

The Endoplasmic Reticulum Stress Sensor, ATF6 α , Protects against Neurotoxin-induced Dopaminergic Neuronal Death^{*S}

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Oxidative stress and endoplasmic reticulum (ER) stress are thought to contribute to the pathogenesis of various neurodegenerative diseases including Parkinson disease (PD), however, the relationship between these stresses remains unclear.

ATF6 α is an ER-membrane-bound transcription factor that is activated by protein misfolding in the ER and functions as a critical regulator of ER quality control proteins in mammalian cells. The goal of this study was to explore the cause-effect relationship between oxidative stress and ER stress in the pathogenesis of neurotoxin-induced model of PD. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin known to produce oxidative stress, activated ATF6 α and increased ER chaperones and ER-associated degradation (ERAD) component in dopaminergic neurons. Importantly, MPTP induced formation of ubiquitin-immunopositive inclusions and loss of dopaminergic neurons more prominently in mice deficient in ATF6 α than in wild-type mice. Cultured cell experiments revealed that 1-methyl-4-phenylpyridinium (MPP⁺)-induced oxidative stress not only promoted phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) but also enhanced interaction between phosphorylated p38MAPK and ATF6 α , leading to increment in transcriptional activator activity of ATF6 α . Thus, our results revealed a link between oxidative stress and ER stress by showing the importance of ATF6 α in the protection of the dopaminergic neurons from MPTP that occurs through oxidative stress-induced activation of ATF6 α and p38MAPK-mediated enhancement of ATF6 α transcriptional activity.

Parkinson disease (PD)² is characterized pathologically by progressive and selective loss of dopaminergic neurons in the

substantia nigra pars compacta (SNc). Recent studies have suggested some pathogenetic mechanisms of PD, including mitochondrial dysfunction (1), oxidative stress induced by dopamine metabolites, and endoplasmic reticulum (ER) stress induced by the accumulation of unfolded proteins (2–4). Dopaminergic neuron loss is often accompanied by formation of intraneuronal inclusion bodies termed Lewy bodies whose major constituent is α -synuclein (5), suggesting the pathogenetic role of unfolded protein accumulation and aggregation.

The unfolded protein response (UPR) is a homeostatic signaling pathway designed to cope with the accumulation of unfolded proteins in the ER lumen. In mammals, the UPR utilize three types of sensor proteins, PERK, IRE1 α , and ATF6 α , which detect protein-misfolding stress in the ER and initiate ER-to-nucleus signaling cascades to maintain the homeostasis of protein quality control system (6). ATF6 is a type 2 transmembrane protein; the C-terminal region is located in the ER, whereas the N-terminal region is located in the cytosolic side. ATF6 consists of two subtypes, ATF6 α and ATF6 β . ATF6 α or ATF6 β single knock-out mice normally develops but mice deficient in both subtypes of ATF6 genes showed embryonic lethal (7, 8). Upon ER stress the N-terminal fragment of ATF6 α designated pATF6 α (N) is cleaved from the parent protein designated pATF6 α (P) and transported into the nucleus, where it binds to cis-acting ER stress response element (ERSE) and UPR element (UPRE), and up-regulates major ER chaperones and ER-associated degradation (ERAD) components (9–14).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted into 1-methyl-4-phenylpyridinium (MPP⁺) in astrocytes and taken up by dopaminergic neurons through the dopamine transporter (DAT). MPP⁺ interferes with the mitochondrial complex-1, generates reactive oxygen species (ROS) and selectively damages dopaminergic neurons (15). Dopamine itself is also a highly-reactive molecule and naturally metabolized to produce ROS including hydrogen peroxide (H₂O₂) via the monoamine oxidase pathway (16). Dopamine-induced ROS promotes phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) (17). MPTP activates p38MAPK pathway in the dopaminergic neuron (18). Other

ER-associated degradation; UPR, unfolded protein response; MPP⁺, 1-methyl-4-phenylpyridinium.

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1 and Figs. S1–S3.

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² The abbreviations used are: PD, Parkinson disease; ER, endoplasmic reticulum; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ERAD,

ATF6 α Protects Dopaminergic Neurons Against MPTP

oxidative neurotoxins such as rotenone and 6-hydroxydopamine (6-OHDA) also enhance the phosphorylation of p38MAPK (19, 20). Interestingly, it was previously reported that phosphorylated p38MAPK can regulate ATF6 α function via phosphorylation (21), suggesting the possibility that oxidative stress and ER stress intersect at the level of p38MAPK and ATF6 α .

Based on these data, the goal of the present study was to explore the cause-effect relationship between oxidative stress and ER stress in the pathogenesis of PD using ATF6 α wild-type (WT) and knock-out (KO) mice treated with MPTP that causes oxidative stress and damages dopaminergic neurons.

EXPERIMENTAL PROCEDURES

Animal and MPTP Treatment—ATF6 α knock-out (KO) mice were generated by gene targeting techniques as previously described (7). Groups of male ATF6 α KO mice and wild-type (WT) littermate controls were injected intraperitoneal with 20 mg/kg of MPTP hydrochloride (Sigma-Aldrich) four times at 2 h intervals on the same day ($n = 10$ for each group). The mice were decapitated 5 days after injection for the subsequent analysis. All surgical procedures were performed according to the rules set forth by the Ethics Committee of Kyoto University.

Quantitative Real-time PCR—The mouse central nervous system (CNS) was dissected into the cerebral cortex, the brainstem, the hippocampus, the striatum, the midbrain, the cerebellum, the olfactory bulb, and the thalamus. Total RNA was isolated from the various parts of the CNS using the RNeasy Kit (Qiagen, Valencia CA) after homogenization (POLYTRON PT10–35). The first strand of cDNA was synthesized from 1 μ g of total RNA using the PrimeScript RT Reagent Kit (Takara Bio, Shiga). Real-time PCR was performed using the LightCycler SYBR Green Master Kit and LightCycler 480 program (Roche) according to the manufacturer's protocol. The sequences of the primers used were listed in the [supplemental Table S1](#). Values were normalized and expressed relative to GAPDH values.

Immunoblotting—Each part of the dissected CNS tissue was weighed and homogenized (POLYTRON PT10–35) in 1 ml/g of ice-cold buffer (50 mM Tris(pH7.5), 5 mM EDTA and 120 mM NaCl, 1% Triton X-100) containing protease inhibitor and phosphatase inhibitor mixture. Samples were centrifuged at $1,000 \times g$ for 5 min, where the supernatant was collected for the additional centrifugation and the resulting pellet was dissolved in 2% SDS buffer for nuclear fraction. The supernatant was centrifuged at $165,000 \times g$ for 60 min. The supernatant was prepared for cytosolic fraction, and the resulting pellet was dissolved in 2% SDS buffer as the ER and vesicle fraction. SH-SY5Y cells, HEK293T cells, ATF6 α WT and ATF6 α KO mouse embryonic fibroblasts (MEFs) were lysed in lysis buffer containing 1% Triton X-100 or 2% SDS buffer containing protease inhibitor and phosphatase inhibitor mixture. 20 or 30 μ g of protein for each sample was separated by SDS-PAGE and electroblotted onto PDGF membrane (Immopore). Immunoblotting analysis was carried out using an enhanced chemiluminescence Western blotting detection system kit (Amersham Biosciences). Rabbit anti-ATF6 α poly-

clonal antibody was prepared as previously described (13). Mouse anti-BiP antibody was purchased from BD Biosciences (San Diego, CA). Rat anti-GRP94 antibody was purchased from Stressgen (Ann Arbor, MI). Rabbit anti-Derlin-3 antibody was purchased from Sigma-Aldrich. β -Actin antibody, anti-p38MAPK antibody, anti-phospho-p38MAPK (p-p38MAPK) antibody and anti-IRE1 α antibody were obtained from Cell Signaling (Boston, MA). Rabbit anti-DAT antibody and mouse anti-tyrosine hydroxylase (TH) antibody were purchased from Millipore (Billerica, MA). Rabbit anti-ubiquitin antibody was purchased from DAKO (Glostrup, Denmark). Goat anti-PERK and rabbit anti-phospho-PERK antibody were obtained from Santa Cruz Biotechnology.

Immunohistochemistry—Mice were injected with pentobarbital and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. Mice were decapitated, and their brains were removed and immersed in 4% paraformaldehyde in PBS for fixation. Serial coronal sections at 20- μ m thickness were collected on slides. Deparaffinized sections were rinsed with PBS containing 0.1% Triton X-100 and then immersed in 0.3% H₂O₂ for 30 min. Sections were stained with primary antibodies against TH (mouse monoclonal; Millipore), BiP (IMGENEX, San Diego, CA) or ubiquitin (DAKO) or p-p38MAPK (Cell Signaling) in 10% normal serum overnight at 4 $^{\circ}$ C. After washing three times in PBST, the sections were stained with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), using the standard avidin-biotin peroxidase method (Elite standard kit SK6100; Vector Laboratories, Burlingame, CA). SH-SY5Y cells, HEK293T cells and primary cultured dopaminergic neurons cultured in 4-well tissue culture chamber were fixed with 10% formaldehyde for 60 min on ice followed by acetone treatment for 5 min. They were then permeabilized with PBS containing 0.2% Triton X-100 for 10 min at room temperature and blocked with 10% goat serum for 30 min. They were then exposed to primary antibody overnight at 4 $^{\circ}$ C for indirect immunofluorescence. Anti-p38MAPK antibody and anti-p-p38MAPK were diluted 25-fold and 100-fold, respectively, with PBS containing 1% BSA before use. For immunofluorescence, primary antibodies were visualized by incubation for 2 h at room temperature with Alexa Fluor488- and Alexa Fluor546-conjugated secondary antibodies (Molecular Probes), followed by confocal laser scanning fluorescence microscopy using an LSM510 microscope (Carl Zeiss, Thornwood, NY).

TH-positive Cell and Ubiquitin-positive Inclusion Counting—Total numbers of TH-positive neurons in the substantia nigra pars compacta (SNc) and ubiquitin-positive inclusions in the striatum were determined using an unbiased optical fractionator method (Stereoinvestigator, MicroBrightField) as previously described with minor modification (4). Three independent sets of immunostained serial sections (sampled as every sixth coronal section throughout the entire range of SNc or the striatum) were analyzed for each animal. Total positive number for each sample was determined in triplicate by the Stereoinvestigator program to obtain averaged values. Location of SNc and the striatum was identified according to established anatomical landmarks (Paxinos mouse brain atlas).

Measurements of Striatal Catecholamines (HPLC)—Tissues dopamine and metabolites were measured according to previously published methods (4).

Plasmids, Cell Culture, Reagent, and Transfection—Various vectors, including pCMVfull-EGFP-ATF6 α (also known as green fluorescent protein (GFP)-ATF6 α fusion gene) (11), pGL3-GRP78 (-132)-Luc carrying the human BiP promoter, pGL3-5 \times UPRE-luc, pDNA3.1 (+)-N-terminal fragment of ATF6 α (1–373) and a dominant-negative form of ATF6 α (171–373) were prepared as previously (13, 14). Flag-p38MAPK was kindly provided by Dr. Katsuji Yoshioka (Kanazawa Univ.). ATF6 α WT/KO primary cultured dopaminergic neurons were prepared as shown under “Primary culture.” ATF6 α WT/KO MEFs were obtained as previously described (7). SH-SY5Y cells were cultured in DMEM supplemented with 10% fetal bovine serum, NEAA (Invitrogen) at 37 °C in a humidified 5% CO₂/95% air atmosphere. HEK293T cells and ATF6 α WT/KO MEFs were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin). Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to each manufacturer’s protocol. 1-Methyl-4-phenylpyridinium iodide (MPP⁺) was purchased from Sigma-Aldrich. SB203585 were purchased from Calbiochem (San Diego, CA). SHSY5Y cells and ATF6 α WT/KO primary cultured dopaminergic neurons were treated with 1 mM MPP⁺ for indicated times in the presence or absence of 5 μ M SB203585. ATF6 α WT/KO MEFs and HEK293T cells were plated in 6-well plates and treated with 50 μ M hydroxyl peroxide (H₂O₂) (Nakalai Tesque, Kyoto) in the presence or absence of 5 μ M SB203585.

Primary Culture—Mesencephalic and striatal cells were prepared from embryonic day 15 ATF6 α WT/KO mice. Both the midbrain and the striatum were mechanically dissected in Neurobasal medium (Invitrogen). The tissues were dissociated with 0.25% trypsin at room temperature for 15 min. The cells were suspended in DMEM supplemented with 10% fetal bovine serum and NEAA and then collected by centrifugation (1000 \times g, 3 min). The cells were resuspended in Neurobasal medium supplemented with B-27(minus Anti-Oxidants) (Invitrogen) and 0.5 mM glutamine at a concentration of 1 million cells/ml, and plated at a density of 0.5 million cells per well into 24 well plate previously coated with poly-L-Lysin (Sigma-Aldrich). One week after plating, the cells were used for reporter assay or immunohistochemistry.

Reporter Assay—SH-SY5Y cells, HEK293T cells and ATF6 α WT/KO primary cultured dopaminergic neurons were transiently transfected with pGL3-5 \times UPRE or pGL3-BiP (-132) along with pRL-SV40 (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were treated with 1 mM MPP⁺ for 24 h or 50 μ M H₂O₂ for 1 h in the presence or absence of 5 μ M SB203585. Luciferase expression was measured in triplicate using a dual luciferase reporter assay system (Promega) on Fluoroskan Ascent FL (Thermo, Waltham, MA). Relative activity was defined as the ratio firefly luciferase activity to *Renilla* luciferase activity to normalize for transfection efficiency.

Immunoprecipitation—After being washed with PBS, SH-SY5Y cells and HEK293T cells cultured in a 6-well plate dish were solubilized for 30 min on ice in 200 μ l of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) with a protease inhibitor mixture and a phosphatase inhibitor. Insoluble fraction was collected by centrifugation (1000 \times g, 3 min), defined as nuclear fraction, and lysed in SDS lysis solution (50 mM Tris-HCl, 10 mM EDTA, 1% SDS) including phosphatase inhibitor. Soluble lysates were incubated overnight with ANTI-FLAG M2 Affinity gel (Sigma-Aldrich). Lysates from nuclear fraction were diluted to 10% by dilution buffer (50 mM Tris-HCl, 167 mM NaCl, 1.1% Triton X-100, and 0.11% sodium deoxycholate) including phosphatase inhibitor and then incubated for overnight by G-Sepharose bound to anti-phospho-p38MAPK antibody (Cell Signaling). Sepharose beads were collected by brief centrifugation and washed with TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH7.4) three times. Immunoprecipitated material was eluted by boiling for 3 min in 2 \times sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.004% BPB).

Chromatin Immunoprecipitation (ChIP) Assay—SH-SY5Y cells were grown to 80% confluence in 6-cm plates and then treated with 1 mM MPP⁺ for 24 h and protein-DNA cross-linking was initiated by adding formaldehyde to the cell culture to a final concentration of 1% and stopped at 15 min by adding 1.5 M glycine to a final concentration of 0.15 M. The cells were lysed in RIPA buffer including a protease inhibitor mixture and a phosphatase inhibitor. Insoluble fraction was collected by centrifugation (1000 \times g, 3 min), defined as nuclear fraction, lysed in SDS lysis solution (50 mM Tris-HCl, 10 mM EDTA, 1% SDS) including phosphatase inhibitor and sonicated for 25 min using Bioruptor (UCD-200TM, COSMO BIO) (Sonication pulse was performed by power high, ON 30 s, OFF 60 s). Diluted to 10% by ChIP dilution buffer (50 mM Tris-HCl, 167 mM NaCl, 1.1% Triton X-100, and 0.11% sodium deoxycholate) including phosphatase inhibitor, lysates were incubated for overnight by G-Sepharose bound to anti-phospho-p38MAPK antibody. Sepharose beads were collected by brief centrifugation, washed with RIPA buffer three times and suspended with ChIP direct elution buffer (10 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 0.5% SDS). Formaldehyde-induced cross linking was reversed (for overnight at 65 °C) and the DNA was purified by phenol-chloroform extraction and ethanol precipitation. Purified DNA was subjected to quantitative PCR by LightCycler 480 program.

Statistical Analysis—All values are presented as the mean \pm S.D. Results were tested for significant differences using one-way ANOVA with the posthoc test or Student’s *t* test. *p* < 0.05 was considered to represent significant difference. In figures, a single asterisk (*) indicates *p* < 0.05, and a double asterisk (**) indicates *p* < 0.01.

RESULTS

ATF6 α Controls the Levels of ER Chaperones and ERAD Component in the Dopaminergic Neuron System in Vivo—To explore the physiological function of ATF6 α *in vivo*, we first investigated where in the central nervous system ATF6 α is

ATF6 α Protects Dopaminergic Neurons Against MPTP

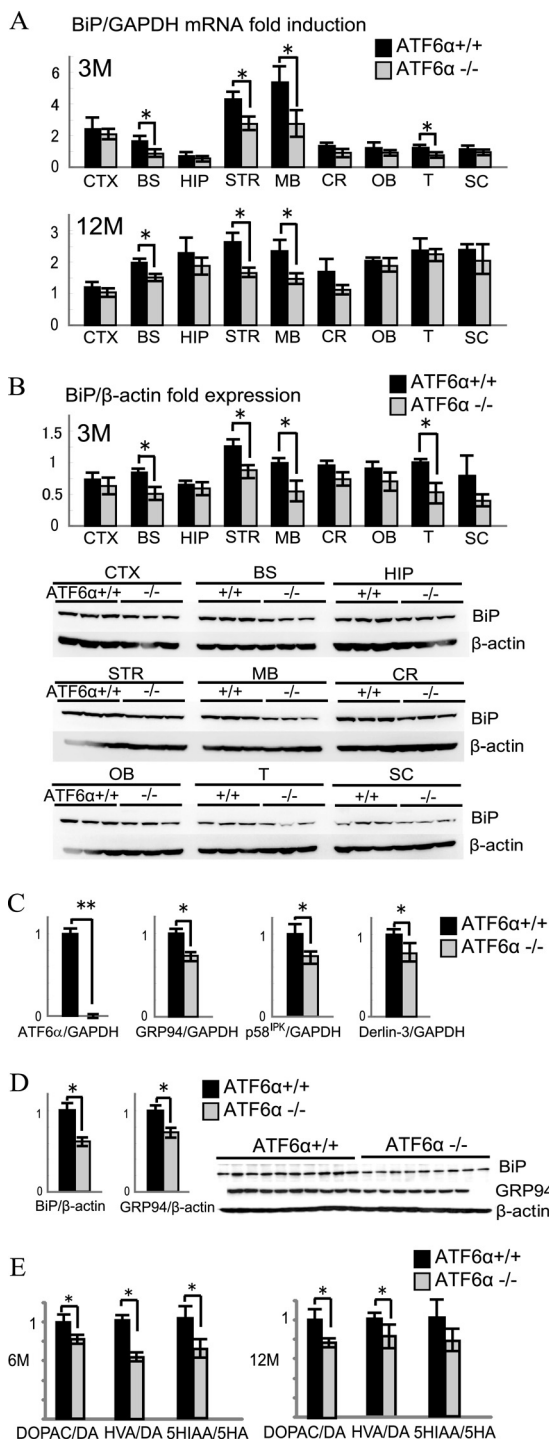


FIGURE 1. ATF6 α controls the levels of ER chaperones and ERAD component in the dopaminergic neuron system under physiological conditions *in vivo*. A, ATF6 α controls the mRNA levels of BiP in the brainstem, the midbrain and the striatum in mice brain *in vivo*. Total RNA was isolated from various sections of brain of 3 and 12-month-old male ATF6 α KO (-/-) mice and ATF6 α WT (+/+) littermates and analyzed by quantitative real-time RT-PCR for detecting the level of BiP mRNA. ($n = 5$ for each group). Fold induction of mRNA level is defined relative to GAPDH mRNA level. Abbreviation: CTX, cerebral cortex; BS, brainstem; HIP, hippocampus; STR, striatum; MB, midbrain; CR, cerebellum; OB, olfactory bulb; T, thalamus; SC, spinal cord; 3 M, 3 months aged group; 12 M, 12-month-old group. The asterisk indicates statistical significance (*, $p < 0.05$; **, $p < 0.01$). Each bar denotes the mean \pm S.D. B, ATF6 α controls the protein levels of BiP in the brainstem, the midbrain and the striatum in mice brain *in vivo*. The tissue lysates of 3-month-old ATF6 α KO mice and ATF6 α WT littermate were

activated under normal physiological conditions by using ATF6 α WT or KO mice. Results of quantitative RT-PCR analysis demonstrated that mRNA level of the ER chaperone BiP was significantly lower in the brainstem, the midbrain and the striatum of ATF6 α KO mice than those of ATF6 α WT mice (Fig. 1A). Accordingly, the protein levels of BiP were also significantly lower in the brainstem, the midbrain and the striatum of ATF6 α KO mice than those of ATF6 α WT mice (Fig. 1B). Other ER stress sensor, IRE1 α , sXBP1, and phosphorylated PERK (p-PERK, indicative of the activation status of another ER stress sensor molecule) are not different in various brain regions of ATF6 α KO mice (supplemental Fig. S1A). The mRNA levels of other ER chaperone GRP94, cochaperone p58^{IPK}, and ERAD component, Derlin-3, in the midbrain were also significantly decreased in ATF6 α KO mice when compared with age-matched ATF6 α WT mice (Fig. 1C). The protein levels of GRP94 in the striatum were significantly decreased in ATF6 α KO mice (Fig. 1D). HPLC analysis of the striatum demonstrated that the metabolisms of dopamine and serotonin were slightly but significantly decreased in ATF6 α KO mice as compared with WT mice (Fig. 1E). Decreased protein levels were not general feature of ATF6 α KO mice because the levels of some proteins involved in synaptic secretion or vesicular transport remained unchanged or rather increased in ATF6 α KO mice as compared with WT mice (supplemental Fig. S1B). Importantly, tyrosine hydroxylase (TH) immunostaining of the midbrain revealed that there was no abnormality in the number and morphology of TH positive neurons in ATF6 α KO mice when compared with WT littermates (supplemental Fig. S1, C and D). TH and DAT protein levels in the striatum of ATF6 α KO mice were similar to those of WT littermates (supplemental Fig. S1E). Therefore, although ATF6 α is not essential for the development and the survival of dopaminergic neurons, ATF6 α up-regulates ER chaperones and ERAD component constitutively in dopaminergic neurons; dopaminergic neurons may experience ER stress under normal physiological conditions.

MPTP Up-regulates the Levels of ER Chaperones and ERAD Component by Activating ATF6 α and Induces the Phosphorylation of p38MAPK in the Dopaminergic Neurons *In Vivo*—To investigate whether ATF6 α plays an important role in dopaminergic cell death of neurotoxin-based PD models, we analyzed dopaminergic neurons of mice treated with MPTP. MPTP was intraperitoneally administered into 6-month-old

subjected to Western blot analysis for detection of BiP and β -actin ($n = 5$). Each protein level was quantified using optical density and estimated relative to β -actin protein level. C, ATF6 α controls the levels of GRP94, p58^{IPK}, and Derlin-3 in the midbrain *in vivo*. Quantitative real-time RT-PCR was performed from the midbrain samples of 12-month-old ATF6 α KO mice and ATF6 α WT littermates using ATF6 α , GRP94, p58^{IPK}, and Derlin-3 primers ($n = 5$ for each group). D, ATF6 α controls the protein levels of BiP and GRP94 in the striatum *in vivo*. ($n = 7-10$). E, metabolisms of dopamine and serotonin were decreased in ATF6 α KO mice as compared with ATF6 α WT mice. Metabolisms of dopamine and serotonin were defined as the relative ratio of DOPAC or HVA to dopamine and 5HIAA to 5HA, respectively. The striatal levels of dopamine, serotonin, and their metabolites were measured by HPLC ($n = 8-10$ for each group). Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5HIAA, 5-hydroxyindole acetic acid; 5HA, 5-hydroxytryptamine; 6 M, 6-month-old group; 12 M, 12-month-old group.

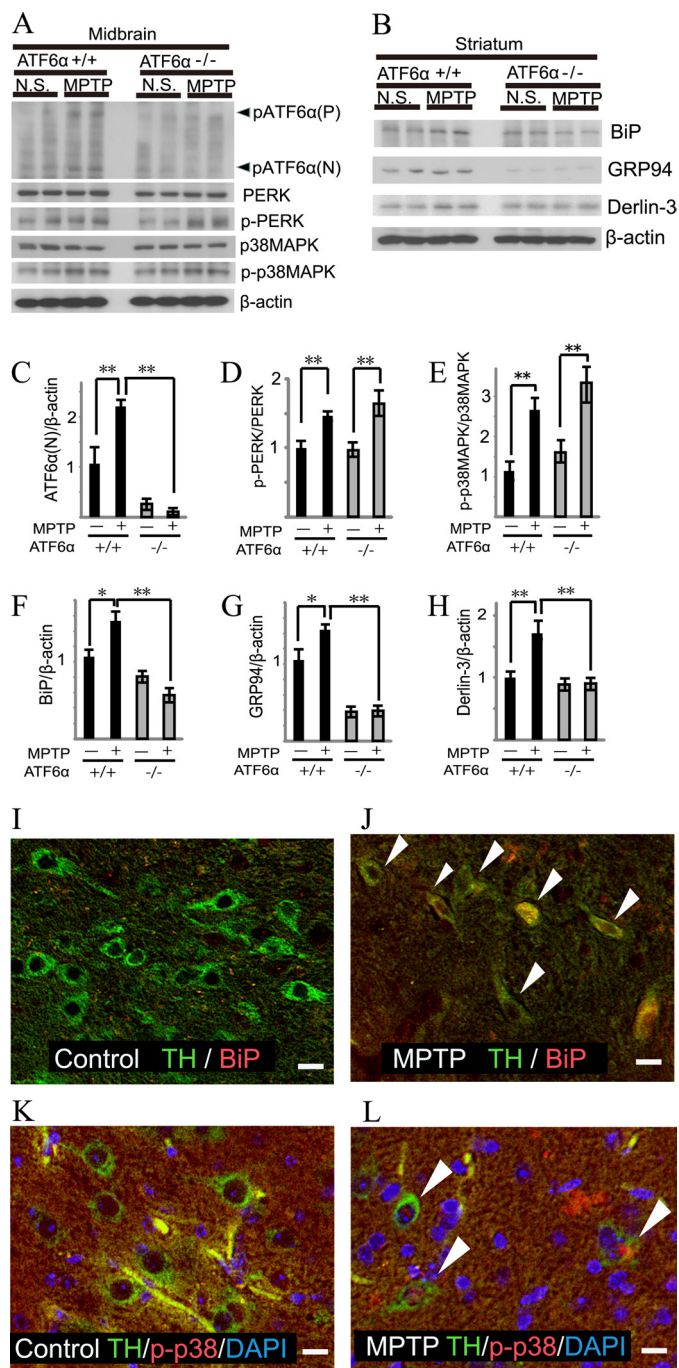


FIGURE 2. MPTP up-regulates the levels of ER chaperones and ERAD component by activating ATF6 α and induces the phosphorylation of p38 MAPK in dopaminergic neurons *in vivo*. A–H, extracts of the midbrain or the striatum of ATF6 α WT (+/+) and ATF6 α KO (–/–) mice were prepared 5 days after intraperitoneal injection with either 20 mg/kg MPTP or normal saline four times every 2 h for Western blot analysis of ATF6 α , PERK, p38MAPK, phospho-PERK (p-PERK), phospho-p38MAPK (p-p38MAPK), BiP, GRP94, Derlin-3, and β -actin ($n = 4$ for each group). The protein levels of p-PERK and p-p38MAPK were quantified using optical density and estimated relative to PERK or p38MAPK protein level, respectively. Other protein levels were estimated relative to β -actin protein level. Each bar denotes the mean \pm S.D. MPTP induces ATF6 α cleavage (A and C), phosphorylation of PERK (A and D) and p38MAPK activation (A and E) in the midbrain. MPTP up-regulates the levels of BiP (B and F), GRP94 (B and G), and Derlin-3 (B and H) in the striatum *in vivo*. Abbreviation: N.S. indicates normal saline injection as control. I–L, MPTP up-regulates the levels of BiP and p-p38MAPK in TH-positive neurons. The midbrains of ATF6 α WT mice treated with MPTP or normal saline (Control) were stained using anti-TH (I–L, green), BiP (I and J,

ATF6 α KO mice and WT littermates. Western blot analysis with antibodies against ATF6 α , PERK, p38MAPK, phosphorylated p38MAPK (p-p38MAPK), p-PERK, BiP, GRP94, and Derlin-3 was performed using midbrain and striatal extracts from WT or ATF6 α KO mice challenged with MPTP. MPTP treatment induced phosphorylation of p38MAPK in the midbrain regardless of ATF6 α genotype as expected (Fig. 2, A and E). Importantly, MPTP treatment not only increased the level of pATF6 α (P), the precursor form of ATF6 α , but also generated pATF6 α (N), the active form produced as a result of the cleavage of pATF6 α (P), in the midbrain of ATF6 α WT mice (Fig. 2, A and C). The levels of ER chaperones, BiP and GRP94, and ERAD component, Derlin-3, were significantly increased in the striatum of MPTP-treated WT mice (Fig. 2, B, F, G, and H). In marked contrast, the induction of these ER chaperones and ERAD components was not observed in ATF6 α KO mice even though MPTP increased phosphorylation of PERK regardless of ATF6 α genotype (Fig. 2, A and D). To identify the cell types in which BiP was up-regulated, we performed double immunostaining for BiP in TH-positive dopaminergic neurons of the midbrain of WT mice. Dopaminergic neurons showed higher BiP expression level in MPTP-treated WT mice than untreated control mice (Fig. 2, I and J). Phosphorylated p38MAPK was detected in the nucleus of the dopaminergic neurons in the midbrain of MPTP-treated mice (Fig. 2, K and L) as previously reported (18). These data clearly indicate that MPTP somehow evokes ER stress and up-regulates the expression of ER chaperones and ERAD component by activating ATF6 α in dopaminergic neurons *in vivo* in addition to previously known effect on the phosphorylation of p38MAPK. One possible cause for induction of ER stress by MPTP is production of ROS as we found that treatment of mouse embryonic fibroblasts (MEFs) derived from ATF6 α WT mice with hydrogen peroxide, a representative ROS, triggered not only phosphorylation of p38MAPK but also cleavage of pATF6 α (P) to produce pATF6 α (N) (supplemental Fig. S2A).

ATF6 α Protects against MPTP-induced Neurotoxicity *in Vivo*—We then asked whether ATF6 α deletion and the sequential decline in the expression levels of ER chaperones and ERAD component have an impact on the ubiquitin proteasome system (UPS)-mediated protein degradation in dopaminergic neurons after MPTP treatment. Immunostaining of the striatum with antibody to ubiquitin revealed no difference in the striatum between ATF6 α WT and KO mice under physiological conditions. However, upon stress with MPTP, the striatum of ATF6 α KO mice showed much higher increase in the formation of clusters of ubiquitin-immunoreactive material than WT mice, suggesting that treatment with MPTP disturbed UPS more profoundly when the levels of ER chaperones and ERAD components were mitigated (Fig. 3, A and B). Next we assessed for neurotoxicity caused by treat-

red), p-p38MAPK (K and L, red) antibodies, and DAPI (K and L, blue). They were visualized by Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies and subjected to confocal fluorescence microscope analysis. White arrowheads in J and L indicate the TH-positive dopaminergic cells of MPTP-treated mice. Scale bars indicate 10 μ m.

ATF6 α Protects Dopaminergic Neurons Against MPTP

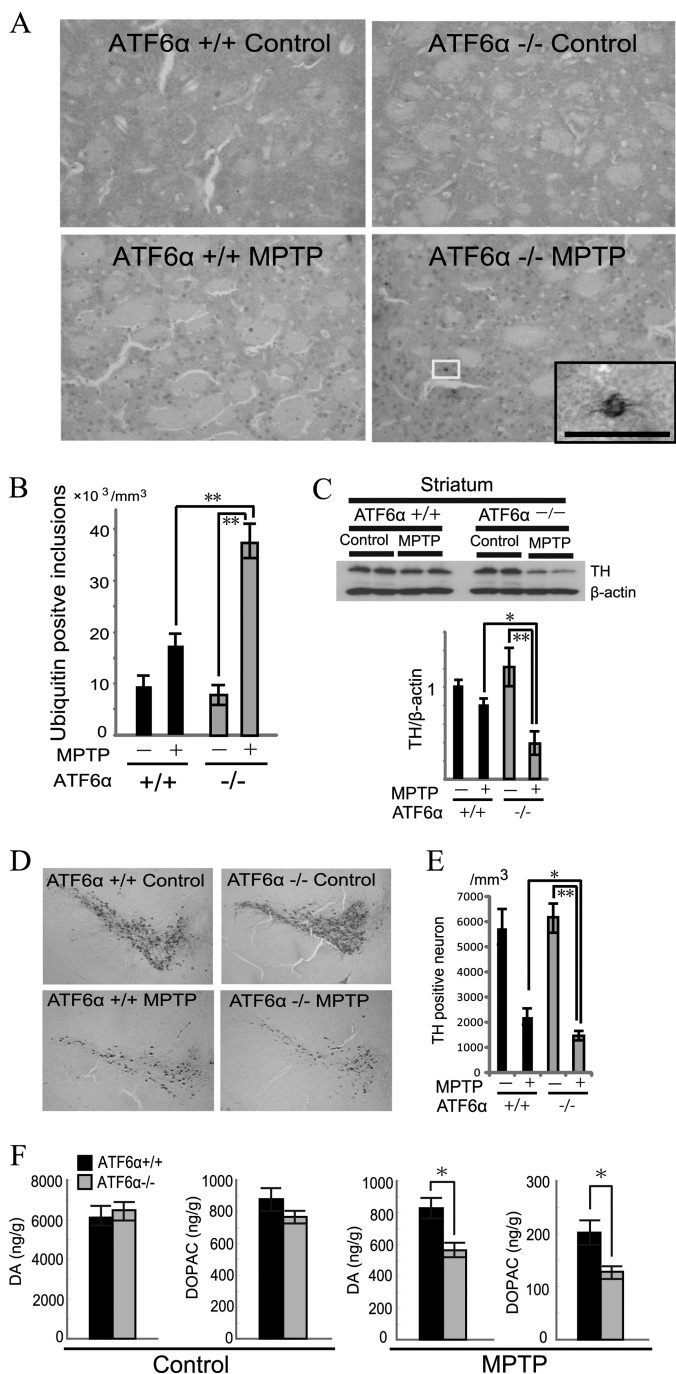


FIGURE 3. ATF6 α protects against MPTP-induced neurotoxicity *in vivo*.

A and B, MPTP induces the formation of ubiquitin-immunopositive clusters in the striatum of ATF6 α KO (-/-) mice. A, representative images of ubiquitin-immunoreactive clusters in the striatum of ATF6 α WT (+/+) and ATF6 α KO mice treated with either 20 mg/kg MPTP or normal saline (Control) four times every 2 h. The white box marks the area enlarged in lower right black box. Scale bars indicate 50 μm . B, numbers of ubiquitin-immunoreactive inclusion per the volume of ROI in the striatum of individual mice were quantified by StereoInvestigator and the density was compared among each indicated group. ($n = 4$ for each group). Each bar denotes the mean \pm S.D. C–F, dopaminergic neurons of ATF6 α KO mice are more vulnerable to MPTP neurotoxicity than ATF6 α WT mice. C, extracts of the striatum of ATF6 α WT and ATF6 α KO mice with MPTP treatment or normal saline (Control) were subjected for Western blot analysis of TH and β -actin ($n = 4$ for each group). TH protein level was quantified using optical density and estimated relative to β -actin protein level. D, representative TH-immunostained images of the midbrain of ATF6 α WT and ATF6 α KO mice treated with normal saline (Control) or MPTP. E, TH-positive numbers in the

midbrain SNc of ATF6 α WT and ATF6 α KO male littermates treated with MPTP or normal saline ($n = 5$ for each group). F, quantification of the striatal levels of dopamine and its metabolite DOPAC in ATF6 α WT and ATF6 α KO mice treated with either MPTP or normal saline (Control) ($n = 5$ for each group). Abbreviations: DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid.

ment with MPTP. Immunoblotting of the extracts from the striatum using TH antibody showed more remarkably decreased level of TH in ATF6 α KO mice than in WT after treatment with MPTP, indicating that more dopaminergic terminal loss takes occurs in ATF6 α KO mice (Fig. 3C). The number of TH-positive neurons in the SNc was significantly decreased in MPTP-treated ATF6 α KO mice when compared with WT littermates (Fig. 3, D and E). These results were correlated with those of HPLC analysis, which showed significant decreases of dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), in the striatum of MPTP-treated ATF6 α KO mice when compared with WT littermates (Fig. 3F). These results indicate that MPTP induces a larger loss of dopaminergic neurons and their terminals under ATF6 α -deleted conditions and further suggest that ATF6 α protects against MPTP-induced neurotoxicity.

Phosphorylated p38MAPK and Activated ATF6 α Cooperate to Enhance the Expression Level of BiP in Dopaminergic Neurons Treated with MPP $^+$ —Next we investigated the possible cooperation between p38MAPK phosphorylated and ATF6 α activated in MPTP-treated dopaminergic neurons. We treated dopaminergic neuroblastoma SH-SY5Y cells with MPP $^+$. Immunoblotting revealed that treatment with MPP $^+$ generated a ~ 60 -kDa protein band representing pATF6 α (N) (Fig. 4, A and B) and induced phosphorylation of PERK (Fig. 4, A and C) as well as that of p38MAPK (Fig. 4, A and D) in SH-SY5Y cells as in the midbrain of ATF6 α WT mice (see Fig. 2A). SB203585, an inhibitor of p38MAPK phosphorylation, attenuated p38MAPK phosphorylation induced by MPP $^+$ (Fig. 4, A and D) but showed little effect on the activation of ATF6 α and PERK phosphorylation (Fig. 4, A–C). We confirmed that SB203585 attenuated p38MAPK phosphorylation without affecting cleavage of pATF6 α (P) in MEFs treated with hydrogen peroxide (supplemental Fig. S2A). However, BiP was up-regulated after MPP $^+$ treatment and this up-regulation was significantly reduced by SB203585 in SH-SY5Y cells (Fig. 4, A and E). This observation was further confirmed by BiP promoter-luciferase reporter system (Fig. 4, F and G). The reporter gene -132/LUC contains a fragment of the BiP promoter driving the luciferase expression. We introduced the BiP promoter-luciferase plasmid into SH-SY5Y cells with or without p38MAPK overexpression vector, and then treated transfected cells with MPP $^+$. Luciferase expression level continuously increased for 24 h after MPP $^+$ treatment and this MPP $^+$ -induced stimulation of reporter expression was synergistically enhanced by p38MAPK overexpression (Fig. 4F). We then examined the effect of MPP $^+$ and SB203585 on reporter expression in the primary co-culture of the midbrain and the striatum prepared from ATF6 α KO or WT mice (supplemental Fig. 2C). We found that MPP $^+$ treatment induced a significant increment in luciferase expression in cells from ATF6 α WT and that this effect was abrogated by the presence

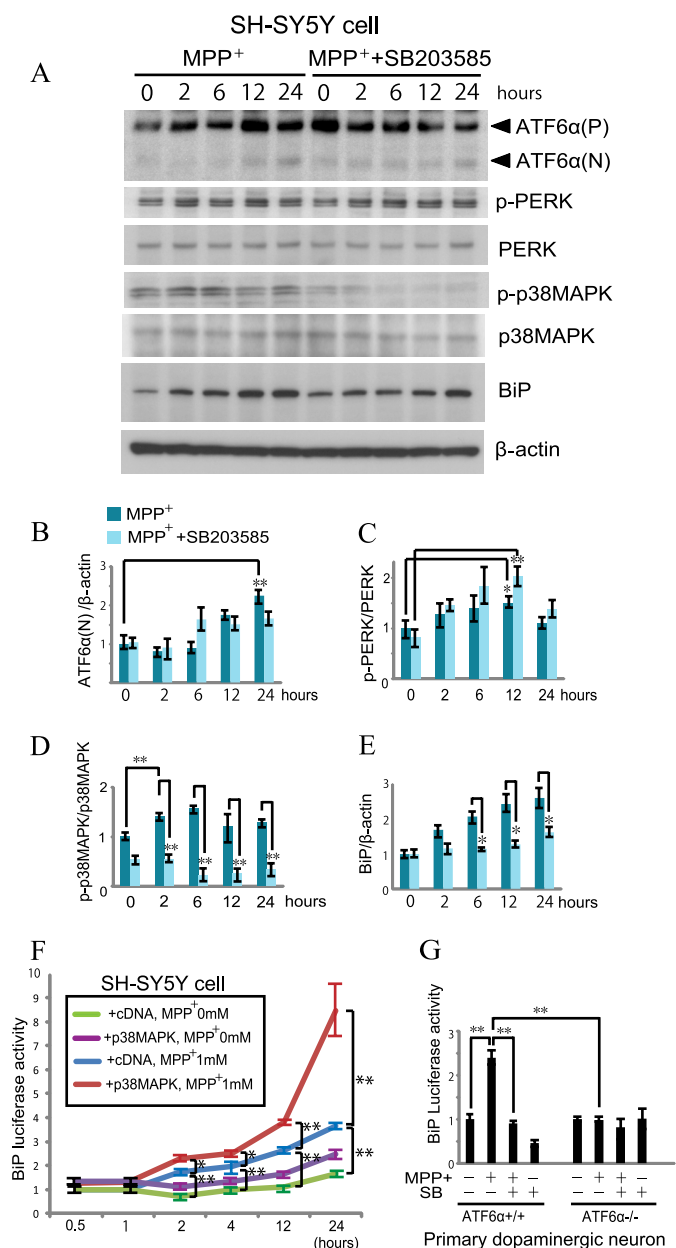


FIGURE 4. Phosphorylated p38MAPK and activated ATF6 α cooperate to enhance the expression level of BiP in dopaminergic neurons treated with MPP $^{+}$. A–E, immunoblot of cell lysates from SH-SY5Y cells treated with 1 mM MPP $^{+}$ for 2, 6, 12, 24 h using anti-ATF6 α , phosphorylated PERK (p-PERK), PERK, p38MAPK, phosphorylated p38MAPK (p-p38MAPK), BiP, and β -actin antibodies. 5 μ M SB203585 was added into the medium prior to MPP $^{+}$ treatment for the indicated times. Densitometry of ATF6 α (N) (B), p-PERK (C), p-p38MAPK (D), and BiP (E) was performed on scanned immunoblots images using the *Image-J* and normalized to β -actin, PERK, p38MAPK, and β -actin, respectively. Each bar denotes the mean \pm S.D. F, BiP promoter-Luciferase plasmid and pRL-SV40 plasmid were transfected into SH-SY5Y cells with p38MAPK overexpression vector or Mock vector. Each group of transfected cells was treated with or without 1 mM MPP $^{+}$ for 0.5, 1, 2, 4, 12, 24 h. Relative activity is defined as the ratio of firefly luciferase activity to *Renilla* luciferase activity in dual-luciferase assay. G, dopaminergic neurons from primary co-culture of the midbrain and the striatum of ATF6 α WT(+/+) or ATF6 α KO(-/-) mice transfected with BiP promoter-Luciferase vector and pRL-SV40 vector and then treated with 1 mM MPP $^{+}$ for 24 h with or without 5 μ M SB203585. Relative activity is defined as the ratio of firefly luciferase activity to *Renilla* luciferase activity in dual-luciferase assay.

of SB203585 (Fig. 4G). In contrast, reporter expression remained unchanged in MPP $^{+}$ -treated cells derived from ATF6 α KO. These data suggest that phosphorylation of p38MAPK induced by treatment with MPP $^{+}$ positively affects the ATF6 α -mediated transcriptional induction of BiP.

MPP $^{+}$ Induces the Translocation of the N-terminal Fragment of ATF6 α to the Nucleus and the Binding of N-terminal ATF6 α with Phosphorylated p38MAPK and Enhances the Transcriptional Activity of ATF6 α —To examine possible interaction between ATF6 α and p38MAPK, we conducted immunofluorescence analysis of primary cultured dopaminergic neurons transfected with vectors to express green fluorescent protein (GFP) fused to the N terminus of full-length ATF6 α (pGFP-ATF6 α (P)), which is cleaved to produce nuclear-translocating pGFP-ATF6 α (N) in response to ER stress (11), using anti-phosphorylated p38MAPK (p-p38MAPK) or anti-p38MAPK antibodies. GFP-ATF6 α (P) was localized in the peri-nuclear region in non-stressed dopaminergic neurons (Fig. 5A) and was partially colocalized with cytosolic p38MAPK (Fig. 5B) in non-stressed dopaminergic neurons. 24 h after the treatment with MPP $^{+}$, pGFP-ATF6 α (N) was translocated into the nucleus and colocalized with both DAPI and p-p38MAPK (Fig. 5C). By treating SH-SY5Y cells with MPP $^{+}$ after expression with both pGFP-ATF6 α (P) and p38MAPK we confirmed that pGFP-ATF6 α (N) was translocated into the nucleus and colocalized with both DAPI and p-p38MAPK (supplemental Fig. S3, A–C). To determine whether ATF6 α physically associates with p-p38MAPK, we cotransfected flag-p38MAPK vector and the N-terminal fragment of ATF6 α vector (1–373), abbreviated to N-ATF6 α , into SH-SY5Y cells and performed immunoprecipitation with an anti-Flag antibody followed by immunoblotting with anti-ATF6 α antibody. As shown in Fig. 5D (upper panel), pATF6 α (N) (60kDa) and pATF6 α (P) (90 kDa) were detected in the immunoprecipitates after treatment of MPP $^{+}$. SB203585 attenuated the intensity of both bands in immunoprecipitates from MPP $^{+}$ -treated SH-SY5Y cells. Both pATF6 α (N) (60 kDa) and pATF6 α (P) (90 kDa) were also increased in the nuclear fraction of MPP $^{+}$ -treated SH-SY5Y lysates after immunoprecipitation with anti-p-p38MAPK antibody as shown in Fig. 5D (middle panel). Endogenous N-ATF6 α can bind to p-p38MAPK in MPP $^{+}$ -treated condition and this binding was attenuated by adding SB203585 (Fig. 5E). Next we examined whether the p-p38MAPK-ATF6 α complex binds to the promoter region of BiP in the nuclear fraction using ChIP assay. As shown in Fig. 5F, SH-SY5Y cells were treated with MPP $^{+}$ for 24 h and then cross-linked with formaldehyde. Extracted nuclear fraction was subjected to immunoprecipitation with p-p38MAPK antibody. Fragmented genomic DNA was extracted from the immunoprecipitates and quantified by QPCR using the primers for ERSEs of BiP promoter regions (–155~–21). MPP $^{+}$ enhanced the binding p-p38MAPK-ATF6 α complex to ERSEs, which was reduced by p38MAPK inhibitor (Fig. 5, G and H). We amplified the other cis-elements on Neurogenin2 (a neural basic-loop-helix (bHLH) transcriptional factor, Ngn2) promoter using the genomic DNA extracted

ATF6 α Protects Dopaminergic Neurons Against MPTP

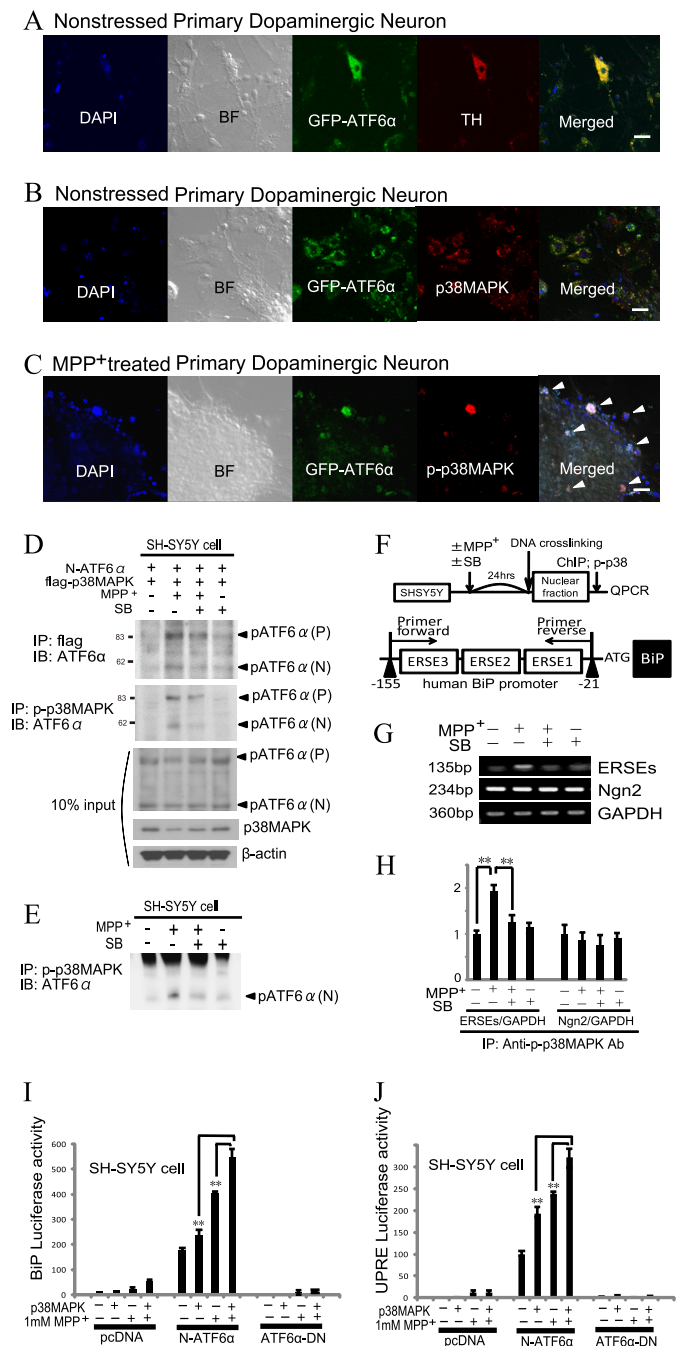


FIGURE 5. Phosphorylated p38MAPK and activated ATF6 α form a protein complex that binds and transactivates promoters containing ERSE and UPRE. A–C, MPP⁺ induces release of N-terminal ATF6 α fragment with subsequent translocation into the nucleus and colocalization with phosphorylated p38MAPK in dopaminergic neurons. 24 h after transfection with pGFP-ATF6 α (P), primary cultured dopaminergic neurons from co-culture of the midbrain and the striatum of ATF6 α KO mice were fixed and stained with anti-TH antibody (A, red), anti-p38MAPK antibody (B, red), and DAPI (blue). 24 h after transfection with pGFP-ATF6 α (P), primary cultured dopaminergic neurons were treated with 1 mM MPP⁺ for 24 h, and then fixed for staining with phosphorylated p38MAPK (p-p38MAPK) antibody (C, red) and DAPI (blue). White arrowheads indicate the nuclear localization of DAPI, GFP, and p-p38MAPK. Scale bars indicate 10 μ m. Abbreviations: TH, tyrosine hydroxylase; BF, bright-field image. D–H, MPP⁺ treatment induces the binding among p-p38MAPK, N-terminal fragment of ATF6 α and ERSEs of BiP promoter regions. SH-SY5Y cells were transfected with Flag-p38MAPK vector and N-terminal fragment of ATF6 α (1–373) vector. They were then cultured with or without 1 mM MPP⁺ in the presence or absence of 5 μ M SB203585 for 24 h. Cells were lysed in RIPA buffer and separated into soluble or

from the same immunoprecipitated samples and found that the amplified PCR products of the Ngn2 promoter regions were not increased in MPP⁺-treated SH-SY5Y cells. Thus, phosphorylated p38MAPK selectively binds to the activated and cleaved N-terminal fragment of ATF6 α that binds to the ERSEs on BiP promoter region in MPP⁺-treated cells.

We finally investigated whether the expression of p38MAPK and/or the treatment with MPP⁺ could increase the transcriptional activity of nuclear-translocating ATF6 α using the BiP promoter-luciferase system. MPP⁺ was added to SH-SY5Y cells cotransfected with Mock or N-ATF6 α encoding transactivator domain (TAD) and the DNA-binding b-ZIP domain or the dominant-negative form lacking TAD of ATF6 α (ATF6 α -DN) in the presence or absence of p38MAPK. MPP⁺ treatment significantly enhanced the stimulative effect of p38MAPK on N-ATF6 α -mediated activation of BiP reporter expression (Fig. 5I). We also found that MPP⁺ treatment increased transcriptional activator activity of N-ATF6 α through UPRE, which has been shown to be present in the promoter region of ERAD components and be functional in the induction of ERAD components (22, 23), and further enhanced it in presence of p38MAPK (Fig. 5J). ATF6 α -DN canceled these enhancement induced by p38MAPK overexpression through ERSE and UPRE. Thus, MPP⁺ promotes phosphorylation of p38MAPK and the binding with the N-terminal ATF6 α , leading to up-regulation of ATF6 α transcriptional activity.

insoluble fraction. D, soluble fraction was immunoprecipitated with anti-Flag antibody and immunoblotted with anti-ATF6 α antibody (upper panel). Insoluble fraction was immunoprecipitated with anti-p-p38MAPK antibody and immunoblotted with anti-ATF6 α antibody (middle panel). Western blot analysis of 10% input of soluble fraction was performed with anti-ATF6 α , p38MAPK and β -actin antibodies (lower panels). Abbreviations: IP, immunoprecipitation; IB, immunoblotting; SB, SB203585; N-ATF6 α , N-terminal fragment of ATF6 α (1–373). E, endogenous N-terminal fragment of ATF6 α can bind to phosphorylated p38MAPK in MPP⁺-treated condition. SH-SY5Y cells were cultured without transfection and treated with or without 1 mM MPP⁺ in the presence or absence of 5 μ M SB203585 for 24 h. Cells were totally lysed in RIPA buffer using BIORUPTOR and immunoprecipitated with anti-p-p38MAPK antibody and immunoblotted with anti-ATF6 α antibody. F, P-p38MAPK-ATF6 α complex binds to ERSEs in response to MPP⁺ treatment. Upper panel shows schematic representation of ChIP assays. SH-SY5Y cells were treated with or without MPP⁺ in the presence or absence of 5 μ M SB203585 for 24 h and then cross-linked with formaldehyde. Cells were lysed in RIPA buffer, and the insoluble fraction defined as nuclear fraction was lysed in SDS buffer. Immunoprecipitated with anti-p-p38MAPK, purified, and input DNAs were analyzed by Quantitative PCR using the primers shown in lower panel. G, primers used for human endogenous BiP promoter, Neurogenin2 (Ngn2) promoter and GAPDH yielded 135, 234, and 360 bp PCR products, respectively. H, values of PCR products were measured by QPCR and fold induction was defined as the value of BiP or Ngn2 promoter regions relative to the value of GAPDH. Each bar denotes the mean \pm S.D. I and J, p38MAPK phosphorylation enhances the transcriptional activity of ATF6 α . I, vectors (1 μ g of BiP promoter-Luciferase vector, 100 ng of pRL-SV40 vector and 1 μ g of control vector or p38MAPK vector) were mixed with 1 μ g of Mock (pcDNA-3.1(+)) or N-terminal fragment (1–373) (N-ATF6 α) or dominant-negative form (171–373) of ATF6 α vector (ATF6 α -DN) for transfection of SH-SY5Y cells in a 6-well dish for 48 h. Cells were challenged with or without 1 mM MPP⁺ for 24 h and lysed for analysis of BiP reporter expression. J, vectors (1 μ g \times UPRE reporter vector, 100 ng pRL-SV40 vector and 1 μ g of control vector or p38MAPK vector) were mixed with 1 μ g of pcDNA-3.1(+) or N-ATF6 α or ATF6 α -DN vector for transfection of SH-SY5Y cells in a 6-well dish for 48 h. Cells were challenged with or without 1 mM MPP⁺ for 24 h and lysed for analysis of UPRE reporter expression.

Hydrogen peroxide also induced the translocation of the N-terminal fragment of ATF6 α into the nucleus, where it co-localizes with p-p38MAPK (supplemental Fig. S3, D–G). Hydrogen peroxide promoted the binding of N-ATF6 α with phosphorylated p38MAPK and enhanced the transcriptional activity of N-ATF6 α through ERSE and UPR (supplemental Fig. S3, H–J).

DISCUSSION

In this study, we concluded that ATF6 α plays an important role in protection from MPTP toxicity in the dopaminergic neurons via up-regulation of ER chaperones and ERAD component. This was based on the following findings: 1) ATF6 α controls the levels of ER chaperones and ERAD component in dopaminergic neurons-containing brain regions (Figs. 1, A–D) and 2). Both MPTP and MPP⁺ upregulate the expression levels of ER chaperones and ERAD component by activating ATF6 α in dopaminergic neurons and SH-SY5Y cells (Fig. 2, A, B, C, F, G, H and 4, A, B, E, F, and G); 3) MPTP triggers more formation of clusters of ubiquitin-immunoreactive material in the striatum of ATF6 α KO mice than in WT mice (Fig. 3, A and B); 4) Dopaminergic neurons under ATF6 α KO conditions are more vulnerable to MPTP neurotoxicity (Fig. 3, C–F). Previous studies showed that MPTP and 6-OHDA induce various ER stress mediators *in vitro* (24–26). However, it has been unclear how these neurotoxins induce UPR.

A plausible mechanism is that MPTP decreases the protein degradation function of striatal UPS (27). A single or intermittent dose of MPTP decreases UPS activity only for a short time and does not lead to the ubiquitinated inclusion bodies in rodent brain (28). In contrast, continuous MPTP administration causes long-lasting impairment of UPS and the production of inclusion bodies immunoreactive for ubiquitin and α -synuclein in the rodent SNc (27). This report suggests that the inhibition of UPS by MPTP causes the accumulation of unfolded proteins followed by activation of UPR, finally leading to ER stress-induced cell death.

Consistent with this hypothesis, our study demonstrated that UPR attenuation caused by ATF6 α deletion accelerates the MPTP-induced formation of ubiquitin immunopositive inclusion body in the striatum as well as dopaminergic neuronal death (Fig. 3). This result suggests that MPTP induces accumulation of ubiquitin-positive aggregates presumably as a result of oxidative protein damage to protein folding and its machineries (29–31) and that activated ATF6 α promotes refolding and/or elimination of ubiquitinated proteins in the nerve terminal of dopaminergic neuron by the action of induced ER chaperones and ERAD components. These findings strongly suggest that both UPS and UPR are involved in MPTP-induced dopaminergic neuronal depletion. In our study we stained the striatal sections from MPTP-treated ATF6 α WT and KO mice with thioflavin T (amyloid-specific dye) and immunostained with anti- α -synuclein antibody and anti-phosphorylated α -synuclein. However we could not detect any stained aggregates for them (supplemental Fig. S2B). Although it remains to be

elucidated what is the major component of the ubiquitinated aggregates in this study, we speculate that oxidative products should be ubiquitinated and accumulated after oxidative stress induced by MPTP.

MPP⁺ interferes with the mitochondrial complex-1, generates ROS. ROS are also generated in the degradation process of dopamine and therefore dopamine is the most oxidative neurotransmitter in brain (16). In the present study, ATF6 α was activated particularly in the dopaminergic neuron system (Fig. 1, A and B) and the metabolism of dopamine is decreased in ATF6 α KO mice (Fig. 1E), suggesting that ROS generated from dopamine metabolic process may play a critical role in the up-regulation of ATF6 α -mediated UPR.

Previous study showed that ROS induced by both MPP⁺ and dopamine metabolism promotes phosphorylation of p38MAPK (17, 18, 32). Phosphorylated p38MAPK phosphorylates ATF6 α and mediates the transcriptional induction of the atrial natriuretic factor gene through a serum response element (21, 33). To explore the relationship between oxidative stress by ROS and UPR by ATF6 α , we focused on p38MAPK.

We demonstrated that: 1) Both MPTP and MPP⁺ induce the phosphorylation of p38MAPK in dopaminergic neurons as well as in SH-SY5Y cells (Figs. 2, A, E, K, L and 4, A and D). 2) MPP⁺ induces ATF6 α -mediated up-regulation of BiP expression level in a p38MAPK-dependent manner (Fig. 4, A, E, F, and G). 3) MPP⁺ triggers the translocation of N-terminal ATF6 α to the nucleus and the binding of N-terminal ATF6 α with phosphorylated p38MAPK to enhance the transcriptional activity of ATF6 α in dopaminergic neurons (Fig. 5, C–J). Hydrogen peroxide, a representative ROS, also activated ATF6 α and phosphorylated p38MAPK (supplemental Fig. S2A). Hydrogen peroxide promoted the formation of N-ATF6 α -p-p38MAPK complex and enhanced the transcriptional activity of ATF6 α to up-regulate ER chaperones and ERAD components (supplemental Fig. S3, D–J).

Based on these data, we propose that MPP⁺-derived oxidative stress results in phosphorylation of p38MAPK, enhancement of ATF6 α transcriptional activity, induction of ER chaperones and ERAD components and degradation of ubiquitinated accumulated proteins in dopaminergic neurons. In contrast, under ATF6 α -deleted condition, MPP⁺ triggers the formation of ubiquitinated aggregates in the striatum, and this protein accumulation lead to apoptosis of the dopaminergic neurons (Fig. 6).

ER stress and oxidative stress are closely linked events. Another oxidative neurotoxin, 6-OHDA, causes rapid generation of ROS, oxidative modification of protein and activates UPR. Antioxidants reduce UPR activation and apoptosis, and improve protein secretion (29). This study suggests that UPR protects against oxidative stress-induced cell death. ER stress preconditioning of BiP attenuates H₂O₂-induced cell injury in LLC-PK1 cells (34). Precondition of ER chaperones in the striatum expressed by ATF6 α under physiological conditions may also suppress dopaminergic neurons depletion induced by MPP⁺-derived oxidative stress.

The p38MAPK pathway is stimulated by cellular stresses, such as free radicals and inflammatory agents, and mediates

ATF6 α Protects Dopaminergic Neurons Against MPTP

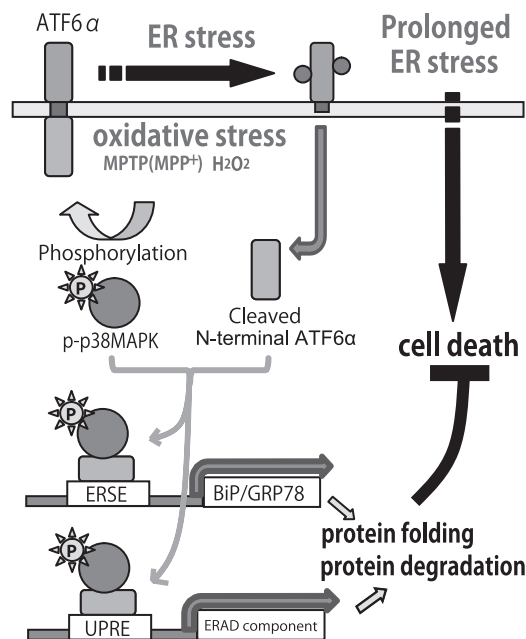


FIGURE 6. A proposed model for oxidative stress-ER stress pathway mediated by p38MAPK-ATF6 α interaction. Oxidative stress induced by MPTP (MPP⁺) or hydrogen peroxide (H₂O₂) cleaves proteolytic N-terminal fragment of ATF6 α and up-regulates the phosphorylation of p38MAPK. Phosphorylated p38MAPK binds to N-terminal fragment of ATF6 α and enhances ATF6 α -mediated induction of ER chaperones and ERAD components. This results in the degradation of accumulated unfolded proteins and prevents ER stress-induced dopaminergic neuronal death.

various kinds of signaling cascades, including; the cell cycle, apoptosis, and cell survival. Previous studies reported that p38MAPK plays a critical role in suppressing ER stress-induced macrophage apoptosis *in vitro* and advanced lesional macrophage apoptosis *in vivo* (35). On the contrary, p38MAPK also has a pro-apoptotic role in maintaining homeostasis under various stresses. Phosphorylation of p53 following phosphorylation of p38MAPK up-regulates transcription of Bax and Puma, leading to apoptotic cell death (18). Whether p38MAPK serves as pro- or anti-apoptotic agent seems to be context-dependent.

It should be noted that p38MAPK does not activate ATF6 α (Fig. 5I) and a p38MAPK phosphorylation inhibitor does not suppress proteolytic cleavage of N-terminal ATF6 α in our *in vitro* study (Fig. 4B and supplemental Fig. S2A). This suggests that neither p38MAPK nor phospho-p38MAPK initiates ATF6 α -mediated UPR. Mechanism underlying oxidative stress-mediated ATF6 α cleavage should be elucidated in the future.

The interaction between phosphorylated p38MAPK and cleaved N-terminal ATF6 α could result in a protective effect against the vicious cycle of oxidative stress-ER stress by induction of ER chaperones and ERAD components. This p38MAPK-ATF6 α interaction provides a new link between oxidative stress and ER stress. ATF6 α -mediated induction of ER chaperones and ERAD components via p38MAPK pathway may provide a therapeutic target for Parkinson disease and other neurodegenerative diseases associated with protein misfolding.

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