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Repeated exposure of cocaine alters mitochondrial dynamics in mouse neuroblastoma Neuro2a

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ABSTRACT

Chronic abuse of psychoactive drugs including cocaine causes addiction, dependence, and brain disorders through the alteration of neuronal plasticity. Mitochondrial fission and fusion, collectively called as mitochondrial dynamics, participates in not only the maintenance of neuronal homeostasis but also reorganization of neuronal circuits. The purpose of this study is to examine effects of direct and repeated exposure of cocaine on mitochondrial dynamics in neuronal cells *in vitro*. Repeated exposure to 600 μM cocaine (2 ~ 3 times per week) of Neuro2a mouse neuroblastoma cells for 3 weeks resulted in decrease of mitochondrial transmembrane potential, activation of autophagy, and upregulation of Parkin, a protein involved in mitochondrial autophagy. Increased expression of mitochondrial fission genes and significant increase in the ser-616 phosphorylated-DRP1, the key regulator of mitochondrial fission, were observed in the cells exposed repeatedly to 600 μM cocaine. Electron microscopy showed significant increase in the number of mitochondria in cocaine-treated cells compared with control cells. Thus, our results show that repeated cocaine exposure not only causes mitochondrial dysfunction but also alters mitochondrial dynamics in Neuro2a cells. Changes in the mitochondrial dynamics might be involved in neural adaptation during repeated cocaine exposure.

1. Introduction

Aberrant and repeated use of psychostimulant drugs, such as cocaine, amphetamine, and methamphetamine, not only induces reorganization of neural circuits as well as brain functions but also leads to dependence and addiction to the drugs (Kelley, 2004). Physical as well as psychological addiction to abused drugs are results from alterations of neural networks, dysfunction of neurons, and subsequent brain disorders. Cocaine abuse leads to a lot of neuronal disorders such as depression (Rounsaville, 2004), anxiety (Vorspan et al., 2015), and decreased appetite (Ersche et al., 2013). Long term chronic cocaine might rise a risk for ischemic stroke (Fonseca and Ferro, 2013; Levine et al., 1990). Emerging roles of mitochondria in the development of addiction, dependence, and damage of brain have been suggested for psychoactive drugs, especially cocaine (de Oliveira and Jardim, 2016). Indeed, it has been demonstrated that cocaine induces dysfunction of mitochondria and subsequent apoptosis of cortical neurons (Cunha-Oliveira et al., 2006). Inhibition of complex I of mitochondria by cocaine was observed in experiments using purified mitochondria from brain *in vitro*, suggesting direct inhibitory effects of cocaine on mitochondrial complex I in the brain (Cunha-Oliveira et al., 2013).

Mitochondria are dynamic organelles that continuously undergo fission and fusion (Friedman and Nunnari, 2014; Westermann, 2010). The balance between fission and fusion is important for proper function of neuronal cells. Mitochondrial fission and fusion are mediated by a panel of GTPase family proteins, and dynamin-related protein 1 (DRP1) play central role in the division process of mitochondria (Friedman and Nunnari, 2014; Westermann, 2010). During the division of mitochondria, DRP1 forms spiral-shaped self-oligomers at the constriction sites of mitochondria and, like dynamin that functions as the molecular scissor for the generation of endosomes from plasma membrane, constricts mitochondrial outer membranes (Ferguson and De Camilli, 2012; Ingerman et al., 2005). Mitochondrial fission 1 (FIS1) is also involved in the division of mitochondria through assisting the recruitment of DRP1 from cytoplasm onto mitochondrial outer membrane (Yoon et al., 2003). Dominant optic atrophy 1 (OPA1), as well as mitofusin 1 and 2 (MFN1 and 2), is another member of GTPase family proteins involved in the fusion of mitochondria. Balance between fission and fusion is inevitable for mitochondrial as well as cellular homeostasis, since mutations in OPA1 and MFN1 result in optic atrophy and Charcot-Marie-Tooth disease type 2a, respectively, through impairment of mitochondrial fusion in neuronal cells (Alexander et al., 2000; Delettre et al.,

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2000; Zuchner et al., 2004).

It should be useful if change of mitochondrial dynamics could be evaluated in *in vitro* system, especially in immortalized neuronal cells, for the following reasons. Studies using experimental animals are time-consuming and difficult to examine mechanistic aspects of neurotoxicity. Primary cultured neurons have lifetime limitations. Indeed, change of mitochondrial dynamics in central nerves system of mice become to be detectable after 1 week of binge cocaine administration (Chandra et al., 2017). Among various neuronal cell lines, we choose Neuro2a murine neuroblastoma since the cells are relatively resistant to cell death by cocaine compared to other neuronal cells (Badisa et al., 2018b). Taking advantage of the use of immortalized cells, we exposed the cells to cocaine for 3 weeks and examined whether repeated cocaine exposure affects mitochondrial dynamics in neuronal cells *in vitro*.

2. Materials and methods

2.1. Cells and repeated cocaine administration

Neuro2a mouse neuroblastoma cells were cultured at 37 °C in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cocaine hydrochloride (hereafter referred to as cocaine) was obtained from SHIONOGI & CO., LTD. (Osaka, Japan). Cocaine was dissolved in water and stored at −20 °C until use. For repeated cocaine treatment, we sub-cultured the cells 2–3 times a week. Every time we sub-cultured the cells, cocaine-containing medium was replenished with a medium freshly added with cocaine. Before harvesting cells, cocaine-containing medium was replenished 6 times. We cultured the cells in cocaine-containing medium for indicated time periods (3 weeks) and used for analysis.

2.2. Cell viability

Cellular viabilities were determined by Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan), which is based on modified MTT assay using a water-soluble tetrazolium salt (WST-8) (Ishiyama et al., 1997). MTT assay estimates cellular viabilities through measuring cellular dehydrogenase activity. WST-8 is reduced in a manner dependent on the generation of NADH through cellular dehydrogenase activity. Reduced WST-8 is an orange formazan product, which could be quantified by measuring absorbance at 450 nm. Cells were grown on 96-well plates and exposed to indicated concentrations of cocaine for 24 h. Then, 10 μl aliquot of CCK-8 solution was added to the medium (100 μl /well), followed by incubation for 30 min. at 37 °C. The absorbance at 450 nm was measured using a microplate reader (ARVO X series, Perkin Elmer, Waltham, MA). Cellular viabilities were calculated as relative ratios of cocaine-exposed cells per control cells.

2.3. Immunoblotting

Neuro2a cells were washed with PBS and lysed by ultrasonic disruption in lysis buffer [10 mM Tris−HCl (pH 7.4), 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), protease inhibitor cocktail (11697498001, Complete, Roche, Mannheim, Germany), 50 mM NaF, and 10 mM Na_3VO_4]. Protein concentrations of the lysates were determined using a Coomassie Protein Assay Kit (23200, Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were denatured in Laemmli's buffer and subjected to sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene difluoride (PVDF) membrane. Immunoblot analysis was performed with anti-cleaved caspase3 (#9661, Cell Signaling Technology, Beverly, CA), anti-caspase3 (#9662, Cell Signaling Technology), anti-ser 227-phosphorylated-receptor interacting protein3 (phosho-RIP3, ab209384, abcam, Cambridge, UK), anti-(total) RIP3 (ab56164, abcam), anti-Parkin (#4211, Cell Signaling Technology), anti-microtubule-associated protein light chain (LC3, #2775, Cell Signaling Technology), anti-p62 (PM045, MBL, Osaka, Japan), anti-ser 616-phosphoylated DRP1 (pDRP1, #4494, Cell Signaling Technology), anti-(total) DRP1 (#8570, Cell Signaling Technology), anti-translocase of outer membrane20 (TOM20, #42406, Cell Signaling Technology), anti-cytochrome c oxidase subunit IV (COX IV, A21348, Thermo Fisher Scientific) or anti-actin (A2066, Sigma-Aldrich, St. Louis, MO) antibodies. Peroxidase-conjugated anti-rabbit and -mouse antibodies (W4011 and W4021, Promega, Madison, MI) were used as second antibodies. Relative protein levels were determined using a software for densitometric analysis (CS Analyzer, ver. 4.0, ATTO, Tokyo, Japan) and normalized to the actin levels.

2.4. Flow cytometric analysis

Mitochondrial transmembrane potential (ΔΨm) was evaluated by flow cytometry using JC-1 cationic dye. In brief, cells treated with or without cocaine were washed with PBS, incubated with JC-1 at a final concentration of 2 μg/ml for 10 min. at room temperature, and analyzed by a flow cytometer (CytoFLEX, Beckman Coulter, Brea, CA). Green fluorescence from JC-1 monomer was observed through FITC channel while Red fluorescence from JC-1 aggregates were observed through PE channel.

2.5. qPCR

For quantitation of relative mRNA abundances, total cellular RNA was extracted from Neuro2a cells using TRIzol reagent (15596026, Thermo Fisher Scientific), and complementary DNA was synthesized by reverse transcription using SuperScript II enzyme (18064-014, Thermo Fisher Scientific) and oligo $(dT)_{15}$. Quantitative reverse-transcriptase-mediated PCR (qPCR) was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) using a GoTaq qPCR master mixture including SYBR green (A6001, Promega). The PCR reaction conditions were: 95 °C for 20 s, followed by 40 cycle of 95 °C for 1 s and 60 °C for 20 s. The primers used are listed in Table S1.

2.6. Fluorescence microscopy

Plasmid vector expressing green fluorescence protein tagged-LC3 (GFP-LC3, kindly provided by Dr. Takeshi Noda, Osaka University) was transfected transiently into Neuro2a cells by use of Lipofectamine2000 (11688-019, Thermo Fisher Scientific). For immunocytochemistry, the cells were fixed with 4% paraformaldehyde (PFA) in PBS, membrane permeabilized by 0.5% Triton-X100 in PBS, and incubated with anti-Parkin antibody at 4 °C over night. Then the cells were further incubated with Alexa488-conjugated anti-mouse IgG and observed under fluorescence microscopy (DMi8, Leica, Wetzlar, Germany).

2.7. Transmission electron microscopy

Transmission electron microscopy was performed as described previously (Funakoshi et al., 2016). In brief, cocaine-treated and untreated Neuro2a cells were washed with 0.1 M phosphate buffer (PB) and fixed with 2.5% glutaraldehyde in PB. After washing in 0.1 M PB, the fixed cells were incubated with 1% osmium tetroxide for 2 h, dehydrated in ethanol and embedded in Epon epoxy resin. Ultrathin sections of the embedded Neuro2a cells were stained with uranyl acetate and lead citrate, and examined under an electron microscope (H7100, Hitachi, Japan). Quantification of mitochondria was performed as follows. Number of mitochondria per field was counted from 10 electron micrographs in each group. Cross-sectional areas of mitochondria (47 for control group and 90 for cocaine group) were counted using ImageJ software (ver1.47).

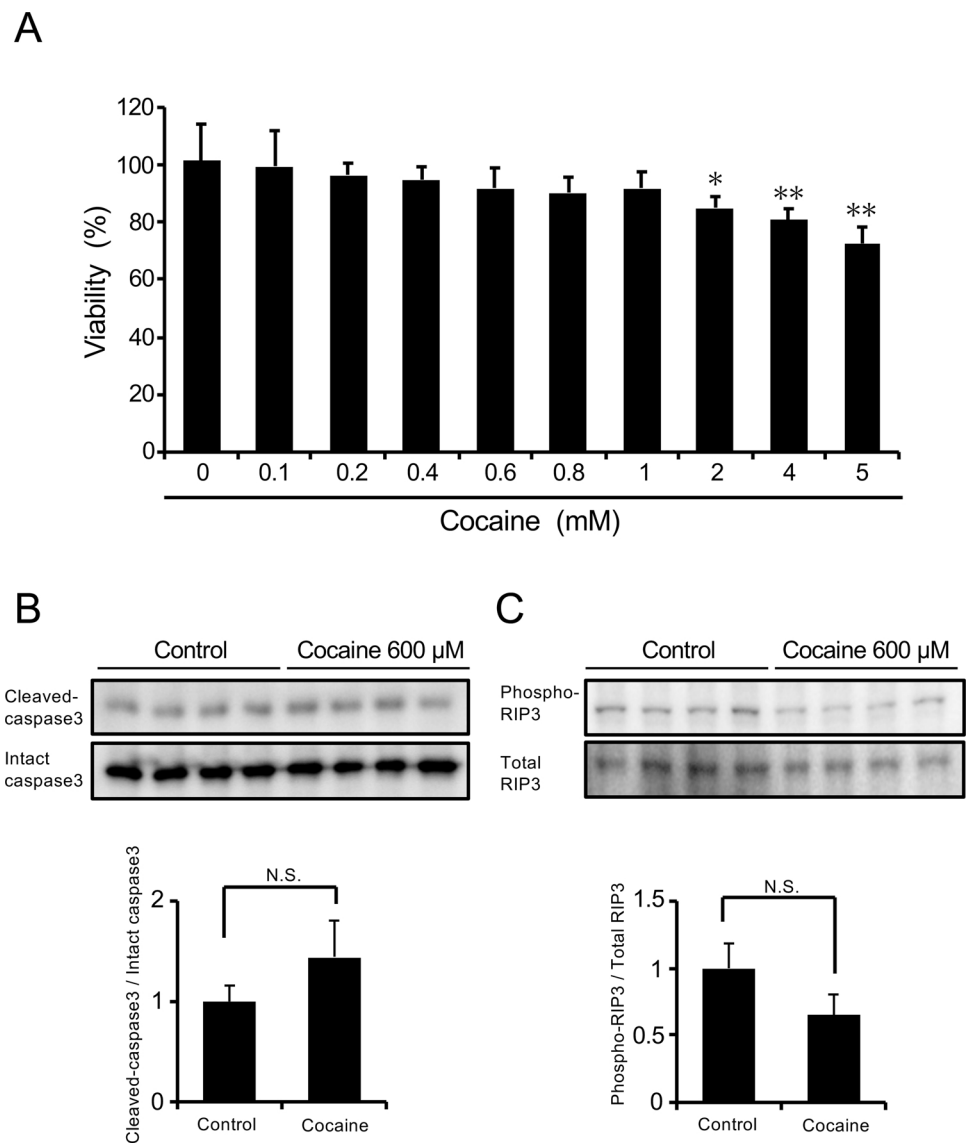

Fig. 1. Repeated cocaine exposure does not induce cell death. (A) Cytotoxicity of cocaine on Neuro2a cells. The cells were exposed to indicated concentrations of cocaine for 24 h and cellular viabilities were determined by CCK-8 assay (modified MTT assay). Each bar represents mean and S.D. of 4 samples. *, $p < 0.05$; **, $p < 0.01$ *versus* 0 mM by Dunnett's test. (B and C) Effects of cocaine on the levels of cleaved- and intact caspase3 (B) and ser-227 phosphorylated- and total RIP3 (C) in Neuro2a cells. Cell lysates were prepared from the cells treated with or without cocaine (600 μM, 3 weeks) and subjected to immunoblot analysis. Each bar represents mean and S.D. of 4 samples. N.S., not significant.

2.8. Statistical analysis

Student's *t*-test and Dunnett's test was used to assess the statistical significance throughout of this study. Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. Neither apoptosis nor necroptosis is induced in Neuro2a cells exposed to 600 μM cocaine for 3 weeks

Before examining the effect of repeated cocaine exposure, we first determined cellular viabilities of Neuro2a cells 24 h after single exposure to indicated concentrations of cocaine. As shown in Fig. 1A, significant decreases of cellular viability were observed in the cells expose to more than 2 mM cocaine. In contrast, cells exposed to less than 1 mM cocaine did not show any decreases in cellular viabilities within 24 h after the treatment (Fig. 1A). To make sure that repeated cocaine exposure would not induce significant loss of cellular viabilities, we adopted 600 μM cocaine for repeated exposure of the cells. The cells were cultured in the medium with or without 600 μM cocaine for 3 weeks. We then examined whether two typical pathways of cell death, apoptosis and necroptosis, were activated or not. For this purpose, we conducted immunoblot analysis to examine the activation of caspase3 and RIP3, executers of apoptosis and necroptosis, respectively. Caspase3 is activated through cleavage from inactive intact form into active form (Porter and Janicke, 1999), whilst RIP3 is activated through phosphorylation at ser 227 (ser 232 for mouse) (Chen et al., 2013). Although slight but not significant increase of cleaved (activated)-caspase3 levels was observed in the cells exposed to 600 μM cocaine for 3 weeks, no increase of phosphorylated (activated)-RIP3 levels was observed in that cells (Fig. 1 B and C). Therefore, apoptosis is negligible and necroptosis is not occurred during repeated cocaine exposure. Thus, we adopted this concentration of cocaine (600 μM) for further analysis.

3.2. Mitochondrial dysfunction and activation of autophagy in Neuro2a cells exposed to 600 μM cocaine for 3 weeks

We next examined whether mitochondrial dysfunction was occurred in the cells exposed repeatedly to 600 μM cocaine for 3 weeks. To evaluate the mitochondrial transmembrane potential (ΔΨm), cells exposed repeatedly to cocaine were loaded with JC-1 and analyzed by flow cytometry. As shown in Fig. 2A, increased green fluorescence from JC-1 monomer, which indicates low concentration of JC-1 in mitochondria due to low ΔΨm, was observed in cocaine-exposed cells

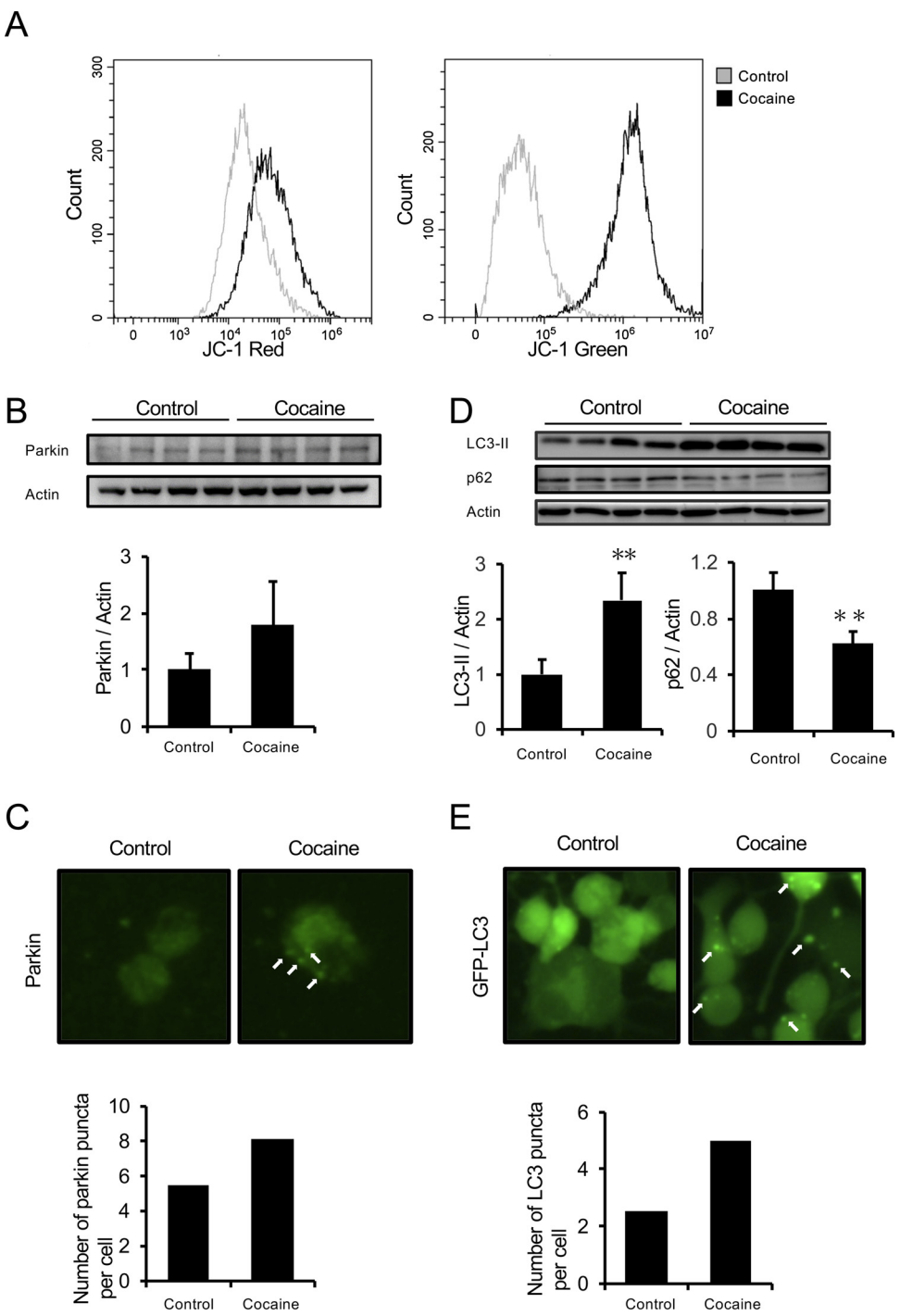

Fig. 2. Repeated cocaine exposure decreases mitochondrial membrane potential and activates autophagy. (A) Effects of repeated cocaine exposure on mitochondrial transmembrane potential (ΔΨm) in Neuro2a cells. The cells were exposed to cocaine (600 μM, 3 weeks) and ΔΨm was determined by flow cytometry using JC-1 fluorescence dye. JC-1 Red and Green fluorescences were observed by use of PE and FITC filters, respectively. (B and D) Effects of repeated cocaine exposure on the levels of Parkin (B), as well as LC3-II and p62 (D), in Neuro2a cells. Cell lysates were prepared from cocaine-exposed and unexposed cells and subjected to immunoblot analysis. Levels of actin were served as internal control. Each bar represents mean and S.D. of 4 samples. **, $p < 0.01$ *versus* control. (C and E) Translocation of Parkin (C) and GFP-LC3 (E) from cytoplasm into punctate structures in Neuro2a cells repeatedly exposed to cocaine (600 μM, 3 weeks). Neuro2a cells exposed to cocaine were stained with anti-Parkin antibody (C) or transfected with GFP-LC3 expression vector (E). Then the cells were observed under a fluorescence microscope. Arrows indicate punctate structures of parkin and GFP-LC3. Average numbers of puncta/cell, which were obtained by examining 60 cells in each experimental group, were also shown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

compared with control cells. This result suggested decrease of ΔΨm in cocaine-exposed cells compared with control cells. In accordance with the result of JC-1 staining, cellular levels of Parkin, an E3 ubiquitin ligase, tended to increase in cocaine-exposed cells (Fig. 2B). Parkin is recruited to mitochondria through PTEN-induced kinase1 (PINK1), which is activated by decrease of ΔΨm (Chan et al., 2011; Narendra et al., 2008). PINK1-Parkin system is involved in the elimination of mitochondria through autophagy as well as proteasome. Immunocytochemical analysis also indicated that Parkin showed dotted localization in cocaine-exposed cells, probably due to its translocation to mitochondria (Fig. 2C). We then examined whether autophagy is induced or not by use of marker proteins, LC3 and p62. LC3-II is lipidated form of LC3 and this lipidation is necessary for autophagosome formation (Kabeya et al., 2000), whereas p62 is a substrate of autophagy and should be degraded in lysosome during the process of autophagy (Komatsu et al., 2007). Thus, LC3-II increases, while p62 decreases, during the progression of autophagy. Autophagy was upregulated in cocaine-exposed cells, since significant increase and decrease of LC3-II and p62, respectively, were observed in cocaine-exposed cells compared to control cells (Fig. 2D). LC3 punctuation was also observed in cocaine-exposed cells, further supporting the results of immunoblotting (Fig. 2E). Taken together, repeated cocaine exposure decreases ΔΨm, alters cellular localization of Parkin, and increases autophagy, which might be involved in the elimination of mitochondria.

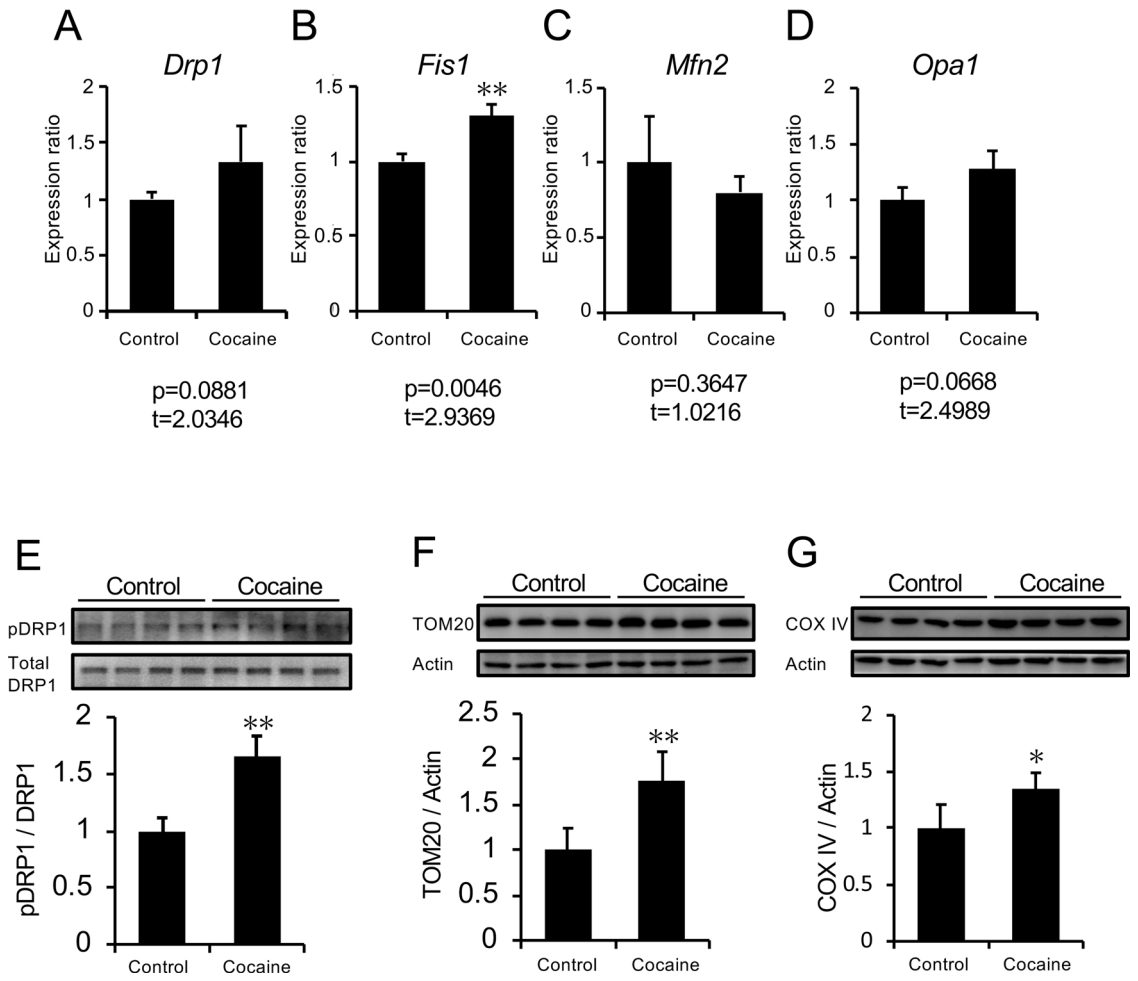

Fig. 3. Repeated cocaine exposure upregulates mitochondrial fission machinery. (A–D) Effects of repeated cocaine exposure (600 μM, 3 weeks) on expression of the genes for mitochondrial fission and fusion in Neuro2a cells. The cells were treated with cocaine and the levels of *Drp1* (A), *Fis1* (B), *Mfn2* (C), and *Opa1* (D) were examined by qPCR. Levels of *GAPDH* were also examined and served as internal control. (E–G) Effects of repeated cocaine exposure on the levels of ser-616 phosphorylated DRP1 (pDRP1) (E), TOM20 (F), and COX IV (G) in Neuro2a cells. Cell lysates were prepared from cocaine-exposed and unexposed cells and subjected to immunoblot analysis. Levels of total DRP1 and actin were served as internal control. Each bar represents mean and S.D. of 4 samples. *, $p < 0.05$, **, $p < 0.01$ *versus* control.

3.3. Activation of mitochondrial fission protein DRP1 in Neuro2a cells exposed to 600 μM cocaine for 3 weeks

We examined whether repeated cocaine treatment affects mitochondrial dynamics or not. Using qPCR analysis, we evaluated relative levels of transcripts involved in the regulation of mitochondrial dynamics (*Drp1*, *Fis1*, *Mfn2*, and *Opa1*). Levels of *Drp1* tended to increase (Fig. 3A), and that of *Fis1* increased significantly (Fig. 3B), in response to repeated cocaine exposure. In contrast, levels of *Mfn2*, which is involved in the fusion of mitochondria, was tended to decrease in response to cocaine exposure (Fig. 3C). Although Opa1 is involved in the process of mitochondrial fusion, its gene expression was tended to increase by repeated cocaine exposure (Fig. 3D). To further evaluate direction of mitochondrial dynamics in the cells exposed repeatedly to cocaine, we examined DRP1 by immunoblotting. DRP1 has been shown activated through phosphorylation at ser-616 (Xie et al., 2015). Immunoblot analysis showed that phosphorylated DRP1 (ser-616) increased in cocaine-exposed cells compared to control cells (Fig. 3E). In addition to increased ser-616-phosphorylated DRP1 levels, immunoblot analysis also showed that both the levels of TOM20 and COX IV, mitochondrial outer and inner membranous proteins, respectively, were significantly increased in cocaine-exposed cells (Fig. 3 F and G). Collectively, these results imply that mitochondrial dynamics is affected by repeated cocaine exposure towards fission of mitochondria.

3.4. Increased number of small mitochondria in Neuro2a cells exposed to 600 μM cocaine for 3 weeks

To obtain direct evidence of increased fission of mitochondria in the cells repeatedly exposed to cocaine, mitochondria were visualized through electron microscopy. As shown in Fig. 4A, there were more numbers of smaller mitochondria in cocaine-exposed cells compared to control cells. Approx. 2-fold increase in the number of mitochondria per areas were observed in cocaine-exposed cells compared to control cells (Fig. 4B). Average cross-sectional areas of mitochondria were 44.5×10^4 and 32.8×10^4 nm^2 in control and cocaine-exposed groups, respectively. The frequency distribution histogram showed that smaller cross-sectional areas of mitochondria were tended to increase in cocaine-exposed cells compared to control cells (Fig. 4C). Thus, these results present robust evidence that repeated cocaine exposure induces mitochondrial fission in the cells.

4. Discussion

In this study, we showed evidences that repeated cocaine exposure alters mitochondrial dynamics in neuronal cells. Mitochondrial damage in the brain has been reported repeatedly in experimental animal models of cocaine abuse. In addition, it has been reported recently that mitochondrial fission is increased in nucleus accumbens of mice

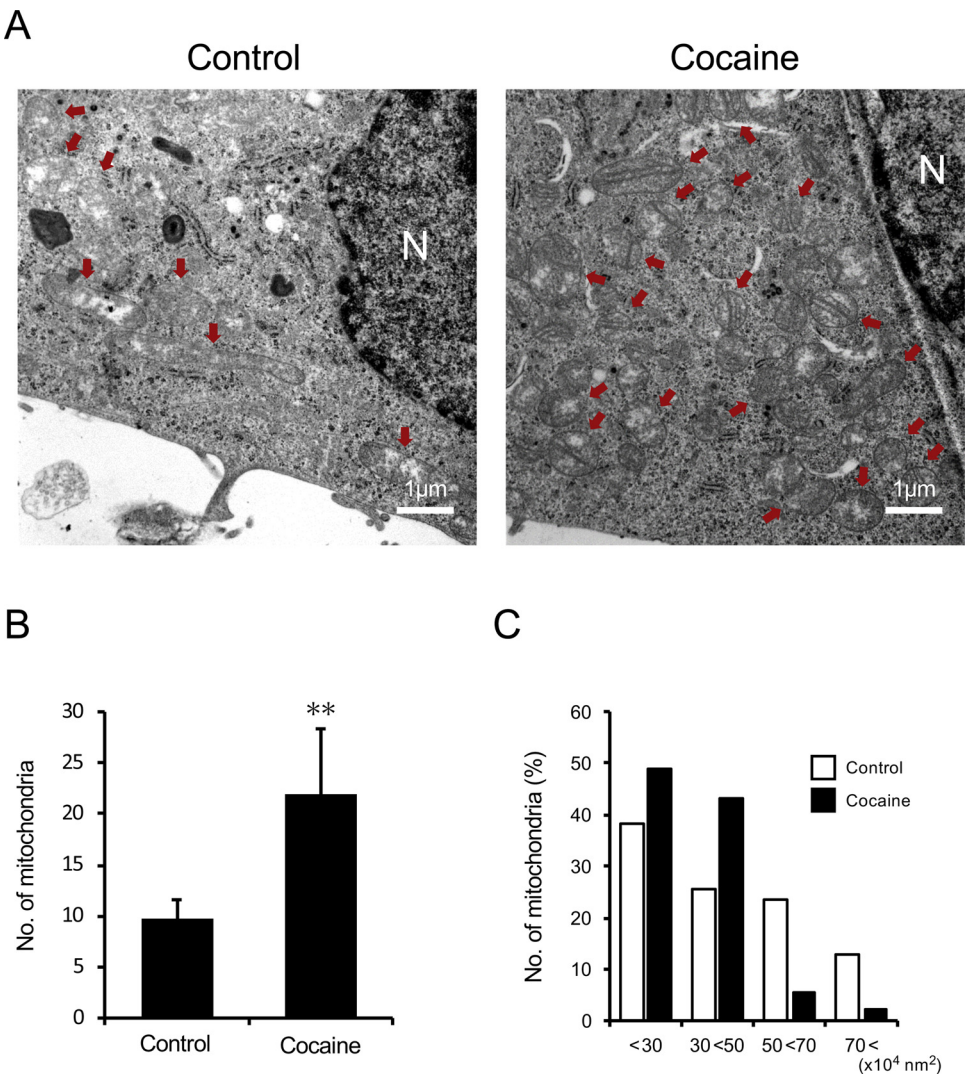

Fig. 4. Repeated cocaine exposure increases number of small mitochondria. (A) Transmission electron micrographs of Neuro2a cells treated with or without cocaine (600 μM, 3 weeks). Magnification, x15,000. N, Nucleus. Red arrows indicate mitochondria. (B) Cocaine increases the number of mitochondria per field in Neuro2a cells. Each bar represents mean and S.D. of 10 areas. **, $p < 0.01$ *versus* control. (C) Cocaine increases smaller mitochondria in Neuro2a cells. Comparison of the distribution of mitochondrial cross-sectional area in control and cocaine-exposed cells were shown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

repeatedly administered cocaine (Chandra et al., 2017). Our current results demonstrate that increased mitochondrial fission in neurons should be direct effect of cocaine and/or its metabolites.

It has been reported that plasma concentrations of cocaine in abusers reach 0.3 μM ~ 1 mM (Yuan and Acosta, 2000). Like other psychoactive drugs, cocaine could enter central nerve system through penetrating and/or destructing blood brain barrier (Kousik et al., 2012; Sharma et al., 2009). Indeed, brain/blood ratio of cocaine concentration was estimated 9.6 in cocaine overdose cases (Spiehler and Reed, 1985). Thus, our results obtained by using 600 μM cocaine should reflect, at least in parts, the events occurred in the neuron of cocaine abusers. In contrast to the change towards fission in mitochondrial dynamics, we observed only negligible levels of cell death (Fig. 1). Although cell death of primary cultured neurons was observed by administration of as low as several 100 μM of cocaine (Guha et al., 2016), previous reports also indicated that neuroblastoma (Neuro2a), pheochromocytoma (PC12), and neuroteratocarcinoma (NT-2) cells are relatively resistant to cocaine cytotoxicity. Badisa et al. reported that within 1 h after the treatment, cocaine did not induce death of Neuro2a even applied at 4 mM (Badisa et al., 2018b). Oliveira et al. also reported that cytotoxicity of cocaine on PC12 cells was observed only when the cells were incubated with several mM concentrations for several days (Oliveira et al., 2002). Significant loss of cell viabilities was observed in NT-2 cells treated with 3 mM cocaine for 24 h, but not observed with 1 mM cocaine (Cunha-Oliveira et al., 2006). Therefore, our results (Fig. 1) are matched well with previous reports and shows that sublethal concentration of cocaine induces mitochondrial fission, rather than mitochondrial apoptosis. In spite of the fact that these neuronal cell lines are not identical to neurons in the brain, crucial characteristics of cocaine cytotoxicity, such as release of dopamine as well as dysfunction of mitochondria, are reproduced in these cells (Badisa et al., 2018a). Our current results represent additional example that neurotoxicity of cocaine is reproduced in neuronal cells *in vitro*.

Increased fission of mitochondria in the brain of mouse repeatedly administered cocaine was observed in dopamine-1 receptor (D1)-containing medium spiny neurons (MSN) but not in D2MSN (Chandra et al., 2017). It has been shown that D1 is not expressed in Neuro2a cells (Takeuchi et al., 1999). However, the cells have also shown producing dopamine at the level approximately 1/2 ~ 1/3 of that in substantia nigra of mouse (Tremblay et al., 2010). Neuro2a cells are also supposed expressing D2/3 (Dziedzicka-Wasylewska and Solich, 2004), as well as tyrosine hydroxylase (TH) which is involved in catecholamine synthesis (Tremblay et al., 2010). Taken together, our current results might indicate that cocaine affects mitochondrial dynamics through D2/3 under some circumstances. Since neurons in the brain receives action of various neurotransmitters, the D1-independent effect of cocaine on mitochondrial dynamics could become observable when it was examined in Neuro2a cells.

Phosphorylation at ser-616 of DRP1 has been shown resulting in the activation of DRP1 (Xie et al., 2015). CDK5, the major neuronal member between cyclin-dependent kinase (CDK) family proteins, has been shown responsible for this phosphorylation (Xie et al., 2015).

Interestingly, CDK5 is also supposed as an important regulator of cocaine action in the brain (Benavides et al., 2007; Bibb et al., 2001). CDK5 is supposed as a target of ΔFosB, which has been shown upregulated in the brain after chronic cocaine (Hope et al., 1994). Therefore, there is a possibility that this ΔFosB-CDK5 axis is also involved in the action of cocaine on Neuro2a cells. Repeated cocaine exposure might increase ser-616 phosphorylation of DRP1 through upregulating ΔFosB-CDK5 axis in Neuro2a cells, though the involvement of ΔFosB-CDK5 in cocaine action in Neuro2A remains to be examined. It should be also noted that Parkin is also reported involving in the increase of mitochondrial fission (Poole et al., 2008). Both DRP1 and Parkin should be involved in the increased mitochondrial fission after repeated cocaine exposure in Neuro2a cells.

Decrease of mitochondrial membrane potential (ΔΨm) results in lower ATP production and subsequent decrease in cellular activities. However, decrease of ΔΨm also suppresses reactive oxygen species (ROS) generation from mitochondria, since ROS is generated from mitochondrial as a byproduct of electron transport chain even in healthy cells (Youle and van der Bliek, 2012). In addition, decrease of ΔΨm is prerequisite for PINK1/Parkin-dependent mitophagy (Matsuda et al., 2010; Narendra et al., 2010). Since mitochondrial fission leads to segregate damaged parts of mitochondria from healthy mitochondria and subsequent elimination of the damaged mitochondria by mitophagy (Youle and van der Bliek, 2012), decrease in ΔΨm should be important for not only suppressing ROS generation from mitochondria but also scavenging dysfunctional mitochondria through mitochondrial fission and subsequent mitophagy. Decrease in ΔΨm observed in this study in cocaine-exposed cells (Fig. 2A) indicates stress in mitochondria by cocaine, and our results also show that mitochondrial fission and autophagy should protect the cells from death by cocaine.

Effect of mitochondrial dynamics on neuronal cells are still under extensive studies (Flippo and Strack, 2017). However, it has been generally accepted that mitochondrial dynamics is essential for mitochondrial as well as cellular homeostasis (Westermann, 2010). It has also been assumed that decrease of mitochondrial function leads to the fission of mitochondria concurrently with the execution of apoptosis (Youle and van der Bliek, 2012). Indeed, it has been proved that suppression of DRP1 protects against neuronal cell death *in vivo* and *in vitro* (Grohm et al., 2012). Thus, increased activation of DRP1 and subsequent division of mitochondria caused by repeated cocaine exposure should decrease both mitochondrial and cellular activities, as observed in the brain of cocaine abusers. Our current results provide simple and useful system to evaluate possible involvement of altered mitochondrial dynamics on neuronal plasticity of cocaine abusers.

To our best of knowledge, this is the first report showing cocaine-induced alteration of mitochondrial dynamics in neuronal cells *in vitro*. Although there might be differences between the changes in mitochondrial dynamics observed in binge cocaine-administrated mouse brain and repeatedly cocaine exposed Neuro2a cells, our results should provide simple system to evaluate mitochondrial dynamics in neuronal cells during cocaine exposure.

Roles of authors

TA and KU designed the study; TF and MF conducted experiments and data analysis; TF and TA wrote the manuscript.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2019.09.001.

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