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P53 knockout mice are protected from cocaine-induced kindling behaviors via inhibiting mitochondrial oxidative burdens, mitochondrial dysfunction, and proapoptotic changes

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ABSTRACT

Previously we demonstrated that p53 mediates dopaminergic neurotoxicity via inducing mitochondrial burdens and proapoptotsis. However, little is known about the role of p53 in the excitotoxicity induced by psychostimulant, such as cocaine. Cocaine-induced kindling (convulsive) behaviors significantly increased p53 expression in the brain. Cocaine-induced p53 expression was more pronounced in hippocampus than in striatum or prefrontal cortex. Genetic depletion of p53 significantly attenuated cocaine-induced convulsive behaviors, followed by c-Fos immunoreactivity, and oxidative burdens in the hippocampus of mice. The antioxidant potentials mediated by genetic depletion of p53 were more pronounced in the mitochondrial-than cytosolic-fraction. Depletion of p53 significantly attenuated the changes in mitochondrial transmembrane potential, intramitochondrial Ca^{2+} level, and mitochondrial oxidative burdens induced by cocaine. Consistently, depletion of p53 significantly inhibited mitochondrial p53 translocation, and cleaved-PKCδ induced by cocaine. In addition, depletion of p53 protected from cytosolic cytochrome c release, and pro-apoptotic changes induced by cocaine. Importantly, the protective/anticonvulsant potentials by genetic depletion of p53 were comparable to those by pifithrin-μ (PFT), a p53 inhibitor. Our results suggest that depletion of p53 offers anticonvulsive and neuroprotective potentials mainly via attenuating mitochondrial oxidative burdens, mitochondrial dysfunction, and pro-apoptotic signalings against cocaine-induced convulsive neurotoxicity.

1. Introduction

Cocaine is a widely abused naturally occurring illicit psychostimulant (Guha et al., 2016; Pradhan et al., 1978), chronic use of cocaine causes convulsions and status epilepticus in rodents and humans, respectively (Dhuna et al., 1991; Purcell et al., 2013; Tella et al., 1992). Cocaine-induced seizures can be life-threatening (Dhuna et al., 1991). Chronic exposure to low doses of cocaine induces drug dependence, whereas repeated sub-convulsive doses enhance sensitivity to its convulsant effect, a phenomenon called "kindling" that is analogous to the kindling of epileptic seizures engendered by repetitive subthreshold electrical stimulation of the limbic system (Goddard et al., 1969; Itzhak and Martin 2000; Mai et al., 2018a; Post et al., 1988). Kindling behaviors induced by cocaine are thought to be relevant animal models to investigate psychopathology of cocaine addiction (Itzhak and Martin 2000; Mai et al., 2018a).

Cocaine metabolism by cytochrome P 450 and flavin adenine (FAD) containing monooxygenases generates reactive oxygen species (ROS)

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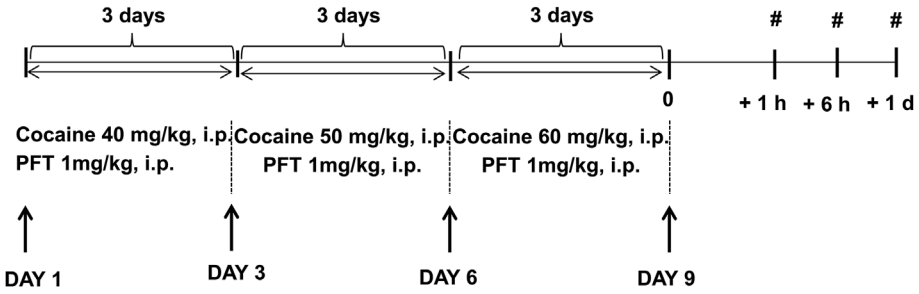

Fig. 1. Experimental design for elucidating on the role of p53 in response to kindling (convulsive) behaviors induced by cocaine in mice. To induce convulsive behaviors, mice received escalating doses of cocaine (i.e., 40 mg/kg i.p. from day 1 to day 3, 50 mg/kg i.p. from day 4 to day 6, and 60 mg/kg i.p. from day 7 to day 9). Wild-type (WT) mice received pifithrin-μ (PFT) 1 mg/kg/day for consecutive 9 days. Control mice received saline/vehicle (5% DMSO). Mice were sacrificed 1 h, 6 h, and 1 d after the final cocaine. # = Sacrifice.

(Kovacic 2005). Indeed, acute or chronic cocaine treatment significantly alters activity of enzymatic antioxidants (i.e., superoxide dismutase, glutathione peroxidase, and catalase) (Dietrich et al., 2005; Macedo et al., 2010; Pomierny-Chamiolo et al., 2013). Thus, it has been well-recognized that cocaine-induced oxidative stress leads to neurotoxic changes (Kovacic 2005; Riezzo et al., 2012; Vitcheva 2012).

P53 is a tumor-suppressor gene, which plays a crucial role in the apoptotic processes associated with neurodegenerative conditions (Camins et al., 2008; Checler and Alves da Costa, 2014; Shin et al., 2016a). Earlier reports demonstrated that p53 mediates methamphetamine-induced neurotoxicity (Hirata and Cadet 1997a; Hirata and Cadet 1997b). Similarly, cocaine alters p53 transcription factor (Novikova et al., 2005). In addition to the transcriptional regulation, p53 caused cell death via mitochondrial translocation (Erster et al., 2004; Shin et al., 2016a).

Cocaine-induced neurotoxicity requires mitochondrial dysfunction and activation of the mitochondrial apoptotic pathway (Cunha-Oliveira et al., 2006a; Cunha-Oliveira et al., 2013). Importantly, mitochondria are principal intracellular organelle for generation of ROS (Gibellini et al., 2015). It has been reported that p53 alters mitochondrial function that causes oxidative stress, which contributes to neurodegeneration (Dai et al., 2016). Moreover, interaction of mitochondrial p53 with Bcl-2 or BclxL disintegrates mitochondrial membrane, and activates apoptotic signals (Chipuk et al., 2004; Endo et al., 2006; Shin et al., 2016a).

In the present study, we investigated whether depletion of p53 affects cocaine-induced kindling (convulsive) behaviors in mice. Here we propose that genetic or pharmacological inhibition of p53 conveys neuroprotective potentials against cocaine-induced convulsive behaviors via rescue of mitochondrial dysfunction, mitochondrial oxidative stress, and pro-apoptotic signal.

2. Material and methods

2.1. Animals

All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Eight-week-old male wild-type (WT; C57BL/6N) mice (Bio Genomics, Inc., Charles River Technology, Gapyung-Gun, Gyeonggi-Do, Republic of Korea), weighing approximately 25 ± 4 g, were randomized and housed in a temperature-controlled animal facility (24 ± 2 °C) under a 12-h/12-h light/dark cycle and fed *ad libitum*. Breeding pairs of p53 gene heterozygous [p53 (±)] mice with C57BL/6N background were obtained from RIKEN BioResource Center (RBRCO1361; Tsukuba, Japan) (Tsukada et al., 1993). P53 knockout (−/−) mice were maintained as heterozygous breeding pairs, and neonates were genotyped.

Polymerase chain reaction (PCR) was performed using DNA extracted from the tail, according to the information provided by the RIKEN BioResource Center. The sequence of primers was as follows; LCB614 in intron 1: 5′- GTTATGCATCCATACAGTACA-3′, LCB651 in exon 3: 5′- CAGGATATCTTCTGGAGGAAG-3′. The products were amplified using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) at 94 °C for 5 min; 34 cycles of 94 °C for 30 s; 57 °C for 30 s; 72 °C for 120 s; and 72 °C for 5 min. Amplification with wild-type DNA yielded a 400-bp product, and amplification with p53 (−/−) DNA yielded a 1500-bp product.

2.2. Drug

Cocaine hydrochloride (Macfarlan Smith Ltd., Edinburgh, UK) was dissolved in saline (1 mg/ml), and pifithrin-μ (PFT, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution, and then stored at 4 °C. Sock solution was diluted in sterile saline (1 ml/kg) immediately before use, and the final DMSO concentration was 5% (v/v). Mice received escalating doses of cocaine (i.e., 40 mg/kg i.p. from day 1 to day 3, 50 mg/kg i.p. from day 4 to day 6, and 60 mg/kg i.p. from day 7 to day 9). PFT 1 mg/kg, i.p. was administered 1 h before every cocaine administration in wild-type mice for consecutive 9 days. Control mice received saline/vehicle, and underwent the same experimental procedures as cocaine-kindled animals (Fig. 1). Mice were euthanized by decapitation, and the hippocampus, striatum, and prefrontal cortex were immediately dissected, and stored in liquid nitrogen for further analyses.

2.3. Assessment of cocaine-induced kindling (convulsive) behaviors

To assess the convulsive behaviors, mice received escalating doses of cocaine (Fig. 1). Mice were placed separately in Plexiglas containers (14 × 25 × 36 cm high) for observation. Convulsive behaviors were assessed after every cocaine treatment.

The presence or absence of convulsions was recorded 30 min following injection. The definition of a cocaine convulsion was the loss of the righting response for at least 5 s and the occurrence of clonic limb movements (characterized by rapid rhythmic contraction and relaxation of muscles in extremities or episodes of violent and dramatic uninhibited running and jumping/bouncing). Kindling was defined as an increase in the percentage of mice exhibiting seizures with repeated exposure to cocaine. The seizure score was generated using the following weighted formula based on latencies to the three different components of the seizure phenotype: Seizure score = [(latency to forelimb or hindlimb clonus)$^{-1}$ × 1000 × 0.2] + [(latency to clonic running seizure) $^{-1}$ × 1000 × 0.3] + [(latency to clonic jumping/bouncing seizure)$^{-1}$ × 1000 × 0.5]; where 0.2, 0.3, and 0.5 are the weights of the respective components. The phenotype components occur in a consistent progression from clonus to jumping/bouncing seizures in mice. The seizure score was calculated as the sum of each component (reciprocal of latency × 1000) times its assigned weight (higher weights given to more severe seizure components). Thus, the weighted seizure score included measures of both seizure latency and seizure severity. A similar weighted seizure score was used in a recent study of kainic acid seizure susceptibility (Ferraro et al., 1997; Golden et al., 2001).

2.4. Preparation of cytosolic and mitochondrial fractions

Cytosolic and mitochondrial fractions were performed as previously described (Mai et al., 2018b; Shin et al., 2016a). Briefly, hippocampal

tissues were homogenized in ice-cold homogenization buffer containing 0.25 M sucrose, 0.5 mM potassium ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM Tris-HCl (pH 7.4), and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) using a Dounce homogenizer. Homogenates were centrifuged at 2000 × *g* for 10 min to remove nuclei and unbroken cells. The clarified supernatants were then centrifuged at 12,000 × *g* for 15 min to obtain crude mitochondrial pellets and cytosolic supernatants. Crude mitochondrial pellets were suspended in 3% Ficoll 400 (Sigma-Aldrich) in Ficoll dilution buffer containing 0.25 M mannitol, 60 mM sucrose, 0.1 mM potassium EGTA, and 10 mM Tris-HCl (pH 7.4). A Ficoll density gradient was constructed by pouring the crude mitochondrial suspension (in 3% Ficoll) over a 6% Ficoll 400 solution. Purified mitochondrial pellets were obtained by centrifugation at 11,500 g for 10 min and then resuspended in buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and a protease cocktail (pH 7.4). For Western blotting, mitochondrial pellets were lysed in 100 mL of lysis buffer.

For measurements of mitochondrial transmembrane potential, animals were anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with 30 mL ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.2). The animals were then decapitated, and the hippocampus was dissected out, rinsed in 10 mL homogenization buffer, and processed using a tissue homogenizer. All subsequent steps were conducted at 4 °C. The resulting homogenate was centrifuged (10 min, 1300 × *g*). The supernatant was removed and centrifuged again (10 min, 10,000 × *g*), and the pellet was gently resuspended (four strokes) in 30 mL homogenization buffer using a hand-held homogenizer and centrifuged (10 min, 10,000 × *g*). The resulting pellet was resuspended and rinsed in EDTA-free homogenization buffer. Then, the mitochondrial pellet was resuspended in 250 mM sucrose to a final concentration of ~20 mg/mL, and placed on ice. The entire mitochondrial preparation took < 1 h to complete.

2.5. Western blot analysis

Western blot analysis was performed as we described previously (Mai et al., 2018a; Shin et al., 2014; Tran et al., 2016b; Tu et al., 2017). Brain (i.e., hippocampus, striatum, and prefrontal cortex) tissues were homogenized in lysis buffer containing 200 mM Tris HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 5 mM EGTA, 5 mM ethylenediaminetetraacetic acid, 10% glycerol, 1 × phosphatase inhibitor cocktail I (Sigma-Aldrich, St. Louis, MO, U.S.A.), and 1 × protease inhibitor cocktail (Sigma-Aldrich). Lysate was centrifuged at 12,000 × *g* for 30 min, and the supernatant fraction was used for western blot analysis. Proteins (20–50 μg/lane) were separated by 8% or 12% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. Following transfer, the membranes were pre-incubated with 5% non-fat milk for 30 min and incubated overnight at 4 °C with primary antibodies against β-actin (42 kDa; 1:50,000; Sigma-Aldrich), COX IV (1:5000; Cell Signaling Technology, Inc.), p53 (1:1000, Santa Cruz), cytochrome c (1:500, Santa Cruz Biotechnology, Inc.), cleaved caspase-3 (1:1000; Cell Signaling Technology, Inc.), PKCδ (Santa Cruz Biotechnology), cleaved-PKCδ (Santa Cruz Biotechnology), Bcl-2 (1:500; Santa Cruz Biotechnology), Bcl-xL (1:1000; Cell Signaling Technology, Inc.), and Bax (1:500; Santa Cruz Biotechnology). Then membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit immunoglobulin G (IgG) (1:1000, GE Healthcare, Piscataway, NJ, U.S.A.), anti-mouse IgG (1:1000, Sigma-Aldrich), or anti-goat IgG (1:1000, Sigma-Aldrich) for 2 h. Subsequent visualization was performed using an enhanced chemiluminescence system (ECL Plus®, GE Healthcare, Arlington Heights, IL, U.S.A.). Relative intensities of the bands were quantified by PhotoCapt MW (version 10.01 for Windows; Vilber Lourmat, Marne la Vallee, France) and then normalized to the intensity of β-actin (Shin et al., 2014).

2.6. Immunocytochemistry

Immunocytochemistry was performed as we described previously (Mai et al., 2018b; Tu et al., 2017). Mice were perfused transcardially with 50 mL of ice-cold PBS (10 mL/10 g body weight) followed by 4% paraformaldehyde (20 mL/10 g body weight). Brains were removed and stored in 4% paraformaldehyde overnight. The brain was cut into 35-μm-thick coronal sections. Sections were blocked with PBS containing 0.3% hydrogen peroxide for 30 min and then incubated in PBS containing 0.4% Triton X-100 and 1% normal serum for 20 min. After 48-h incubation with primary antibody against c-Fos (1:5000; Merck Millipore, Billerica, MA, U.S.A.) (Inagaki et al., 2014; Tran et al., 2016a), sections were incubated with the biotinylated secondary antibody (1:1000; Vector Laboratories, Burlingame, CA, U.S.A.) for 1 h. Then, sections were immersed in an avidin-biotin peroxidase complex (Vector Laboratories) containing solution for 1 h, and 3,30-diaminobenzidine was used as the chromogen. Digital images were acquired under an upright microscope (BX51; Olympus) using an attached digital microscope camera (DP72; Olympus) and an IBM-compatible PC. ImageJ version 1.47 software (National Institutes of Health, Bethesda, MD, U.S.A.) was utilized to count c-Fos-immunoreactive cells as described previously (Tran et al., 2016a). Briefly, hippocampus (CA1, CA3, and DG) was selected as the region of interest from each section.

2.7. Mitochondrial transmembrane potential

Briefly, 250 μg aliquots of isolated mitochondrial protein was suspended in respiration buffer [250 mM sucrose, 20 mM HEPES, 2 mM $MgCl_2$, 2.5 mM inorganic phosphates (pH 7.2), and 10 mM succinate (5 mM glutamate and 2.5 mM maleate produced similar results in all paradigms)] in a final volume of 200 μL. Then, the energized mitochondria were incubated at 37 °C in the presence of 10 μM JC-1 for 30 min, after which fluorescence was measured with a fluorescent plate reader (Molecular Devices). The relative amount of mitochondrial polarization was quantified by taking the ratio of emission from 590 to 535 nm, respectively, with excitation at 490 nm (Mai et al., 2018b).

2.8. Intramitochondrial Ca^{2+} levels

Intramitochondrial Ca^{2+} levels were measured as described previously (Mai et al., 2018b; Shin et al., 2016a; Shin et al., 2014). Mitochondrial fractions (250 μg) from hippocampus were incubated in the presence of Rhod-2-AM (5 μM) for 30 min at 37 °C and washed three times with Ca^{2+}-free Locke's solution. This reduced form of Rhod-2-AM is a colorless, non-fluorescent dye that has a net positive charge, which promotes sequestration into mitochondria. Then, the dye is oxidized in the mitochondria where the AM ester is cleaved from Rhod-2-AM, trapping the dye in the mitochondria. Fluorescence was quantified with a fluorescent plate reader (Molecular Devices) with excitation and emission wavelengths of 549 and 581 nm, respectively.

2.9. Determination of reactive oxygen species (ROS)

The extent of reactive oxygen species (ROS) formation in the hippocampus was assessed by as previously described (Lebel and Bondy 1990; Mai et al., 2018b; Shin et al., 2014). Ten percent (w/v) homogenates, in phosphate buffered saline (PBS), of hippocampal tissues were incubated with 5 mM 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, U.S.A.) for 3 h at 37 °C. The excess unbound probe and the flocculent precipitate were removed by centrifugation at 12,500 × *g* for 10 min. The fluorescent intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm. 20,70-Dichlorofluorescein (DCF) was used as a standard. Protein level in each homogenate was measured using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL,U.S.A.).

2.10. Determination of 4-hydroxy-2-nonenal (HNE)

The amount of lipid peroxidation was determined by measuring the level of HNE using the OxiSelect™ HNE adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's manual (Mai et al., 2018b; Shin et al., 2014). One hundred μL of the cytosolic and mitochondrial fraction at a protein concentration of 10 μg/mL was incubated in the 96-well protein binding plate at 4 °C for overnight. After protein adsorption, HNE adducts in each well were labeled with HNE antibody, followed by the HRP-conjugated secondary antibody. And then, colorimetric development was performed with the substrate solution. Absorbance was recorded at 450 nm using a microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA), and an amount of HNE adduct in each sample was calculated from the standard curve of HNE-BSA (Shin et al., 2016a).

2.11. Determination of protein carbonyl

The extent of protein oxidation was assessed by measuring the content of protein carbonyl groups, which was determined spectrophotometrically with the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure (Tran et al., 2016a). The results are expressed as nanomole of DNPH incorporated/mg protein based on the extinction coefficient for aliphatic hydrazones of 21 mM^{-1} cm^{-1} (Mai et al., 2018b; Shin et al., 2016a; Shin et al., 2014; Xu et al., 2013).

2.12. Statistical analysis

Data were analyzed by commercially available software IBM SPSS ver.23.0 (IBM, Chicago, IL, U.S.A). Statistical analysis was performed using one-way, two-way or three-way analysis of variance (ANOVA), followed by Post hoc Fisher's least significant difference pairwise comparisons. All values were expressed as the mean ± standard error of mean (S.E.M). p-values less than 0.05 ($p < 0.05$) were considered to be statistically significant.

3. Results

3.1. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 in response to kindling (convulsive) behaviors induced by cocaine in mice

According to experimental design of Fig. 1 mice received cocaine for consecutive 9 days. As shown in Fig. 2, repeated cocaine treatment significantly induced convulsive behaviors in wild-type (WT) mice. In contrast, genetic depletion of p53 significantly attenuated cocaine-induced convulsion ratio ($P = 3.5 \times 10^{-4}$) (F [2, 27] = 9.104, $P = 9.5 \times 10^{-4}$, Fig. 2A), and severity scores ($P = 5.1 \times 10^{-5}$) (F [2, 27] = 11.82, $P = 2 \times 10^{-4}$, Fig. 2B) in mice. The anticonvulsive potentials by p53 knockout in mice were comparable to those by PFT (Fig. 2A and B). In addition, a single dose of cocaine (60 mg/kg, i.p.) also induced seizures in wild-type (WT) mice. In contrast, PFT or genetic depletion of p53 appeared to attenuate cocaine-induced seizure ratio without reaching the statistical significance (Supplementary Figs. S1B and C).

3.2. Changes in p53 expression induced by cocaine in the hippocampus, striatum and prefrontal cortex of wild-type mice

As shown in Fig. 3, repeated cocaine treatment significantly enhanced p53 expression in hippocampus (6 h; $P = 0.025$, 1d; $P = 1.5 \times 10^{-4}$) (F [3, 24] = 10.020, $P = 3 \times 10^{-4}$, Fig. 3A), and striatum (1d; $P = 0.014$) (F [3, 24] = 3.206, $P = 0.05$, Fig. 3B), whereas did not significantly change in prefrontal cortex (F [3, 24] = 1.098, $P = 0.373$, Fig. 3C) of wild-type mice. Cocaine-induced p53 expression appeared to be more pronounced in hippocampus than in striatum or prefrontal cortex. Hippocampal p53 was maximally induced 1 d post-cocaine. Therefore, we focused on 1 d time-point for further study. Moreover, a single dose of cocaine significantly increased p53 expression (1 h; $P = 0.002$, 6 h; $P = 0.035$) (F [3, 24] = 7.19, $P = 0.002$, Supplementary Fig. S2) in the hippocampus.

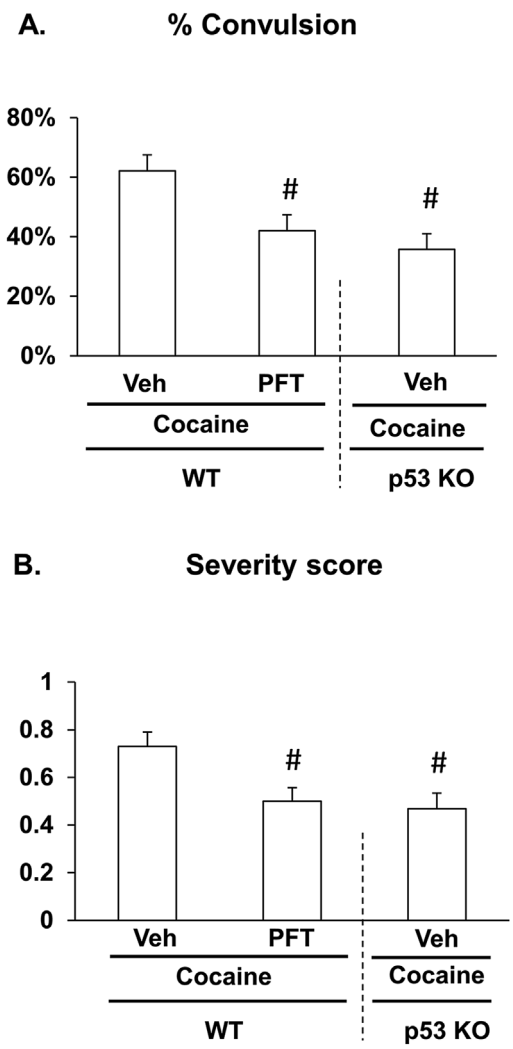

Fig. 2. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against kindling (convulsive) behaviors induced by cocaine in mice. (A) Convulsion ratio (%). (B) Seizure score. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of ten animals. $^{\#}P < 0.01$ vs. Veh/Cocaine/WT (two-way ANOVA with Fisher's LSD pairwise comparisons).

3.3. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on the cocaine-induced increase in c-Fos immunoreactivity (c-Fos-IR) in the hippocampus of mice

As shown in Fig. 4, we investigated if PFT or p53 knockout modulates cocaine-induced c-Fos-IR in the hippocampus of mice. A very little c-Fos-IR in the CA1 (F [5, 60] = 17.274, $P = 3.54 \times 10^{-10}$, Fig. 4B), CA3 (F [5, 60] = 18.537, $P = 1.1 \times 10^{-10}$, Fig. 4C), and dentate gyrus (DG) (F [5, 60] = 67.373, $P = 5.6 \times 10^{-22}$, Fig. 4D) regions of the hippocampus was observed in the absence of cocaine. However, we found that cocaine treatment significantly increased c-Fos-IR in the CA1 ($P = 6.9 \times 10^{-10}$), CA3 ($P = 3.4 \times 10^{-10}$), and DG ($P = 2.5 \times 10^{-20}$) of mice. Cocaine-induced c-Fos-IR appeared to be more pronounced in DG than CA1 or CA3 region. In contrast, genetic depletion of p53 significantly attenuated c-Fos-IR induced by cocaine in the CA1 ($P = 4.4 \times 10^{-8}$), CA3 ($P = 3.5 \times 10^{-8}$), and DG

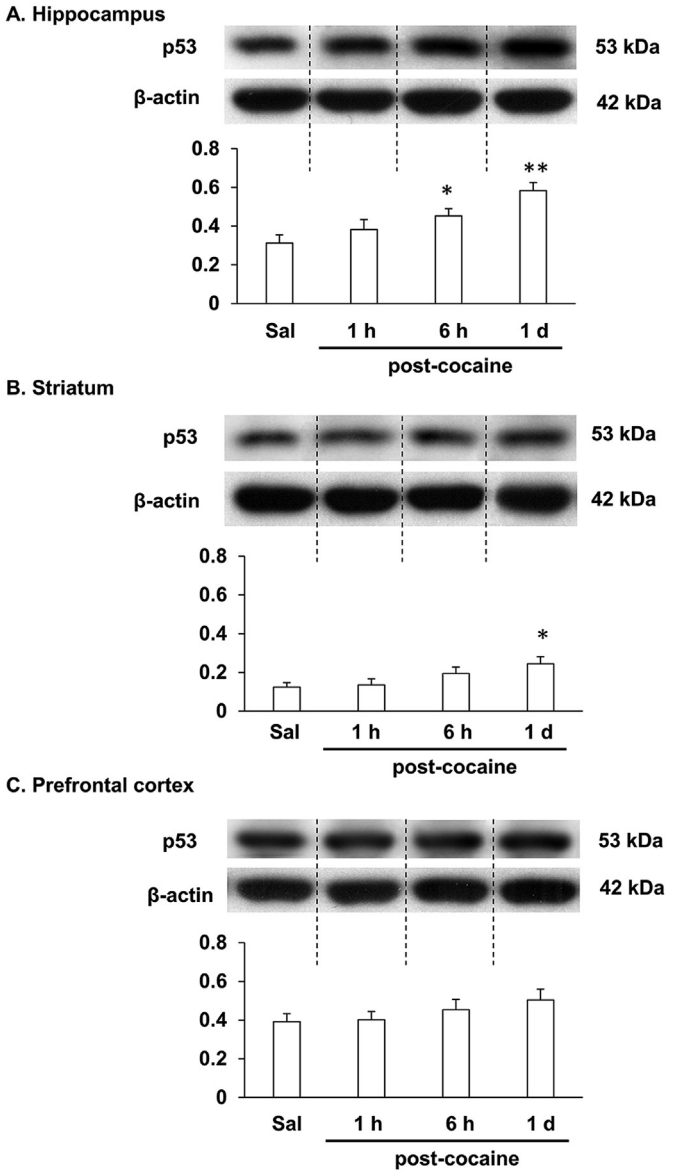

Fig. 3. Changes in p53 expression induced by cocaine in the hippocampus, striatum, and prefrontal cortex of wild-type mice. Sal = Saline. Each value is the mean ± SEM of six animals. *$P < 0.05$, **$P < 0.01$ vs. respective Sal (one-way ANOVA with Fisher's LSD pairwise comparisons).

($P = 2.3 \times 10^{-19}$). Consistently, the attenuation by p53 knockout was comparable to that by PFT (Fig. 4A–D). Moreover, a single injection of cocaine tended to increase c-Fos-IR without reaching the statistical significance, while inhibition of p53 appeared to attenuate cocaine effect in the CA1, CA3, and DG regions of the hippocampus (Supplementary Figs. S3A–C).

3.4. *Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on the cocaine-induced increases in cytosolic and mitochondrial expression of p53 in the hippocampus of mice*

As shown in Fig. 5, we examined the effect of PFT (or p53 knockout) in response to cocaine-induced mitochondrial translocation of p53. We found that repeated cocaine treatment significantly increases cytosolic ($P = 0.007$) ($F\ [5,\ 60] = 28.429$, $P = 5.3 \times 10^{-14}$, Fig. 5A) and mitochondrial ($P = 4.2 \times 10^{-4}$) ($F\ [5,\ 60] = 52.349$, $P = 1.7 \times 10^{-19}$, Fig. 5B) expressions of p53. PFT selectively and significantly attenuated ($P = 4.3 \times 10^{-4}$) mitochondrial translocation of p53 induced by

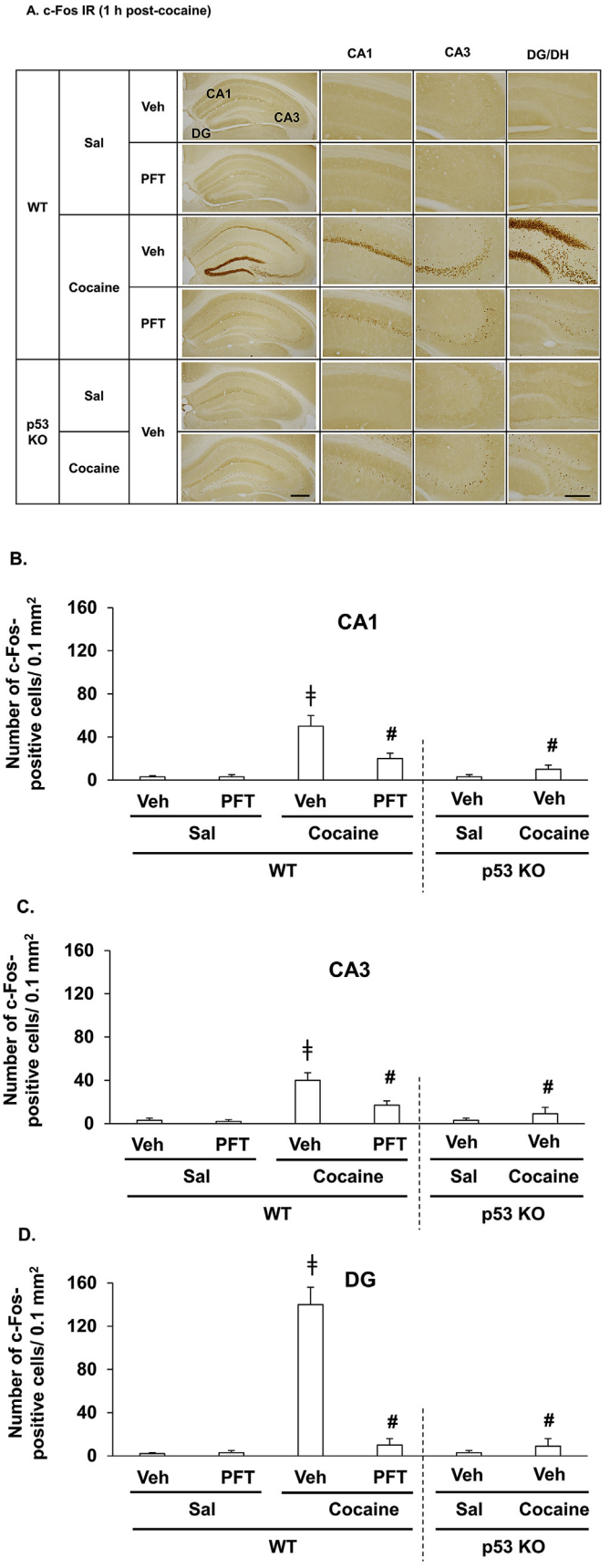

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Fig. 4. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on the cocaine-induced increase in c-Fos immunoreactivity (IR) in the hippocampus of mice. (A) Representative c-Fos-IR. (B) c-Fos-IR in CA1. (C) c-Fos-IR in CA3. (B) c-Fos-IR in dentate gyrus (DG). Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of ten animals. $^{‡}P < 0.01$ vs. Veh/Sal, $^{\#}P < 0.01$ vs. respective Veh/Cocaine/WT (three-way ANOVA with Fisher's LSD pairwise comparisons). Scale bar = 400 μm.

cocaine (Fig. 5B).

3.5. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on cytosolic and mitochondrial oxidative parameters induced by cocaine in the hippocampus of mice

As shown in Fig. 6, we examined if PFT or genetic depletion of p53 positively modulates cocaine-induced cytosolic and mitochondrial oxidative parameters (i.e., ROS, HNE, and protein carbonyl) in mice.

Cocaine treatment initially and consistently increased oxidative

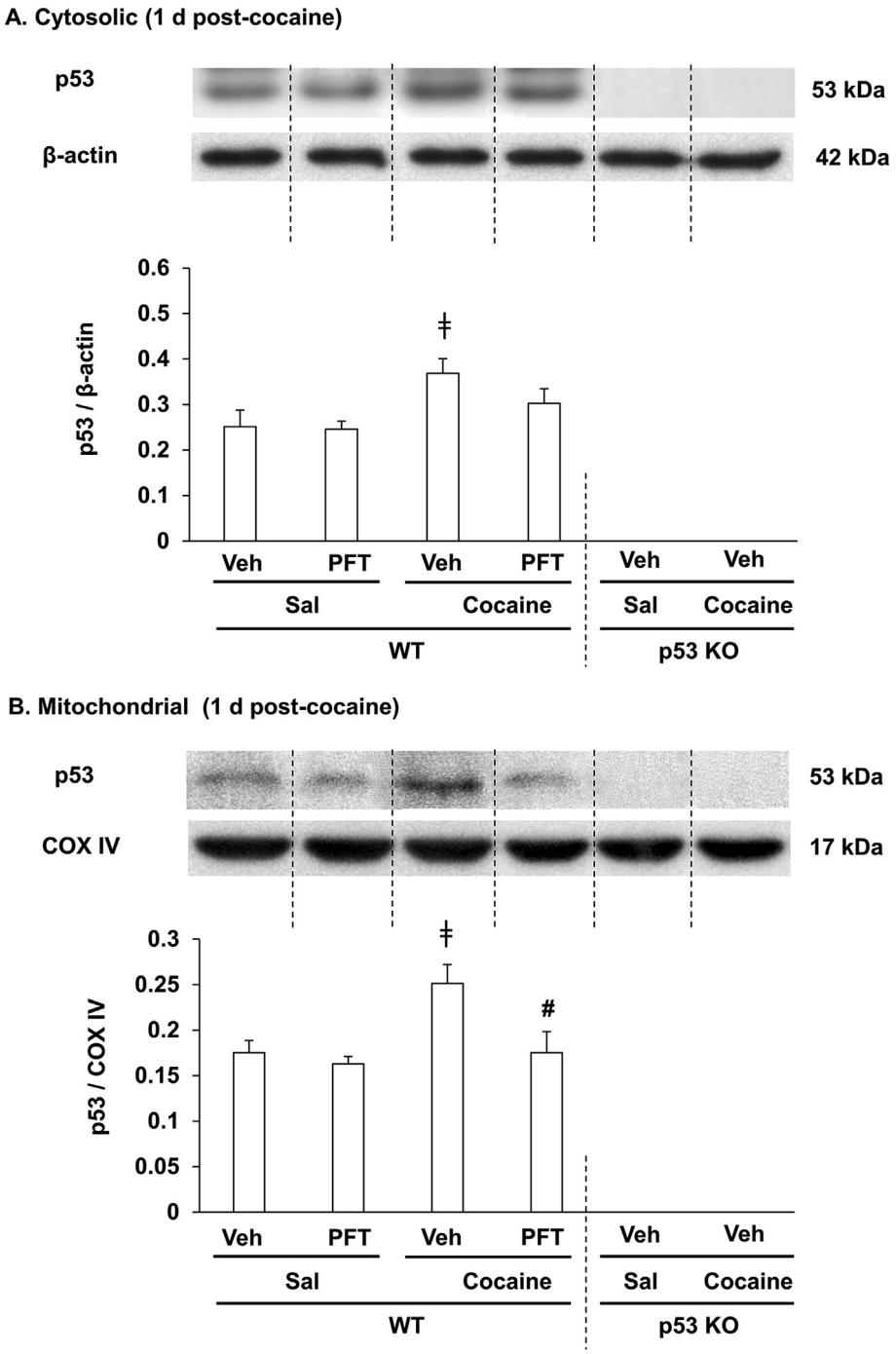

Fig. 5. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on the cocaine-induced increase in cytosolic and mitochondrial expressions of p53 in the hippocampus of mice. (A) Effect on cocaine-induced cytosolic p53 expression. (B) Effect on cocaine-induced mitochondrial translocation of p53. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of six animals. $^{‡}P < 0.01$ vs. Veh/Sal/WT; $^{\#}P < 0.01$ vs. Veh/Cocaine/WT (three-way ANOVA with Fisher's LSD pairwise comparisons).

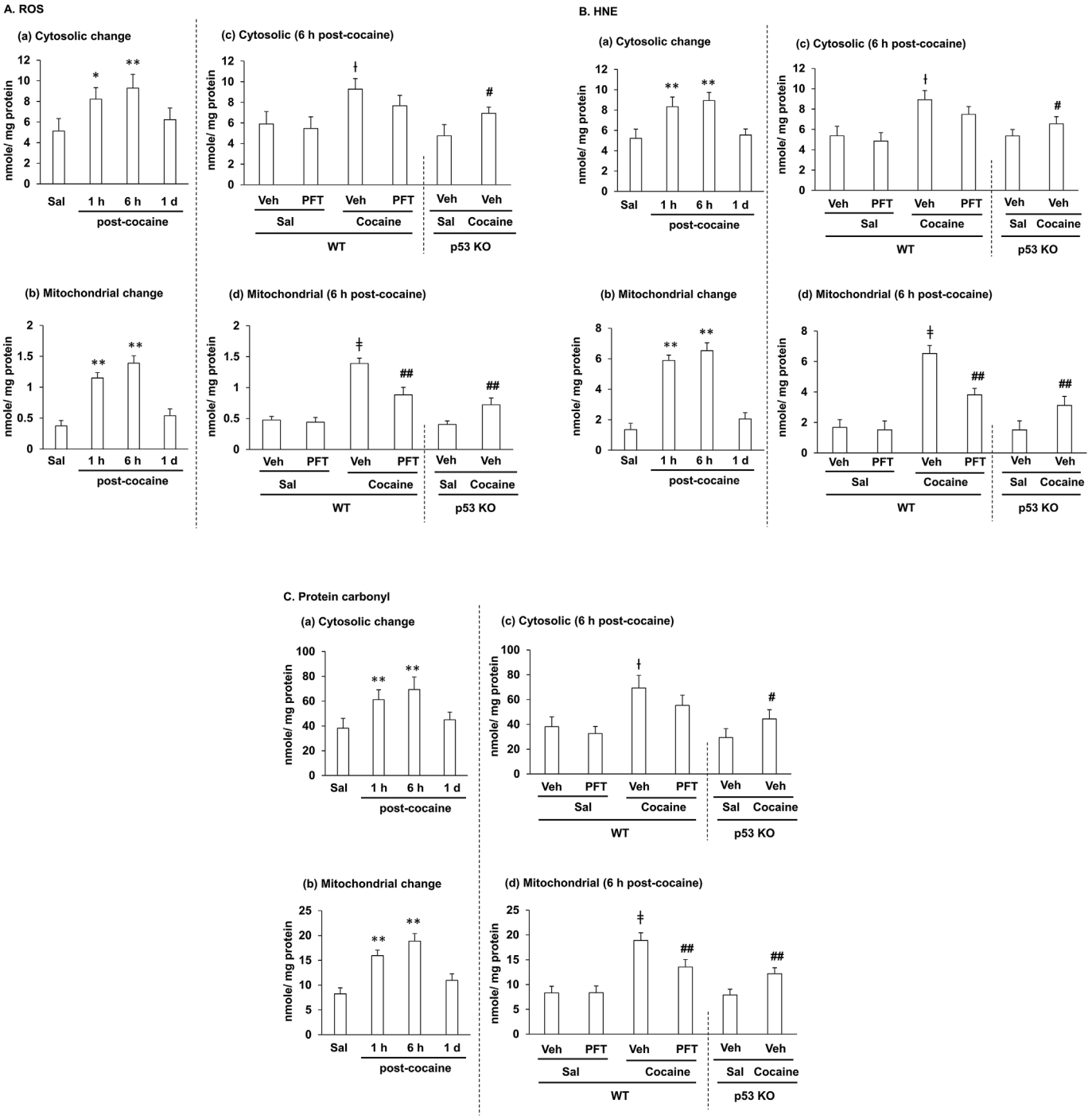

Fig. 6. Effect of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 on the cytosolic and mitochondrial oxidative parameters induced by cocaine in the hippocampus of mice. (Aa-d) Effect on cytosolic and mitochondrial reactive oxygen species (ROS). (Ba-d) Effect on cytosolic and mitochondrial 4-hydroxy-2-nonenal (HNE). (Ca-d) Effect on cytosolic and mitochondrial protein carbonyl. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of ten animals. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sal, $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ Veh/Sal/WT, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. Veh/Cocaine/WT (one way or three-way ANOVA with Fisher's LSD pairwise comparisons).

parameters 1 h later, and maximally increased 6 h later. The increase appeared to be more pronounced in mitochondrial-than cytosolic-fraction. The increase consistently returned to near control (saline) level 1 d later. Significant increases in cytosolic [ROS (1 h; $P = 0.05$, 6 h; $P = 0.009$, respectively) (F [3, 40] = 3.094, $P = 0.039$, Fig. 6A–a), HNE (1 h; $P = 2.2 \times 10^{-4}$, 6 h; $P = 4.02 \times 10^{-5}$, respectively) (F [3, 43] = 9.632, $P = 6.8 \times 10^{-5}$, Fig. 6B–a), and protein carbonyl (1 h; $P = 0.002$, 6 h; $P = 1.8 \times 10^{-4}$, respectively) (F [3, 43] = 6.765, $P = 8.7 \times 10^{-4}$, Fig. 6C–a)], and mitochondrial [ROS (1 h; $P = 1.7 \times 10^{-6}$, 6 h; $P = 7.7 \times 10^{-9}$, respectively) (F [3, 40] = 25.481, $P = 5.09 \times 10^{-9}$, Fig. 6A and b), HNE (1 h; $P = 3.8 \times 10^{-10}$, 6 h; $P = 1.3 \times 10^{-11}$, respectively) (F [3, 43] = 48.717, $P = 9.3 \times 10^{-13}$, Fig. 6B–b), and protein carbonyl (1 h; $P = 1.8 \times 10^{-6}$, 6 h; $P = 2.71 \times 10^{-9}$, respectively) (F [3, 40] = 24.981, $P = 6.5 \times 10^{-9}$, Fig. 6C–b)] oxidative burdens were observed in the hippocampus of cocaine-treated WT mice. Because this increase appeared to be more evident 6 h than 1 h post-cocaine, we focused on 6 h time-point for further study. Genetic depletion of p53, but not PFT, significantly

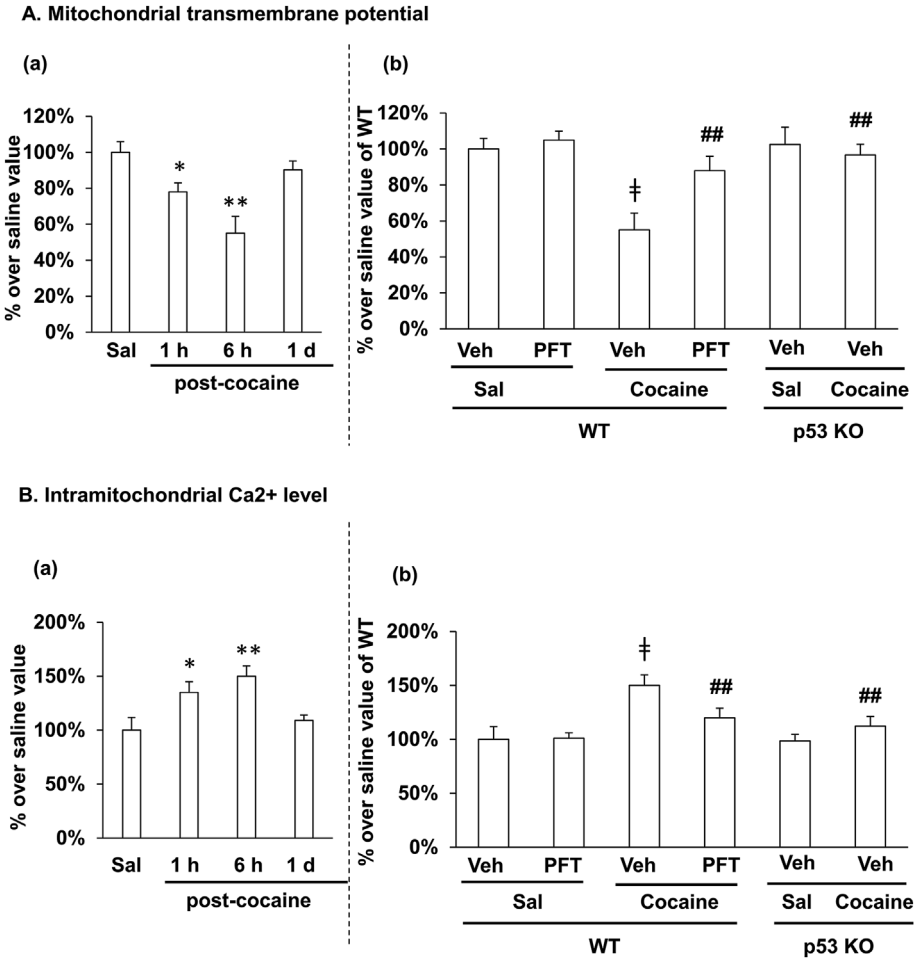

Fig. 7. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced changes in mitochondrial transmembrane potential and intramitochondrial Ca^{2+} accumulation in the hippocampus of mice. (Aa-b) Effects on mitochondrial membrane potential. (Ba-b) Effects on intramitochondrial Ca^{2+} accumulation. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of ten animals. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sal; $^{‡}P < 0.01$ Veh/Sal/WT, $^{\#}P < 0.01$ vs. Veh/Cocaine/WT (one way or three-way ANOVA with Fisher's LSD pairwise comparisons).

attenuated cocaine-induced cytosolic oxidative burdens [ROS ($P = 0.035$) (F [5, 60] = 2.742, $P = 0.028$, Fig. 6A–c), HNE ($P = 0.027$) (F [5, 60] = 4.404, $P = 0.002$, Fig. 6B and c), and protein carbonyl ($P < 0.006$) (F [5, 60] = 6.023, $P = 1.7 \times 10^{-4}$, Fig. 6C–c), respectively]. However, genetic depletion of p53 or PFT significantly attenuated [ROS (genetic depletion of p53, $P = 2.6 \times 10^{-5}$; PFT, $P = 9.6 \times 10^{-4}$) (F [5, 60] = 2.609, $P = 0.035$, Fig. 6A–d), HNE (genetic depletion of p53, $P = 4.7 \times 10^{-7}$; PFT, $P = 2.9 \times 10^{-5}$) (F [5, 60] = 21.762, $P = 7.2 \times 10^{-12}$, Fig. 6B–d), and protein carbonyl (genetic depletion of p53, $P = 2.5 \times 10^{-13}$; PFT, $P = 1.9 \times 10^{-5}$) (F [5, 60] = 28.324, $P = 5.7\ 10^{-14}$, Fig. 6C and d)] mitochondrial oxidative burdens induced by cocaine. The antioxidant potentials mediated by PFT or p53 knockout appeared to be more pronounced in mitochondrial-than cytosolic-fraction. (Fig. 6A, B, and C).

3.6. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced changes in mitochondrial transmembrane potential and intramitochondrial Ca^{2+} accumulation in the hippocampus of mice

As shown in Fig. 7, we examined whether p53 depletion affects cocaine-induced mitochondrial dysfunction. Decreases in mitochondrial transmembrane potential was observed 1 h ($P = 0.02$), and 6 h ($P = 1.9 \times 10^{-5}$) post-cocaine (F [3, 40] = 8.995, $P = 1.4 \times 10^{-5}$, Fig. 7A–a), and increased intramitochondrial Ca^{2+} level was also was observed 1 h ($P = 0.024$), and 6 h ($P = 0.002$) post-cocaine (F [3, 40] = 4.819, $P = 0.006$, Fig. 7B–a). These phenomena were most pronounced 6 h post-cocaine administration. Therefore, we focused on 6 h time-point for further study. In contrast, changes in mitochondrial transmembrane potential and intramitochondrial Ca^{2+} accumulation returned to near control (saline) level 1d post-cocaine (Fig. 7A and B). Genetic depletion of p53 significantly attenuated cocaine-induced changes in mitochondrial transmembrane potential ($P = 1.2 \times 10^{-4}$) (F [5, 60] = 6.870, $P = 5.01 \times 10^{-5}$, Fig. 7A and b), and increase in intramitochondrial Ca^{2+} ($P = 4.3 \times 10^{-4}$) (F [5, 60] = 7.487, $P = 2.1 \times 10^{-5}$, Fig. 7B–b), 6 h post-cocaine. Consistently, the attenuation by genetic depletion of p53 was comparable to that by PFT.

3.7. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced cleaved-PKCδ in the hippocampus of mice

As shown in Fig. 8, we investigated whether cocaine treatment alters cleaved-PKCδ expression over time. We found that cocaine treatment significantly increased cytosolic cleaved-PKCδ 1 d ($P = 0.003$) (F [3, 40] = 4.621, $P = 0.008$, Fig. 8A) post-cocaine and mitochondrial cleaved-PKCδ expression initially increased 6 h ($P = 0.05$), and reached a maximum 1 d ($P = 2.9 \times 10^{-6}$) post-cocaine (F [3, 40] = 14.208, $P = 2.8 \times 10^{-6}$, Fig. 8B). Therefore, we focused on 1 d time-point to investigate whether p53 depletion affects cocaine-induced mitochondrial cleaved-PKCδ. Consistently, genetic depletion of p53 significantly attenuated cytosolic ($P = 9.6 \times 10^{-5}$) (F [5, 60] = 4.88, $P = 9.3 \times 10^{-4}$, Fig. 8C) and mitochondrial ($P = 2.9 \times 10^{-8}$) (F [5, 60] = 17.86, $P = 2.05 \times 10^{-10}$, Fig. 8D) cleaved-PKCδ 1 d post-

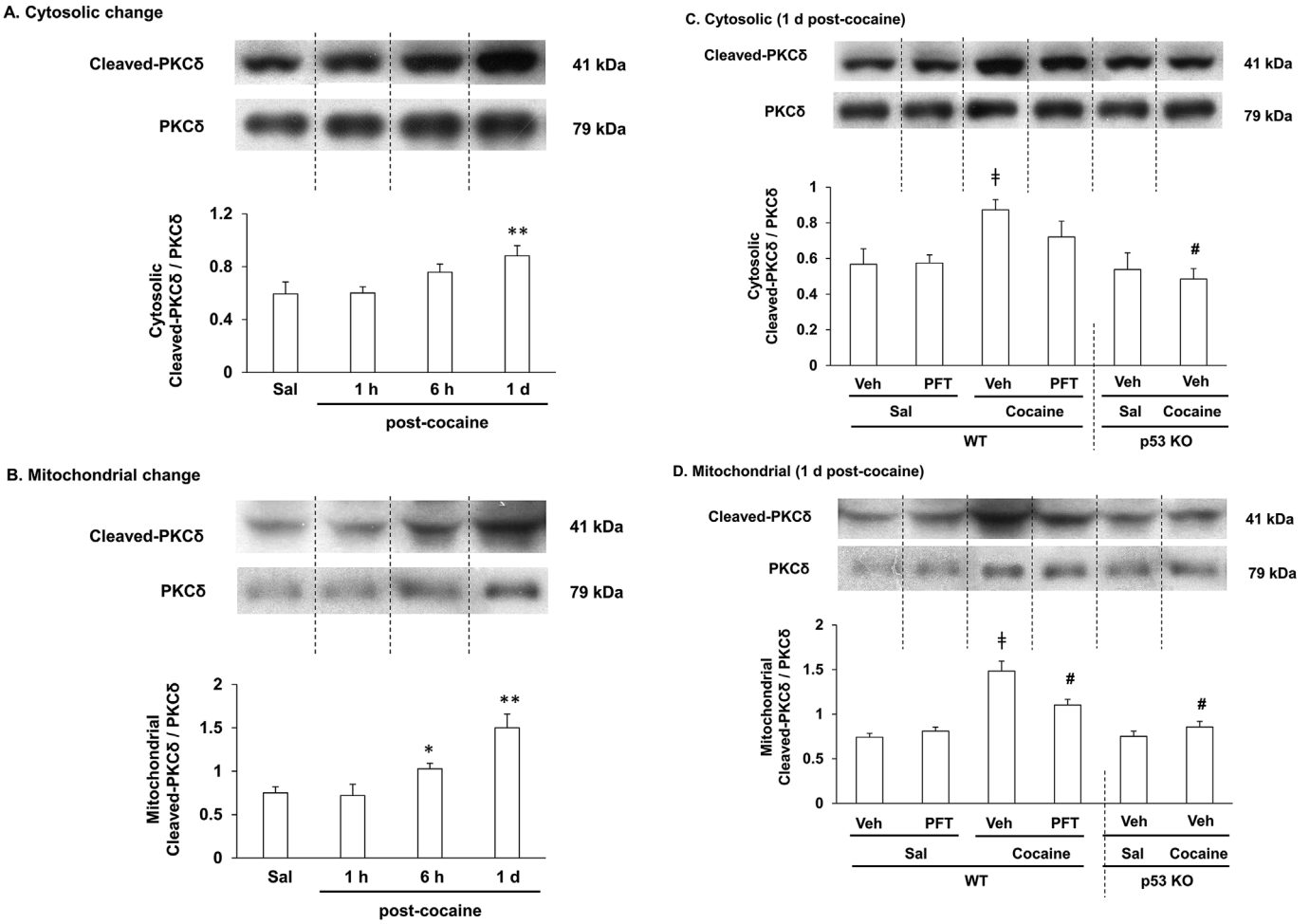

Fig. 8. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced mitochondrial translocation of cleaved PKCδ in the hippocampus of mice. (A) Time-course of changes in cytosolic cleaved-PKCδ expression. (B) Time-course of changes in mitochondrial cleaved-PKCδ expression. (C) Effect of p53 depletion on cytosolic cleaved-PKCδ expression. (D) Effect of p53 depletion on mitochondrial cleaved-PKCδ expression. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of ten animals. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sal; $^{‡}P < 0.01$ vs. Veh/Sal/WT, $^{\#}P < 0.01$ vs. Veh/Cocaine/WT (one way or three-way ANOVA with Fisher's LSD pairwise comparisons).

cocaine (Fig. 8C and D). Attenuation of cleaved-PKCδ by genetic depletion of p53 was more evident in mitochondrial-than cytosolic-fraction. The inhibition of mitochondrial translocation by genetic depletion of p53 was comparable to that by PFT against cocaine insult.

3.8. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced pro-apoptotic signals

We investigated whether p53 depletion affects cocaine-induced pro-apoptotic potentials. As shown in Fig. 9, cocaine treatment significantly increased cytosolic cytochrome c release from mitochondria ($P = 3.1 \times 10^{-17}$) (F [5, 60] = 53.142, $P = 1.2 \times 10^{-9}$, Fig. 9A), cleaved caspase-3 ($P = 1.1 \times 10^{-9}$) (F [5, 60] = 48.289, $P = 9.9 \times 10^{-19}$, Fig. 9B), and Bax ($P = 1.014 \times 10^{-14}$) (F [5, 60] = 37.54, $P = 2.1 \times 10^{-9}$, Fig. 9C) expression (Fig. 9A–D), whereas significantly decreased anti-apoptotic factors i.e., Bcl-2 ($P = 7.6 \times 10^{-10}$) (F [5, 60] = 17.4, $P = 3.02 \times 10^{-10}$, Fig. 9E), and Bcl-xL ($P = 1.09 \times 10^{-11}$) (F [5, 60] = 24.37, $P = 9.58 \times 10^{-13}$, Fig. 9F). PFT or p53 knockout significantly attenuated cocaine-induced increases in cytosolic cytochrome c release (PFT; $P = 1.8 \times 10^{-4}$, p53 knockout; $P = 4.02 \times 10^{-9}$), cleaved caspase-3 (PFT; $P = 1.8 \times 10^{-4}$, p53 knockout; $P = 3.24 \times 10^{-9}$), and Bax (PFT; $P = 7.8 \times 10^{-8}$, p53 knockout; $P = 1.75 \times 10^{-8}$) expression. Consistently, PFT or p53 knockout attenuated cocaine-induced decreases in Bcl-2 (PFT; $P = 0.001$, p53 knockout; $P = 0.002$), and Bcl-xL (PFT; $P = 7.8 \times 10^{-5}$, p53 knockout; $P = 5.04 \times 10^{-4}$), respectively (Fig. 9B–F).

4. Discussion

We demonstrated in this study that pharmacological or genetic inhibition of p53 protects against cocaine-induced kindling (convulsive) neurotoxicity. Here we observed that cocaine-induced expression of p53 gene was more pronounced in the hippocampus than other brain regions. Cocaine treatment significantly facilitated c-Fos-IR, mitochondrial dysfunction, mitochondrial translocation of p53, cleaved-PKCδ, and pro-apoptotic changes. These neurotoxic changes were attenuated by inhibition of p53, suggesting that p53 is a therapeutic target against cocaine insult. In current study, cocaine-induced convulsive behaviors occurred within 80 s (Supplementary Fig. S4), it remains to be further elucidated on the biological events during the pre-convulsive period.

Cocaine-induced convulsive behaviors have been associated with high mortality (Spivey and Euerle 1990). Earlier studies demonstrated that different strains of animals exhibit differences in their response to sub-convulsive doses of cocaine for producing convulsive behaviors (Marley et al., 1992; Marley et al., 1991). In this study, repeated escalating doses of cocaine-induced kindling effect, which was paralleled

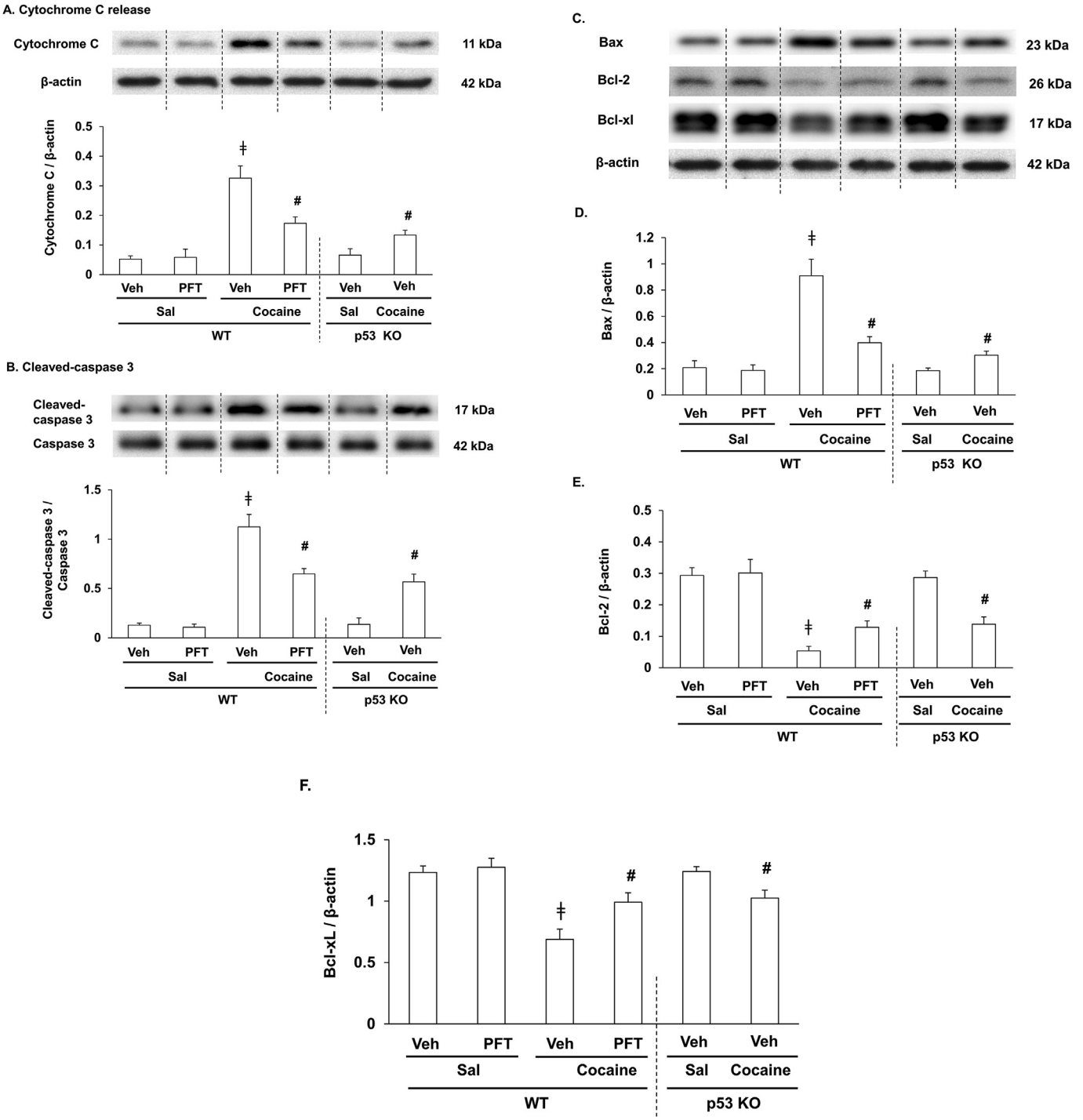

Fig. 9. Effects of pifithrin-μ (PFT), a p53 inhibitor, or genetic depletion of p53 against cocaine-induced pro-apoptotic potentials in the hippocampus of mice. (A) Effect on cytosolic cytochrome C release. (B) Effect on Cleaved-caspase-3 expression. (C) Representative expression in Bax, Bcl-2, and Bcl-xL. (D) Effect on Bax expression. (E) Effect on Bcl-2 expression. (F) Effect on Bcl-xL expression. Sal = saline, Veh = Vehicle (5% DMSO), WT = wild-type mice, p53 KO = p53 knockout mice. Each value is the mean ± SEM of 10 animals. $^{‡}P < 0.01$ vs. corresponding Veh/Sal, $^{\#}P < 0.01$ vs. Veh/Cocaine/WT (three-way ANOVA with Fisher's LSD pairwise comparisons).

to our previous demonstration (Mai et al., 2018a). Moreover, the paradigm is at least in line with other kindling models, such as amygdala- or pentylenetetrazol-kindled models (Goddard et al., 1969; Pinel and Cheung 1977).

Cocaine treatment triggers nuclear events such as chromatin remodeling linked with increased expressions of immediate-early genes, such as c-*fos* (Larson et al., 2010; Xu et al., 2013). Cocaine treatment induces c-Fos expression in brain (Buffalari and Rinaman 2014; Carbo-Gas et al., 2014), which possibly contributes to an imbalance in enzymatic antioxidant system in rodent (Dietrich et al., 2005; Kitano et al., 1989). Consistently, cocaine treatment significantly impaired glutathione system in the hippocampus (Muriach et al., 2010). Although a single dose of cocaine (60 mg/kg, i.p.) also induces seizure by 60%, and induces p53 expression 6 h later, it does not induce c-Fos level in the hippocampus. Therefore, neither PFT nor p53 knockout significantly affects seizures induced by a toxic dose of cocaine. Therefore, it is

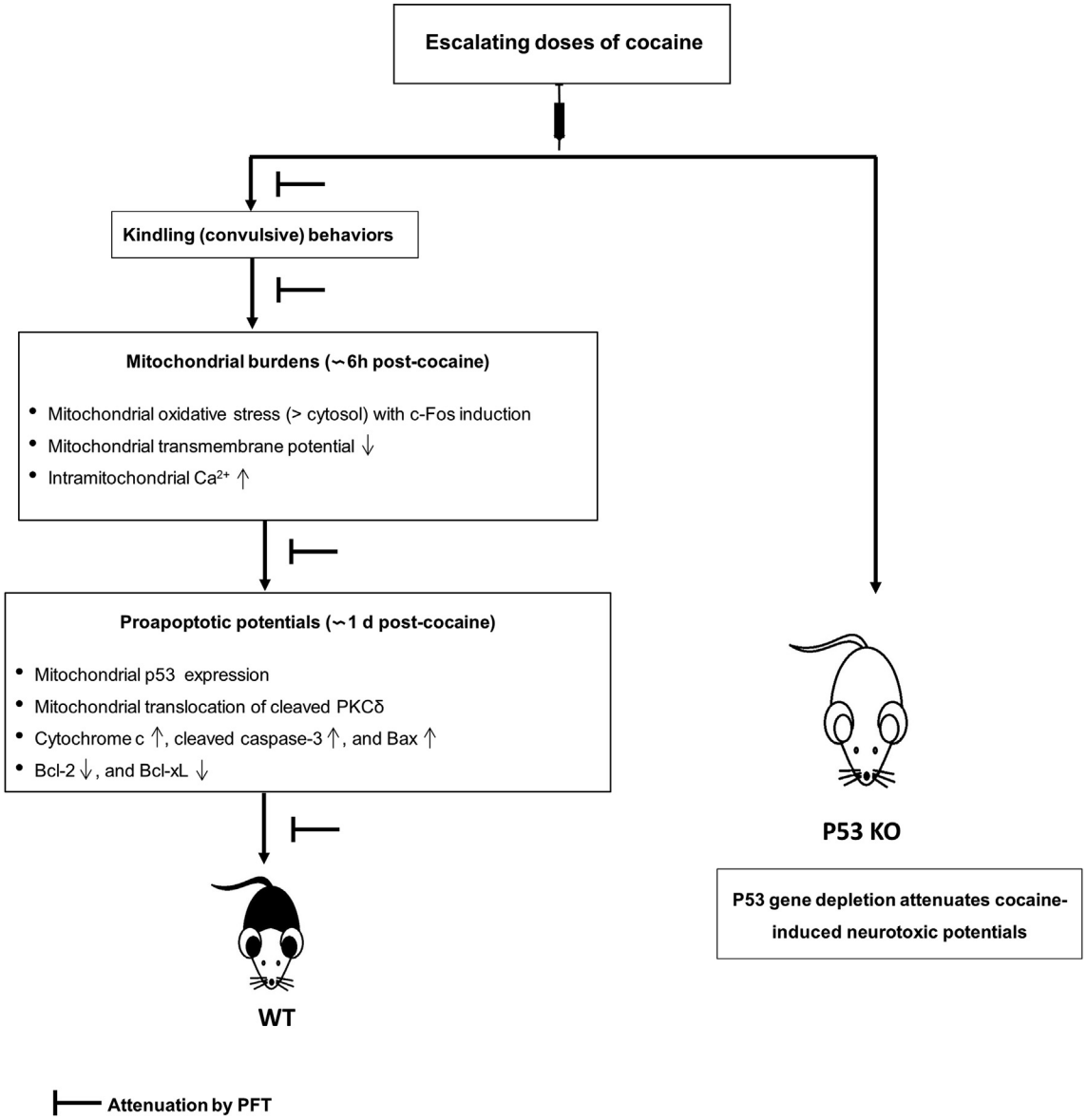

Fig. 10. A schematic depiction on p53 gene depletion-mediated protective effects against cocaine-induced convulsive neurotoxicity. Escalating doses of cocaine potentiated kindling behaviors. Kindling behaviors resulted in an early induction (6 h post-cocaine) of c-Fos, and in mitochondrial burdens. Kindling behaviors facilitated p53 and cleaved PKCδ expressions in the mitochondria followed by proapoptotic changes (1d post-cocaine). Genetic depletion (KO) or pharmacological inhibition (PFT) of p53 significantly attenuated cocaine-induced convulsive neurotoxicity. We propose here that p53 gene might be a potential therapeutic target against cocaine-induced kindling behaviors of neurotoxicity.

plausible that excitotoxic and proapoptotic potentials are more pronounced in case of multiple cocaine treatments (kindling model) than in case of a single cocaine treatment. However, this phenomenon remains to be further characterized.

P53 is a tumor-suppressor gene, and also a redox-sensitive transcription factor (Seemann and Hainaut 2005). Although it is recognized that targeting p53 increases the sensitivity for cancer risk (Ozaki and Nakagawara 2011; Uehara and Tanaka 2018), p53 in non-cancerous cells also involved in oxidative stress-induced apoptosis (Argiolas et al., 1985; Ma et al., 2018; Ma et al., 2013). In addition, p53 is an important regulatory molecule for different cellular signaling networks, such as cell cycle arrest or apoptosis (Beyfuss and Hood 2018; Gudkov and Komarova 2007; Jebelli et al., 2012; Ozaki and Nakagawara 2011). Indeed, it is well-recognized that p53 plays an active role in proapoptotic processes in neurodegenerative disorders, including Parkinson's disease-like conditions (Camins et al., 2008; Checler and Alves da Costa, 2014; Shin et al., 2016a; Speidel 2010).

Moreover, altered p53 expressions have been well-recognized in the different brain regions, such as frontal cortex (Guilarte et al., 2008), hippocampus (Araki et al., 2004), and striatum (Xu et al., 2013). In addition, previous studies suggested that alteration in hippocampal p53 could be a major cause of seizure generation (Araki et al., 2004; Morrison et al., 1996). Consistently, kainic acid (KA)-induced seizures facilitated p53 expression and neuronal degeneration. Whereas, genetic depletion of p53 attenuated KA-induced neuronal damage (Morrison et al., 1996). Therefore, it is proposed that p53 induction might be responsible for epileptogenesis (Araki et al., 2004; Engel et al., 2007; Morrison et al., 1996), suggesting that current study supports their finding.

Evidence suggested that synaptic action of dopamine is critical for cocaine-induced behavioral effects (Amara and Kuhar 1993; Amara and Sonders 1998). Xu et al. (2013) demonstrated repeated high doses of cocaine increase the phosphorylation of cAMP response element-binding protein (CREB) kinase, c-Fos, and p53 signaling systems.

Moreover, stimulation of dopamine-receptors activates cAMP-dependent protein kinase A pathway (Calabresi et al., 2000; Mahajan et al., 2009), which might further lead to phosphorylation of DARPP-32, an important regulator of cocaine-induced signaling (Greengard et al., 1999; Svenningsson et al., 2005). However, the role of dopamine receptor in cocaine-induced enhanced p53 expression remains to be further elucidated.

Previously, it has been demonstrated that mitochondrial dysfunction might also cause seizure-induced brain damage (Kudin et al., 2002; Liang et al., 2000; Liang and Patel 2004; Shin et al., 2008a; Shin et al., 2011; Shin et al., 2008b). Consistently (Cunha-Oliveira et al., 2006b), reported that cocaine treatment alters cell viability, mitochondrial membrane potential, and cytochrome c release in cultured neurons. Therefore, it is plausible that alteration in mitochondrial functions might lead to epileptogenesis or seizure generation (Kudin et al., 2002; Kunz 2002).

It has been reported that mitochondrial dysfunction requires a loss of homeostasis in Ca^{2+}, and leads to seizure generation (Zsurka and Kunz 2015). In mitochondria, p53 bind to Mn-SOD (SOD-2) and thereby suppress its antioxidant activity (Zhao et al., 2005), confirming that p53-induced mitochondrial dysfunction is important for seizure generation. These findings are in line with our previous studies (Shin et al., 2016a), indicating that recovery of mitochondrial function may be critical for the protective potentials mediated by depletion of p53 against cocaine insult.

Mitochondrial translocation of p53 is contributable to transcription-independent apoptosis (Chi 2014; Speidel 2010), possibly by interaction with anti-apoptotic factors such as Bcl-2 or BclxL (Chipuk et al., 2004; Mihara et al., 2003; Shin et al., 2016a), and might facilitate caspase 3 activation, and then apoptotic signaling. We demonstrated that PFT selectively attenuated p53 mitochondrial translocation as well as apoptosis (Shin et al., 2016a; Vaseva and Moll 2009). In our previous study, we showed that inhibition of p53 mitochondrial translocation is essential for N-methyl, N-propynyl-2-phenylethylamine (MPPE)-mediated neuroprotective potentials (Shin et al., 2016a). Hirata and Cadet (1997a) showed that genetic depletion of p53 protects against MA-induced dopaminergic toxicity. Therefore, it is plausible that genetic depletion or pharmacological inhibition of p53 might be effective against neurotoxicity induced by certain psychostimulants. However, p53 depletion conveyed positive modulation in mitochondrial function against cocaine excitotoxicity remains to be further elucidated.

PKCδ is well-known redox-sensitive kinase (Ryer et al., 2005; Shin et al., 2017; Ward et al., 1998) and has been associated with seizures generation (Kim et al., 2010; Shin et al., 2016b). We previously demonstrated that activation of PKCδ is essential for inducing convulsive behaviors, (Shin et al., 2016b). It is recognized that direct phosphorylation of N-terminal of p53 by PKCδ causes stabilization and accumulation of p53 protein in cells, which finally stimulates apoptotic signaling (Lee et al., 2006; Rogoff et al., 2002). In contrast, inhibition of p53 attenuated cleaved-PKCδ, which further inhibited cocaine-induced pro-apoptotic changes, indicating that PKCδ activation may be important for cocaine-induced convulsive neurotoxicity.

Taken together, we observed that genetic or pharmacological inhibition of p53 alleviates cocaine-induced convulsive behaviors. In addition, p53 depletion attenuates mitochondrial p53 translocation, oxidative stress, mitochondrial dysfunction, and pro-apoptotic signaling. Our findings indicate that recovery of mitochondrial function by genetic or pharmacological inhibition of p53 may be essential for protective potentials against cocaine-induced convulsive behaviors (Fig. 10). Finally, we propose that p53 gene might be a potential therapeutic target against cocaine-induced convulsive behaviors.

Conflicts of interest

Authors reported no potential conflicts of interest relevant to this article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.12.017.

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