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ORIGINAL ARTICLE

Pifithrin-Alpha Reduces Methamphetamine Neurotoxicity in Cultured Dopaminergic Neurons

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Abstract

Methamphetamine (Meth) is a widely abused stimulant. High-dose Meth induces degeneration of dopaminergic neurons through p53-mediated apoptosis. A recent study indicated that treatment with the p53 inhibitor, pifithrin-alpha (PFT-α), antagonized Meth-mediated behavioral deficits in mice. The mechanisms underpinning the protective action of PFT-α against Meth have not been identified, and hence, their investigation is the focus of this study. Primary dopaminergic neuronal cultures were prepared from rat embryonic ventral mesencephalic tissue. High-dose Meth challenge reduced tyrosine hydroxylase immunoreactivity and increased terminal deoxynucleotidyl transferase-mediated dNTP nick-end labeling (TUNEL) labeling. PFT-α significantly antagonized these responses. PFT-α also reduced Meth-activated translocation of p53 to the nucleus, an initial step before transcription. Previous studies have indicated that p53 can also activate cell death through transcription-independent pathways. We found that PFT-α attenuated endoplasmic reticulum (ER) stressor thapsigargin (Tg)-mediated loss of dopaminergic neurons. ER stress was further monitored through the release of Gaussia luciferase (GLuc) from SH-SY5Y cells overexpressing GLuc-based Secreted ER Calcium-Modulated Protein (GLuc-SERCaMP). Meth or Tg significantly increased GLuc release in to the media, with PFT-α significantly reducing GLuc release. Additionally, PFT-α significantly attenuated Meth-induced CHOP expression. In conclusion, our data indicate that PFT-α is neuroprotective against Meth-mediated neurodegeneration via transcription-dependent nuclear and -independent cytosolic ER stress pathways.

Keywords Methamphetamine · p53 · Pifithrin-alpha · Dopaminergic · Degeneration

Abbreviations

BSA	Bovine serum albumin
CHOP	CCAAT/enhancer-binding protein (C/EBP) homologous protein
DIV	Day in vitro
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
GLuc	Gaussia luciferase
Meth	Methamphetamine
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PFT-α	Pifithrin-alpha
SERCaMP	Secreted ER calcium-modulated protein
Tg	Thapsigargin
TH	Tyrosine hydroxylase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dNTP nick-end labeling
VM	Ventral mesencephalon

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Introduction

Methamphetamine (Meth) is a highly addictive and popular psychostimulant (UNODC 2018). Abuse of Meth can lead to degeneration of dopaminergic neurons in animals (Cadet et al.

1998; Reiner et al. 2014; Chou et al. 2008) and patients (Volkow et al. 2001). Meth-mediated neurotoxicity has been linked to various molecular mechanisms, including excitotoxicity (Yu et al. 2016), autophagy (Xu et al. 2018), oxidative stress (Kita et al. 2003), endoplasmic reticulum stress (ER stress) (Jayanthi et al. 2009; Takeichi et al. 2012), and apoptosis (Deng et al. 2001).

p53 is a key nuclear transcription factor modulating cell apoptosis and death. After injury, p53 protein translocates from the cytosolic to the nuclear compartment (Uberti et al. 1999) followed by activation of proapoptotic target genes (i.e., PUMA, PTEN, Apaft-1, PERP) (Sullivan et al. 2018). p53 also induces apoptosis/cell death via a transcription-independent pathway by caspase-dependent apoptosis and ER stress in cytoplasm.

Increasing scientific evidence suggests that p53 may be a major factor in Meth-mediated neurodegeneration. Specifically, Meth increased the expression of p53 mRNA (Imam et al. 2001) and p53 DNA-binding activity (Asanuma et al. 2002). Similar to p53, Meth activated proapoptotic genes (Asanuma et al. 2007) and increased apoptotic protein production (Shen et al. 2016). Knocking out p53 significantly reduced Meth-induced damage of dopaminergic cells (Hirata and Cadet 1997). Finally, a recent study demonstrated that DA-specific p53 gene deletion in DAT-p53 knockout mice had less decline in TH protein levels in striatum and locomotor activity after binge Meth compared to the wide-type controls (Lu et al. 2017). These data support the notion that the apoptosis-inducing factor p53 is involved in the regulation of Meth neurotoxicity in dopaminergic neurons.

Pifithrin-alpha (PFT-α) is a small synthetic p53 inhibitor initially identified from a screening library to mitigate doxorubicin-induced apoptosis (Komarov et al. 1999) that, together with its analogs, are now widely used in neuroscience to block neuronal apoptotic cell death (Zhu et al. 2002). We and others previously demonstrated that PFT-α reduced terminal deoxynucleotidyl transferase-mediated dNTP nick-end labeling (TUNEL) labeling in stroke brain (Luo et al. 2009; Leker et al. 2004) and ischemia-mediated PUMA activation in the SVZ (Luo et al. 2009). PFT-α also reduced dopaminergic neurodegeneration in animal models of Parkinson's disease (Liang et al. 2007; Duan et al. 2002), prevented cell death of dopaminergic transplants in 6-OHDA-lesioned rats (Chou et al. 2011), improved the survival of neuroprogenitor cells in subventricular zone of stroke rats (Luo et al. 2009), and reduced traumatic brain injury (Huang et al. 2018; Yang et al. 2015). These data suggest that PFT-α has multiple neuroprotective actions. The interaction of PFT-α and Meth has not been well examined. Although PFT-α has been demonstrated to mitigate behavioral deficits induced by binge Meth in rodents (Lu et al. 2017), the protective action of PFT-α against Meth toxicity has not been identified.

We previously reported a method to monitor ER calcium homeostasis through the secreted ER calcium-monitoring proteins (SERCaMPs) in SY5Y cells (Henderson et al. 2014). These proteins were designed based on the structure of an ER stress-sensitive trophic factor named Mesencephalic Astrocyte-derived Neurotrophic Factor (MANF) (Apostolou et al. 2008). The C-terminal ASRTDL sequence in MANF is essential for the ER stress-mediated secretion (Henderson et al. 2013). A Gaussian luciferase (GLuc) reporter with a modified carboxy terminal containing ASRTDL (GLuc-SERCaMP) has been successfully developed. GLuc-SERCaMP can respond to the ER stressor (i.e., thapsigargin or Tg) and release GLuc into the culture media (Henderson et al. 2014). This reporter has also been used in vivo to show that systemic delivery of Tg significantly increased plasma GLuc levels in rats receiving intrahepatic injection of AAV-GLuc-SerCaMP (Wires et al. 2017a). The technique has been further applied to monitor ER stress of liver by high-fat diet in rodents (Wires et al. 2017b) and to screen therapeutics for the treatment of ER stress. Recent work shows that elevated levels of extracellular GLuc-SERCaMP reflect a loss of ER resident proteins from the ER lumen (Trychta et al. 2018).

The goal of this study was to examine the protective effects of PFT-α against Meth neurotoxicity in cultured dopaminergic neurons. We found that treatment with PFT-α reduced Meth-mediated dopaminergic degeneration in ventral mesencephalic (VM) cultures. We further demonstrated that PFT-α reduced Meth-mediated p53 nuclear translocation in primary dopaminergic neurons and reduced GLuc release in SY5Y cells over-expressing GLuc-SerCaMP. Our data support the concept that PFT-α is neuroprotective against Meth-mediated neurodegeneration via transcription-dependent nuclear and -independent ER stress pathways.

Materials and Methods

Primary dopaminergic neuronal cultures were prepared from VM tissues of E14-E15 embryos obtained from timed-pregnant Sprague-Dawley rats as described previously (Reiner et al. 2014), in accordance with protocols approved by the Animal Research Ethics Board at the National Health Research Institutes, Taiwan. The dissected brain VM tissues were pooled and then incubated with pre-warmed trypsin-EDTA (0.25%) with gentle agitation for 15 min at 37 °C. The digested tissues were washed three times with pre-warmed Dulbecco's modified Eagle medium (DMEM)/F12 and then triturated. The dissociated cells were diluted in the neuron culture medium and seeded at different density for further analysis. (1) For immunocytochemical analysis, cells were seeded at a density of 6×10^4 viable cells/well into 96-well cell culture plates. (2) For confocal imaging analysis, cells were seeded at a density of 1×10^5 viable cells/well in

12-well slice culture plates. (3) For Western blot analysis, dissociated cells were diluted in the neuron culture medium and seeded at a density of 1×10^6 viable cells/well into 6-well cell culture plates. All culture plates or coverslips were coated with poly-D-lysine (Sigma-Aldrich). The VM neuronal culture medium was composed of DMEM/F12, 10% heat-inactivated fetal bovine serum, 2% B27 supplement, and 1 mM $_L$-glutamine. VM cultures were maintained at 37 °C in a humidified incubator with 5% CO_2. On days in vitro (DIV) 3 and 5, one-half volume of media was replaced with fresh Neurobasal media (Invitrogen), supplemented with 0.5 mM $_L$-glutamate and 2% B27 supplement with antioxidants. On DIV 7 and 10, cells were kept in supplemented Neurobasal media without antioxidants. To evaluate the effects of Meth and PFT-α on the dopaminergic neurons, vehicle, we added Meth (1 mM, from the National Bureau of Controlled Drugs, Taiwan FDA, Department of Health, Taiwan), PFT-α (10 μM, from the National Institute of Aging, NIH), or both Meth and PFT-α to the neuron cultures on DIV 10. The doses of Meth and PFT-α were selected based on previous reports (Chou et al. 2008; Reiner et al. 2014; Rachmany et al. 2013). After incubation at 37 °C for 48 h, cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for immunocytochemistry.

Immunocytochemistry

The PFA-fixed cultures were washed with PBS and then incubated with the blocking solution (0.1% Triton X-100, 5% BSA in PBS) for 1 h. Cells were then stained with a TH-specific mouse monoclonal or rabbit polyclonal antibody (Cat#MAB318 or AB152, Millipore), CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) specific mouse monoclonal antibody (Cat#2895, Cell Signaling Technology), and a p53-specific mouse monoclonal antibody (Cat#sc-126, Santa Cruz Biotechnology) at 4 °C for overnight. After washing with PBS three times, cells were incubated with an Alexa-fluor-488-conjugated goat anti-mouse secondary antibody (Cat#A11001, Invitrogen) and an Alexa-fluor-568-conjugated goat anti-mouse or anti-rabbit secondary antibody (Cat#A11031 or A11011, Invitrogen) for 1 h, followed by washing with PBS three times. Cell nuclei were stained with DAPI at room temperature for 10 min. Cells were examined by a NIKON TE2000 inverted microscope (Nikon) and D-ECLIPSE 80i confocal laser-scanning microscope (Nikon) equipped with a ×100 oil-immersion objective; fluorescence images were obtained by EZ-C1 3.90 software (Nikon). TH-positive cells in each well of a 96-well plate were manually counted from ×10 images of four microscopic fields, and data were expressed as a percentage of the vehicle group. The intensity of intracellular green fluorescence was individually measured by the NIS Elements AR 3.2 Software (Nikon).

Separation of Cytosolic and Nuclear Fractions and Western Blot Analysis

On DIV12, cells were harvested. The nuclear and cytoplasmic fractions of whole cell lysates were extracted by using the NE-PER extraction reagent (#78833, Thermo Scientific), according to the manufacturer's instructions. The obtained samples were mixed with 4X NuPAGE LDS sample buffer (#NP0008, Thermo Scientific) and heated at 95 °C for 5 min. Samples were then subjected to electrophoretic separation on a 12% NuPAGE gel (#NP0321PK2, Thermo Scientific), and the separated proteins were electrophoretically transferred from the gel to a PVDF membrane (#RPN303F, GE Healthcare). The membrane was incubated with the blocking buffer (5% skim milk and 0.1% Tween-20 in PBS) at 4 °C overnight and then incubated with appropriate antibodies against p53 (1:500; #2524, Cell Signaling), GADPH (1:50,000; #GTX100118, GeneTex), or HDAC2 (1:1500; #GTX112957, GeneTex) in 10 mL blocking buffer for 1 h with gentle agitation. After washing with 0.1% Tween-20 (in PBS) three times for 10 min each, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat polyclonal antibodies against mouse IgG (1:5000; #GTX21311-01, GeneTex) or rabbit IgG (1:5000; #GTX21310-01, GeneTex) in 10 mL blocking buffer for 1 h, followed by the washing procedure as described above. The light emission signals of the target proteins on the membrane were generated using an enhanced chemiluminescence reagent (#ORT2655, PerkinElmer) and detected by X-ray films (#NEF596, Kodak). The intensity of detected signals was quantified using ImageJ software.

SH-SY5Y-GLuc-SERCaMP Cell Culture

SH-SY5Y-GLuc-SERCaMP were cultured in a 37 °C humidified incubator with 5% CO_2 in DMEM (4.5 g/L D-gLucose) containing 2 mM GlutaMAX, 10% bovine growth serum (Sigma-Aldrich), 10 U/mL penicillin (Thermo Fisher Scientific), and 10 μg/mL streptomycin (Thermo Fisher Scientific). Cells were plated at 5×10^4 cells per well (100 μL volume). On the day, media were exchanged into DMEM (4.5 g/L D-gLucose) containing 2 mM GlutaMAX, 1.5% BGS, 10 U/mL penicillin, and 10 μg/mL streptomycin before 16-h drug pre-treatment. Cells were incubated for 48 h prior to adding drugs. Media was collected (5 μL) prior to and at indicated time points post-drug treatment for enzymatic assay, as described above.

Gaussia Luciferase Secretion Assay

Five microliters of culture medium were transferred to white 96-well plates. Coelenterazine (Cat# 1-361204-200, Regis Technologies) stock solutions were prepared at 20 mM in acidified methanol (10 μL of 10 N HCl/1 mL of methanol)

and stored at − 80 °C as single-use aliquots. The prepared substrate was incubated at room temperature 30 min prior to use. One-hundred microliters of the diluted substrate were injected into each well followed by immediate luminescence reading. The amount of luciferase was determined using a plate reader with an injector setup (Biotek Synergy HT, Winooski, VT) to immediately read the sample after injection. For secretion assays, vehicle controls were used in all experiments under conditions equivalent to the treatments.

TUNEL

Cultures were assayed for DNA fragmentation using a TUNEL-based method (In Situ Cell Death Detection Kit; Roche, Indianapolis, IN). Briefly, 4% PFA fixed cells were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. To label damaged nuclei, 50 μL of the TUNEL reaction mixture was added to each sample and kept at 37 °C in a humidified chamber for 60 min. Procedures for positive and negative controls were carried out as described in the manufacturer's manual (Roche). Controls consisted of not adding the label solution (terminal deoxynucleotidyl transferase) to the TUNEL reaction mixture. Material was examined using a Nikon TE2000 inverted microscope equipped with fluorescence. TUNEL (+) cells were manually counted in ×20 images (4 fields per well).

Statistical Analysis

All data were presented as means ± standard error of the mean (SEM) and analyzed by GraphPad Prism 6.0 software. Differences between two groups were evaluated by Student's *t* test. Comparisons between multiple groups were performed by one-way or two-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc test. Statistical significance was considered in cases where two-tailed analysis yielded $p < 0.05$.

Results

PFT-α Reduces Meth-Induced Degeneration of Dopaminergic Neurons

The protective effects of PFT-α against Meth neurotoxicity were examined in the primary dopaminergic VM neuronal cultures. Cells were treated with vehicle, Meth (1 mM), PFT-α (10 μM), or Meth (1 mM) + PFT-α (10 μM) on DIV 10 for 48 h. Similar to previous reports (Chou et al. 2008; Reiner et al. 2014), Meth significantly reduced TH immunoreactivity ($p < 0.001$). Whereas PFT-α did not alter TH immunoreactivity in the absence of Meth ($p = 0.861$), however significantly antagonized Meth-mediated dopaminergic neuronal loss ($F_{3,20} = 79.32$, one-way ANOVA; $p = 0.004$, post hoc Newman-Keuls test). Meth increased TUNEL labeling (Fig. 1c), which was also significantly mitigated by the co-treatment with PFT-α (Fig. 1c, d; $p < 0.001$, ANOVA on Rank; $p < 0.05$, post hoc Newman-Keuls). These results suggest that PFT-α has protective actions against Meth-induced neurotoxicity in cultured VM dopaminergic neurons.

PFT-α Reduces Meth-Mediated Translocation of p53 to Nuclei

To investigate the effects of PFT-α on Meth-induced nuclear translocation of p53 in dopaminergic neurons, we treated rat VM neuron cultures with Meth (1 mM), PFT-α (10 μM) + Meth (1 mM), or vehicle for 48 h, and then examined nuclear p53 levels in each group by Western blot analysis. First, the whole cell lysates were separated into nuclear and cytoplasmic fractions and then probed for GAPDH (a cytoplasm protein) and HDAC2 (a nuclear protein). As shown in Fig. 2a, the majority of GAPDH was identified in the cytoplasmic fractions, while the majority of HDAC2 was identified in the nuclear fractions, indicating that both sample fractions were only minimally contaminated with each other. In the nuclear fractions, p53 levels were significantly increased in the Meth-challenged groups, compared to the vehicle groups (Fig. 2b, c; $p = 0.025$, $F_{2,6} = 7.082$, one-way ANOVA + Newman-Keuls test). However, when cells were treated with PFT-α + Meth, p53 was significantly reduced to a level similar to that in the vehicle groups (Fig. 2b, c; $p = 0.039$, one-way ANOVA + Newman-Keuls test). Taken together, these results suggest that PFT-α can attenuate Meth-induced nuclear translocation of p53 in dopaminergic neurons.

To cross-validate our Western blot data, the translocation of p53 within dopaminergic neurons was further examined by confocal microscopy. VM cells were plated at low density on a cover slice cultures (1×10^5 viable cells/well in 12-well slice culture plates) to clearly illustrate p53 immunoreactivity in individual TH neurons. In vehicle (control) culture (in the absence of Meth challenge), p53 immunoreactivity resided in the cytosol of dopaminergic neurons (Fig. 3) and non-dopaminergic cells (Supplemental Fig. 1). Following Meth administration, p53 immunoreactivity was evident within the nucleus of TH neurons. Co-treatment with PFT-α prevented the migration of p53 into the nucleus, as p53 immunoreactivity was mainly localized in the cytosol (Fig. 3). These data suggest that PFT-α suppressed Meth-mediated nuclear translocation of p53 in dopaminergic neurons.

PFT-α Suppresses Meth-Induced CHOP Expression and ER Stress

p53 has known interactions with ER stress. For example, ER stress stimulates p53 expression; a loss of p53 hinders ER

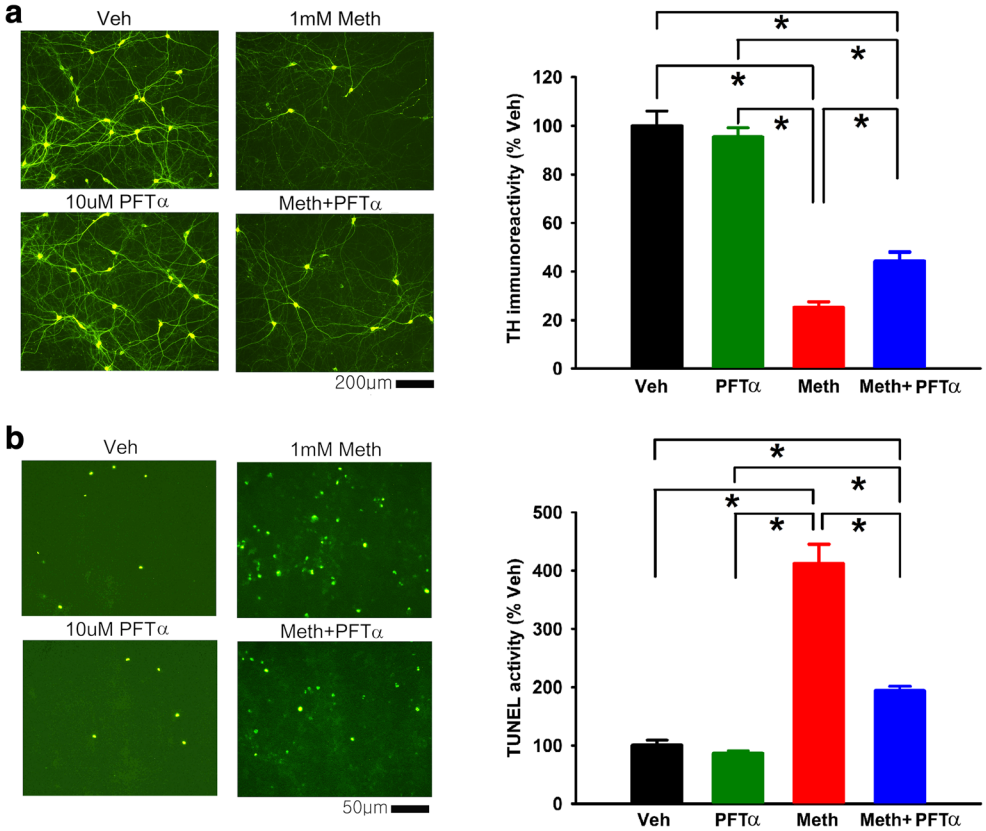

Fig. 1 PFT-α attenuated Meth-induced degeneration of TH (+) cells in cultured ventromesencephalic cells. Cells were treated with vehicle, Meth (1 mM), 10 μM PFT-α, or both on DIV10 for 48 h and then subjected to **a**, **b** TH immunostaining or **c**, **d** TUNEL labeling. **a** Representative photomicrographs illustrate that Meth reduced the number of TH (+) cells. PFT-α alone did not change the number of TH (+) cells but ameliorated Meth-mediated loss of TH (+) cells. Scale bar = 200 μm. **b** TH (+) cells were normalized to the mean of vehicle group. Meth induced a reduction in TH immunoreactivity (*$p < 0.0001$ Meth vs. vehicle) that was significantly antagonized by PFT-α ($p = 0.004$, PFTα + Meth vs. Meth; one-way ANOVA + Newman-Keuls test). **c** Representative photomicrographs demonstrate that Meth increased TUNEL activity, which was antagonized by PFT-α. Scale bar = 50 μm. **d** PFT-α significantly antagonized Meth-induced TUNEL activity ($p < 0.05$, one-way ANOVA on Rank)

stress and enhances ER function (Byun et al. 2015). To further examine the interaction of PFT-α and Meth on ER stress, the expression of CHOP was examined after Meth treatment. We found that Meth treatment increased immunoreactivity of

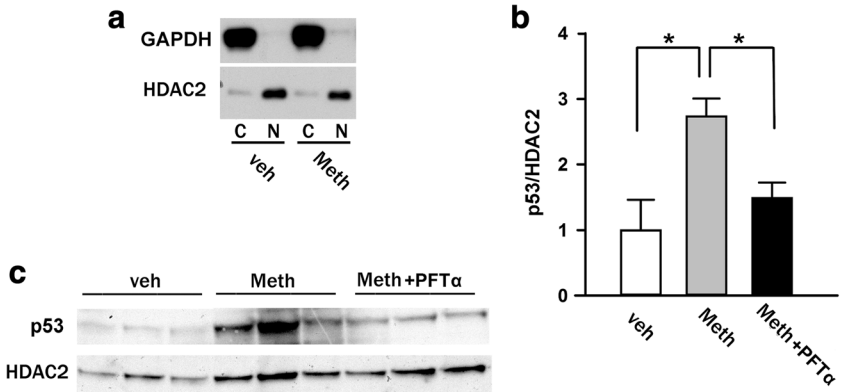

Fig. 2 PFT-α attenuated Meth-induced nuclear translocation of p53. Primary neuron cultures of rat ventral mesencephalon were treated with Meth (1 mM), PFT-α (10 μM) + Meth (1 mM), or vehicle on days in vitro 10 (DIV10) for 48 h. **a** The nuclear (N) and cytoplasmic (C) fractions of whole cell lysates were then subjected to Western blot analysis using antibodies against GAPDH (a cytoplasm protein) and HDAC2 (a nuclear protein). **b** The nuclear fractions with three replications were examined for the expression of p53 and HDAC2 by Western blot analysis. **c** The p53 levels were normalized to HDAC2 levels, and relative optical density values were expressed as folds of vehicle groups. The results represent mean values ± SEM of three replications from a representative result. Significant differences are indicated (*$p < 0.05$; one-way ANOVA)

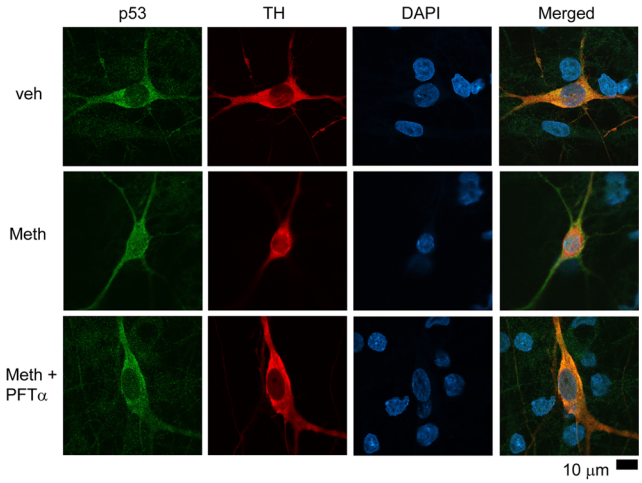

Fig. 3 PFT-α suppresses Meth-induced translocation of p53 to the nucleus of dopaminergic neurons. Primary ventral mesencephalon cultures were treated with vehicle, Meth, or Meth + PFT-α on DIV 10 for 24 h, followed by immunostaining for p53 (green) and TH (red). In the TH (+) cells of vehicle-treated group, p53 was predominantly localized in the cytosol. In contrast, an increase of p53 immunoreactivity was found within the nucleus of TH (+) cells after Meth challenge. Notably, co-treatment with PFT-α reduced p53 within the nucleus

CHOP, an apoptotic marker induced by ER stress (Zinszner et al. 1998), in VM culture ($p < 0.001$, $F_{3,19} = 638.03$, one-way ANOVA; $p < 0.001$, post hoc Newman-Keuls test); this response was significantly mitigated by PFT-α (Fig. 4; $p < 0.001$).

PFT-α mitigates Tg-mediated ER stress and neurodegeneration

Similar to Meth, Tg also induced dopaminergic neurodegeneration. Specifically, TH immunoreactivity was significantly suppressed by Tg in primary VM cultures (Fig. 5; $p < 0.001$, $F_{2,15} = 271.564$, one-way ANOVA + Newman-Keuls test). This Tg-induced loss of TH immunoreactivity was significantly antagonized by co-administration of PFT-α (Fig. 5d; $p < 0.001$).

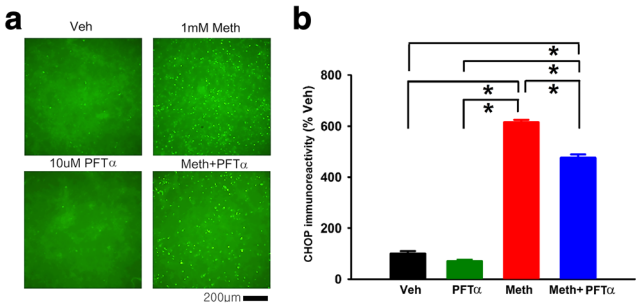

Fig. 4 PFT-α reduced Meth-mediated CHOP immunoreactivity in VM cultures. **a** Representative photomicrographs indicated that Meth challenge increased CHOP expression, which was antagonized by the co-treatment with PFT-α. **b** CHOP immunoreactivity was quantified and normalized to the mean of the vehicle control. PFT-α significantly reduced Meth-mediated CHOP expression ($p < 0.001$)

Meth- and Tg-mediated ER stress was further examined in SY5Y cells overexpressing GLuc-SERCaMP, a reporter of ER stress that is triggered by ER calcium depletion (Henderson et al. 2014). The level of extracellular GLuc-SERCaMP has been used as an indicator of a change in ER proteostasis (Trychta et al. 2018). Culture media was collected before, as well as at 24 and 48 h after drug treatment. Meth and Tg time-dependently increased GLuc release into the media (Fig. 6a: Meth vs. veh: $p < 0.001$, $F_{3,75} = 64.857$; Fig. 6b: Tg vs. veh: $p < 0.001$, $F_{3,102} = 11.843$, two-way ANOVA + Newman-Keuls test). Both responses were significantly attenuated by PFT-α (Fig. 6a: $p < 0.001$, Meth + PFT-α vs. Meth; Fig. 6b: $p < 0.001$, Tg + PFT-α vs. Tg). Combined together, our data indicate that PFT-α significantly inhibited Meth-mediated ER stress.

Discussion

The principal finding of the present study was that PFT-α antagonized Meth-mediated dopaminergic neurodegeneration by inhibiting p53 translocation into the nucleus and ER stress within the cytoplasm. Similar to previous reports (Reiner et al. 2014; Chou et al. 2008), we demonstrated that Meth reduced TH immunoreactivity while it increased TUNEL labeling in primary dopaminergic neuron cultures. Meth-induced cell death has been linked to apoptosis (Deng et al. 2001) and ER stress (Jayanthi et al. 2004). In this study, we demonstrated that PFT-α antagonized Meth-mediated changes in TH immunoreactivity, TUNEL, p53 nuclear translocation in dopaminergic neuronal culture, and expression of CHOP. Our data indicate that PFT-α is a potent neuroprotective agent against Meth-mediated toxicity in dopaminergic neurons through multiple pathways.

p53 plays an important role in Meth neurotoxicity. High doses of Meth have been reported to increase p53 expression in striatum while reducing DAT mRNA level and TH cell numbers in nigra (Imam et al. 2001; Hirata and Cadet 1997). Knocking out p53 (Hirata and Cadet 1997) or suppressing the p53-activated target gene PAG608 by antisense (Asanuma et al. 2007) reduced Meth-mediated neurodegeneration. Selectively knocking out the p53 gene in dopaminergic neurons reduced Meth-mediated neurodegeneration (Lu et al. 2017). These data suggest that Meth can induce dopaminergic neuronal injury by acting through p53. p53 is a nuclear transcriptional factor that is vital in maintaining cellular genomic integrity and controlled cell growth, and is widely considered as a cellular gatekeeper to programmed cell death (Pietsch et al. 2008). After cell injury, p53 translocates to the nucleus and activates apoptotic genes, including PUMA, PTEN, Apaft-1, and PERP (Beyfuss and Hood 2018). Selectively deleting the p53 gene in dopamine neurons reduced the expression of p53 target genes after binge Meth (Lu et al. 2017). In this study, we demonstrated that PFT-α reduced Meth-mediated translocation of p53 protein to nucleus. As seen by confocal imaging, the change in p53 translocation was mainly

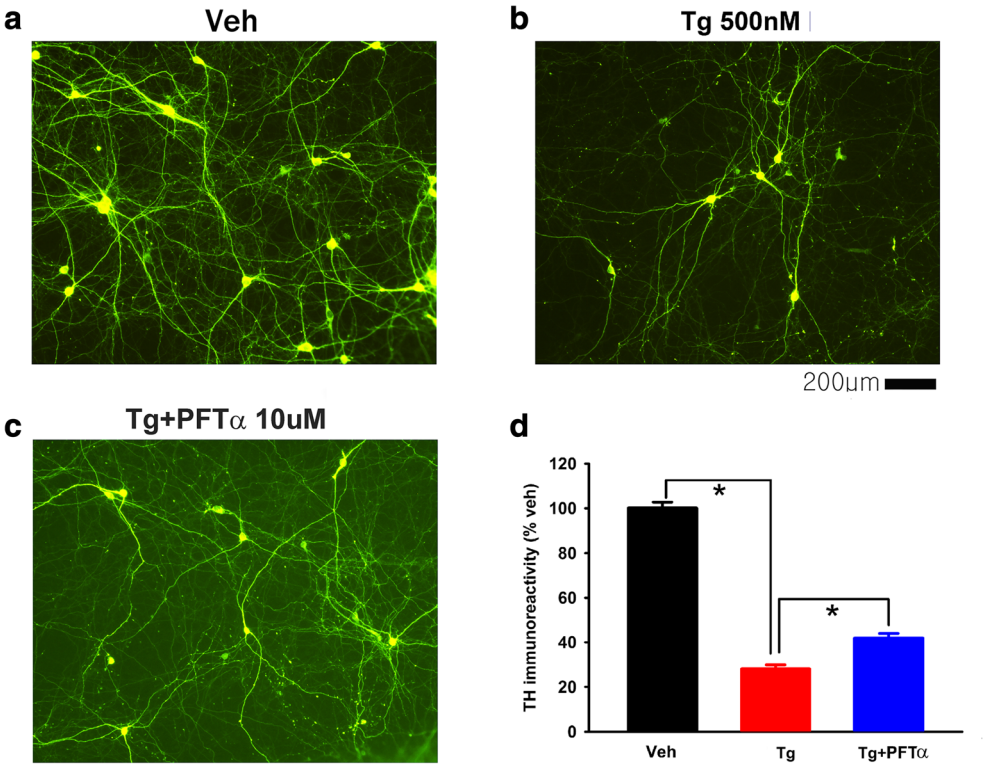

Fig. 5 PFT-α antagonized Tg-mediated dopaminergic neurodegeneration in primary ventromesencephalic cultures. **a** Treatment with Tg at 500 nM reduced TH immunoreactivity. Co-administration of PFT-α partially restored TH immunoreactivity. **b** TH immunoreactivity was quantified and normalized to the mean of the veh control group. Tg significantly suppressed TH immunoreactivity. The loss of TH immunoreactivity after Tg was significantly antagonized by PFT-α (*$p < 0.001$)

located in the TH neurons, suggesting that PFT-α selectively inhibits Meth-mediated p53 nuclear translocation, which would reduce the subsequent transcription-dependent neurodegeneration in dopaminergic neurons. This is in accordance with a study by Leker et al. (2004)), demonstrating that the administration of PFT-α to rats challenged with transient middle cerebral artery occlusion, inhibited p53 and p21WAF translocation to the nucleus of neuronal cells, as likewise evaluated by immunostaining, resulting in reduced neuronal cell loss, stroke volume, and motor disabilities.

Increasing evidence supports the concept that Meth induces neurodegeneration through ER stress. Meth increased the phosphorylation and production of ER stress sensor proteins (i.e., p-IRE1, p-PERK, ATF6, and the pro-apoptotic protein CHOP) in BMVEC (bEND3) cells (Qie et al. 2017) and induced an upregulation of ER stress genes (Bip/Grp-78, Atf2, C/EBP homologous protein) in striatum after binge administration of Meth in rats (Beauvais et al. 2011). Silencing ATF6, IRE1a, PERK, or CHOP by siRNA reduced Meth-mediated cell death in cultured astrocytes (Shah and Kumar 2016).

p53 can activate apoptosis through changes of ER Ca^{++}; a loss of p53 hindered ER stress and enhanced ER function (Byun et al. 2015). Intracellular Ca^{++} homeostasis plays important roles in cell signaling and function. The cytosolic Ca^{++} level under resting condition is approximately 100 nM. The majority of Ca^{++} is stored in the ER at a much higher concentration of Ca^{++} (i.e., 100–800 μM) and is kept in the ER through three Ca^{++} regulator proteins on the ER membrane (Sacro ER Ca^{++} ATPase protein or

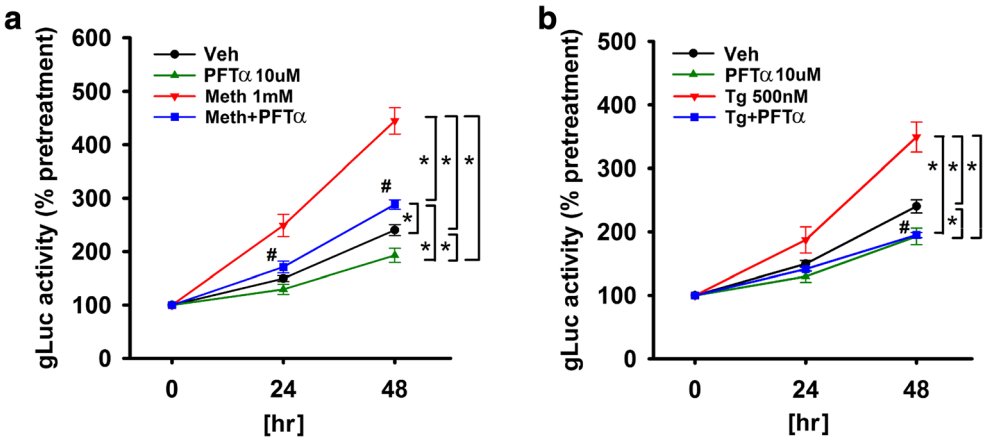

Fig. 6 PFT-α antagonized Meth- and Tg-mediated ER stress in SY5Y cells overexpressing GLuc-SERCaMP. Culture media was collected before, 24, and 48 h after Meth and Tg challenge. Meth and Tg time-dependently increased GLuc release to the media. Both responses were significantly attenuated by PFT-α co-administration ($p < 0.001$)

SERCA, Ryanodine receptor (RyR), and inositol triphosphate receptor (IP3R)) (Raffaello et al. 2016). p53 binds to the SERCA upon injury, followed by depleting ER Ca^{++}, the unfolded protein response, and cell apoptosis (Giorgi et al. 2015). Similar responses can be found after challenging with the ER stressor Tg. Tg inhibits SERCA, depletes ER Ca^{++}, increases the formation of unfolded or misfolded protein, and causes cell apoptosis and death (Giorgi et al. 2016). In this study, we monitored ER calcium homeostasis through GLuc-SERCaMP in SY5Y cells (Henderson et al. 2014). In response to low Ca^{++} in ER, GLuc is released into the culture media (Henderson et al. 2014). Recently, it was shown that the secretion of GLuc-SERCamp reflects a departure of ER resident proteins containing an ER retention signal (ERS) into the extracellular space when ER calcium is decreased, a phenomenon referred to as "exodosis" (Trychta et al. 2018). We found that both Meth and Tg significantly elevated GLuc levels in the media indicating, for the first time, that Meth may trigger exodosis. These responses were antagonized by PFT-α. Previous studies have shown that knockdown of molecules within the ER stress pathways reduced Meth-mediated CHOP expression (Shah and Kumar 2016). We additionally demonstrated that the p53 inhibitor PFT-α reduced Meth-mediated CHOP expression in primary dopaminergic neurons. Our data hence support the view that PFT-α is neuroprotective against Meth toxicity through inhibition of ER stress.

In response to death stimuli, p53 can also mediate apoptosis through translocation to mitochondria (Culmsee et al. 2001; Endo et al. 2006) where it induces permeabilization of the outer mitochondrial membrane, resulting in cytochrome C release and cascades of caspase-dependent apoptosis (Wang et al. 2014). Further work is required to delineate the mechanisms which underline PFT-α-mediated protection in mitochondria.

The protective effect of PFT-α against Meth toxicity has been documented in a behavioral study in rodents. Treatment with PFT-α reduced Meth-mediated rearing behavior deficits (Lu et al. 2017). Our work provides a mechanistic underpinning the prior research and, to our knowledge, is the first to demonstrate that PFT-α antagonizes Meth-induced neurodegeneration of dopaminergic neurons. In this regard, we demonstrated that PFT-α reduced Meth-mediated p53 translocation, apoptosis, and ER stress, which are closely associated with p53 action in nucleus and ER. One report has indicated that PFT-α induces protection after silencing the p53 gene in hippocampal HT-22 neuronal cells (Neitemeier et al. 2014). It is possible that non-p53 mechanisms may also be involved in the PFT-α protective action, which may require further investigation.

The use of p53 inhibitors for the treatment of p53-related pathologies, such as Meth-induced neurodegeneration, may raise a safety concern because an increased risk of tumor development is observed in mice and humans with p53 deficiency (Donehower et al. 1992; Varley et al. 1997). However, accumulating evidence based on pharmacological and genetic experiments has indicated that temporary suppression of p53 by PFT-α and genetic silencing does not increase the frequency of cancer (Christophorou et al. 2006; Gudkov and Komarova 2010; Komarov et al. 1999). Although there are no currently available FDA-approved p53 inhibitors for any clinical use, PFT-α is a potential agent worth evaluating for the treatment of acute Meth intoxication.

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