PARKINSON'S DISEASE

Nigral dopaminergic PAK4 prevents neurodegeneration in rat models of Parkinson's disease

So-Yoon Won,[1] Mee-Hee Park,[1] Soon-Tae You,[1] Seung-Won Choi,[1] Hyong-Kyu Kim,[2] Catriona McLean,[3] Suk-Chul Bae,[4] Sang Ryong Kim,[5,6] Byung Kwan Jin,[7] Kun Ho Lee,[8,9] Eun-Young Shin,[1] Eung-Gook Kim[1]*

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic (DA) neurons in the substantia nigra. No neuroprotective treatments have successfully prevented the progression of this disease. We report that p21-activated kinase 4 (PAK4) is a key survival factor for DA neurons. We observed PAK4 immunoreactivity in rat and human DA neurons in brain tissue, but not in microglia or astrocytes. PAK4 activity was markedly decreased in postmortem brain tissue from PD patients and in rodent models of PD. Expression of constitutively active PAK4$^{S445N/S474E}$ (caPAK4) protected DA neurons in both the 6-hydroxydopamine and α-synuclein rat models of PD and preserved motor function. This neuroprotective effect of caPAK4 was mediated by phosphorylation of CRTC1 [CREB (adenosine 3′,5′-monophosphate response element–binding protein)–regulated transcription coactivator] at S215. The nonphosphorylated form of CRTC1^{S215A} compromised the ability of caPAK4 to induce the expression of the CREB target proteins Bcl-2, BDNF, and PGC-1α. Our results support a neuroprotective role for the PAK4-CRTC1^{S215}-CREB signaling pathway and suggest that this pathway may be a useful therapeutic target in PD.

INTRODUCTION

Parkinson's disease (PD) affects 1 to 2% of the population older than 65 years of age (*1*). The pathological feature of PD is degeneration of the dopaminergic (DA) neurons in the substantia nigra (SN) that decreases striatal dopamine, leading to motor dysfunction, resting tremors, bradykinesia, and rigidity (*2*). Most PD patients start to experience motor symptoms when 50% or more of their DA neurons have already died (*3*, *4*), meaning that diagnosis is usually delayed until this point. Despite substantial efforts, methods for early diagnosis of PD remain unsatisfactory. Moreover, nine gene therapy clinical trials for PD have failed because of poor clinical efficacy and there is no cure for this disease (*5*). Consequently, there is an urgent need for the development of neuroprotective therapies that can prevent or delay the irreversible loss of DA neurons. To achieve this goal, it is critical to understand the etiology of PD and identify efficacious targets for therapeutic interventions.

The p21-activated kinases (PAKs) are a family of protein serine/threonine kinases. There are six mammalian PAKs that are classified into group I (PAK1 to PAK3) and group II (PAK4 to PAK6) based on their structural and biochemical properties (*6*). Group II PAKs regulate a wide range of cellular processes including actin cytoskeleton reorganization, tumorigenesis, and neuronal dysfunction (*6*–*9*). Of group II PAKs, PAK5 and PAK6 have been implicated in brain functions such as learning and regulation of locomotion (*10*). Initial studies on PAK4 have revealed its role in the reorganization of the actin cytoskeleton, neuronal development, and regulation of axonal outgrowth in neural progenitor cells (*11*, *12*). PAK4-deficient mice die at embryonic day 10.5 and display abnormalities in the heart and nervous system, with the most striking defects observed in the brain and neural tube (*11*). Together, these studies implicate deregulation of PAKs in neuronal pathophysiology.

The oligomerization/aggregation of α-synuclein has been mechanistically linked to neurodegeneration in PD (*13*). Danzer *et al.* discovered that α-synuclein oligomers, but not monomers, inhibited PAK4 kinase activity in vitro (*14*), but no further investigation of the role of PAK4 in DA neurons of the SN has been conducted. The serine/threonine kinase AKT has also been implicated in PD (*15*, *16*). AKT activation has been shown to protect DA neurons in cellular and animal models of PD (*15*, *16*). PAK4 activity is linked to AKT signaling (*17*, *18*), and PAK4 could have a potential role in promoting the survival of DA neurons through AKT.

CREB (adenosine 3′,5′-monophosphate response element–binding protein)–regulated transcription coactivators (CRTCs) are indispensable coactivators of CREB that regulate essential physiological functions such as glucose homeostasis, long-term fear memory formation, and neuronal survival (*19*–*23*). CRTC has three isoforms: CRTC1, CRTC2, and CRTC3; CRTC1 is expressed mainly in the brain, whereas CRTC2 is a major isoform in the liver. CRTC1 contributes to neuroprotection by inducing the expression of CREB target genes, such as those encoding brain-derived neurotrophic factor (BDNF) (*24*), peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α) (*25*), and B cell lymphoma 2 (Bcl-2) (*26*). Deregulation of CRTC1-dependent CREB transcriptional activity is implicated in Alzheimer's disease, Huntington's disease, ischemia, addiction, and circadian clock activity (*20*, *21*, *24*, *27*, *28*). CRTC1-deficient mice show depression-related behaviors (*29*). These mice display a decrease in dopamine metabolites, which may implicate CRTC1 in dopamine metabolism in PD. However, the potential role of CRTC1 in PD remains undefined. CRTC activates

[1]Department of Biochemistry and Signaling Disorder Research Center, College of Medicine, Chungbuk National University, Cheongju 28644, South Korea. [2]Department of Medicine and Microbiology, College of Medicine, Chungbuk National University, Cheongju 28644, South Korea. [3]Department of Pathology, The Alfred Hospital, Melbourne, Victoria 3004, Australia. [4]Department of Biochemistry, School of Medicine, Institute for Tumor Research, Chungbuk National University, Cheongju 28644, South Korea. [5]School of Life Sciences, BK21 plus KNU Creative BioResearch Group, Kyungpook National University, Daegu 41566, South Korea. [6]Brain Science and Engineering Institute, Kyungpook National University, Daegu 41944, South Korea. [7]Department of Biochemistry & Molecular Biology, Department of Neuroscience, Neurodegeneration Control Research Center, School of Medicine, Kyung Hee University, Seoul 02447, South Korea. [8]National Research Center for Dementia, Chosun University, Gwangju 61452, South Korea. [9]Department of Biomedical Science, Chosun University, Gwangju 61452, South Korea.
*Corresponding author. Email: egkim@chungbuk.ac.kr

CREB through a phosphorylation-independent interaction (*30*). To act as a coactivator, CRTC1 must translocate to the nucleus, and translocation is facilitated by its dephosphorylation at S151 and S245 (*23*, *31*); phosphorylation at S151 and S245 inhibits the coactivator activity of CRTC1 by maintaining its localization in the cytoplasm (*23*).

We previously demonstrated that PAK4 activates the transcriptional activity of CREB through an unknown mechanism (*32*). PAK4 did not phosphorylate CREB in vitro, suggesting that activation of CREB by PAK4 occurs through a phosphorylation-independent mechanism that involves coactivators of CREB such as CRTC1. Given that PAK4 regulates CREB activity and that CREB plays a key role in neuronal survival, maintaining PAK4 activity is expected to be critical for preventing the degenerative loss of DA neurons. It is possible that decreased PAK4 activity may in part contribute to the pathogenesis of PD. Here, we investigated the role of PAK4 in PD and elucidated downstream signaling that may be neuroprotective. PAK4 can be constitutively activated by the S445N mutation in the catalytic domain, which enhances catalytic activity by stabilizing the PAK4-substrate complex (*33*), and by autophosphorylation of S474 in the activation loop. For gain-of-function studies, we used this mutant form of constitutively active PAK4$^{S445N/S474E}$ (caPAK4). Lentiviral-mediated transduction of caPAK4 prevented neurodegeneration in two rodent models of PD: one induced by the toxin 6-hydroxydopamine (6-OHDA) and the other by overexpression of α-synuclein (genetic model). We found that CRTC1 is a new PAK4 substrate whose phosphorylation at S215 is required for the neuroprotective effect of PAK4. Finally, we found an impairment of PAK4-CRTC1 signaling in human postmortem brain tissue from PD patients.

RESULTS

DA neurons express both PAK4 and PAK6

To determine whether PAK4 and phospho-PAK4^{S474} (hereafter referred to as pPAK4), an index of PAK4 activation (*33*), can be detected in DA neurons, we performed immunofluorescence analyses of human (table S1) and rat midbrain tissues. We observed strong immunoreactivity for both pPAK4 and PAK4 in DA neurons, but not microglia or astrocytes, in human (Fig. 1, A to D) and rat (Fig. 1, E to H) SN brain tissue, as assessed by costaining with tyrosine hydroxylase (TH), a DA neuronal marker. Blocking with the phosphopeptide used for immunization indicated that pPAK4 staining was specific (fig. S1). We also detected PAK6 but not PAK5 staining in DA neurons (fig. S2), but did not pursue PAK6. (Our study provided data on the expression of group II PAK isoforms in the rat SN; information on the differential expression of group II PAK isoforms in the mouse brain is available at http://brain-map.org/.)

PAK4 may be a neuroprotective kinase in PD

Next, we investigated whether the expression of pPAK4 and PAK4 was altered in PD. Both pPAK4 and PAK4 expression was decreased in lysates of SN tissue from postmortem brain samples from patients with PD compared with age-matched controls (Fig. 2, A and B, and table S1). We observed similar expression of TH except for two age-matched controls and one PD sample, which showed variable expression (Fig. 2A). Although TH is widely used as a marker for DA neurons, TH expression does not always directly correlate with overt DA neuronal cell death (*34*–*36*). Thus, the reduced TH expression in two age-matched control samples and increased TH expression in the PD sample may not exactly reflect DA neuronal cell death. Although analysis of postmortem brain tissue provided useful information, it was not clear whether decreased PAK4 activity was a driver of PD pathogenesis or whether it was simply a consequence of DA neuron loss. Thus, we carefully evaluated the expression of both pPAK4 and PAK4 in living TH-positive DA neurons in postmortem brain tissue from PD patients, age-matched controls, and young controls (table S1). We found that pPAK4 and PAK4 expression was lower in PD patient DA neurons than in DA neurons derived from age-matched controls (Fig. 2, C to E). The pPAK4/PAK4 ratio was greater in the PD DA neurons than in the age-matched control and young control DA neurons (Fig. 2F). We therefore traced the

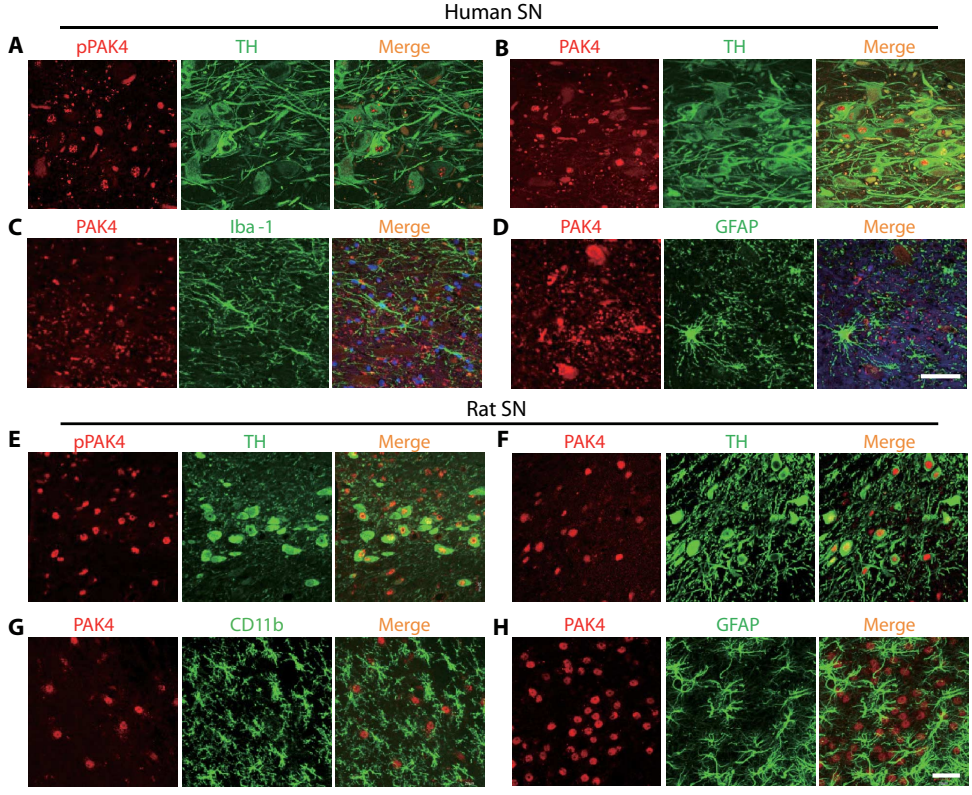

Fig. 1. Detection of pPAK4 and PAK4 in DA neurons of the SN. Confocal microscopy images of pPAK4 (**A** and **E**) and PAK4 (**B** to **D** and **F** to **H**) in healthy human (A to D) and rat SN (E to H). Coimmunostaining for pPAK4 (A and E) or PAK4 (B and F) and TH, a DA neuron marker. Coimmunostaining for PAK4 and the microglial marker Iba-1 (C) or CD11b (G). Coimmunostaining for PAK4 and GFAP, an astrocyte marker (D and H). Scale bars, 20 μm (A to D); 25 μm (E to H).

fate of pPAK4-positive and pPAK4-negative neurons in postmortem brain tissue from PD patients by costaining for pPAK4 and Bcl-2 (a marker of survival) or for terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL; a marker of apoptosis). Neuromelanin-positive cells can represent DA neurons because oxidation of dopamine and its metabolites generates neuromelanin (*37*). We conducted a costaining analysis for neuromelanin-positive cells. In age-matched control postmortem brain tissue, most of the neuromelanin-positive neurons exhibited pPAK4 immunoreactivity; however, in postmortem brain tissue from PD patients, less than 25% of the neuromelanin-positive neurons were pPAK4-positive (fig. S3A and table S2). All the pPAK4-positive DA neurons in PD patients stained positive for Bcl-2 (Fig. 2G, inset 1; fig. S3B; and table S2), but were negative for TUNEL staining (Fig. 2H, inset 1; fig. S3C; and table S2). Conversely, pPAK4-negative neurons in PD patient postmortem brain tissue were typically Bcl-2-negative (Fig. 2G, inset 2; fig. S3B; and table S2), but 33 to 57% of these neurons were TUNEL-positive (Fig. 2H, inset 2; fig. S2C; and table S2).

Pharmacological inhibition of PAK4 sensitizes DA neurons to 6-OHDA in vivo

Next, we examined alterations in pPAK4 expression in a unilateral 6-OHDA rat model of PD, in which the loss of DA neurons in the SN on one side of the brain was induced by stereotactic injection of the toxin 6-OHDA. In the SN on day 2 after 6-OHDA injection, pPAK4 expression was lower by 44% than in the SN of control rats injected with PBS, whereas neither PAK4 nor TH was altered, suggesting that a reduction in pPAK4 preceded DA neuron loss in this model (Fig. 3, A to C).

To further examine the effect of decreased PAK4 activity in PD, we performed a loss-of-function study. We stereotactically injected the lentiviral–green fluorescent protein (GFP)–small hairpin control (shCtrl) or lentiviral-GFP-shPAK4 construct into the right striatum of rat brain (Fig. 3D and fig. S4, A and B). GFP-shPAK4 colocalized with TH but not with integrin α-M (CD11b) or glial fibrillary acidic protein (GFAP), indicating that the lentivirus was transduced mainly into TH-positive DA neurons rather than into glial cells (fig. S4, C to E). PAK4 shRNA efficiently depleted PAK4 in the SN (Fig. 3, E and F, and fig. S4F). To exclude off-target effects of rat PAK4 shRNA, lentivirus expressing human caPAK4 was introduced into cultured rat mesencephalic neurons. Human caPAK4 was resistant to rat PAK4 shRNA (fig. S5A) and rescued 6-OHDA–induced neurotoxicity (fig. S5B). Two weeks after the injection of lentivirus constructs, the rats were injected with a suboptimal dose of 6-OHDA (1 μg instead of the usual 10 μg) into the ipsilateral medial forebrain bundle (*38*). This treatment resulted in nigrostriatal destruction in rats in which PAK4 had been knocked down, but only had slight effects on control rats, as measured by stereological counts of TH-positive and Nissl-stained DA neurons (Fig. 3, G and H) and measurements of striatal TH-positive fiber density (Fig. 3, I and J). Along with the defects in the nigrostriatal DA system, the rats treated with shPAK4 lentivirus developed motor symptoms, such as defective use of the contralateral paws as monitored by the cylinder test (Fig. 3K) and elevated amphetamine-induced ipsilateral rotations (Fig. 3L).

To obtain pharmacological evidence for a neuroprotective role of PAK4, we examined the effect of PF-3758309, a potent pan-PAK inhibitor (*39*), which has been reported not to cross the blood-brain barrier (*8*). We stereotactically injected PF-3758309 at three

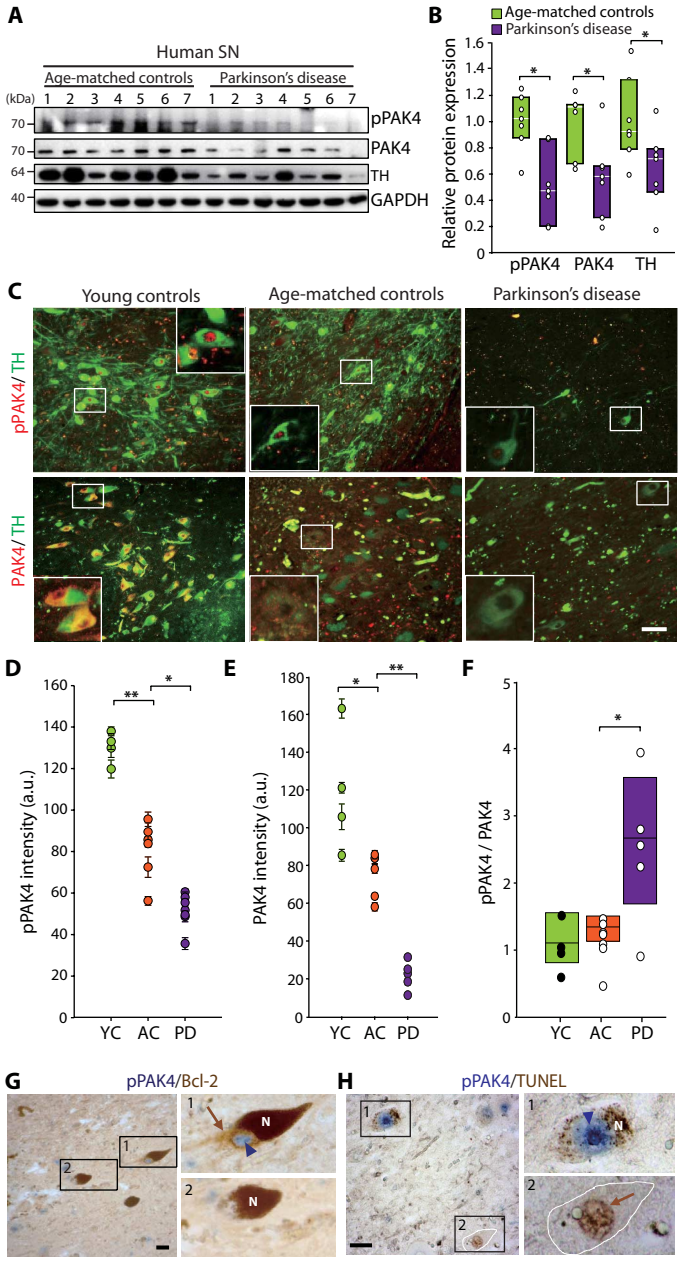

Fig. 2. PAK4 activity may promote the survival of DA neurons. (**A** to **F**) pPAK4 and PAK4 expression in the SN of postmortem brain tissue from PD patients. (A) Immunoblotting of SN lysates from age-matched controls ($n = 7$) and PD patients ($n = 7$). (B) Quantification of the blot in (A) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C) Confocal microscopy images: red, pPAK4 and PAK4; green, TH. Insets provide a high-magnification view of individual TH-positive neurons. Scale bar, 50 μm. (D and E) Quantification of pPAK4 (D) and PAK4 (E) staining intensity in DA neurons (a.u., arbitrary units). YC, young controls ($n = 4$); AC, age-matched controls ($n = 6$); PD, PD patients ($n = 5$). (F) pPAK4/PAK4 ratio in DA neurons. (**G** and **H**) Costaining in the PD SN for pPAK4 (blue arrowhead) and Bcl-2 (brown arrow) ($n = 4$) (G) and for pPAK4 (blue arrowhead) and TUNEL (brown arrow) ($n = 3$) (H). N, neuromelanin. Boxed areas 1 and 2 in the left panel are shown at a higher magnification in the right panel. Scale bars, 25 μm. Error bars, mean ± SEM. *$P < 0.01$, **$P < 0.001$, Student's t test (B) or analysis of variance (ANOVA) with Student-Newman-Keuls post hoc analysis (D to F).

different doses (0.25, 0.5, and 2 μg) into the right SN of rats (fig. S6A). We selected 0.5 μg of PF-3758309 for the next experiment (fig. S6F) because pPAK4 expression was reduced at this dose but not at a dose of 0.25 μg (fig. S6, B to E). We next asked whether PF-3758309 treatment sensitized rats to a suboptimal dose of 6-OHDA (1 μg). PF-3758309 (0.5 μg) alone decreased the number of DA neurons by ~30% and the striatal TH-positive fiber density by ~25% compared to vehicle-treated controls (fig. S6, G to J). Stereotactic injection of 6-OHDA alone also showed cytotoxicity (~20% cell loss and ~5% decrease in striatal TH-positive fiber density) compared to PBS-treated controls (fig. S6, G to J). When used together with PF-3758309, 6-OHDA treatment resulted in a ~80% decrease in both the number and the density of TH-positive fibers (fig. S6, G to J). Collectively, these results showed that PAK4 deficiency or inhibition of its activity rendered DA neurons more vulnerable to 6-OHDA–mediated neurotoxicity.

caPAK4 protects DA neurons from 6-OHDA neurotoxicity in vivo

For the gain-of-function study, we first examined wild-type PAK4 ($PAK4^{WT}$), caPAK4, and $PAK4^{S474E}$ for their protective effects against 6-OHDA–induced cell death. We measured 6-OHDA–induced cytotoxicity and expression of Bcl-2, an anti-apoptotic marker. SH-SY5Y cells expressing $PAK4^{WT}$ or caPAK4, but not those expressing $PAK4^{S474E}$, showed resistance to 6-OHDA (fig. S7A). Moreover, $PAK4^{WT}$ and caPAK4 prevented the 6-OHDA–induced suppression of Bcl-2 expression (fig. S7, B and C). Thus, $PAK4^{WT}$ was protective, although to a lesser extent than caPAK4. However, $PAK4^{S474E}$ had only a modest effect and thus did not work as an active phosphomimetic form, which is consistent with previous studies (*33*, *40*). Therefore, we used caPAK4 to investigate the neuroprotective role of PAK4 in the PD rat model. We tested the effects of caPAK4 in the 6-OHDA PD model, as outlined in Fig. 4A. After stereotactic injection into the striatum, lenti-caPAK4 was transduced mainly into TH-positive DA neurons in the SN, as detected by GFP fluorescence (fig. S8). Rats were then injected with a high dose (10 μg) of 6-OHDA into the medial forebrain bundle. caPAK4 protected TH-positive and Nissl-stained DA neurons in the SN (Fig. 4, B and C), retained striatal TH-positive fiber density (Fig. 4, D and E), and protected the production of dopamine and its metabolites—3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)—compared to rats injected with lentivirus-control (Fig. 4F). Consistent with these findings, in comparison to lentivirus-control–injected rats, lenti-caPAK4–injected rats showed improved motor function. Improved motor function included a reduced preference for contralateral forelimb use (Fig. 4G) and lower ipsilateral turning 2 and 4 weeks after 6-OHDA treatment in response to amphetamine administration (Fig. 4H). These results suggested that PAK4 activity may be protective against 6-OHDA–induced nigrostriatal DA neurodegeneration in rats.

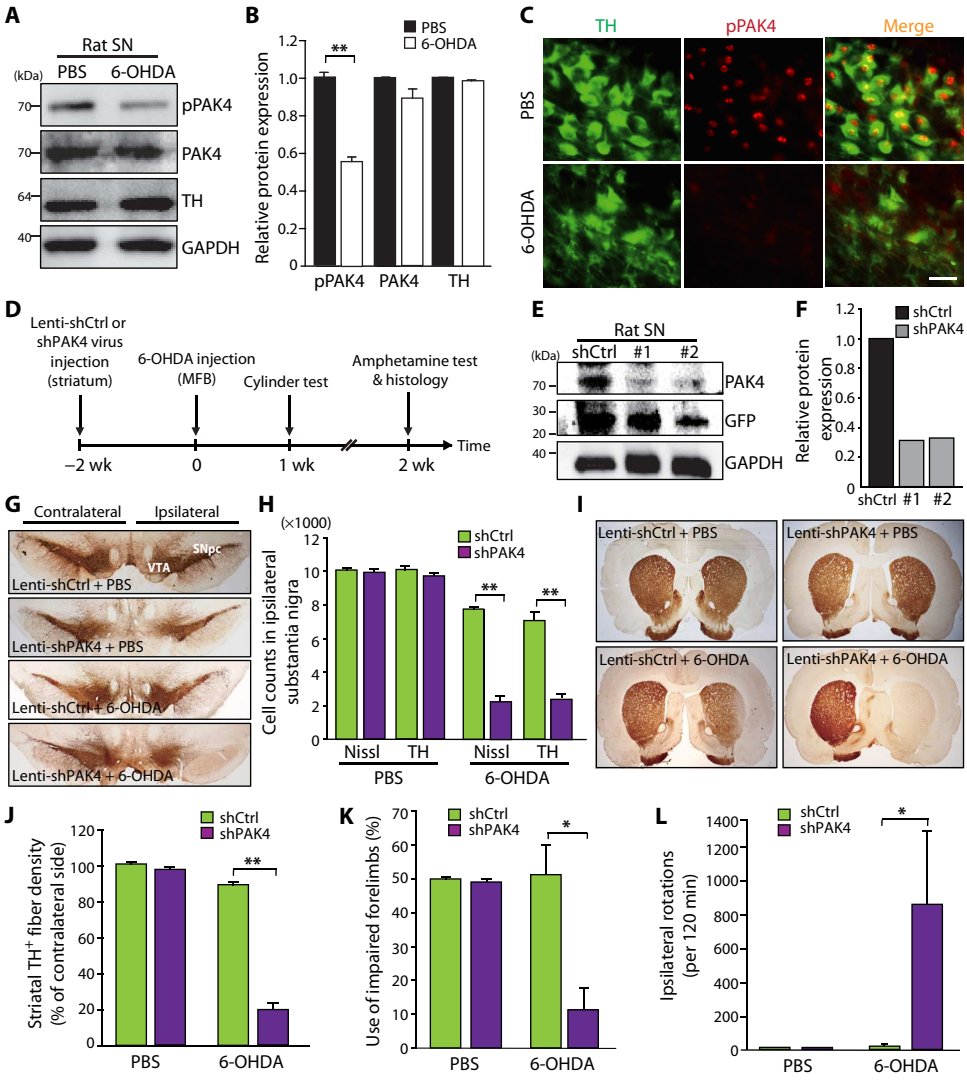

Fig. 3. PAK4 knockdown sensitizes rats to 6-OHDA–induced neurotoxicity. (**A** to **C**) pPAK4 and PAK4 expression in the 6-OHDA rat model of PD. (A) Immunoblotting for pPAK4, PAK4, and TH in SN lysates from rats injected with PBS (n = 3) or 6-OHDA (n = 3). (B) Quantification of the blot results in (A) normalized to GAPDH. (C) Double-labeling for TH (green) and pPAK4 (red) after treatment with PBS or 6-OHDA. Scale bar, 50 μm. (**D**) Experimental scheme. MFB, medial forebrain bundle. (**E**) Immunoblotting for validation of knockdown efficiency in rats injected with shPAK4. (**F**) Quantification of the blot results in (E) normalized to GAPDH. (**G**) Representative images of TH immunostaining in the SN of rats injected with lenti-shCtrl or lenti-shPAK4 followed by PBS or 6-OHDA treatment. VTA, ventral tegmental area; SNpc, substantia nigra pars compacta. (**H**) Stereological counting of TH-positive and Nissl-stained DA neurons (n = 6 per group). (**I**) Representative images of TH immunostaining in the rat striatum. (**J**) Quantification of the density of TH-positive striatal fibers (n = 6 per group). (**K**) Quantification of performance in the cylinder test (n = 6 per group). (**L**) Quantification of cumulative amphetamine-induced ipsilateral rotations (n = 6 per group). Error bars, mean ± SEM. *P < 0.01, **P < 0.001, unpaired two-tailed Student's t test.

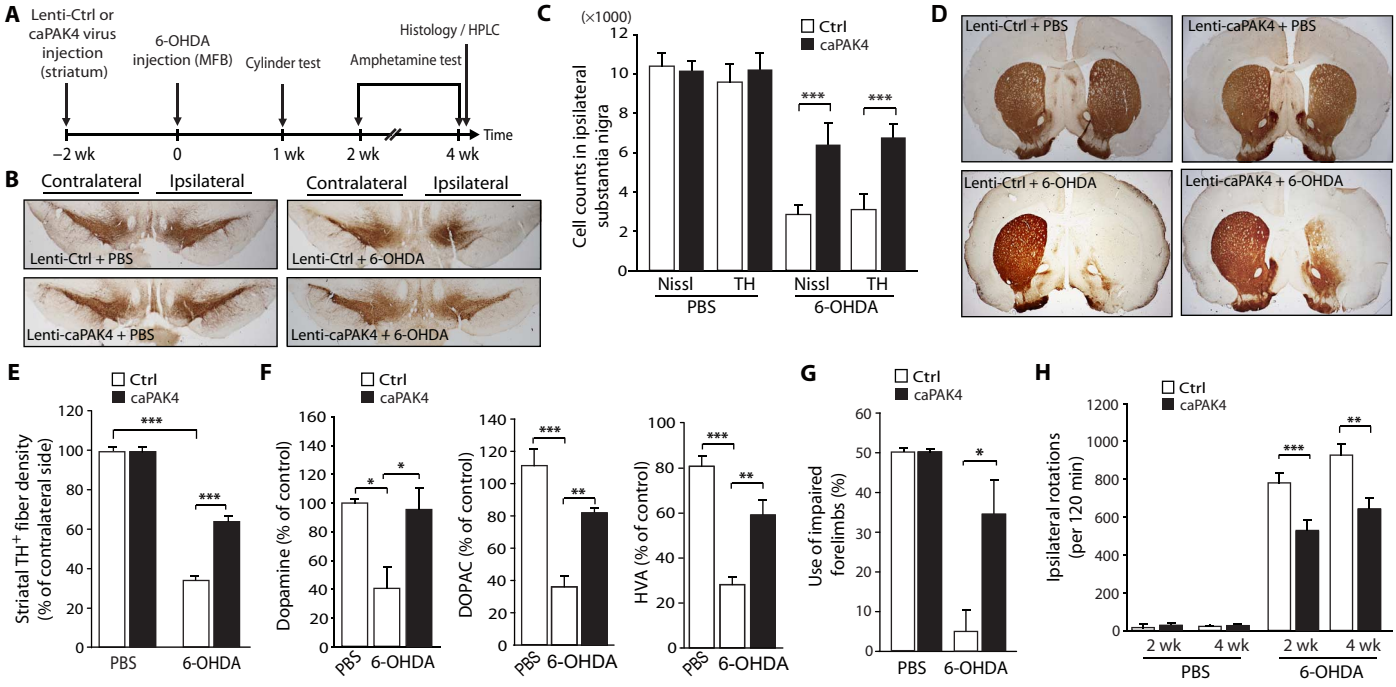

Fig. 4. caPAK4 protects rats from 6-OHDA–induced neurotoxicity. (**A**) Experimental scheme. HPLC, high-performance liquid chromatography. (**B**) Representative images of TH immunostaining in the SN of rats injected with lenti-Ctrl or lenti-caPAK4 followed by PBS or 6-OHDA treatment. (**C**) Stereological counting of TH-positive and Nissl-stained DA neurons ($n = 7$ per group). (**D**) Representative images of TH immunostaining in the striatum. (**E**) Quantification of TH-positive striatal fiber density ($n = 7$ per group). (**F**) Quantification of dopamine and its metabolites in the ipsilateral striatum ($n = 3$ per group). (**G**) Quantification of performance in the cylinder test ($n = 7$ per group). (**H**) Quantification of cumulative amphetamine-induced ipsilateral rotations ($n = 7$ per group). Error bars, mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Student-Newman-Keuls post hoc analysis (E to G) or Student's t test (C and H).

caPAK4 protects DA neurons in the α-synuclein rat model of PD

To determine whether these findings extended to genetic models of PD, we examined the rat unilateral α-synuclein lesion model (*41*). Recombinant adeno-associated virus type 2 (AAV2) containing the human WT α-synuclein gene (AAV2-α-syn) or the enhanced GFP gene (AAV2-eGFP) was stereotactically injected into the SN (Fig. 4A). AAV2-mediated overexpression of α-synuclein was detected in the rat SN and striatum 5 weeks after injection of the AAV2-α-syn construct (fig. S9, A and B). α-Synuclein immunoreactivity was also observed in the cytoplasm of TH-positive neurons (fig. S9C). Five weeks after AAV2-α-syn injection, histological analysis revealed that AAV2-α-syn–lenti-caPAK4–injected rats had more TH-positive and Nissl-stained DA neurons than AAV2-α-syn–lenti-Ctrl–injected rats (Fig. 5, B and C). We also found a higher TH-positive fiber density in the AAV2-α-syn–lenti-caPAK4–injected group than in the AAV2-α-syn–lenti-Ctrl–injected group (Fig. 5E). Administration of lenti-caPAK4 alleviated α-synuclein–induced behavioral impairment, as assessed by amphetamine-induced ipsilateral rotation (Fig. 5F) and defective use of the contralateral forelimb in the cylinder test (Fig. 5G).

CRTC1-CREB signaling mediates caPAK4-induced neuroprotection

Our previous study implicated PAK4 as a critical regulator of CREB (*32*). Thus, we tested whether PAK4 also regulates CREB in neuronal cells. In the SH-SY5Y neuronal cell line, the expression of caPAK4 markedly increased CRE reporter activity (fig. S10A) and prevented the 6-OHDA–induced suppression of the expression of the CREB target proteins PGC-1α, BDNF, and Bcl-2 (fig. S10, B and C). To determine whether caPAK4-mediated neuroprotection required CREB, we selectively inhibited CREB function by silencing with small interfering RNA (siRNA). Accordingly, the ability of caPAK4 to rescue 6-OHDA cytotoxicity was compromised in CREB siRNA-treated SH-SY5Y cells compared to siRNA-treated control cells (fig. S10D). These data suggest that CREB may mediate the neuroprotective effects of caPAK4.

The molecular mechanism by which PAK4 regulates CREB-dependent transcription is unknown. Because PAK4 does not phosphorylate CREB (fig. S11A) (*32*), we assumed that PAK4 might regulate CREB indirectly through its coactivators such as CRTC1, the major CRTC isoform in the brain. We examined CRTC1 expression in DA neurons from postmortem brain tissue. Immunostaining showed the presence of CRTC1 in DA neurons from human postmortem brain tissue and rat brain (Fig. 6, A and B). Notably, depletion of CRTC1 by siRNA inhibited stimulation of CRE reporter activity by caPAK4 (fig. S10E). We next examined the involvement of CRTC1 in caPAK4-mediated neuroprotection. CRTC1 knockdown reduced the caPAK4-mediated prevention of 6-OHDA toxicity in SH-SY5Y cells (fig. S10F). These results strengthened the conclusion that the CRTC1-CREB pathway was playing a role in mediating neuroprotection of 6-OHDA–treated neurons by caPAK4 in vitro. To confirm our findings in vivo, we tested whether CRTC1 knockdown compromised the ability of caPAK4 to protect DA neurons against 6-OHDA toxicity in the rat model of PD (Fig. 6C). Striatal injection of lenti-shCRTC1 efficiently reduced CRTC1 protein expression in the SN (Fig. 6, D and E). Knocking down CRTC1 suppressed the ability of caPAK4

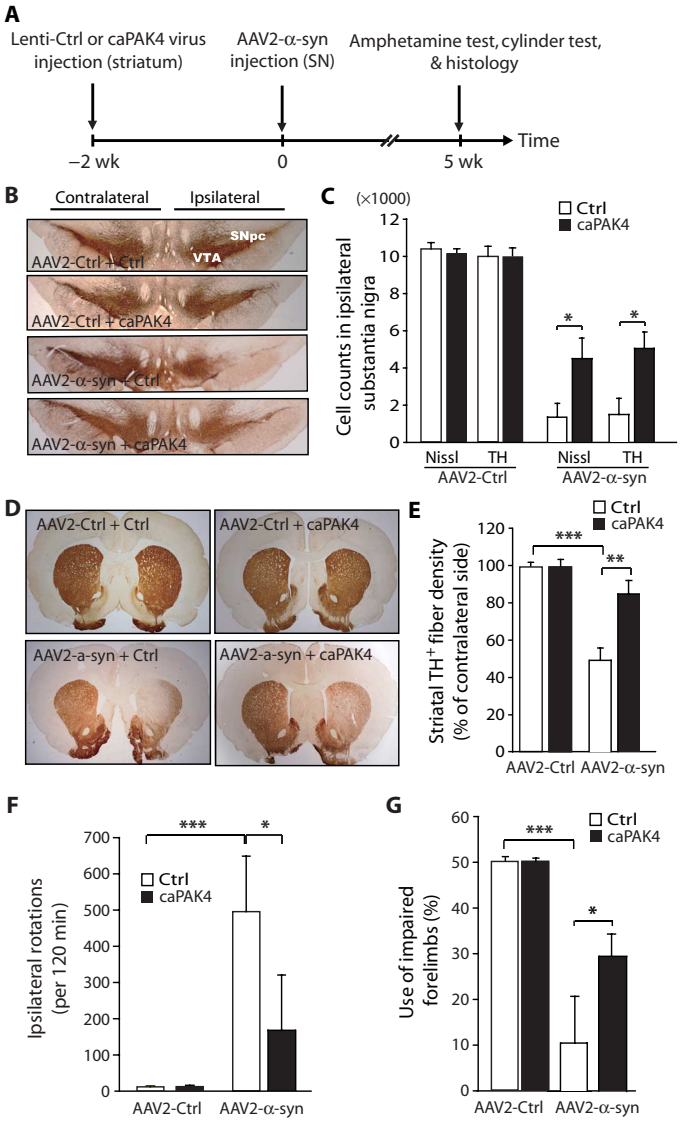

Fig. 5. caPAK4 protects rats from α-synuclein–induced PD. (**A**) Experimental scheme. (**B**) Representative images of TH immunostaining in the SN and striatum of rats injected with lenti-Ctrl or lenti-caPAK4 followed by injection of AAV2-α-syn. (**C**) Stereological counting of Nissl-stained and TH-positive DA neurons (n = 5 per group). (**D**) Representative images of TH immunostaining in striatum. (**E**) Quantification of TH-positive striatal fiber density (n = 5 per group). (**F**) Quantification of cumulative amphetamine-induced ipsilateral rotations (n = 5 per group). (**G**) Quantification of performance in the cylinder test (n = 5 per group). Error bars, mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, Student's t test (C) or ANOVA with Student-Newman-Keuls post hoc analysis (E to G).

to protect the TH-positive and Nissl-stained DA neurons in the SN (Fig. 6, F and G) and TH-positive fiber density in the striatum (Fig. 6, H and I) against 6-OHDA toxicity. Moreover, CRTC1 depletion hindered the caPAK4-mediated improvement in motor function in the 6-OHDA rat model of PD, as assessed by the cylinder test (Fig. 6J) and the amphetamine-induced rotation test (Fig. 6K).

CRTC1 phosphorylation at S215 mediates caPAK4-induced neuroprotection

CRTC1 is localized mainly in the nucleus of DA neurons (Fig. 6, A and B). A major fraction of PAK4, including pPAK4, is also localized in the nucleus (Fig. 1). We therefore examined their interaction. PAK4 immunoprecipitated with CRTC1 and CREB, indicating a physical association (Fig. 7A). Pulldown assays with purified PAK4 and CRTC1 recombinant proteins revealed that this interaction was direct (Fig. 7B). Furthermore, in vitro kinase assays with purified proteins revealed that CRTC1 is a substrate of caPAK4 but not of protein kinase A (fig. S11A). These results showed that PAK4 acts by phosphorylating CRTC1. Sequence analysis suggested that S215 and T449 of CRTC1 might be potential PAK4 phosphorylation sites (fig. S11B); dephosphorylation of CRTC1 at S151 contributed to its activation (*31*). Therefore, we generated mutant forms of CRTC1 that could not be phosphorylated at these sites ($CRTC1^{S215A}$, $CRTC1^{S151A}$, and $CRTC1^{T449A}$). We expressed these mutant forms with active PAK4 and examined their mobility shifts after electrophoresis. Both $CRTC1^{S151A}$ and $CRTC1^{S151A/T449A}$, but not $CRTC1^{S151A/S215A}$, had a slowly migrating band (fig. S11, C and D). Consistent with this result, active PAK4 recombinant proteins barely phosphorylated $CRTC1^{S215A}$ (Fig. 7C). Thus, S215 appeared to be the primary PAK4 phosphorylation site. To examine the functional significance of this phosphorylation, we coexpressed caPAK4 with either $CRTC1^{WT}$ or $CRTC1^{S215A}$ and analyzed CRE reporter activity. Expression of $CRTC1^{S215A}$ strongly inhibited CRE activation by caPAK4 (Fig. 7D). Consistent with this result, $CRTC1^{S215A}$ decreased caPAK4-induced expression of the CREB target proteins PGC-1α, BDNF, and Bcl-2 (Fig. 7, E and F). The presence of $CRTC1^{S215A}$ also decreased caPAK4-induced protection against 6-OHDA cytotoxicity (Fig. 7G). These results demonstrated that CRTC1 phosphorylation at S215 may be a critical event in PAK4-mediated CREB activation and neuroprotection.

Next, to obtain in vivo evidence that CRTC1 is phosphorylated at S215 and to investigate its potential relevance in PD, we produced an antibody that specifically recognized phospho-$CRTC1^{S215}$ ($pCRTC1^{S215}$) (Fig. 8A). SN lysates from rats injected with 6-OHDA showed markedly reduced amounts of $pCRTC1^{S215}$ concomitant with a decrease in the CREB target proteins compared to phosphate-buffered saline (PBS)–injected control rats (Fig. 8, B and C). AAV2-mediated overexpression of α-synuclein also reduced the amounts of $pCRTC1^{S215}$, PGC-1α, BDNF, and Bcl-2 in the SN, and this reduction was reversed by caPAK4 (Fig. 8, D and E).

Finally, to gain a better understanding of the $pCRTC1^{S215}$ status in PD patients, we examined postmortem human brain tissue. Immunoblotting analysis revealed a significant decrease in $pCRTC1^{S215}$ in the SN from PD patient postmortem brain tissue versus that from age-matched controls (Fig. 8, F and G). At the cellular level, most neuromelanin-positive DA neurons in age-matched controls exhibited nuclear $pCRTC1^{S215}$ reactivity (Fig. 8, H and K), which was completely abolished by preincubation with the blocking phosphopeptide used for immunization (Fig. 8I). In contrast, $pCRTC1^{S215}$ could not be detected in most DA neurons in postmortem brain tissue from PD patients; even in positively stained neurons, the signal was weak (Fig. 8, J and K). These findings suggested that the PAK4-$CRTC1^{S215}$-CREB pathway might be impaired in PD.

DISCUSSION

Development of a neuroprotective therapy to delay or prevent the progression of PD and other neurodegenerative diseases remains an unmet goal. Accumulating evidence indicates a large gap between preclinical studies and clinical treatments. This might be due to poor

efficacy of therapeutic genes delivered by gene therapy (*5*) and disproportionate use of toxin-based PD models for evaluation of neuroprotective effects (*42*). Here, we report that PAK4 and its downstream signaling pathway may be a new potential target for neuroprotective therapies based on results from two different rat models of PD: the 6-OHDA toxin model and the α-synuclein genetic model. Furthermore, we recapitulated these findings in human postmortem brain tissue from PD patients.

One of the most striking features of PD is that it primarily affects a restricted neuronal population in the midbrain (*43*). However, the molecular mechanism underlying this high vulnerability of midbrain DA neurons remains elusive. Our findings demonstrate that PAK4 is present in SN DA neurons in the human and rat brain. Notably, knocking down PAK4 increased susceptibility of rat DA neurons to 6-OHDA–induced neurotoxicity. In this context, notably, PAK4 expression and its activity declined in the human midbrain during aging (Fig. 1, C to E). This decline might induce a pre-parkinsonian state (a PD-prone state), which could be easily converted to PD by chronic exposure to unidentified environmental factors (*44*). Decreased PAK4 activity thus could explain not only the selective vulnerability of DA neurons but also why aging is the greatest risk factor for PD (*34*–*36*, *45*). In light of the chronic and progressive nature of PD in human patients, a decrease in PAK4 activity might occur early in PD progression, and reduced PAK4 activity may contribute to PD pathogenesis.

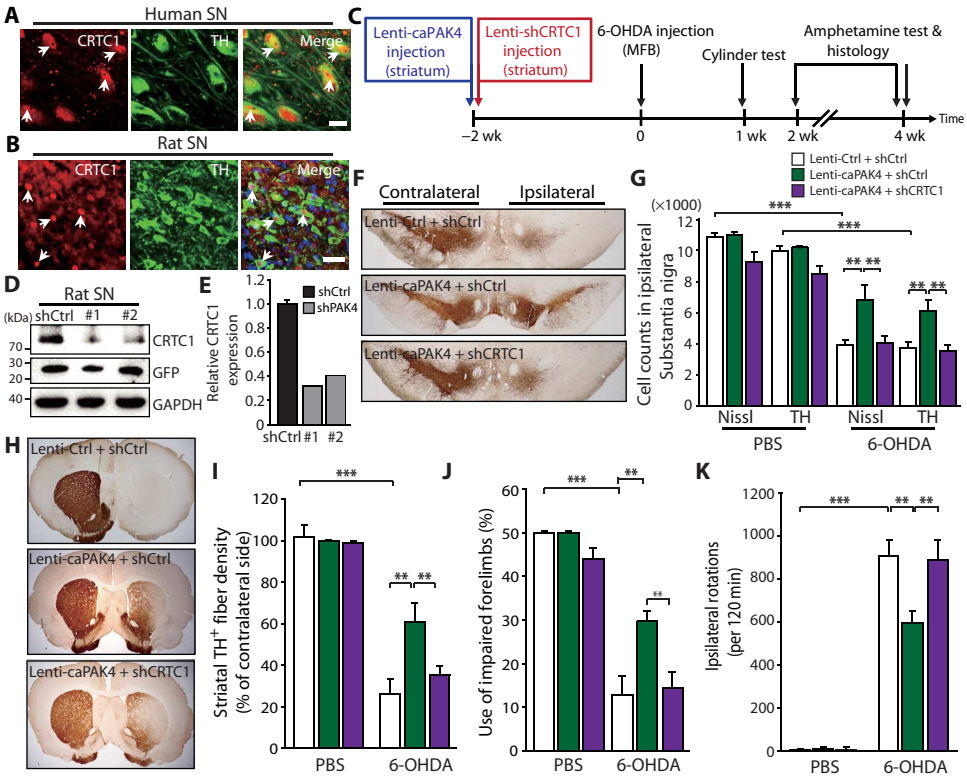

Fig. 6. CRTC1 is required for PAK4-mediated neuroprotection. (**A** and **B**) Fluorescence images of CRTC1 (red) in TH-positive (green) DA neurons in the SN from healthy human (A) and rat (B) brain. White arrows indicate nuclear CRTC1. Scale bars, 50 μm. (**C**) Experimental scheme. (**D**) Immunoblotting for validation of knockdown efficiency in shCRTC1-injected rats. (**E**) Quantification of the blot shown in (D) normalized to GAPDH. (**F**) Representative images of TH staining in the SN of rats coinjected with lenti-caPAK4 and either shCtrl or shCRTC1 followed by 6-OHDA injection. (**G**) Stereological counting of TH-positive and Nissl-stained DA neurons (n = 5 per group). (**H**) Representative images of TH immunostaining in the rat striatum. (**I**) Quantification of TH-positive striatal fiber density (n = 5 per group). (**J**) Quantification of performance in the cylinder test (n = 5 per group). (**K**) Quantification of cumulative amphetamine-induced ipsilateral rotations (n = 5 per group). Error bars, mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA with Student-Newman-Keuls post hoc analysis.

There is strong evidence that mitochondrial dysfunction is linked to PD (*46*). PAK4 inactivates the apoptotic protein BAD (Bcl-2–associated death promoter) by phosphorylating S112 (*40*). This phosphorylation prevents BAD localization in mitochondria, thereby inhibiting apoptosis. Both overexpression of α-synuclein and 6-OHDA treatment inhibited PAK4 activity in parallel with down-regulation of the CREB target proteins Bcl-2 and PGC-1α. Conversely, caPAK4 suppressed down-regulation of Bcl-2, PGC-1α, and BDNF in the 6-OHDA and α-synuclein rat models of PD. Moreover, pPAK4-positive DA neurons in postmortem brain tissue from PD patients stained positive for Bcl-2, but not for TUNEL, suggesting that PAK4 was protecting these neurons through up-regulation of Bcl-2. Considering that the expression of Bcl-2 and PGC-1α is closely related to mitochondrial health (*47*, *48*), PAK4 may protect the mitochondria from noxious stimuli in a transcription-dependent manner. These dual transcription-dependent and transcription-independent activities of PAK4 might explain the mechanisms that prevent mitochondrial dysfunction in the presence of neurotoxic stimuli.

PD patients initially suffer from motor symptoms that result from low striatal dopamine; thus, initial treatments focus on elevating dopamine through administration of drugs such as L-dopa. TH is the rate-limiting enzyme in dopamine synthesis. The gene encoding TH contains a CRE in its promoter (*49*), and its expression is thus regulated by CREB (*50*). Because caPAK4 phosphorylates CRTC1 and activates CREB, it is plausible that caPAK4 could up-regulate dopamine expression. Even in the 6-OHDA rat model of PD, caPAK4 maintained dopamine expression similar to that in control rats (Fig. 3F). α-Synuclein aggregates inhibit PAK4 (*14*) and suppress transactivation of CREB and nuclear factors of activated T cells (*51*). As expected, decreased dopamine was observed in the brain of the α-synuclein rat model of PD (*52*). Whether caPAK4 can restore decreased dopamine in this model remains to be determined. The potential protective effect of PAK4 on mitochondria combined with a potential role in dopamine synthesis may contribute to caPAK4's ability to improve motor dysfunction in PD rat models.

Dephosphorylation-coupled nuclear translocation of CRTC1 is a well-established mechanism for its activation (*23*, *31*). This mechanism involves S151 and S245, whose phosphorylation sequesters CRTC1 in the cytoplasm by promoting its binding to 14-3-3, thus rendering CRTC1 inactive (*23*). In contrast, CRTC1 dephosphorylation at S151 and S245 leads to its nuclear translocation and activation (*23*, *31*). CRTC1 is also activated by Sirt1 in a deacetylation-dependent

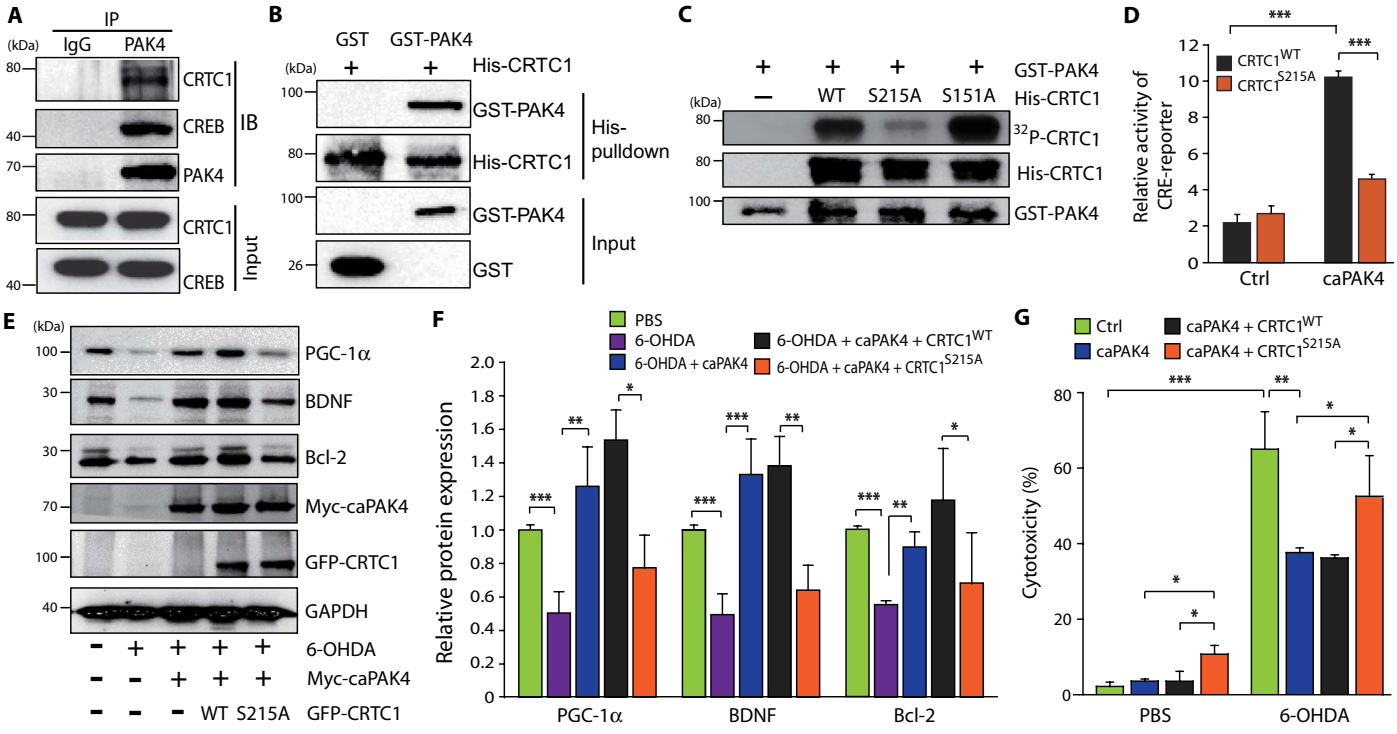

Fig. 7. CRTC1 phosphorylation at S215 mediates PAK4-induced CREB activation. (**A**) Immunoprecipitation (IP) and immunoblotting (IB) for the association of PAK4 with CRTC1 and CREB in the SH-SY5Y neuronal cell line. (**B**) Histidine (His)–pulldown assays were used to assess the direct binding of PAK4 with CRTC1. (**C**) In vitro kinase assay with WT and mutant CRTC1. (**D**) CRE reporter assay in the SH-SY5Y neuronal cell line (n = 3). (**E**) Immunoblotting for the CREB downstream target proteins PGC-1α, BDNF, and Bcl-2 in the SH-SY5Y neuronal cell line (n = 3). (**F**) Quantification of the blots shown in (E) normalized to GAPDH. (**G**) Lactate dehydrogenase activity in SH-SY5Y cells (n = 3). Error bars, mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA with Student-Newman-Keuls post hoc analysis.

manner (*24*). Here, we propose a mechanism for the activation of CRTC1 that involves phosphorylation at S215. PAK4 directly interacts with CRTC1, and pPAK4 is located primarily in the nucleus. Therefore, it is tempting to speculate that the dephosphorylation-induced nuclear import of CRTC1 might be linked to its subsequent phosphorylation at S215. Whether this coupling fully activates CRTC1 as a CREB coactivator remains to be resolved.

Considering the therapeutic potential of caPAK4, its persistent expression in the brain after delivery by gene therapy might provoke concerns about tumorigenesis because PAK4 is regarded as a potent oncogene. However, we did not observe neoplasia in animals in which caPAK4 was expressed, but did not follow the animals long-term. No tumorigenesis might be due to a poor microenvironment for cell proliferation in the midbrain of PD rat models. At the onset of symptoms in human PD, about 50% of DA neurons have already died (*3*, *4*), suggesting that the midbrain would not provide a proliferative environment. Moreover, brain tumors develop mostly from non-neuronal cells such as glial and meningeal cells. Thus, although PAK4 is oncogenic, if an efficient local delivery method guaranteed its targeted application to the midbrain, this could enable prevention of undesirable effects. In this regard, it is notable that lentivirus injection into the striatum delivered caPAK4 in a DA neuron–specific manner, thereby excluding PAK4 expression from microglia and astrocytes. This DA neuron–specific targeting may not only minimize the risk of tumorigenesis but also provide an opportunity for therapeutic intervention. We can also consider the active form of CRTC1$^{S151A/S215D}$ as a second target for modulation of PAK4 signaling. CRTC1 may be less potent than caPAK4 because CRTC1 has a more restricted role than caPAK4, but may have the advantage of enhancing specificity of CREB transcriptional activity with a reduced risk of tumorigenesis.

Group I PAKs play vital roles in X-linked mental retardation (*9*, *53*), fragile X syndrome (FXS) (*54*), and neurodegenerative diseases such as Alzheimer's disease (*55*) and Huntington's disease (*7*, *8*). Successful rescue of abnormal behavior in FXS model mice by FRAX485, a small-molecule inhibitor of group I PAKs, validated a proof of concept for the use of PAKs as therapeutic targets (*56*). A recent study implicated PAK6 in PD by demonstrating its function as a downstream regulator of LRRK2 (*57*); mutations in the LRRK2 gene frequently cause PD. Here, we have demonstrated a physiological role for PAK4 as an endogenous neuroprotective kinase. Together with previous studies (*7*, *9*, *53*–*55*), our findings suggest dysregulation of PAKs as a pathogenic mechanism in diverse neurological disorders. Therefore, development of new and more selective interventions targeting PAKs may hold promise as a therapeutic strategy

This study demonstrates a potential neuroprotective role for PAK4 in PD animal models. A limitation of this study is the short-term observations of the beneficial effects of caPAK4. A further limitation is the small number of human postmortem brain samples due to limited availability. We have demonstrated the importance of CRTC1 as a mediator of caPAK4, but the mechanism for pCRTC1^{S215}-induced CREB activation remains unclear. A better understanding of the PAK4 neuroprotective pathway will be needed before PAK4 can be exploited therapeutically to slow neurodegeneration in PD.

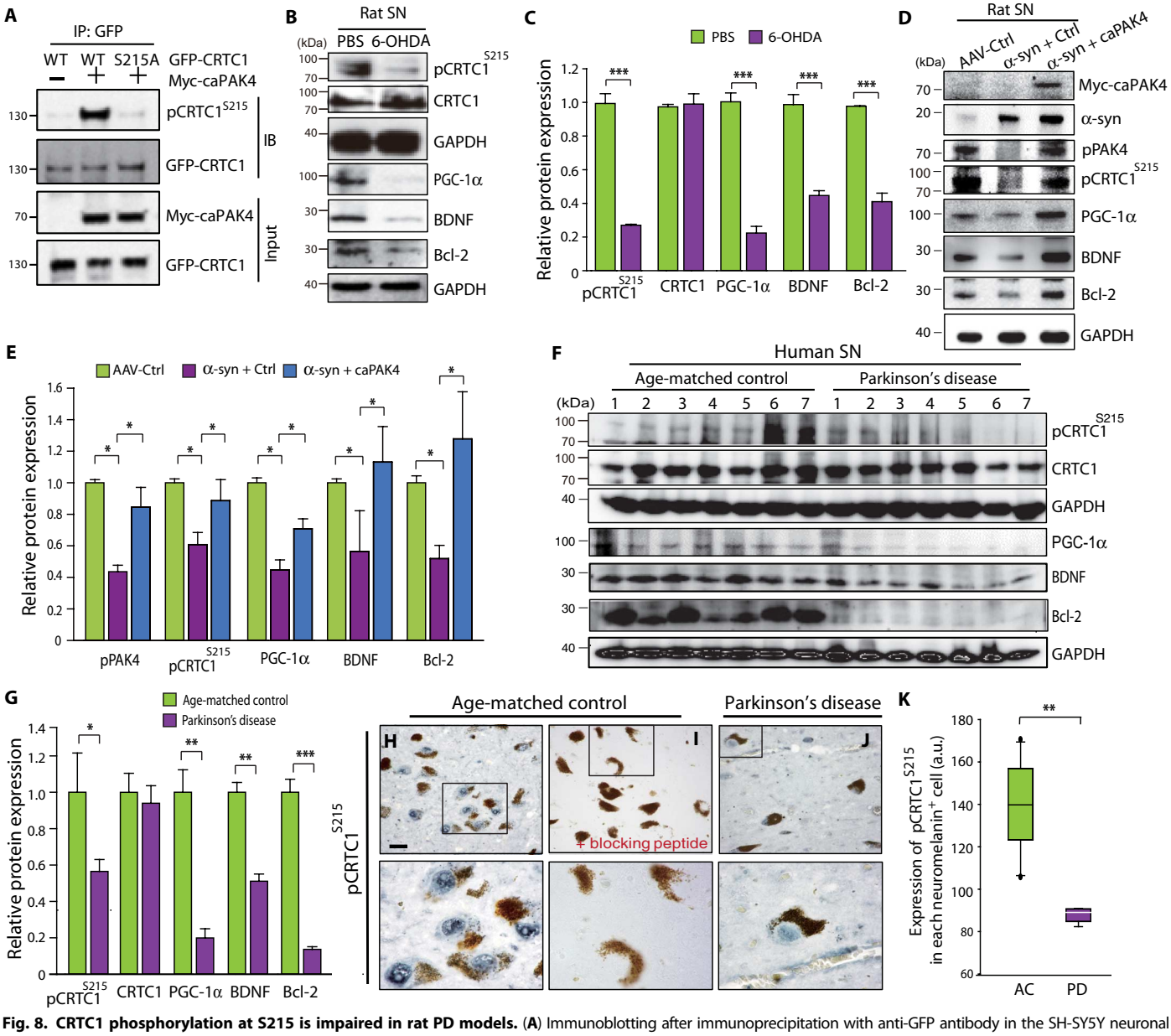

Fig. 8. CRTC1 phosphorylation at S215 is impaired in rat PD models. (**A**) Immunoblotting after immunoprecipitation with anti-GFP antibody in the SH-SY5Y neuronal cell line. (**B**, **D**, and **F**) Immunoblotting for the indicated proteins in SN lysates from rat PD models (B and D) and from postmortem brain tissue from PD patients ($n = 7$) and age-matched controls (AC) ($n = 7$) (F). (**C**, **E**, and **G**) Quantification of the blots shown in (B), (D), and (F), respectively, normalized to GAPDH. (**H** to **K**) Immunohistochemical analysis of pCRTC1^{S215} in the human SN: AC (H and I) and PD patients (J). Boxed areas on the top panel are shown at a higher magnification on the bottom panel. (I) Effect of preincubation of the pCRTC1^{S215} antibody with an excess of the blocking phosphopeptide used for immunization. Scale bar, 25 μm. (K) Quantification of pCRTC1^{S215} signal intensity in each neuromelanin-positive DA neuron. Neuromelanin-positive cells were analyzed in control (cell number, 82) and PD (cell number, 27) human brain tissue samples. Error bars, mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ANOVA with Student-Newman-Keuls post hoc analysis (E) or Student's t test (C, G, and K).

MATERIALS AND METHODS

Study design

Previous studies demonstrated that PAK4 increases the activity of CREB (*32*), which promotes neuronal survival (*24*, *28*). The primary objective of the present study was to investigate the neuroprotective effect of PAK4 in PD. To test this effect, we stereotactically injected PD rats with lenti-GFP-shPAK4 for the loss-of-function study or lenti-GFP-caPAK4 for the gain-of-function study and analyzed their motor functions. Tissue samples from patients with PD were obtained from the Victoria Brain Bank Network (VBBN). Experiments were carried out in accordance with a protocol approved by the Ethics Review Committee of the Institutional Review Board of Chungbuk National University (approval number: CBNU-IRB-2011-T01). Animals were allocated to experimental and control groups by random numbers generated by MS Excel. No data were included or excluded from the analysis. The investigators carrying out a behavior test, biochemical analyses, and quantitative comparisons were blinded to the information about treatment

groups. Patient information is provided in table S2. The number of experimental replicates is indicated in each figure legend. Additional Materials and Methods are available in the Supplementary Materials.

Experimental animals

The rats were obtained from Taconic. Female Sprague-Dawley rats, 260 to 280 g at the time of surgery, were housed two to three per cage with ad libitum access to water and food during a 12-hour light/dark cycle. All reasonable efforts were made to minimize animal suffering and to use the minimum number of animals necessary to perform statistically valid analyses. All experiments were performed in accordance with approved animal protocols and the guidelines established by the Ethics Review Committee of Chungbuk National University for Animal Experiments (approval number: CBNUA-400-12-02).

Human postmortem brain tissue

Human tissue was obtained from the VBBN. Experiments were carried out in accordance with a protocol approved by the Ethics Review Committee of the Institutional Review Board of Chungbuk National University (approval number: CBNU-IRB-2011-T01). For immunohistochemistry, we studied samples from seven PD patients, six age-matched controls, and four young controls. For immunoblotting analysis, we analyzed specimens from seven PD patients and seven controls (table S1). PD was diagnosed using neuropathological criteria in conjunction with the clinical history (*58*). The age-matched controls tissue used was from patients in whom there was no neuropathological evidence of PD or any other neurodegenerative disease or any other significant pathology on neuropathologic examination, which included macroscopic and microscopic analysis.

Lentivirus injection

Lentivirus particles can be transported to the SN from the striatum by retrograde transport (*59*, *60*). Thus, the stereotactic injection site was the striatum [anteroposterior (AP), 1.0 mm; mediolateral (ML), −3.0 mm; and dorsoventricular (DV), −5.0 mm from bregma] (*61*). In a single unilateral injection, 5 μl of lentivirus [shPAK4, 1×10^8 transducing units (TU)/ml; shCRTC1, 1.5×10^8 TU/ml; and caPAK4, 1.5×10^8 TU/ml] was delivered at a constant rate (0.25 μl/min) using a Hamilton removable needle (RN) syringe (33-gauge needle) with a Neuro adapter attached to a syringe pump (K. D. Scientific). A lentivirus expressing GFP alone was used as the control. AAV2-WT human α-synuclein and AAV2-GFP were provided by the University of North Carolina Vector Core. The AAV2 viruses used here are driven by a chicken β-actin promoter. Adenoviral vectors were stereotactically injected into the right SN. The injection site coordinates in the right SN were AP −4.3 mm, ML −2.0 mm, and DV −8.0 mm from bregma (*61*). In a single unilateral injection, 4 μl of AAV2 [AAV2-α-syn, 1.5×10^{13} viral genomes (vg)/ml; AAV2-GFP, 8.1×10^{12} vg/ml] was delivered at a constant rate (0.25 μl/min) using Hamilton RN syringe (33-gauge needle) with a Neuro adapter attached to a syringe pump. After the injection, the needle was maintained in place for an additional 5 min to prevent retrograde flow along the needle track.

Statistical and image analysis

ImageJ was used to quantify the intensity of the immunofluorescence signals and band densities from immunoblots. The data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (version 11.0). Statistical significance was assessed using an unpaired two-tailed Student's *t* test or an ANOVA with Student-Newman-Keuls post hoc analysis. Quantitative data are presented as means ± SEM. Differences were considered significant at $P < 0.05$.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Confirmation of the specificity of the anti-pPAK4 antibody.

Fig. S2. Immunohistochemical analysis of PAK5 and PAK6 in nigral dopamine neurons.

Fig. S3.Representative images of costaining for pPAK4/Bcl-2 and pPAK4/TUNEL in the brains of PD patients.

Fig. S4. Monitoring lentivirus-mediated delivery of PAK4 shRNA into the rat SN.

Fig. S5. Exclusion of the off-target effects of rat PAK4 shRNA.

Fig. S6. PF-3758309 sensitizes rats to 6-OHDA–induced neurotoxicity.

Fig. S7. caPAK4 and WT PAK4 protect SH-SY5Y cells against 6-OHDA–induced toxicity.

Fig. S8. Monitoring lentivirus-mediated delivery of caPAK4 into the rat SN.

Fig. S9. Monitoring the expression of human α-synuclein in the rat striatum and SN.

Fig. S10. caPAK4 requires CREB and CRTC1 for neuroprotection.

Fig. S11. Identification of S215 of CRTC1 as a major phosphorylation site.

Table S1. Summary of parameters for experimental groups.

Table S2. Immunohistochemical analysis of neuromelanin-positive cells in human PD.

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Nigral dopaminergic PAK4 prevents neurodegeneration in rat models of Parkinson's disease

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Editor's Summary

PAKing a punch in Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is caused by the loss of dopaminergic (DA) neurons in the substantia nigra. The resulting decrease in dopamine leads to resting tremor and rigidity in patients with this disease. PAK4 (p21-activated kinase 4) is known to play a key role in neuronal survival. Now, Won *et al*. show that constitutively active PAK4 S445N/S474E protects DA neurons from neurodegeneration in two rodent models of PD. The neuroprotective effect of constitutively active PAK4 required CREB and its brain-specific coactivator CRTC1. These findings may provide an avenue for developing neuroprotective interventions for PD.

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