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Original Article

Experimental Neurobiology

Abnormal Mitochondria in a Non-human Primate Model of MPTP-induced Parkinson's Disease: Drp1 and CDK5/p25 Signaling

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Mitochondria continuously fuse and divide to maintain homeostasis. An impairment in the balance between the fusion and fission processes can trigger mitochondrial dysfunction. Accumulating evidence suggests that mitochondrial dysfunction is related to neurodegenerative diseases such as Parkinson's disease (PD), with excessive mitochondrial fission in dopaminergic neurons being one of the pathological mechanisms of PD. Here, we investigated the balance between mitochondrial fusion and fission in the substantia nigra of a non-human primate model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD. We found that MPTP induced shorter and abnormally distributed mitochondria. This phenomenon was accompanied by the activation of dynamin-related protein 1 (Drp1), a mitochondrial fission protein, through increased phosphorylation at S616. Thereafter, we assessed for activation of the components of the cyclin-dependent kinase 5 (CDK5) and extracellular signal-regulated kinase (ERK) signaling cascades, which are known regulators of Drp1(S616) phosphorylation. MPTP induced an increase in p25 and p35, which are required for CDK5 activation. Together, these findings suggest that the phosphorylation of Drp1(S616) by CDK5 is involved in mitochondrial fission in the substantia nigra of a non-human primate model of MPTP-induced PD.

Key words: Cyclin-dependent kinases, Mitochondria, Mitochondrial dynamics, Non-human primate, Parkinson disease

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INTRODUCTION

Parkinson's disease (PD) is the most common age-related neurodegenerative disease affecting motor control. Clinically, it is characterized by four cardinal signs: rigidity, bradykinesia, resting tremor, and postural instability. The motor symptoms are accom-

panied by dopaminergic neuron degeneration in the substantia nigra pars compacta, leading to a dopamine deficit in the striatum, including the caudate and putamen [1, 2]. The causes of PD pathogenesis are complex, with various contributors, such as genetic susceptibility and environmental factors. Recently, accumulating evidence has suggested a link between PD pathogenesis and mitochondrial dysfunction [3, 4].

Mitochondria are the main subcellular organelles responsible for production of adenosine triphosphate (ATP) and regulation of metabolite synthesis, intracellular calcium homeostasis, and programmed cell death. In particular, the central nervous system (CNS) has a high demand for mitochondrial ATP as an energy source to maintain ionic gradients across the axonal membrane, a process that is essential for neurotransmission [5, 6]. Mitochondria are highly dynamic; they continuously undergo fission, which is regulated by Drp1 and Fis1, and fusion, which is regulated by Mfn1, Mfn2, and Opa1 [7-9]. The balance between mitochondrial fission and fusion significantly affects the role of mitochondria in the maintenance of cellular process [7, 8, 10]. Excessive mitochondrial fission triggers mitochondrial fragmentation and dysfunction, subsequently leading to a reduction in the mitochondrial membrane potential, depletion of ATP, accumulation of reactive oxygen species (ROS), and release of apoptotic factors [11, 12]. In view of this, abnormal mitochondrial dynamics is also thought to be involved in various neurodegenerative diseases, including PD [13, 14]. Indeed, a change in Drp1 activity has been implicated in various neurodegenerative disorders [15, 16]. Drp1-dependent mitochondrial morphology and distribution are key factors in modulating mitochondrial homeostasis in dopaminergic neurons in models of PD [17, 18]. Drp1 activity is controlled by post-translational modifications, including phosphorylation [19]. Specifically, phosphorylation of a serine residue, S616, results in increased Drp1 activity, reflecting variant pathological processes [20, 21]. However, more information is needed on the precise relationship between abnormal mitochondrial dynamics and the causative factors of PD.

CDK5 is a proline-directed serine-threonine kinase that is mainly expressed in post-mitotic neurons [22, 23]. CDK5 activity is mainly controlled by neuron-specific activators, p35 and p39, which are activated after being cleaved into p25 and p29, resulting in CDK5 hyperactivity [24, 25]. CDK5 plays an important role in the regulation of CNS development and synaptic plasticity [26, 27]. However, inappropriate activation of CDK5 plays an early role in the cell death cascade, even before the initiation of mitochondrial dysfunction, and CDK5 inhibition prevents mitochondrial damage and cell death in a model of PD [28-30]. Interestingly, CDK5 modulates mitochondrial morphology during neuronal apoptosis as an upstream signaling kinase [31, 32]. Furthermore, CDK5-mediated phosphorylation of Drp1 is related to mitochondrial morphology control during neuronal injury [33]. However, the mechanisms via which CDK5 regulates mitochondrial fission by phosphorylation of Drp1 at S616 during dopaminergic neuronal loss are still not completely understood.

The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can trigger parkinsonism in non-human primates, and has been used extensively in experimental models of PD [34-36]. However, it is difficult to develop macaque models of MPTP-induced chronic parkinsonism owing to symptomatic variation. To induce a stable non-human primate PD model, adjustments of MPTP administration at an individual-level are required according to the severity of behavioral symptoms [37]. Recently, we established and verified a primate model of chronic stable PD by repeated low-dose MPTP administration based on automatic quantification of individual global activity in cynomolgus monkeys (*Macaca fascicularis*) [38]. In our MPTP-treated monkeys, parkinsonian symptoms and decreased dopamine transporter activity persisted until 1 year. Dopaminergic neuronal cell death was confirmed by immunohistochemistry and western blotting [38]. Although the clinical features in human chronic PD patients can be observed in this model, further investigation is needed to support its use for chronic PD research and drug discovery. In the present study, we investigated pathological alterations and molecular mechanisms of mitochondrial dynamics in the substantia nigra of MPTP-treated cynomolgus monkeys at 1 year after the first MPTP administration.

MATERIALS AND METHODS

Animals

All experimental animals were derived from our previous study [38]. Briefly, four female adult cynomolgus monkeys were obtained from the Zhaoqing Laboratory Animal Research Centre (Guangdong Province, China). They were maintained in individual indoor cages (60×80×80 cm) at the National Primate Research Center of the Korea Research Institute of Bioscience and Biotechnology (KRIBB) at a temperature of 24±2℃, a relative humidity of 50±5%, and under a 12-h light/12-h dark cycle. The monkeys were able to have visual contact and voice interaction with neighbors but no physical contact (to avoid aggression), as described previously [39, 40]. The dimensions of the cages met that provided by the guidelines of the USA National Institutes of Health. The monkeys were fed commercial monkey chow (Harlan Teklad, Indianapolis, IN, USA) supplemented with various fruits and were given water ad libitum. They were also given various rubber and

plastic toys and fruits as environmental enrichment. The attending veterinarian monitored the monkeys' health in accordance with the recommendations of the Weatherall report on the use of non-human primates in research [41]. They were also monitored through a once yearly administration of microbiological tests for B virus, simian retrovirus, simian immunodeficiency virus, simian virus 40, and simian T-cell lymphotropic virus. All procedures were approved by the KRIBB Institutional Animal Care and Use Committee (Approval No. KRIBB-AEC-16068). All animal experiments complied with the ARRIVE guidelines [42].

MPTP administration

MPTP (0.2 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline to a final concentration of 2 mg/mL and intramuscularly injected into the left femoral region of the cynomolgus monkeys daily, from Monday to Friday each week, as described previously [38]. The total number of MPTP injections were commensurate with each individual animal's global activity intensity. The stop point thresholds for MPTP administration were indicated by a global activity intensity lower than 8% (arbitrary) of baseline intensity.

Tissue preparation

Four monkeys were transcardially perfused with 400 mL of 100 mM phosphate-buffered solution (PBS) under deep anesthesia induced by an intramuscular injection of ketamine (1 mg/kg) at 48 weeks following the first MPTP administration. Whole brains were removed from the skull, washed in cold PBS, and bilaterally separated. For immunohistochemical staining, the left hemispheres were post-fixed with 4% paraformaldehyde and incubated in 30% sucrose solution at 4℃.

Western blot analysis

The tissues were harvested from the substantia nigra of the monkey brains using punches on 4-mm-thick slices, snap-frozen, and stored at -80℃. Whole protein lysates of the substantia nigra were prepared using the PRO-PREP protein extraction solution (Intron Biotechnology, Seongnam, Korea). Equal amounts of proteins were separated by electrophoresis on 10~15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes (BD Biosciences, Franklin Lakes, NJ, USA). The membranes were blocked using incubation in blocking buffer (BD Biosciences) and primarily blotted with primary antibodies against anti-TH (MAB318; Merck Millipore, Darmstadt, Germany), anti-GFAP (AB5804), anti-β-actin (A5316; Sigma-Aldrich, St. Louis, MO, USA), anti-Iba-1 (ab108539) anti-Mfn1 (ab57602; Abcam, Cambridge, MA, USA), anti-Drp1 (#8570), anti-phospho(p)-Drp1 (#3455), anti-Mfn2 (#9482), anti-Opa1 (#67589), anti-CDK5 (#2506), anti-ERK (#9102), anti-p-ERK (#9101; Cell Signaling, Danvers, MA, USA), anti-Fis1 (PA1-41082), and anti-p35 (MA5-14834; Thermo Scientific, Waltham, MA, USA) antibodies at 4℃ overnight. The membranes were washed with 10 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween-20 (TBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) for 1 h at room temperature. After the removal of excess antibodies by washing with TBST, specific binding was detected using a chemiluminescence detection system (Thermo Scientific) according to the manufacturer's instructions.

Immunohistochemistry and mitochondrial imaging

The left hemispheres of the brains were sectioned in the coronal plane at 30 μm of thickness using a cryostat (Leica Biosystems, Wetzlar, Germany). For blocking, 30-μm free-floating tissue sections were incubated with 4% normal horse serum (S-2000; Vector Laboratories, Burlingame, CA, USA) in 0.3% Triton X-100 for 2 h at room temperature. For immunohistochemistry and immunofluorescent staining, the tissue sections were incubated with anti-TH (AB152; Merck Millipore), anti-GFAP (AB5804; Sigma-Aldrich), anti-Iba-1 (ab108539; Abcam), and anti-TOM20 (#42406; Cell Signaling) antibodies at 4℃ overnight. The appropriate secondary antibodies (Vector Laboratories and Thermo Scientific) were incubated for 2 h at room temperature to allow binding to the primary antibody. Immunohistochemistry staining was visualized using the ABC method (Vector Laboratories) with 3, 3′-diaminobenzidine as the peroxidase substrate. The tissue sections were observed using the Precipoint M8 digital microscope (PreciPoint, Freising, Germany). Fluorescent images were acquired using the LSM-710 confocal microscope (Carl Zeiss, Jena, Germany). Measurement of mitochondrial length was performed as described previously [43].

Statistical analysis

The data represent the mean and standard deviation (SD) from three independent experiments (n=3). Experimental differences were tested for statistical significance using two-way analysis of variance (ANOVA) using GraphPad Prism 5 software (San Diego, CA, USA). A p-value <0.05 was deemed to be statistically significant and is indicated on graphs by an asterisk; p-values <0.01 and <0.001 are indicated by two and three asterisks, respectively.

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RESULTS

Loss of dopaminergic neurons in the basal ganglia region of monkeys with MPTP-induced PD

We previously developed a model of chronic PD in non-human primates using a novel strategy of MPTP administration that was based on global activity evaluation in individual cynomolgus monkeys [38]. In this model, we first confirmed damage of dopaminergic neurons in the basal ganglia region of the monkey brain by determining the protein level of tyrosine hydroxylase (TH), a

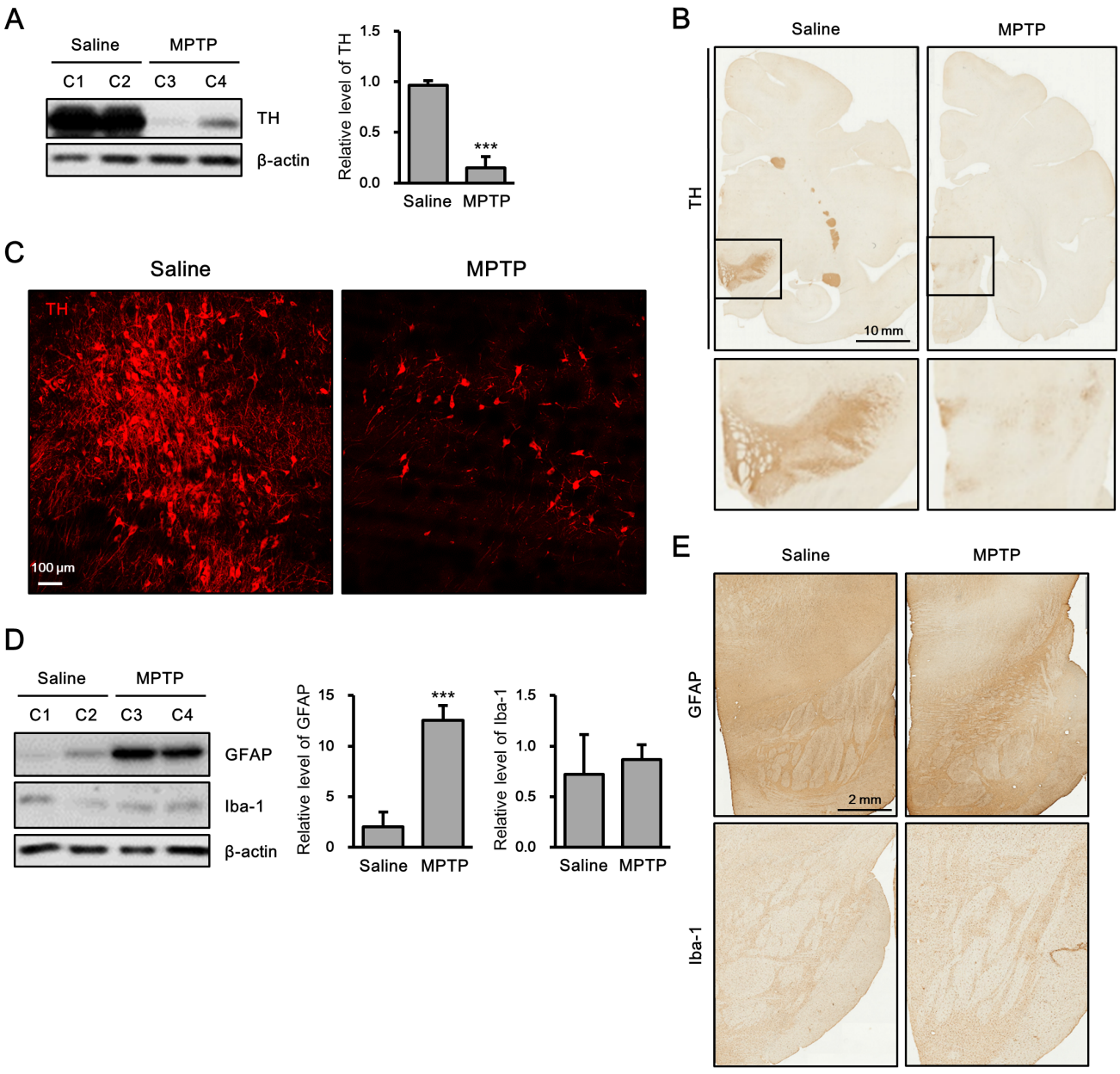

Fig. 1. Effect of MPTP on dopaminergic neuronal loss and neuroinflammation in the substantia nigra of cynomolgus monkeys. (A) TH protein expression level in the substantia nigra of saline- or MPTP-injected cynomolgus monkeys was determined by using western blotting. (B) Immunohistochemistry staining of TH-positive neurons in the cynomolgus monkey brain injected with saline or MPTP was performed using anti-TH antibody. The bottom panels show magnified images of the substantia nigra pars compacta (SNpc) region indicated by the black squares in the top panels; scale bars=10 mm. (C) Fluorescent imaging results of TH proteins were validated using anti-TH antibody in the substantia nigra of saline- or MPTP-injected cynomolgus monkeys; scale bars=100 μm. (D) GFAP and Iba-1 protein expression in the substantia nigra of saline- or MPTP-injected cynomolgus monkeys was confirmed using western blotting. (E) Expression of GFPA and Iba-1 proteins were identified in the SNpc region of saline- or MPTP-injected cynomolgus monkeys using immunohistochemistry; scale bars=2 μm. C1 and C2 indicate the saline-injected group, and C3 and C4 indicate the MPTP-injected group. The data are presented as mean values±SD (n=2). ***denotes p<0.001.

marker of dopaminergic neurons, using immunoblotting. Our results showed that the protein level of TH was dramatically reduced in the substantia nigra than in the saline group (Fig. 1A, 1B, and 1C). We also investigated neuroinflammation, an important physiological alteration in PD, by determining the protein level of GFAP (a marker of astrocytes) and Iba-1 (a marker of microglia), as described in our earlier study [39]. Our results indicated that the protein level of GFAP in the substantia nigra was higher in the MPTP group than in the saline group, whereas there was no significant difference in Iba-1 between the two groups (Fig. 1D and 1E). Altogether, we demonstrated that our MPTP-induced PD model successfully reflected dopaminergic neuronal loss and neuroinflammation in the substantia nigra.

MPTP-induced increase of abnormal mitochondria in the substantia nigra

Abnormal mitochondrial dynamics significantly affect dopaminergic neuronal loss in patients with PD [3]. Therefore, we first observed dopaminergic mitochondrial morphology by immunohistochemistry for TOM20, a mitochondria outer membrane protein and a marker of mitochondria, co-stained with TH. Our observation indicated that the mitochondria of the dopaminergic neurons in the substantia nigra contained a high number of interconnected structures and were widely distributed throughout the whole cell, including the perinuclear and synaptic regions in the saline group. On the other hand, the number of mitochondria in the MPTP group was markedly reduced; moreover, mitochondria

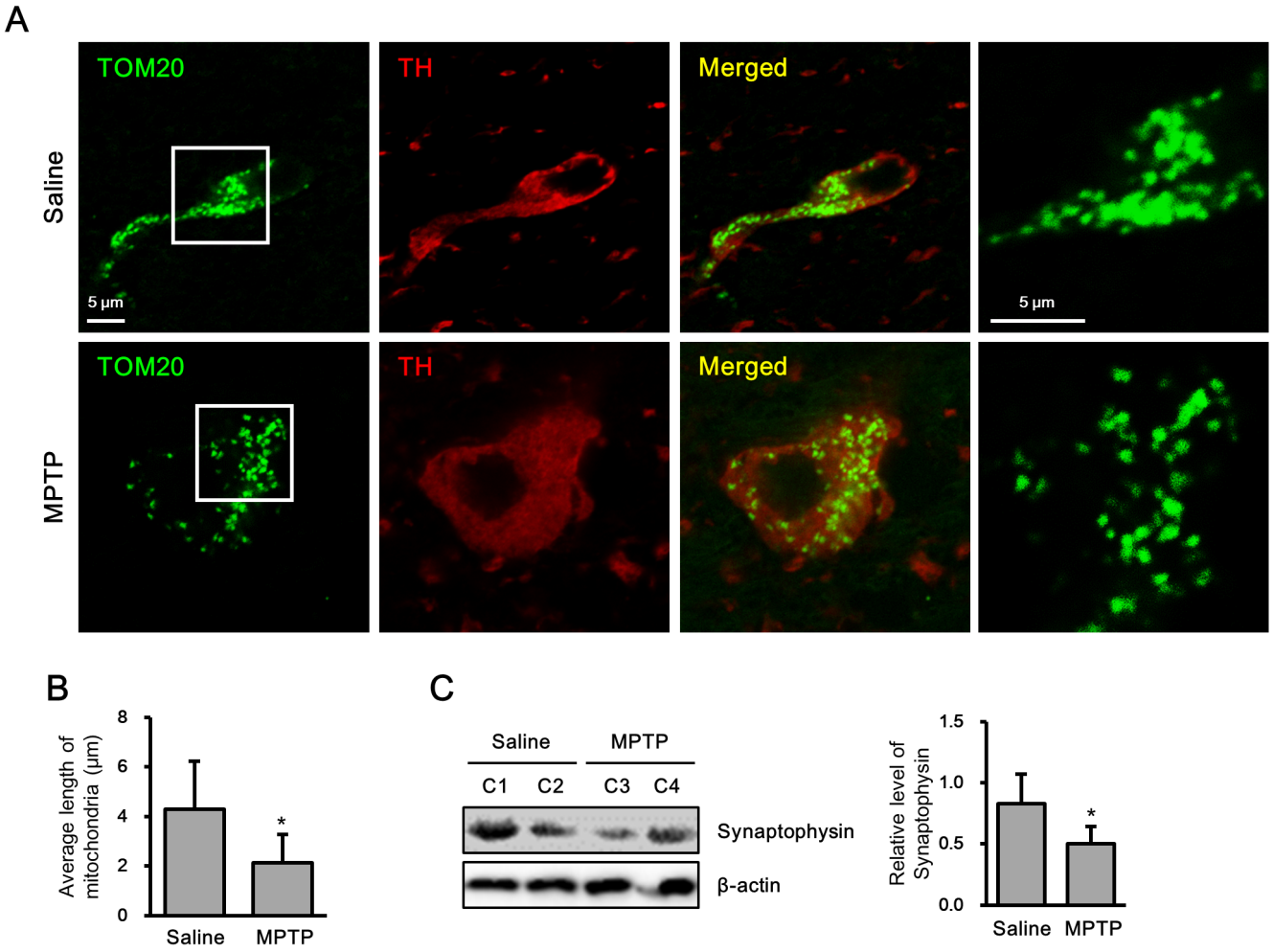

Fig. 2. Effect of MPTP on mitochondrial morphology and synaptic function in the substantia nigra. (A) Mitochondrial morphology in the substantia nigra of saline- or MPTP-injected cynomolgus monkeys was observed using immunofluorescent staining with anti-TOM20 and anti-TH antibodies. The right end panels show magnified images of the regions indicated by white squares in the left end panels; scale bars=5 μm. (B) The graph shows the average mitochondrial length in the substantia nigra of saline- and MPTP-injected cynomolgus monkeys. (C) The expression of synaptophysin, a presynaptic marker, was determined using western blotting with anti-synaptophysin antibody. The data are presented as mean values±SD (n=2). *denotes $p<0.05$.

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were distributed around the nuclear region in a punctate manner (Fig. 2A). The average length of mitochondria in the MPTP group was significantly shorter than that in the saline group (Fig. 2B). Moreover, the neurite structure in the saline-injected group was more developed than that in the MPTP-injected group. Therefore, we determined the protein level of synaptophysin, a pre-synapse marker, using immunoblotting to verify its possible decrease induced by MPTP in the substantia nigra. We noted that synaptophysin levels was decreased after MPTP than after saline injection (Fig. 2C). Our findings indicated that MPTP induced abnormal mitochondrial morphology and distribution in the substantia nigra of the monkey brain.

MPTP-induced phosphorylation-mediated Drp1 activation in the substantia nigra

Drp1-mediated control of mitochondrial morphology and distribution is crucial for modulating dopaminergic neurons in models of PD [17]. Thus, we assessed the mitochondrial fission and fusion proteins, including the phosphorylation level of Drp1(S616), using immunoblotting. Our results showed that phosphorylation of Drp1(S616) was markedly increased by MPTP injection, with no change in the expression level of the mitochondrial fission proteins, Drp1 and Fis1 (Fig. 3A). The expression of mitochondrial fusion proteins, Mfn1, Mfn2, and Opa1 were not significantly changed by MPTP (Fig. 3B). Although the expression of mitochondrial fusion proteins was independent of MPTP, there were differences among individuals. Taken together, our data suggested that abnormal mitochondrial phenotype in the substantia nigra of MPTP-injected monkeys was accompanied by an increase in Drp1(S616) phosphorylation.

MPTP-induced activation of the CDK5/p35 signaling pathway in the substantia nigra

Drp1-mediated excessive mitochondrial fission was mainly induced by increased Drp1 phosphorylation. Drp1 can be phosphorylated by various kinases, such as CDK5 and ERK [44, 45]. Therefore, we confirmed the activity of kinases upstream of Drp1(S616) phosphorylation using immunoblotting. Our data showed that the protein level of CDK5 was unchanged, but those of p35 and p25, the neuron-specific activators of CDK5, were increased in the substantia nigra of the MPTP group (Fig. 4A). In contrast, other upstream kinases of Drp1(S616) phosphorylation, ERK, were not different between the two groups (Fig. 4B). ERK phosphorylation level was lower in the MPTP group than in the

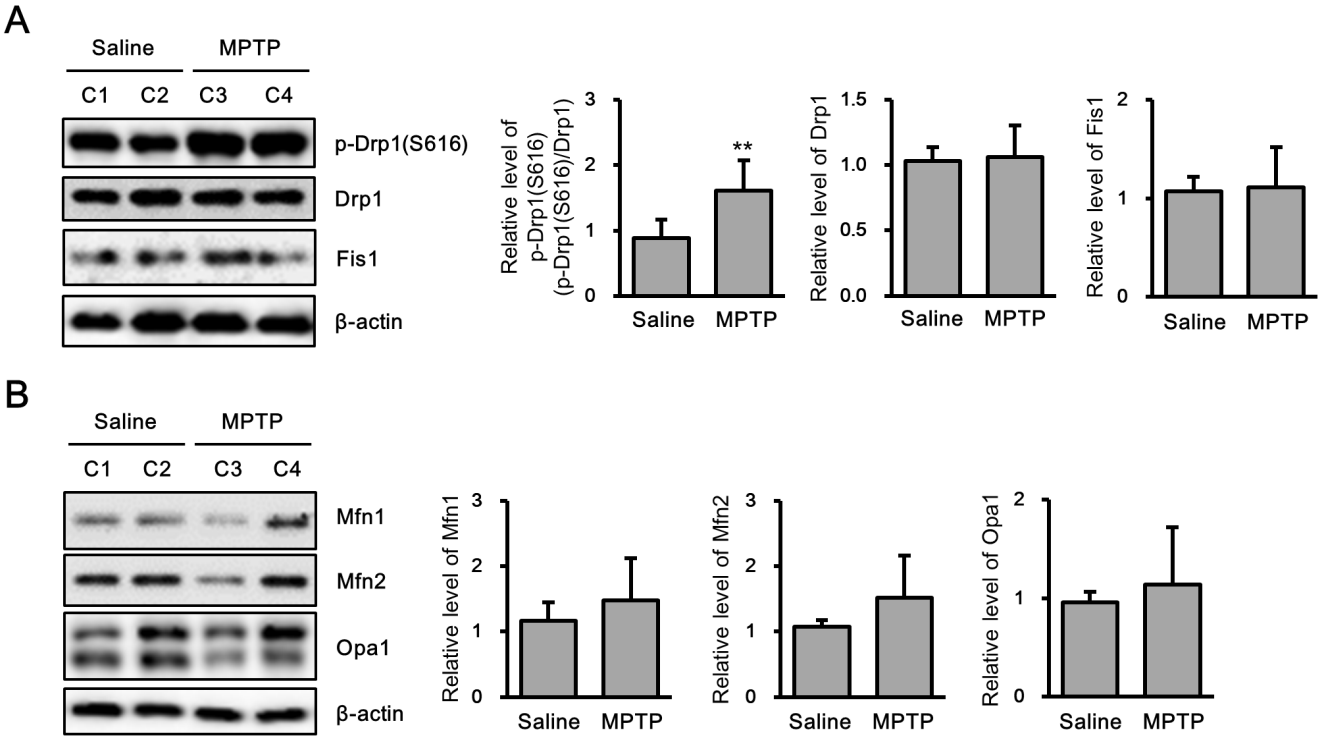

Fig. 3. Expression level of proteins involved in mitochondrial dynamics. (A) The levels of the mitochondrial fission proteins, p-Drp1(S616), Drp1, and Fis1, and (B) the mitochondrial fusion proteins, Mfn1, Mfn2, and Opa1, in the substantia nigra of saline- or MPTP-injected cynomolgus monkeys were identified using western blot analysis. Drp1 was used as the loading control for p-Drp1(S616). The data are presented as mean values±SD (n=2). **denotes p<0.01.

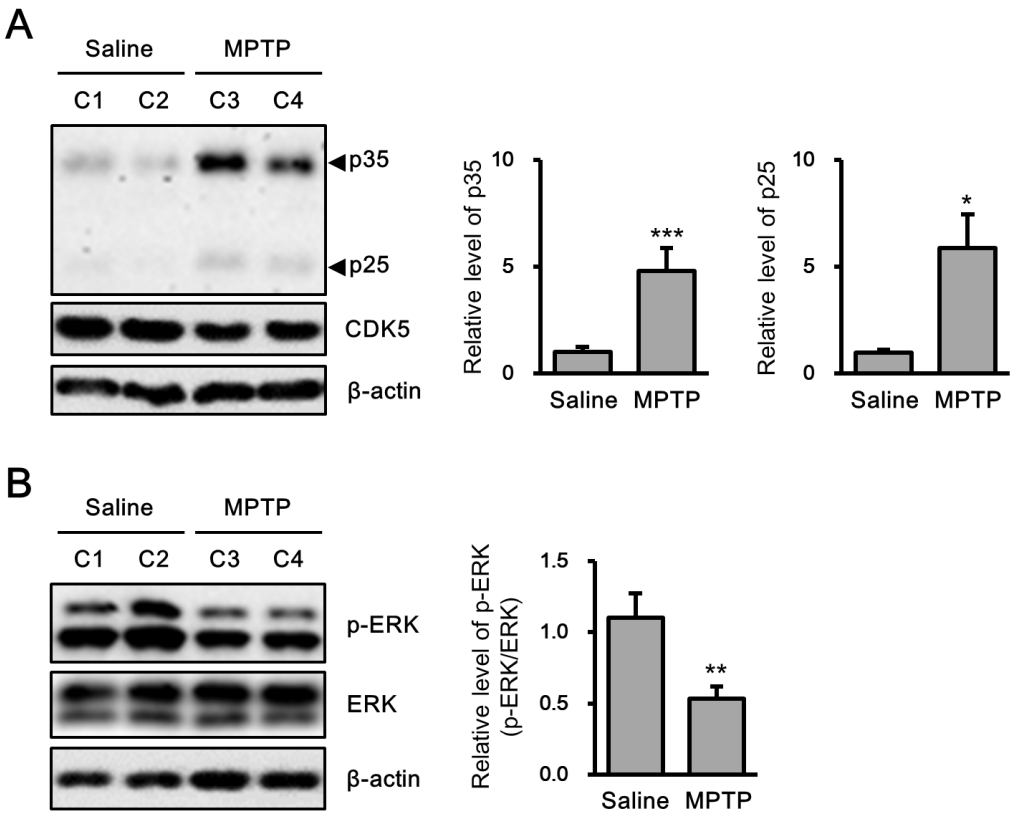

Fig. 4. The CDK5/p25 signaling pathway in the substantia nigra of MPTP-injected cynomolgus monkeys. (A) The protein levels of CDK5, p35, and p25, and (B) p-ERK in the substantia nigra of saline- and MPTP-injected cynomolgus monkeys were determined using western blotting. ERK was used as the loading control for p-ERK. The data are presented as mean values±SD (n=2). *denotes $p<0.05$, **denotes $p<0.01$, and ***denotes $p<0.001$.

saline group. These results suggested that Drp1-mediated abnormal mitochondrial morphology involved CDK5 activation via elevated p35 and p25 levels.

DISCUSSION

Mitochondria are important organelles in PD, and dopaminergic neurons appear to be particularly sensitive to mitochondrial dysfunction. One of the possible reasons for such vulnerability is the lower basal level of mitochondria in dopaminergic neurons than in other midbrain neurons [46, 47]. Therefore, emphasis has been placed on maintaining mitochondrial function in dopaminergic neurons. The homeostasis of mitochondrial dynamics is not only associated with the maintenance of mitochondrial function, but also with an imbalance between mitochondrial fission and fusion, which can trigger dopaminergic neuronal loss [14, 15, 48]. However, little is known regarding the molecular mechanisms underlying the mitochondrial dynamics in PD.

MPTP has been commonly used to induce stable PD in non-human primates, with bilateral clinical features closely resembling idiopathic PD [35]. Therefore, we investigated the mechanisms of mitochondria dynamics in a non-human primate model of MPTP-induced PD. First, we confirmed the loss of dopaminergic neurons and an increase of neuroinflammation in the basal ganglia region of cynomolgus monkeys injected with MPTP using our own strategy based on global activity evaluation [38]. In this model of PD, mitochondrial fission as well as unusual mitochondrial distribution were observed in the MPTP group. In MPTP-injected monkeys, mitochondria were located closer to the nucleus than was observed in the saline group. Mitochondrial distribution within the regions of high energy demand is critical for various functions, and impaired mitochondrial transport and distribution have been linked to abnormal neuronal synaptic functions as in PD [6, 49-53]. In addition, we found a decrease in the protein level of synaptophysin, a marker of synaptic number and function. Accordingly, we showed that mitochondrial distribution and synaptic function were disrupted in our experimental model. These findings were consistent with those of earlier studies, which showed loss of dopaminergic synapses followed by substantia nigra cell bodies in mice treated with MPTP [54, 55].

Recent evidence has suggested that the balance between mitochondrial fission and fusion is correlated with axonal mitochondrial transport and distribution [8, 14, 48, 56]. Although the mitochondrial fission process is essential for axonal mitochondrial transport and the degradation of damaged mitochondria [57, 58], excessive mitochondrial fission is an early event of synaptic

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degradation [59]. Furthermore, Drp1 activity has been closely associated with the fate of dopaminergic neurons [17, 18], and inhibition of Drp1 activation attenuates disrupted synaptic function in diverse neurodegenerative models, including PD [16, 60, 61]. Our results indicated that excessive mitochondrial fission in MPTP-induced PD in monkeys was accompanied by phosphorylation of Drp1(S616), which triggers Drp1 activation. These results are consistent with other published results that an increase in Drp1(S616) phosphorylation is associated with various neurodegenerative diseases involving dopaminergic neurons [18, 62, 63]. However, the precise molecular mechanisms of excessive mitochondria fission mediated by Drp1 phosphorylation in experimental PD models are still unclear.

CDK5 has been identified as a regulator of mitochondrial fragmentation during neuronal apoptosis by modulating Drp1 phosphorylation, and its suppression attenuates excessive mitochondrial fission leading to apoptosis [31, 32, 45]. However, the precise mechanism underlying the relationship between mitochondrial morphology and activated CDK5 in PD is not fully understood. Our findings indicated that Drp1(S616) phosphorylation was induced by CDK5 activation, which was accompanied by an increased level of p35 and p25 in the substantia nigra of MPTP-injected monkeys. On the other hand, another kinase of Drp1, ERK, remained unchanged after MPTP injection. Our results indicated that MPTP-induced CDK5 activation regulates mitochondrial fragmentation by modulating the phosphorylation of Drp1(S616). In PD, CDK5 hyperactivation is a classical pathology that is associated with loss of dopaminergic neurons in the substantia nigra [64]. Inhibition of CDK5 hyperactivation provides a neuroprotective effect in experimental PD models [65, 66]. Furthermore, hyperactivation of CDK5 is involved in pre-synaptic loss, and ultimately neurodegeneration, by regulating neuronal actin cytoskeleton remodeling [67]. Therefore, our model of MPTP-induced PD indicated that CDK5-mediated increase of Drp1 phosphorylation at the S616 residue may trigger mitochondrial fission, ultimately inducing dopaminergic neuronal loss in the substantia nigra.

Human PD symptoms were observed in our non-human primate model of MPTP-induced PD. However, the degree of physical response to MPTP varies according to each individual monkey. Therefore, we developed a new strategy for MPTP-induced chronic PD, with consistent symptoms [38]. In this chronic PD model, we evaluated the molecular pathology more precisely, focusing on altered mitochondrial morphology, which is a marker of various genetic and pharmacological mechanisms of PD [68]. Thus, our model showed that CDK5-mediated increase of Drp1(S616) phosphorylation triggers mitochondrial fission, and ultimately induces dopaminergic neuronal loss in the substantia nigra. Therefore, inhibition of CDK5-relative signaling and excessive mitochondrial fission may provide therapeutic strategies. Altogether, our MPTP-mediated non-human primate PD model reflects PD pathology with both behavioral symptoms and molecular mechanisms. Therefore, our findings could contribute to the development of therapeutic strategies.

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