. Myung Ae Lee (Ajou University Medical Center, Suwon, Korea) (Kim et al., 2003) and Renilla luciferase (20 ng). The Renilla luciferase vector was used as an internal control. The reporter assay was performed in 24-well plates in triplicate. Luciferase activity was measured using a Dual-Luciferase Reporter assay system (Promega, USA) according to the manufacturer's protocol.

Immunoprecipitation

Cells were harvested in lysis buffer supplemented with protease inhibitors. Lysates were rotated at 4°C for 1 h and then centrifuged at 14,000 rpm for 20 min. The supernatants were then immunoprecipitated using an anti-PAK4 antibody at 4°C for 18 h. Immunoprecipitants were collected by adding protein-G agarose and washed five times with lysis buffer. Immunoblotting analysis was performed using an anti-TH antibody.

In vitro kinase assay

Expressed His-TH mutants were purified using a Ni-NTA column. His-TH was incubated with or without GST-PAK4 (Active Motif, USA) in kinase assay buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 1 mM dithiothreitol) containing 100 mM ATP and 5 cCi [γ-32P] ATP (PerkinElmer, USA) for 30 min at 30°C. To discriminate between autophosphorylated PAK4 and phosphorylated TH, GST-PAK4 (active-PAK4) alone was included in the reaction. Following electrophoretic separation of the proteins, phosphorylation of the target proteins was evaluated using autoradiography and immunoblotting.

Dopamine analysis

PC12 cells were seeded in 6-well culture plates at a density of 1×10^5 cells per well. Cells were transfected with CREB siRNA (100 nM), WT-PAK4 (0.5 μg) or caPAK4 (0.5 μg) and incubated for 48 h. Culture media were collected and centrifuged at 10,000*g* for 20 s at 4°C. The supernatants were used to measure the amount of extracellular dopamine. Cell pellets were used for measurement of intracellular dopamine. Dopa-

mine was measured using a dopamine ELISA Kit (BioVision, USA).

Immunoblotting and quantification

Protein extracts were prepared as previously described. The immunoblots were visualized using HRP-conjugated secondary antibodies against IgG and a chemiluminescent substrate (Thermo Fisher Scientific, Norway). For quantification of protein levels, the density of each band on the immunoblots was quantified using the ImageJ software (National Institutes of Health, USA). Background values were subtracted from all images.

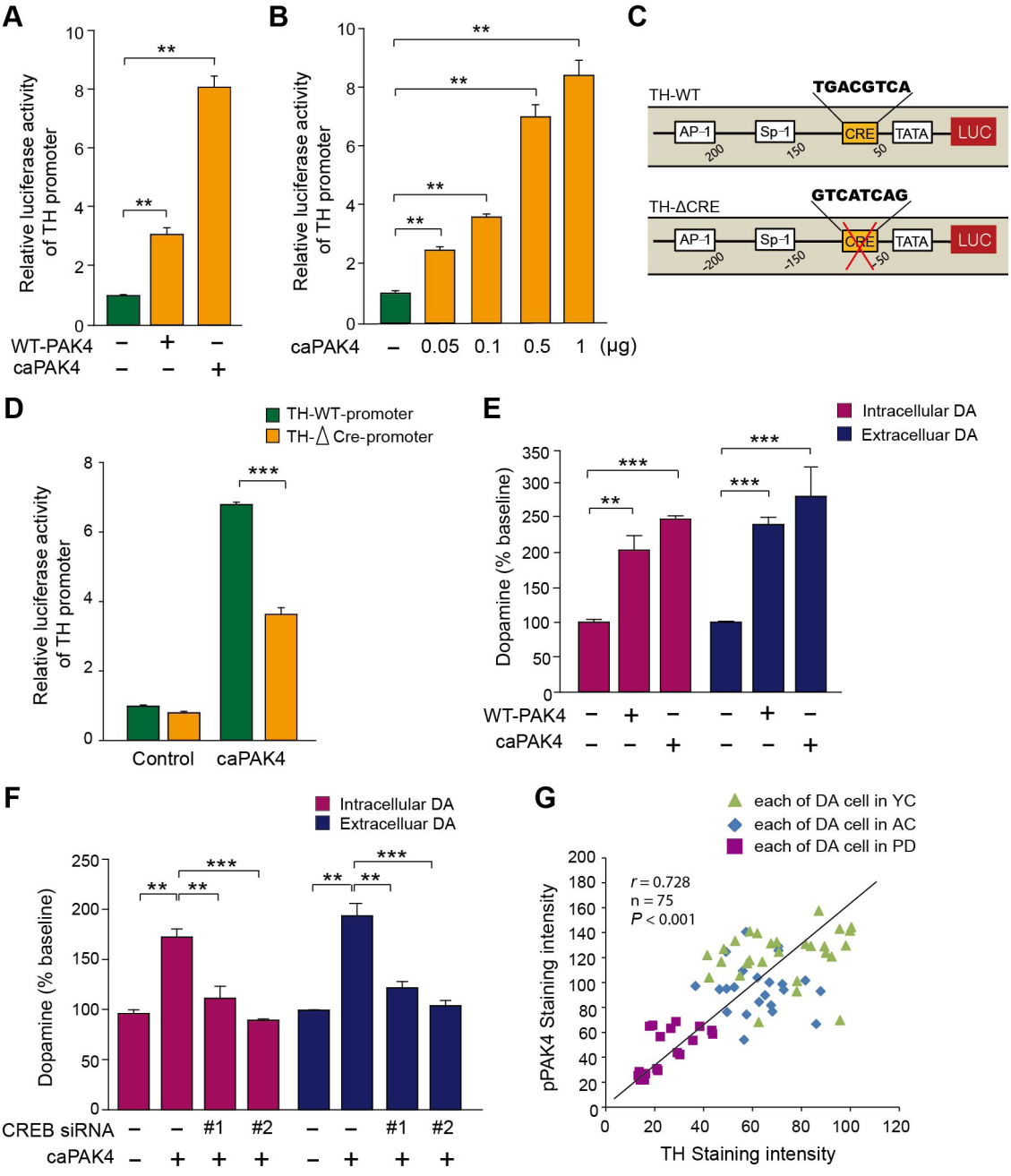

Fig. 1. caPAK4 stimulates TH promoter activity via CREB. (A and B) TH promotor activity assay in wild type PC12 cells (n = 3), WT-PAK4 (0.5 μg), and caPAK4 (0.5 μg). (C) Illustration of the structure of the TH promoter linked to a firefly luciferase reporter gene harboring no mutations (TH-WT) or a CRE site deletion (TH-△CRE). (D) TH promotor activity assay in PC12 cells (n = 3). (E and F) Quantification of DA levels in wild type PC12 cells (n = 3), WT-PAK4 (0.5 μg), and caPAK4 (0.5 μg). Data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Student-Newman-Keuls analysis (A, B, E, and F) or Student's *t*-test (D). (G) Spearman correlation analysis of the correlation between pPAK4 and TH levels in neuromelanin-positive DA neurons of the SN from humans. YC (n = 4), AC (n = 7), PD patients (n = 7), neuromelanin-positive cells, n = 25 each ($r = 0.728$, $P < 0.001$).

Statistical and image analysis

ImageJ was used to quantify the intensity of immunofluorescence signals and band densities from immunoblots. All data were analyzed using GraphPad Prism 6 software (GraphPad Software, USA). Data are presented as the mean ± SEM from at least three biologically independent experiments. Representative images were taken from at least three biologically independent experiments with similar results. Student's *t*-tests or ANOVA with Student–Newman–Keuls analysis tests were used to assess statistical significance.

RESULTS

caPAK4 increases the promoter activity of the TH gene via the CREB transcription factor

PAK4 stimulates transcriptional activity of CREB via CREB regulated transcription coactivator 1 (CRTC1) (Won et al., 2016), and the TH promoter contains a CRE site for CREB (Piech-Dumas and Tank, 1999). Thus, PAK4 might upregulate TH levels via CREB. To test this idea, we examined the effect of PAK4 on the activity of the TH promoter. Constitutively active PAK4 (caPAK4) elevated the activity of the TH promoter ~8-fold compared to controls in PC12 cells, as measured by luciferase reporter activity (Fig. 1A). Wild type PAK4 (WT-PAK4) also increased activity ~3-fold but to a lesser extent than caPAK4 (Fig. 1A). caPAK4 increased TH promoter activity in a dose-dependent manner (Fig. 1B). CREB binds to the CRE on the TH promoter and regulates its transcription in rat midbrain DA cells (Ghee et al., 1998). To examine the involvement of CREB in the effect of caPAK4 on TH transcription, we used a TH promoter in which the CRE site was deleted (TH-△CRE), as illustrated in Fig. 1C.

When the TH promoter containing this CRE mutant was expressed, the reporter activity for this promoter was decreased to approximately half of that for the WT TH promoter (Fig. 1D). Collectively, these results indicate that PAK4 upregulates transcription of TH via CREB. Next, we examined whether PAK4 increases DA production because TH is the rate-limiting enzyme in DA synthesis. Expression of WT-PAK4 or caPAK4 increased levels of both intracellular and extracellular secreted DA (Fig. 1E). When CREB was knocked down by two different siRNAs, caPAK4-induced DA synthesis was reduced to a basal level (Fig. 1F). Finally, to determine whether pPAK4 and TH are correlated at the cellular level, we performed costaining for pPAK4, an index of PAK4 activity, and TH in postmortem brain samples from patients with PD. PD patients displayed low intensities of pPAK4 and TH, whereas young controls (YC) and age-matched controls (AC) exhibited higher pPAK4 and TH levels (Fig. 1G). Overall, levels of pPAK4 and TH were positively correlated (r = 0.728) (Fig. 1G), suggesting that PAK4 also regulates TH transcription *in vivo*.

PAK4 upregulates dopamine synthesis through TH phosphorylation at Ser^{40}

TH activity is regulated by phosphorylation on serine residues S^{19}, S^{31}, and S^{40} (Haycock and Haycock, 1991; Zhu et al.,

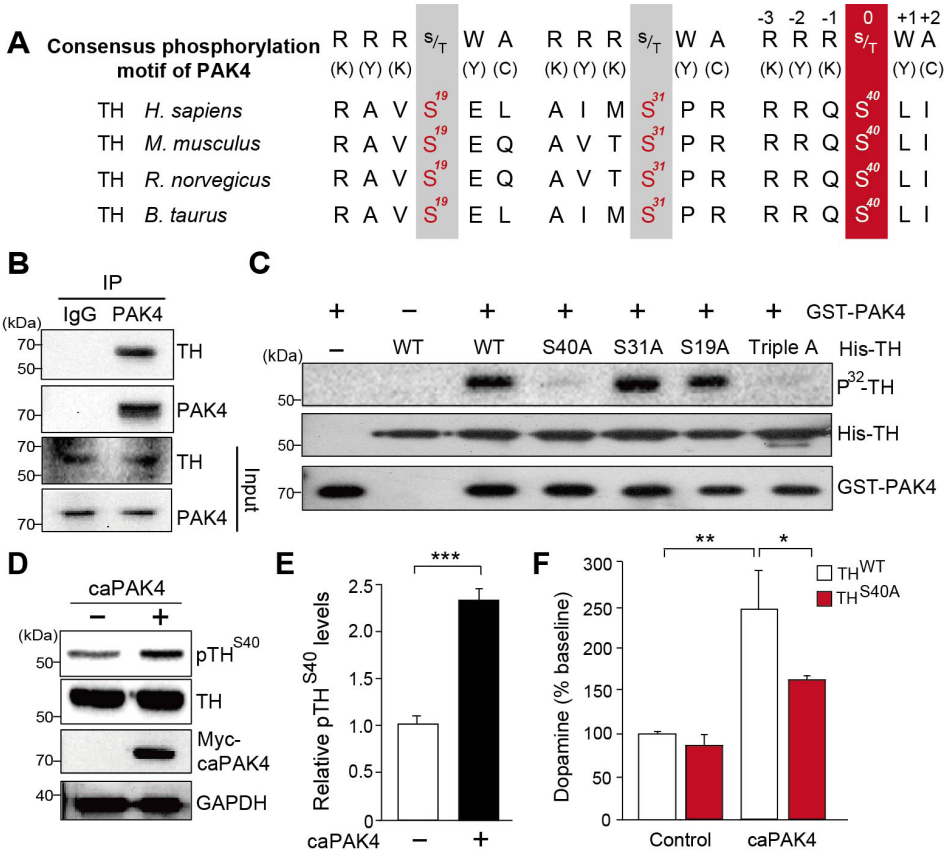

Fig. 2. PAK4 phosphorylates at S^{40} and upregulates dopamine synthesis. (A) Alignment of TH amino acids surrounding potential PAK4 phosphorylation residues S^{19}, S^{31} and S^{40}. (B) Immunoprecipitation (IP) of rat SN lysates. (C) *In vitro* PAK4 kinase assay. (D) Immunoblotting for pTH^{S40} in lysates from primary mesencephalic cultures (n = 3) and caPAK4 (1 μg). (E) Quantification of the blot in (D); pTH^{S40} levels. (F) Quantification of DA levels in PC12 cells (n = 3) and caPAK4 cells (1 μg). Data are presented as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, ANOVA with Student-Newman-Keuls analysis (F) or Student's *t*-test (E).

2012). A search for a PAK4-mediated phosphorylation motif in TH revealed S^{40} and its flanking sequences in human TH as a potential candidate (Fig. 2A) (Rennefahrt et al., 2007). To determine whether PAK4 directly interacts with TH, we first performed a coimmunoprecipitation assay using rat SN lysates. As shown in Fig. 2B, PAK4 physically interacts with TH, suggesting that TH is a substrate of PAK4. To determine whether PAK4 does indeed phosphorylates TH on S^{40}, we generated nonphosphorylatable mutant forms of TH in which each of the previously known phosphorylated residues (Haycock and Haycock, 1991), S^{19}, S^{31}, or S^{40}, was replaced by alanine. An *in vitro* PAK4 kinase assay using recombinant proteins revealed a loss of phosphorylation in TH^{S40A} but not TH^{S19A} or TH^{S31A} mutants (Fig. 2C), identifying S^{40} as a target residue of PAK4. Consistent with this result, forced expression of caPAK4 in primary cultured mesencephalic neurons elevated the levels of phosphorylated TH^{S40} (pTH^{S40}) (Fig. 2D; quantified in Fig. 2E). Next, we examined the functional significance of this phosphorylation in the regulation of DA levels. To this end, we coexpressed caPAK4 with either TH^{WT} or TH^{S40A} and analyzed DA levels in PC12 cells. Coexpression of caPAK4 with TH^{WT} elevated DA levels ~2.4-fold compared to controls (Fig. 2F). However, expression of TH^{S40A} significantly blocked the caPAK4-induced increase in DA levels in PC12 cells (Fig. 2F). These results support the notion that PAK4 stimulates DA synthesis, at least in part, through phosphorylation of TH at S^{40}.

Positive correlation of pPAK4 and pTH^{S40} in human PD brain tissues

To examine the clinical relevance of the pPAK4/pTH^{S40} axis, we performed immunostaining for pTH^{S40} in SN tissues from the human brain. Most DA cells showed strong signals for pTH^{S40} in the age-matched control tissues (Fig. 3A; quantified in Fig. 3B). However, the signals were heterogeneous, though overall relatively weak, in the remaining DA neurons in the PD brain (Fig. 3A; quantified in Fig. 3B). This heterogeneity in pTH^{S40} levels may reflect alterations in pPAK4 levels. We therefore examined phosphorylation of TH^{S40} in relation to pPAK4 levels by double immunostaining. As shown in Fig. 3C, neuromelanin-positive DA neurons with strong (inset 1) or weak (inset 2) pPAK4 reactivity (red arrowheads) displayed

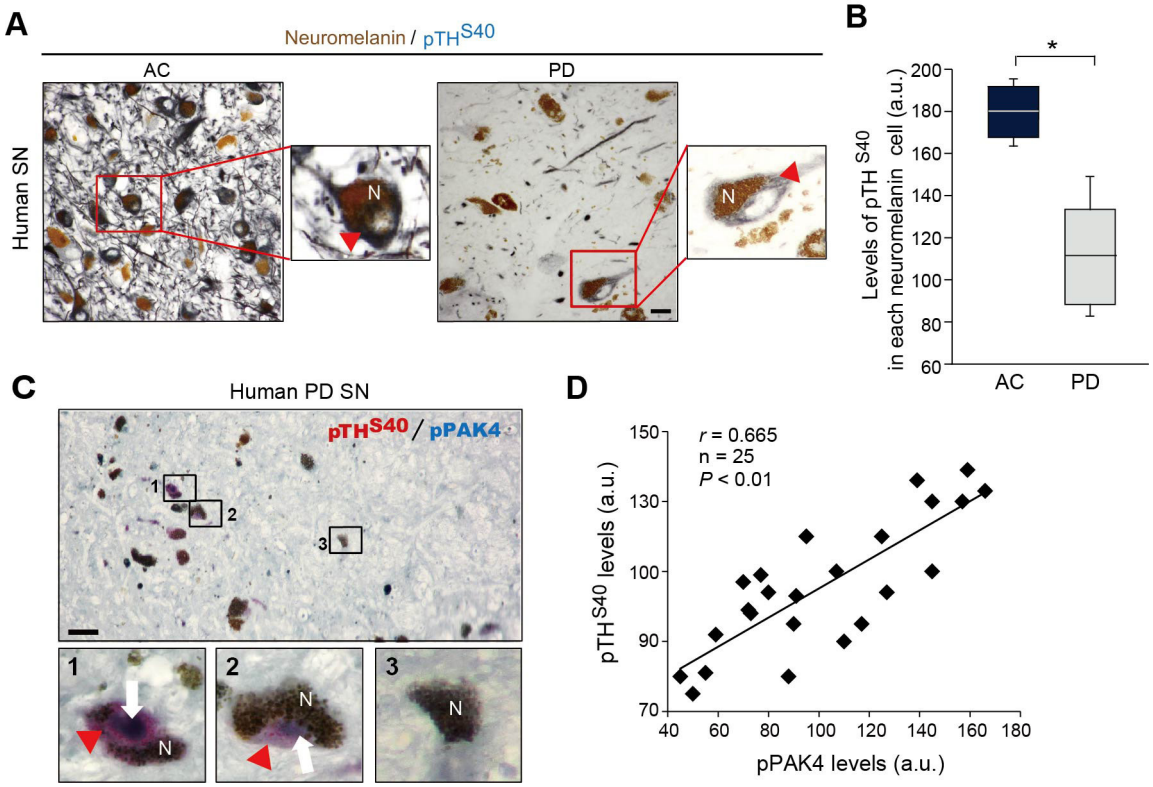

Fig. 3. Expression of pTH^{S40} and pPAK4 in the SN of the human Parkinson's disease brain. (A) Representative images of pTH^{S40} staining in neuromelanin-positive DA neurons of the SN from AC and patients with PD. Boxed areas are shown at a higher magnification in the right panel. pTH^{S40}, red arrowhead; N, neuromelanin. Scale bar = 25 μm. (B) Quantification of the signal intensity of pTH^{S40} in each neuromelanin-positive DA neuron. AC (n = 7), neuromelanin-positive cells, n = 56; PD patients (n = 7), neuromelanin-positive cells, n = 30. Data are presented as the mean ± SEM. $*P < 0.05$, Student's *t*-test. (C) Representative images of costaining for pTH^{S40} (red; red arrowheads) and pPAK4 (blue; white arrows) in neuromelanin-positive DA neurons of the SN from patients with PD. Boxed areas 1, 2, and 3 (C) are shown at a higher magnification in the bottom panel. Scale bar = 50 μm. (D) Spearman correlation analysis for the correlation between pPAK4 and pTH^{S40} levels in neuromelanin-positive DA neurons from the SN from patients with PD. PD patients (n = 7), neuromelanin-positive cells, n = 25 ($r = 0.665$, $P < 0.01$).

corresponding signals for pTH^{S40} (white arrows), although no signals for either pPAK4 or pTH^{S40} were detected in some cells (Fig. 3C; inset 3). The levels of pPAK4 and pTH^{S40} showed a positive correlation (r = 0.665) (Fig. 3D), suggesting that decreased PAK4 activity in PD may partially explain the low TH activity and the resultant low DA levels. These results provide *in vivo* evidence for the correlation of activities between pPAK4 and pTH^{40}. Taken together, our data demonstrate that caPAK4 can act as a stimulator of DA synthesis by elevating TH transcription and activity.

DISCUSSION

The present study unravels a novel mechanism underlying PAK4-mediated modification of PD in animal models in which caPAK4 upregulates DA levels. Expression of caPAK4 stimulated transcription of TH via the CREB transcription factor. Moreover, caPAK4 increased the catalytic activity of TH through its phosphorylation of S^{40}, which is essential for TH activation. More importantly, these findings were recapitulated in brain tissues from PD patients; levels of pPAK4 and pTH^{S40} were positively correlated. Collectively, our data support the therapeutic effects of PAK4 and suggest that targeting PAK4 is a viable approach for symptomatic treatment of PD.

Previously, we demonstrated that caPAK4 elevates levels of dopamine and its metabolites in a 6-hydroxydopamine (6-OHDA)-induced PD model compared to controls (Won et al., 2016). Our interpretation was that this effect was solely due to the neuroprotective function of PAK4. In the present study, we unraveled its hidden function, demonstrating that PAK4 controls both TH activity and levels. Together, it seems likely that PAK4 may elevate dopamine levels in the 6-OHDA PD model through its neuroprotective effect on dopaminergic neurons and its regulatory effect on TH. It is thus tempting to speculate that these dual functions of PAK4 may work synergistically, resulting in significant rescue of the impaired motor behavior of 6-OHDA rats.

PAK4 regulates a number of target genes through CREB, resulting in pleiotropic effects, such as promotion of cell proliferation and neuroprotection (Won et al., 2019), but these two proteins are ubiquitously expressed. In contrast, TH is not widely expressed but only exists in certain cell types, such as DA neurons in the brain and melanocytes in the skin (Won et al., 2016; Yun et al., 2015). This restricted expression of TH may confer specificity to its PAK4-dependent regulation. Additionally, the finding that PAK4 interacts with TH in the cytoplasm adds to the specificity. Together, decreases in PAK4 and pPAK4 levels in PD may contribute to impaired movement.

Currently, the gold standard of PD treatment is DA replacement using L-dopa. However, long-term treatment with this drug frequently causes motor complications, such as an increase in motor fluctuations and dyskinesia over time. Most likely, progressive degeneration of the nigrostriatal axis explains these side effects; thus, disease-modifying treatment is critically needed. Gene therapy could be an available option for PD treatment (Wood, 2020). Current gene therapy focuses on either neuroprotective interventions that employ trophic factors, such as GDNF, BDNF, and neurturin, or DA replacement by introduction of a gene(s) for TH or amino acid decarboxylase (Axelsen and Woldbye, 2018; Bjorklund and Kordower, 2010; Denyer and Douglas, 2012). Clinical trials have shown that gene therapy for PD is safe, although its efficacy remains a hurdle. Considering its beneficial effect on DA synthesis, PAK4 might be an alternative target for DA replacement in patients with PD. Together with our previous study that defined a key role for PAK4 in dopaminergic neuron survival (Won et al., 2016), the current findings support a dual effect of PAK4 involving both neuroprotection and elevation of DA levels. In this regard, PAK4-based gene therapy may offer a disease-modifying effect for successful PD treatment.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (Ministry of Science, ICT and Future Planning; 2017R1A2B3005714, 2017R1C-1B2006193, 2020R1A5A2017476).

AUTHOR CONTRIBUTIONS

S.Y.W. designed and performed experiments and wrote the manuscript. S.T.Y., S.W.C., and E.Y.S. performed research. C.M. interpreted immunohistochemical data. E.G.K. supervised the entire project and wrote the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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