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Impairment of Atg5-Dependent Autophagic Flux Promotes Paraquat- and MPP+-Induced Apoptosis But Not Rotenone or 6-Hydroxydopamine Toxicity

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Controversial reports on the role of autophagy as a survival or cell death mechanism in dopaminergic cell death induced by parkinsonian toxins exist. We investigated the alterations in autophagic flux and the role of autophagy protein 5 (Atg5)-dependent autophagy in dopaminergic cell death induced by parkinsonian toxins. Dopaminergic cell death induced by the mitochondrial complex I inhibitors 1-methyl-4-phenylpyridinium (MPP+) and rotenone, the pesticide paraquat, and the dopamine analog 6-hydroxydopamine (6-OHDA) was paralleled by increased autophagosome accumulation. However, when compared with basal autophagy levels using chloroquine, autophagosome accumulation was a result of impaired autophagic flux. Only 6-OHDA induced an increase in autophagosome formation. Overexpression of a dominant negative form of Atg5 increased paraquat- and MPP+-induced cell death. Stimulation of mammalian target of rapamycin (mTOR)-dependent signaling protected against cell death induced by paraquat, whereas MPP+-induced toxicity was enhanced by wortmannin, a phosphoinositide 3-kinase class III inhibitor, rapamycin, and trehalose, an mTOR-independent autophagy activator. Modulation of autophagy by either pharmacological or genetic approaches had no effect on rotenone or 6-OHDA toxicity. Cell death induced by parkinsonian neurotoxins was inhibited by the pan caspase inhibitor (Z-VAD), but only caspase-3 inhibition was able to decrease MPP+-induced cell death. Finally, inhibition of the lysosomal hydrolases, cathepsins, increased the toxicity by paraquat and MPP+, supporting a protective role of Atg5-dependent autophagy and lysosomes degradation pathways on dopaminegic cell death. These results demonstrate that in dopaminergic cells, Atg5-dependent autophagy acts as a protective mechanism during apoptotic cell death induced by paraquat and MPP+ but not during rotenone or 6-OHDA toxicity.

Key Words: **autophagy; apoptosis; Atg5; cathepsins; paraquat; rotenone; MPP+; 6-hydroxydopamine; neurodegeneration; Parkinson's disease.**

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, and its prevalence increases exponentially from 65 to 90 years of age. A fraction of PD occurrence is related to specific mutations, whereas over 90% of PD occurs most commonly in a sporadic (idiopathic) form, without a clearly defined genetic basis, and only a vaguely delineated pathogenesis that is thought to be linked to unknown environmental causes. However, it is clear that not a single environmental toxicant can be the cause of PD. Thus, it is now considered that sporadic PD arises from the convergence of genetic susceptibility, environmental exposures, and aging (Horowitz and Greenamyre, 2010; Vance *et al.*, 2010). A dysfunction in the electron transport chain has been found in PD brains. Thus, inhibitors of complex I activity are well-accepted toxicological models to understand dopaminergic cell death pathways (Cannon and Greenamyre, 2010). Recent epidemiological data also suggest a link between the exposure to environmental toxicants, such as paraquat and rotenone, and an increased risk of developing PD (Franco *et al.*, 2010; Tanner *et al.*, 2011). PD is characterized by the irreversible loss of dopaminergic neurons in the substantia nigra pars compacta (Schapira, 2009), and although the underlying mechanisms and etiology of PD are not fully understood, mitochondrial dysfunction, oxidative stress, and protein aggregation are suggested as causative factors of neuronal death (Levy *et al.*, 2009; Przedborski, 2005; Yao and Wood, 2009).

Macroautophagy (here referred as autophagy) is an essential pathway for macromolecule and organelle turnover. It is initiated by an isolation membrane/phagophore surrounding cytoplasmic constituents in an enclosed double-membraned structure called autophagosome. Subsequently, the autophagosome fuses with a lysosome forming an autolysosome, whose content undergoes degradation by lysosomal hydrolases (He and Klionsky, 2009). Autophagy in neurodegenerative diseases has gained attention in the past years. Pioneer studies evidenced

that impairment of autophagy, specifically in the nervous system, leads to neurodegeneration accompanied by polyubiquitinated protein aggregation in cytoplasmic inclusion bodies (Hara *et al.*, 2006; Komatsu *et al.*, 2006). Moreover, morphological features of autophagy have been observed in postmortem human samples of PD patients (Anglade *et al.*, 1997).

Autophagy and chaperone-mediated autophagy have been proposed to prevent α-synuclein accumulation (Cuervo *et al.*, 2004; Webb *et al.*, 2003), whereas Parkin and PTEN-induced putative kinase 1 have been shown to regulate mitochondrial targeted autophagy (mitophagy) (Dagda *et al.*, 2009; Lee *et al.*, 2010; Narendra *et al.*, 2010; Vives-Bauza *et al.*, 2010). On the other hand, less is known regarding the role of autophagy in dopaminergic cell death induced by environmental/mitochondrial toxins (parkinsonian mimetics). Autophagy acts as a protective mechanism promoting cell survival in response to a variety of stressors, whereas only in very few cases, autophagy participates as a programmed cell death mechanism (Shen *et al.*, 2012). The pesticide paraquat, the mitochondrial complex I inhibitors 1-methyl-4-phenylpyridinium (MPP^+) and rotenone, and the dopamine oxidation product 6-hydroxydopamine (6-OHDA), which are used *in vitro* and *in vivo* as experimental PD models, have been shown to induce the accumulation of autophagosomes. However, whether this is associated with an increase in or a disruption of autophagic flux has not been addressed in detail. Furthermore, conclusions regarding the role of autophagy in dopaminergic cell death induced by these parkinsonian mimetics have been reached, in many cases, only by the use of nonspecific pharmacological stimulators or inhibitors. Contradictory, both autophagy-dependent cell death and survival mechanisms have been reported to regulate dopaminergic cell death induced by MPP^+ (Chu *et al.*, 2007; Dehay *et al.*, 2010), whereas both stimulation and inhibition of autophagy have been reported to be induced by paraquat (Gonzalez-Polo *et al.*, 2007; Wills *et al.*, 2012).

In this work, we determined the role of autophagy in dopaminergic cell death induced by parkinsonian mimetics. We demonstrated that paraquat and complex I inhibition by MPP^+ and rotenone impair autophagic flux. Autophagy protein 5 (Atg5)-dependent autophagy and lysosomal hydrolase activity were observed to act as protective mechanisms during dopaminergic cell death in response to paraquat and MPP^+. Only 6-OHDA was shown to stimulate autophagy flux. However, neither pharmacological inhibition of autophagy nor impairment of Atg5-dependent autophagy regulated rotenone or 6-OHDA toxicity.

MATERIALS AND METHODS

Cell culture and reagents. The human dopaminergic neuroblastoma cell line SK-N-SH was purchased from the American Type Culture Collection. SK-N-SH cells were originally established from a biopsy of a neuroblastoma patient. SK-N-SH cells, from which SH-SY5Y cells were derived, have been reported to present significant levels of dopamine β-hydroxylase activity (Biedler *et al.*, 1973) and low levels of tyrosine hydroxylase activity (Klongpanichapak *et al.*, 2008; West *et al.*, 1977). SK-N-SH cells express significant levels of the dopamine transporter (SLC6A3), which are not affected by retinoic acid-induced differentiation (unpublished data). Cells were cultured as explained in Rodriguez-Rocha *et al.*, (2012). Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride, 856177), MPP^+ (D048), rotenone (R8875), 6-OHDA (Cat No. 162957), and chloroquine (CQ, C6628) were obtained from Sigma/Aldrich. Rapamycin was from LC Laboratories (R-5000). Wortmannin (AC32859) and D-trehalose (AC30987) were from Acros Organics. Staurosporine, Z-VAD-FMK (219007), Ac-DEVD-CMK (218750), Ac-DMQD-CHO (235421), and Z-FG-NHO-Bz (219415) were from Calbiochem EMD Millipore.

Western immunoblotting (WB). Cells were lysed in either 1% SDS or radio immunoprecipitation assay (RIPA) buffer (20mM Tris-HCl, 150mM NaCl, 1mM Na_2EDTA, 1mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100) containing protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo/Pierce). Samples were sonicated and centrifuged, and pellets were discarded. Twenty-five to fifty micrograms of protein per sample were loaded separated by SDS-polyacrylamide gel electrophoresis, and transferred to PVDF or nitrocellulose membranes. Blots were incubated with the appropriate primary antibody (1:1000): LC3B (Sigma/Aldrich, L7543), Atg5 (Cell Signaling, 8540), and cleaved caspase-3 (Cell Signaling, 9664) at 4°C overnight. Peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (1:2000 or 1:5000, Thermo/Pierce or Cell Signaling) were used, and bands were detected using enhanced chemiluminescence (ECL) WB substrate (GE Life Sciences or Thermo/Pierce). Blots were subsequently reprobed for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 2118) or β-actin (ThermoScientific, MA5-15739) to verify equal protein loading.

EGFP-LC3 stable transfection. EGFP-LC3 plasmid generously donated by Dr Tamotsu Yoshimori (Osaka University, Japan) was linearized with *Nco I/Stu I* and transfected into SK-N-SH cells using FuGENE HD reagent (Promega). Stable cells overexpressing EGFP-LC3 were selected in complete medium containing 0.3 mg/ml geneticin (G418, Acros Organics), and subsequently sorted out in a BD FACSaria cell sorter (BD Biosciences).

Recombinant adenoviral vectors. The adenovirus Ad-EGFP-LC3 was a gift from Dr Aviva M. Tolkovsky (Cambridge Centre for Brain Repair, Cambridge, United Kingdom). The Ad-dnAtg5 was provided by Dr Gökhan S. Hotamisligil (Harvard School of Public Health, Boston, Massachusetts). Control adenovirus containing only the cytomegalovirus promoter (Ad-Empty) was used as control. Adenoviruses were amplified and titered in HEK293T cells according to previously established protocols (Barde *et al.*, 2010; Rodriguez-Rocha *et al.*, 2012). SK-N-SH cells were infected with adenoviral vectors at a multiplicity of infection (MOI) of 1.5 (Ad-Empty and Ad-dnAtg5) or 2.5 (Ad-EGFP-LC3) and treated with experimental conditions as indicated.

Fluorescence live-cell imaging. Cells were grown on glass-bottom dishes (MatTek Corp) coated with poly-D-lysine (0.1 mg/ml, Sigma/Aldrich). EGFP-LC3 was used to monitor autophagosome formation and LysoTracker Red DND-99 (500nM, Invitrogen, L-7528) to label lysosomes, and their fluorescence pattern was analyzed using an inverted (Olympus IX 81) confocal laser scanning microscope as follows: GFP, excitation laser wavelength: 488 nm, emission filter wavelength: 505–525 nm; and LysoTracker Red DND-99, ex laser wavelength: 543 nm, em filter wavelength: 560–600 nm. LysoTracker was removed after 15 min of incubation, and fresh phenol red–free medium was added for image acquisition.

Transmission electron microscopy. Cells were fixed with 2% glutaraldehyde in 0.2M 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) (pH 7.4) for 2 h at room temperature. Fixed cells were collected and postfixed with 1% OsO_4 and stained in 2% uranyl acetate (Eskelinen, 2008). The pellets were dehydrated through a graduated ethanol series and embedded in Epon 812 (Electron Microscopic Sciences) for sectioning and observed under a transmission electron microscope (Hitachi H7500). A series of ultrastructural images were collected with a bottom-mount digital camera for the analysis of autophagosomes.

Evaluation of cell death and apoptosis. Loss of cell viability was determined by propidium iodide (PI, 1 μg/ml, Sigma/Aldrich, P4170) uptake as a marker for loss of plasma membrane integrity using flow cytometry (FACS, fluorescence-activated cell sorting). PI was detected using FL-3 (488 nm ex,

695/40 nm em) or L2-4 (561 nm ex, 615/25 nm em). Cell death was represented as either percentage of cells with increased PI or mean PI fluorescence (AU). Externalization of phosphatidylserine was determined using Annexin V conjugated to Fluorescein isothiocyanate (FITC) (Trevigen). FITC fluorescence was analyzed on FL1 (488 nm ex, 530-30 nm em). Early apoptotic cells are defined as having Annexin V-positive (+), PI-negative (−) staining. Late-apoptotic and nonviable cells are both Annexin V and PI positive. The colorimetric measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant was also used for the quantification of cell death according to the manufacturer's protocol (Roche, 11644793001). After treatments, cell-free culture supernatant was assessed for LDH activity. Cells were lysed with a Triton X-100 solution at a final concentration of 1%, and LDH activity was also assessed to determine total LDH content. Absorbance (Abs) was measured at 490 and 630 nm of reference in an ELx800 Absorbance Microplate Reader (Biotek). Total LDH activity was determined using the following equation: LDH release (%) = [Supernatant Abs × 2]/[Supernatant Abs + (2 × Lysed Abs)]. Results are expressed as percentage of LDH release and were normalized to control (untreated) cells.

Statistical analysis. All experiment replicas were independent and performed on separate days. Collected data were analyzed according to statistical criteria by using paired or unpaired *t* test or 2-way ANOVA, and the appropriate parametric or nonparametric normality posttest using SIGMA-PLOT/STAT package. A probability value of $p < .05$ was considered statistically significant. Data were plotted as mean values of at least 3 independent experiments ± SEM using the same statistical package for data analysis. Flow cytometry plots and WB presented are representative of at least 3 independent experiments. Densitometry analysis of immunoblots was performed using ImageJ (NIH) v3.91 software (http://rsb.info.nih.gov/ij).

RESULTS

Autophagic Flux Is Impaired by Paraquat, MPP⁺, and Rotenone, and Increased by 6-OHDA

Accumulation of autophagosomes has been reported to parallel dopaminergic cell death induced by the parkinsonian mimetics paraquat, MPP⁺, rotenone, and 6-OHDA (Dagda *et al.*, 2008; Gao *et al.*, 2012; Gonzalez-Polo *et al.*, 2007; Zhu *et al.*, 2007). We observed that indeed, paraquat, MPP⁺, rotenone, and 6-OHDA induced a significant increase in autophagosome accumulation in dopaminergic cells in a dose-dependent manner, depicted by WB as the conversion of the microtubule-associated protein light chain 3 (LC3-I) to the autophagosomes marker LC3-II (Fig. 1A). Autophagosome accumulation was corroborated by electron microscopy (Fig. 1B). All neurotoxins induced, to a different extent, the accumulation of double-membrane vesicles (autophagosomes) and autolysosomes, in some of which their content could be evidenced under degradation.

Accumulation of autophagosomes might be associated with either an increase in or an impairment of autophagic flux, defined as the complete process of autophagy, beginning with the formation of the phagophore and ending after the fusion of the autophagosome with the lysosome for the subsequent degradation of the lysosomal cargo. Autophagic flux is inferred by WB analysis of LC3-II turnover in the presence and absence of inhibitors of lysosomal or vacuolar degradation. CQ is usually described in the literature as an inhibitor of the fusion of autophagosomes with lysosomes. However, several reports demonstrate that, as a lysosomotropic agent, CQ actually inhibits the acid-dependent degradation of the autolysosome content without affecting autophagosome-lysosome fusion, resulting in an accumulation of autophagolysosomes that cannot be cleared (Amaravadi *et al.*, 2007; Glaumann and Ahlberg, 1987; Maclean *et al.*, 2008; Poole and Ohkuma, 1981) (Fig. 7). As shown in Figure 1C, CQ leads to the accumulation of lysosomes in cells overexpressing EGFP-LC3 and stained with lysotracker, but it does not impair autophagosome-lysosome fusion.

Autophagic flux was blocked with CQ to evaluate basal autophagy levels and contrast them with the effects of neurotoxins. CQ induces the accumulation of autolysosomes as early as 2–4 h of incubation (data not shown). Cells were treated with the parkinsonian mimetics for 24 h, and CQ was added 2 h before LC3 analysis by WB. In the presence of CQ, paraquat, MPP⁺, and rotenone induced a decrease in LC3-II accumulation compared with CQ alone (Figs. 1C and 1D). Only 6-OHDA increased LC3-II levels in the presence of CQ. These results demonstrate that paraquat and the complex I inhibitors, MPP⁺ and rotenone, induce an accumulation of autophagosomes by impairment of autophagy flux, whereas 6-OHDA does it by increasing autophagy rate.

MODULATION OF AUTOPHAGY BY PHARMACOLOGICAL AND GENETIC APPROACHES

Rapamycin is a well-established stimulator of autophagy via the inhibition of the serine/threonine kinase mammalian target of rapamycin (mTOR). Recently, a novel function of trehalose as an mTOR-independent autophagy activator was described (Sarkar *et al.*, 2007) (Fig. 7). Rapamycin and trehalose induced the accumulation of autophagosomes and an increase in autophagic flux in dopaminergic cells (Figs. 2A–C). The formation of autophagosome precursors requires class III phosphoinositide 3-kinases (PI3Ks) and is prevented by PI3K inhibitors such as 3-methyladenine (3-MA) and wortmannin (Petiot *et al.*, 2000; Rubinsztein *et al.*, 2007) (Fig. 7). 3-MA, but not wortmannin, has been shown to both stimulate and inhibit autophagy depending on the concentration used; therefore, we decided to use wortmannin in our studies (Wu *et al.*, 2010). Wortmannin reduces the levels of basal autophagy compared with CQ, as well as the accumulation of autophagosomes upon rapamycin treatment (Fig. 2C). Most of the studies addressing the role of autophagy in dopaminergic cell death induced by parkinsonian mimetics have used similar pharmacological approaches. However, it is important to state that there are a number of secondary signaling pathways modulated by such pharmacological tools (see Supplementary Table 1).

The Atg12-Atg5 complex conjugation is essential for autophagosome formation, and functions as an E3-like ligase for LC3 lipidation (Hanada *et al.*, 2007; Tanida, 2011) (Fig. 7). Recently, Atg5-independent autophagy has been reported to occur in response to starvation and DNA damage (Nishida *et al.*,

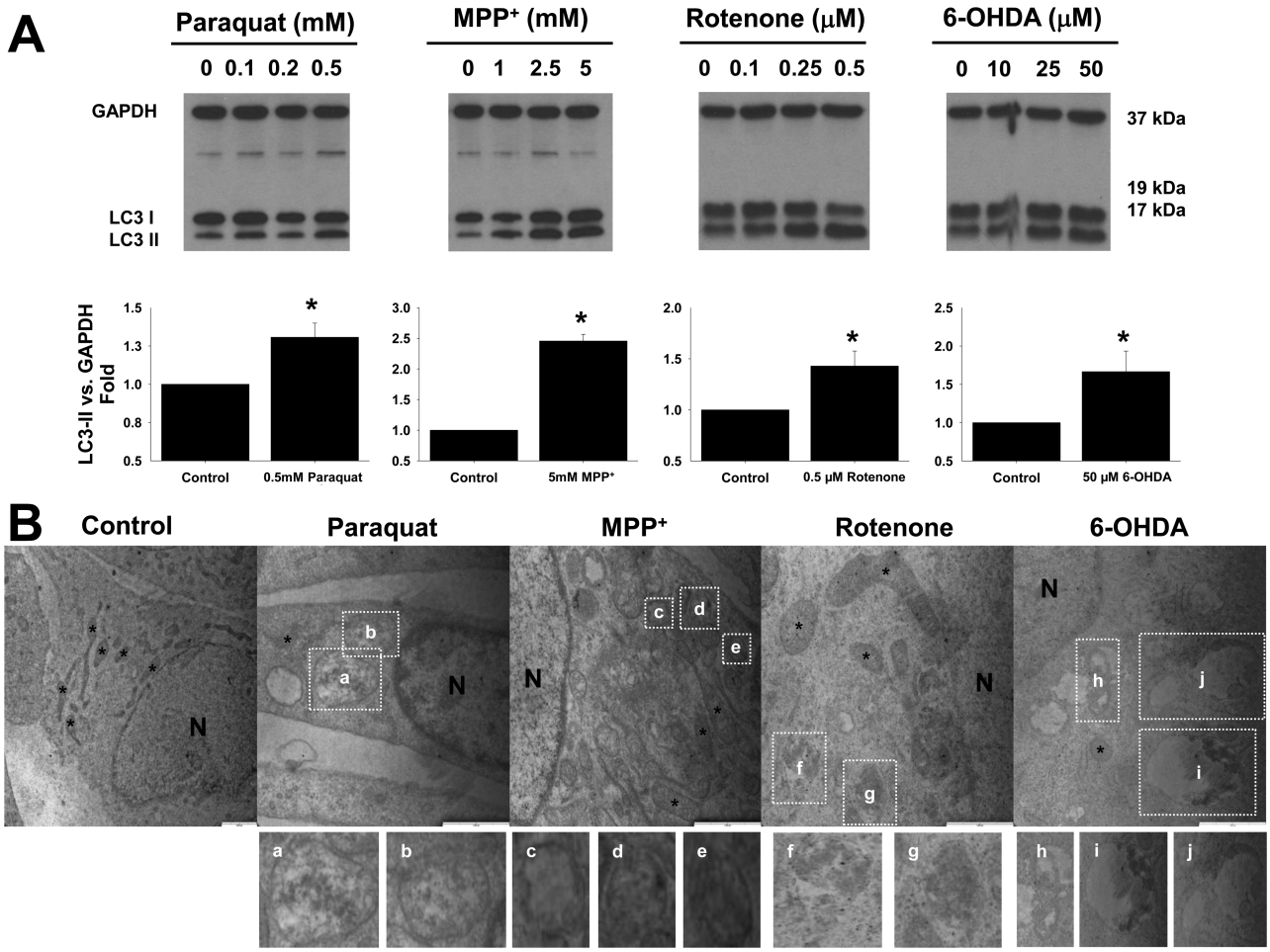

FIG. 1. Alterations in autophagic flux induced by PD toxins. A, Human dopaminergic SK-N-SH cells were treated with PD neurotoxins at the indicated concentrations for 24 h. Accumulation of LC3-II was analyzed by WB in whole-cell lysates. B, Autophagy morphology was evaluated by TEM. Representative electron micrographs show typical features of double-membraned vesicles (autophagosomes [c–g]) and autolysosomes (a, b, h–j), which exhibited late autophagic compartment containing partially degraded, electron-dense materials, as evidenced in the magnified images of boxed areas. N, nucleus; *, mitochondria. Bars indicate 1-μm scale. Normal SK-N-SH (D) or cells transduced with 2.5 MOI Ad-EGFP-LC3 (C) were treated with PQ (0.5mM), MPP^+ (2.5mM), rotenone (Rot, 4μM), or 6-OHDA (50μM) for 24 h and 40μM CQ was added 2 h before analysis. In (C), cells were stained with LysoTracker Red (500nM) for 15 min prior to confocal microscopy imaging. Boxed areas (B and C) in the merged panels are enlarged and represented in the insets. Autophagolysosome formation is indicated by white arrowheads highlighting EGFP-LC3 and LysoTracker colocalization. D, WB is representative of 3 independent experiments. Data in bar graphs represent the ratio of LC3-II/GAPDH or /β-actin normalized against control (untreated) samples (A) or CQ-treated cells (D) and are means ± SEM of 3 independent experiments. *$p < .05$, significant difference between values of the corresponding drug-treated and untreated cells in the absence (A) or presence of CQ (D). Abbreviations: 6-OHDA, 6-hydroxydopamine; CQ, chloroquine; MOI, multiplicity of infection; MPP^+, 1-methyl-4-phenylpyridinium; PQ, paraquat; PD, Parkinson's disease; TEM, transmission electron microscopy; WB, Western immunoblotting.

2009). Overexpression of a dominant negative (dn) form of Atg5 (dnAtg5) has been previously demonstrated to effectively inhibit autophagy (Yang *et al.*, 2010). We overexpressed dnAtg5 in cells treated with the parkinsonian toxins (Fig. 2D). dnAtg5 significantly reduced autophagic flux (in the presence of CQ) in control cells and in cells treated with the parkinsonian toxins (Figs. 2E–I).

Atg5-Dependent Autophagy Has a Protective Role in Response to Paraquat- and MPP^+-Induced Cell Death but Not in Response to Rotenone- or 6-OHDA-Induced Cell Death

Using the pharmacological and genetic tools described above, we next decided to evaluate the role of autophagy in dopaminergic cell death induced by parkinsonian mimetics. Cell death was evaluated by FACS using PI uptake and by determining the activity of released LDH, both reliable markers of compromised membrane integrity and cell death. Rapamycin significantly decreased the toxicity induced by paraquat (Figs. 3A and 3B). Neither trehalose (data not shown) nor wortmannin showed an effect over paraquat toxicity (Figs. 3A and 3B). Blockage of Atg5-dependent autophagy by dnAtg5 increased cell death induced by paraquat (Fig. 3C).

Paraquat and MPP^+ are structurally similar. However, it has been clearly demonstrated that the toxicity exerted by these

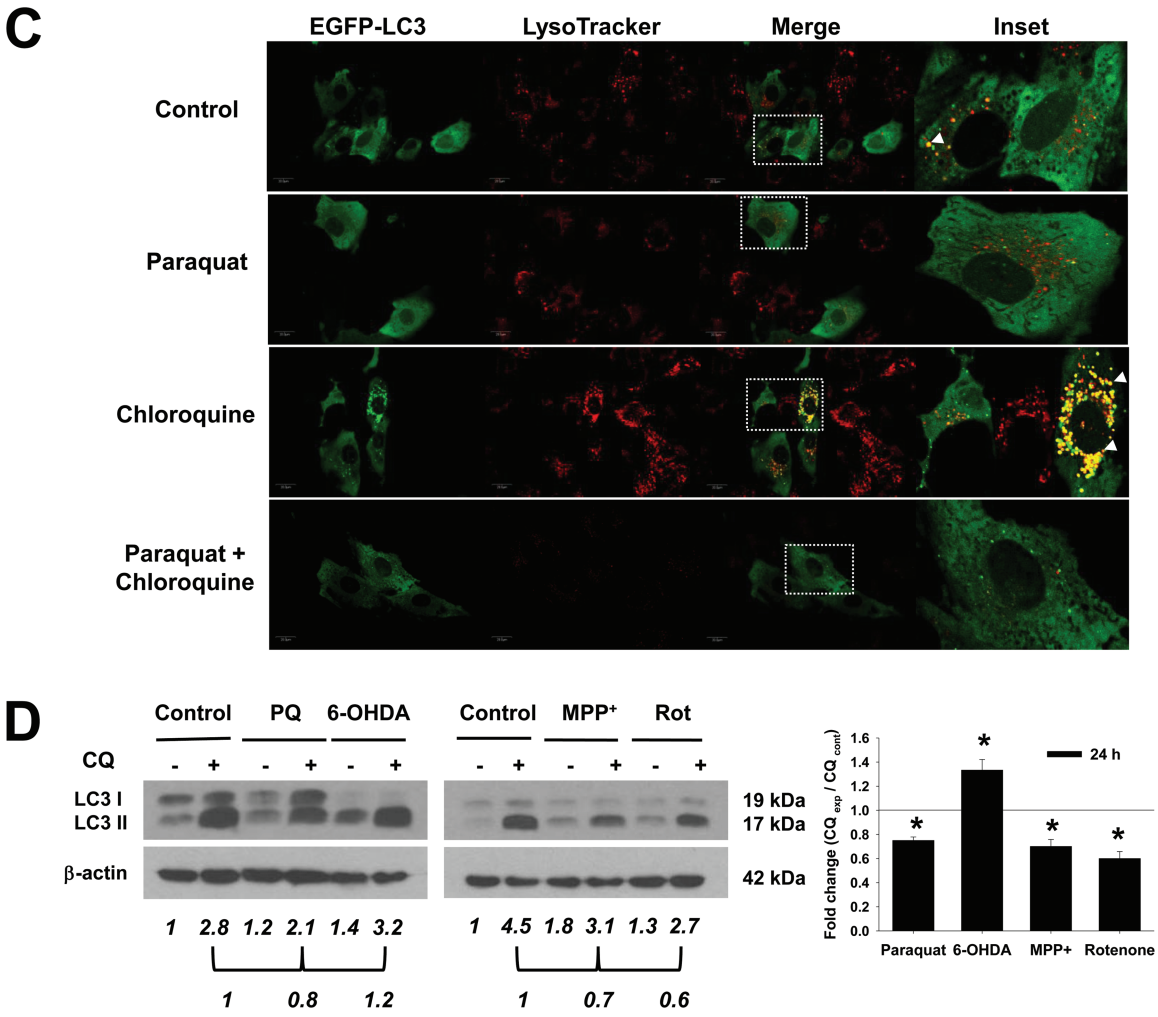

FIG. 1. Continued

drugs is mediated by divergent mechanisms (Ramachandiran *et al.*, 2007). Rapamycin and trehalose (Figs. 4A and 4B) augmented MPP+-induced cell death. Contradictory, wortmannin (Fig. 4A) or dnAtg5 (Fig. 4C) also led to increased cell death. These results demonstrate that inhibition of Atg5-dependent autophagy potentiates paraquat- and MPP+-induced cell death and thus autophagy seems to exert a protective effect against the toxicity of these neurotoxins. The discrepancy between these results and those obtained using both pharmacological stimulators (rapamycin and trehalose) inhibitors (wortmannin) can be ascribed to secondary or non-specific effects of these agents (see Discussion section and Supplementary Table 1).

As complex I inhibitors, it is usually considered that both MPP+ and rotenone share similar mechanisms to induce dopaminergic toxicity (Martinez and Greenamyre, 2012) (Fig. 7). However, recent reports suggest that rotenone and MPP+ induce distinct effects on cellular bioenergetics and cell death (Giordano *et al.*, 2012). In contrast to MPP+ and paraquat, neither the pharmacological modulators of autophagy, nor overexpression of dnAtg5 had an effect on rotenone toxicity (Figs. 5A–D). 6-OHDA is a hydroxylated analog of dopamine used to induce selective cell death of dopaminergic cells. Similar to rotenone, 6-OHDA was affected by neither treatment with rapamycin, trehalose (not shown), or wortmannin, nor by overexpression of dnAtg5 (Figs. 5E–H).

CASPASE-DEPENDENT CELL DEATH INDUCED BY PARKINSONIAN MIMETICS IS REGULATED BY ATG5-DEPENDENT AUTOPHAGY AND CATHEPSIN ACTIVITY

Dopaminergic cell death induced by parkinsonian neurotoxins has been previously demonstrated to be mediated by caspase-dependent apoptosis (Ahmadi *et al.*, 2003; Fei *et al.*, 2008; Hartley *et al.*, 1994; Hartmann *et al.*, 2000; Lotharius *et al.*, 1999; Viswanath *et al.*, 2001). However, some reports have questioned this idea suggesting that either caspase-independent apoptosis or autophagic cell death mediate the toxicity of parkinsonian mimetics (Chu *et al.*, 2005; Han *et al.*,

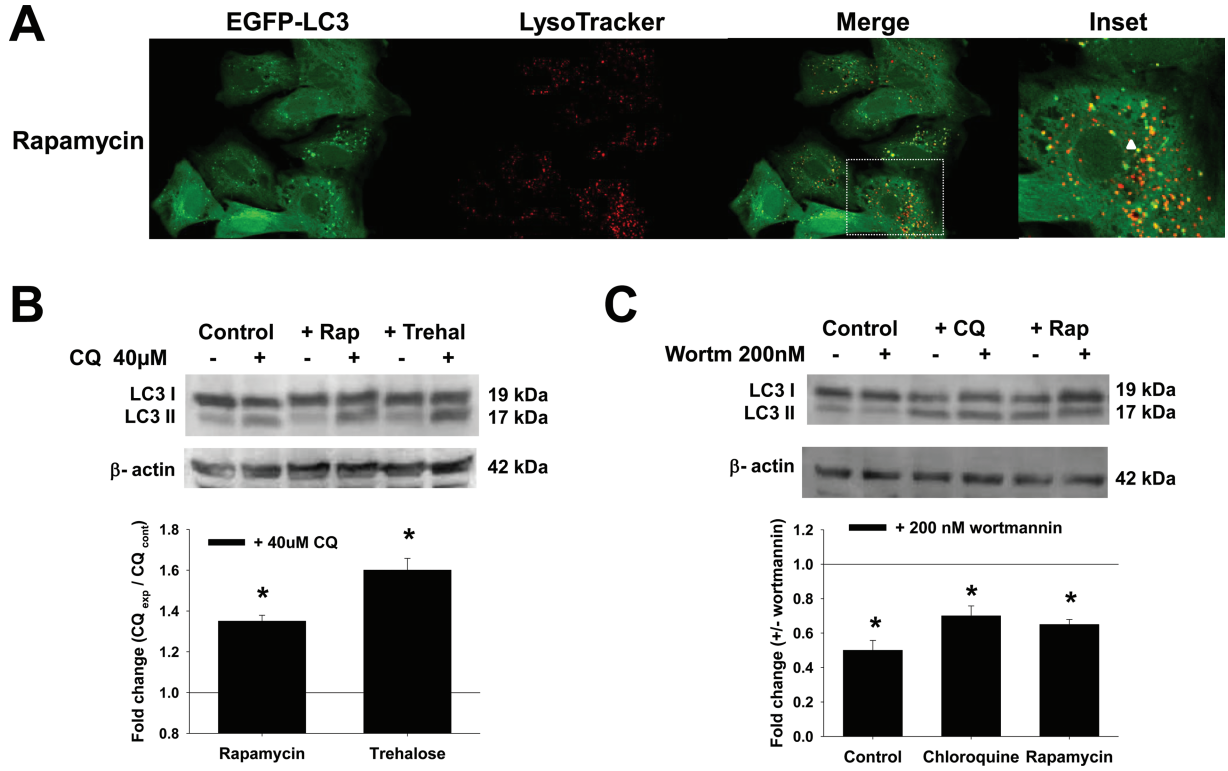

FIG. 2. Pharmacological and genetic approaches used to modulate autophagy flux and Atg5-dependent autophagy. A, Cells were transduced with Ad-EGFP-LC3 (2.5 MOI) for 24 h and then treated with 5μM rapamycin for 24 h. Cells were stained with LysoTracker and analyzed by confocal microscopy. The boxed area in the merged panel is enlarged and represented in the inset. Autophagolysosome formation is indicated by white arrowheads highlighting EGFP-LC3 and LysoTracker colocalization. B and C, Cells were treated with rapamycin (5μM) and trehalose (100mM) for 24 h in the presence or absence of CQ (40μM for 4 h before analysis) or wortmannin (200nM, 24 h). D–H, Cells were infected for 24 h with Ad-Empty or Ad-dnAtg5 at 1.5 MOI followed by treatment with PQ (0.5mM), MPP^+ (2.5mM), rotenone (Rot, 4μM), or 6-OHDA (50μM) for 24 h. LC3-II accumulation and Atg12-Atg5 complex were evaluated by WB. Blots are representative experiments. I, Autophagic flux was evaluated in cells treated with CQ (40μM) 4 h prior to collection. Changes in LC3-II/β-actin signal induced by dnAtg5 with respect to cells infected with Empty virus in the presence of CQ, was evaluated in controls (dotted lines) or cells treated with parkinsonian mimetics (broken lines). *$p < .05$, significant difference between cells infected with dnAtg5 and Empty viruses. Abbreviations: 6-OHDA, 6-hydroxydopamine; CQ, chloroquine; dnAtg5, dominant negative form of Atg5; MOI, multiplicity of infection; MPP^+, 1-methyl-4-phenylpyridinium; PQ, paraquat; WB, Western immunoblotting.

2003; Kang *et al.*, 2012; Li *et al.*, 2005; Lotharius *et al.*, 1999; Ramachandiran *et al.*, 2007; Rodriguez-Blanco *et al.*, 2012; Zhu *et al.*, 2007). Thus, we wanted to evaluate the role of apoptosis in dopaminergic cell death induced by neurotoxins and the role of Atg5-dependent autophagy. Apoptosis was determined by detection of phosphatidylserine externalization (Annexin V-FITC) in the absence of lost plasma membrane integrity (PI staining). As shown in Figure 6A (Annexin V-FITC/PI contour plots), all neurotoxins induced apoptotic cell death to a different extent, evidenced by the appearance of an Annexin V + / PI - population (lower right quadrant in broken line). Inhibition of the executioner caspase-3 with DMQD-CHO peptide only decreased the cell death triggered by MPP^+ treatment. Similar results were obtained with the irreversible caspase-3/-7–like inhibitor DEVD-FMK (not shown). In contrast, the irreversible pan-caspase inhibitor Z-VAD-FMK significantly reduced cell death induced by all neurotoxins (Fig. 6B). Because dnAtg5 increased the cell death induced by paraquat and MPP^+, we examined the influence of autophagy in the activation of caspases. Both paraquat- and MPP^+-induced caspase-3 activation/cleavage was increased by blocking autophagy with dnAtg5 (Fig. 6C), and this effect was more prominent in MPP^+ toxicity, which correlates with the selective effect of the caspase-3 inhibitor on MPP^+-induced cell death.

The degradation of cellular constituents by autophagy is executed inside the lysosomal (autolysosomal) compartment, and the efficiency of lysosomal degradation determines autophagic flux (Fig. 7). Lysosomes contain more than 50 acid hydrolases, including peptidases, phosphatases, nucleases, glycosidases, proteases, and lipases, which digest most macromolecules in the cell (Kaminskyy and Zhivotovsky, 2012). The major function of lysosomal proteases is not to kill the cell but to maintain cellular homeostasis by recycling cellular content (Turk and Turk, 2009). Cathepsins B, L, and D are the most abundant lysosomal proteases (Kaminskyy and Zhivotovsky, 2012). Inhibition of lysosomal enzymes with Z-FG-NHO-Bz augmented paraquat- and MPP^+-mediated cell death (Fig. 6D) suggesting that

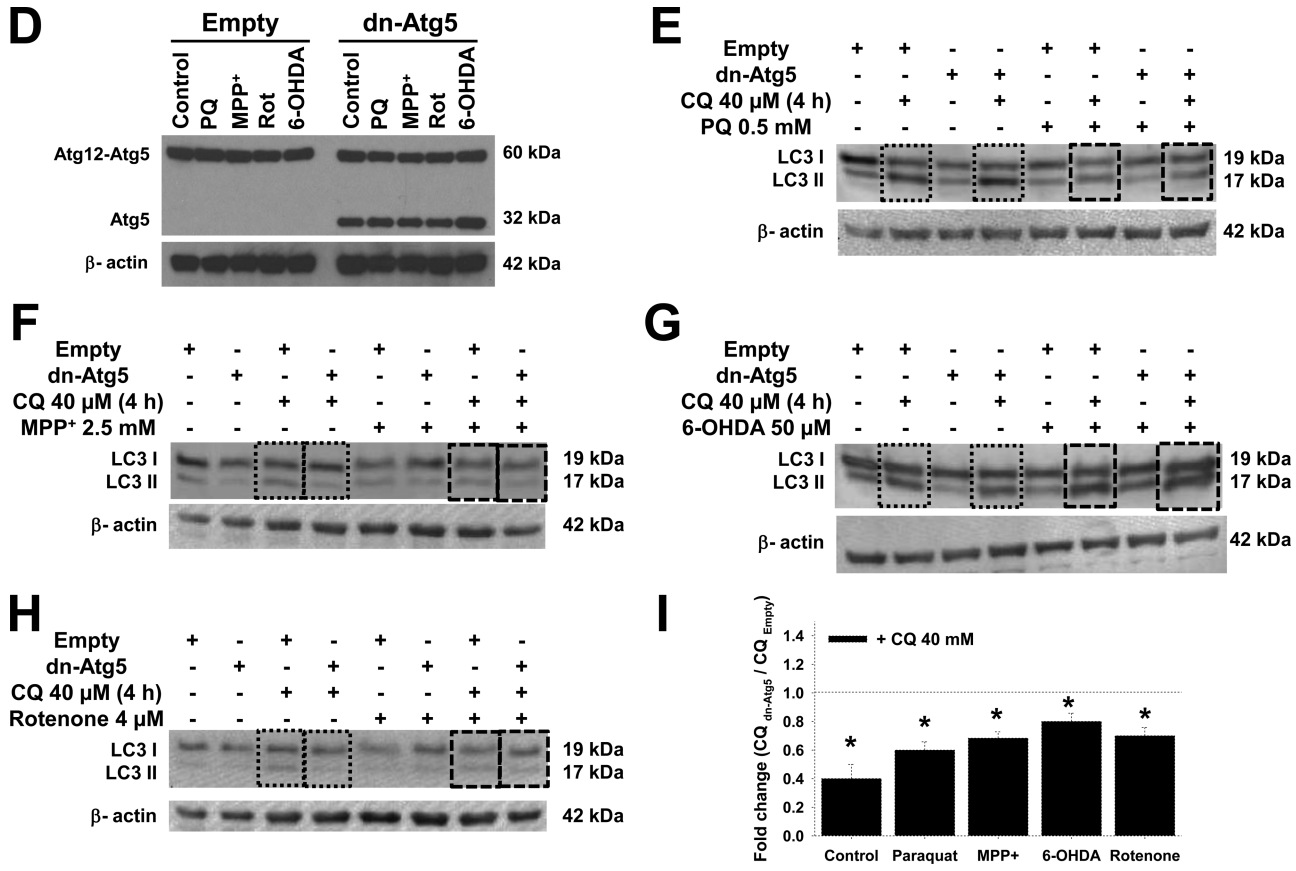

FIG. 2. Continued

lysosomal degradation/turnover of subcellular components is involved in the protective effects of Atg5-dependent autophagy against MPP^+ and paraquat toxicity.

DISCUSSION

The observation that accumulation of autophagosome structures is found in postmortem PD brains has prompted researchers to study the role of autophagy in PD progression (Anglade *et al.*, 1997). A number of findings have demonstrated the importance of autophagy pathways in regulating the accumulation of aggregated proteins, mitochondrial function, and cell survival/death in experimental PD models, highlighting the complexity of the interrelationship between autophagy pathways and dopaminergic neurodegeneration (Harris and Rubinsztein, 2012; Wong and Cuervo, 2010). However, a number of important questions still remain unsolved. Parkinsonian mimetics such as dopamine analogs (6-OHDA), environmental toxicants (pesticides and metals), and mitochondrial toxins (complex I inhibitors) have been widely used to understand the sensitivity of dopaminergic cells to the prooxidant properties of dopamine, environmental exposures, and mitochondrial dysfunction, respectively. To date, contradictory results still exist regarding the alterations in autophagy flux induced by parkinsonian mimetics. Furthermore, the role of autophagy in cell death induced by parkinsonian neurotoxins and the exact signaling mechanisms involved in the regulation of autophagy in dopaminergic cell death are still unclear. In this work, we demonstrated that autophagy is regulated distinctively by neurotoxins. Although complex I inhibitors and paraquat decrease autophagic flux, the toxicity of 6-OHDA is associated with a clear increase in the autophagic rate. Atg5-dependent autophagy and lysosomal hydrolase activity were shown to exert a protective effect against MPP^+ and paraquat toxicity. In contrast, cell death induced by rotenone and 6-OHDA did not seem either to be regulated by Atg5-dependent signaling or to respond to pharmacological regulation of autophagy.

Autophagic flux refers to the entire process of autophagy including the delivery of autophagosomes' cargo to lysosomes, its subsequent degradation, and the release of the resulting macromolecules back into the cytosol (Fig. 7). By responding to perturbations in the extracellular environment, cells tune autophagic flux to meet intracellular metabolic demands. Accumulation of autophagosomes can be associated with increased autophagic flux, impaired autophagosolysosome clearance, or both. Paraquat has been reported to stimulate or inhibit autophagy (Gonzalez-Polo *et al.*, 2007; Wills *et al.*, 2012). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),

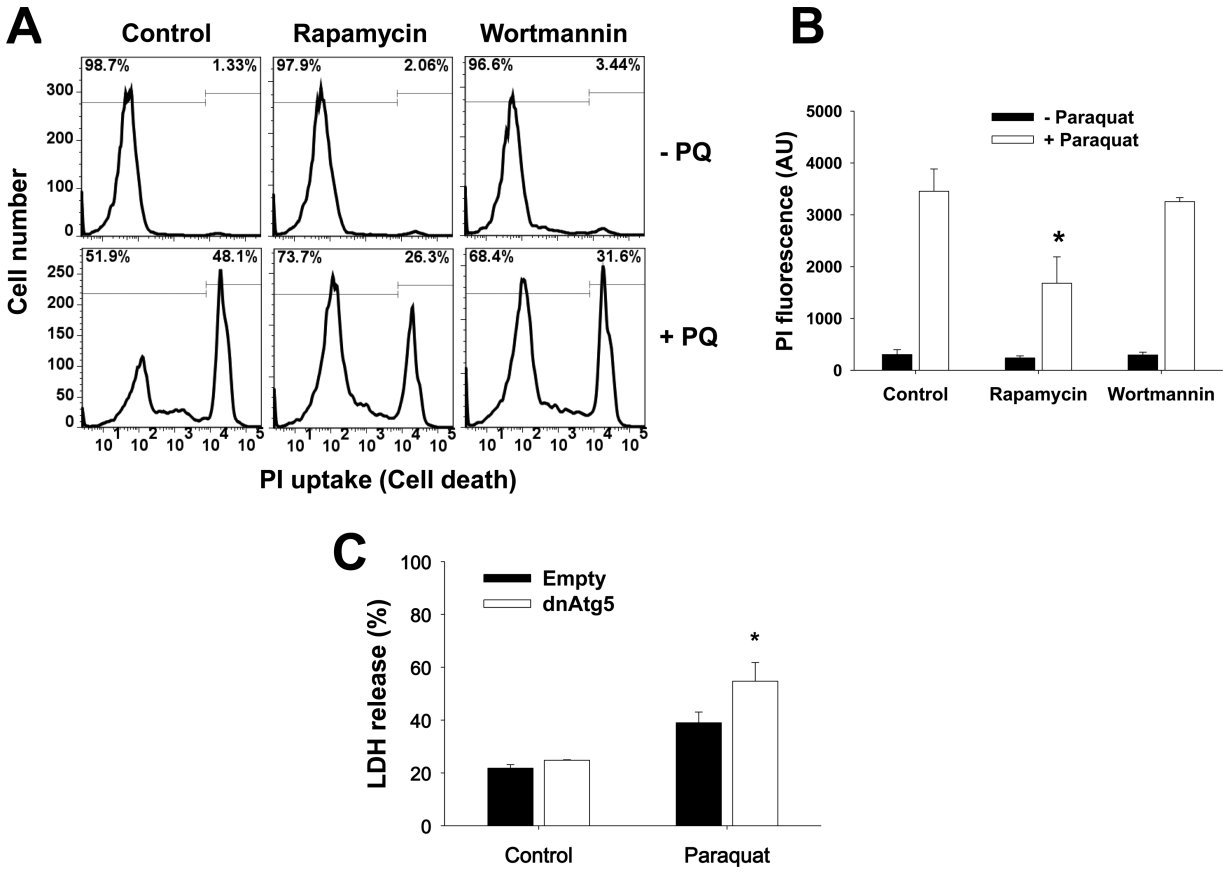

FIG. 3. Atg5-dependent autophagy protects against dopaminergic cell death induced by PQ. A and B, Cells were subjected to 1 h pretreatment with rapamycin (5μM) or wortmannin (200nM), followed by exposure to PQ (0.5mM) for 48 h. Loss of cell viability or plasma membrane integrity was analyzed by flow cytometry and is reflected as an increase in the number of cells with high PI fluorescence (histograms in A and bar graphs in B). C, Cells were infected with Ad-Empty or Ad-dnAtg5 at 1.5 MOI for 24 h followed by treatment with PQ (0.5mM) for additional 48 h. LDH activity was also assessed to evaluate cell death. Data in bar graphs represent changes in PI fluorescence intensity in AU (B), or percentage of released LDH (C), and are means ± SEM of 3–4 independent experiments. *$p < .05$, significant difference with respect to PQ-only treatment. Abbreviations: dnAtg5, dominant negative form of Atg5; LDH, lactate dehydrogenase; MOI, multiplicity of infection; PI, propidium iodide; PQ, paraquat.

rotenone, and 6-OHDA have also been reported to increase autophagy (Dadakhujaev *et al.*, 2010; Dehay *et al.*, 2010; Gao *et al.*, 2012; Li *et al.*, 2011; Meredith *et al.*, 2009; Pan *et al.*, 2009; Rodriguez-Blanco *et al.*, 2012; Solesio *et al.*, 2012). However, conclusions in these studies have been reached without proper analysis of the autophagic rate that requires the presence of autophagic flux inhibitors. Here, we have clearly demonstrated that paraquat and the complex I inhibitors MPP^+ and rotenone impair autophagy flux. Similar to what was reported by Dagda *et al.* (2008), we also found that 6-OHDA induced a clear increase in autophagic flux. In contrast, a previous study reported an increase in autophagic flux in response to MPP^+ in the presence of bafilomycin (Zhu *et al.*, 2007).

A number of complex I inhibitors have been described to date (Friedrich *et al.*, 1994; Hollingworth *et al.*, 1994; Miyoshi, 1998; Schapira, 2010) (see http://www.oxphos.org/index.php?option=com_content&task=view&id=77&Itemid=75 for a comprehensive list of inhibitors). Unfortunately, very little research has been done on the effect on autophagy flux of other complex I inhibitors besides MPP^+ or rotenone. For example, ranolazine, capsaicin, deguelin, and the oxidative phosphorylation uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone induce an increase in autophagosome formation, but these studies did not evaluate if this effect was associated with an increase in autophagy rate or an impairment in the autophagy flux (Huang *et al.*, 2010; Kwon *et al.*, 2011; Oh and Lim, 2009; Yang *et al.*, 2013). Similar to the observations made here that MPP^+ and rotenone inhibit autophagy flux, haloperidol, another complex I-inhibitor, was shown to reduce autophagolysosome formation in primary neuronal cultures (Park *et al.*, 2012).

We have demonstrated that the toxicity of paraquat, MPP^+, and rotenone is associated with a decrease in autophagy flux. However, the mechanisms involved remain unclear. Contradictory results exist on the effect of parkinsonian mimetics on mTOR signaling, a major signaling regulator of basal autophagy. For example, paraquat has been shown to increase (Niso-Santano *et al.*, 2011) autophagy by dephosphorylation of mTOR, whereas a different study reports on the inhibitory

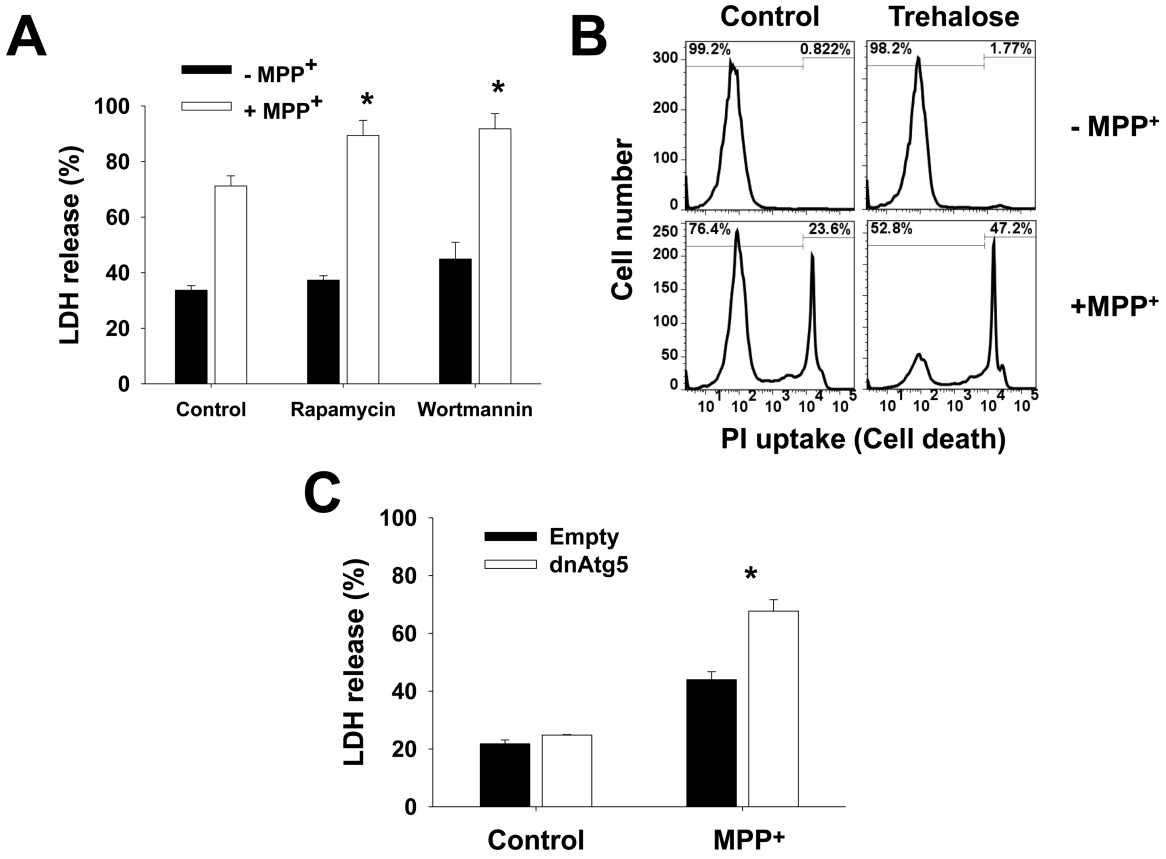

FIG. 4. Atg5-dependent autophagy protects against MPP+-induced dopaminergic cell death. A and B, Cells were subjected to 1 h pretreatment with rapamycin (5μM), wortmannin (200nM), or trehalose (100mM). In (C), cells were infected with Ad-Empty or Ad-dnAtg5 at 1.5 MOI for 24 h. Then, cells were treated with MPP+ (2.5mM) for additional 48 h. Loss of cell viability or plasma membrane integrity was determined by measurement of released LDH activity (A and C) or by PI uptake by flow cytometry (B) as explained in Figure 3. Histograms in (A) are representative of 3 independent experiments. Data in bar graphs represent percentage of released LDH activity, and are means ± SEM of 3 independent experiments. *$p < .05$, significant difference with respect to MPP+-only treatment. Abbreviations: dnAtg5, dominant negative form of Atg5; LDH, lactate dehydrogenase; MOI, multiplicity of infection; MPP+, 1-methyl-4-phenylpyridinium; PI, propidium iodide.

effect of paraquat on autophagy by an increase in mTOR signaling (Wills *et al.*, 2012). MPTP/MPP+ has been consistently reported to inhibit mTOR signaling (Deguil *et al.*, 2007; Rieker *et al.*, 2011; Rodriguez-Blanco *et al.*, 2012). An interesting study showed that chronic rotenone exposure impairs the ability of rapamycin to induce autophagy, suggesting that alterations in cellular metabolism by rotenone impair autophagic activity (Yu *et al.*, 2009). Multiple signaling pathways regulate/trigger autophagy (He and Klionsky, 2009; Yang and Klionsky, 2010); thus, the identification of the mechanisms by which paraquat and complex I inhibitors impair autophagy requires a more thorough study. Autophagy has been demonstrated to require ATP as energy source (Sakai and Ogawa, 1982; Schellens and Meijer, 1991). Thus, it is plausible that inhibition of autophagy by these neurotoxins is linked directly to ATP depletion (Kang *et al.*, 2012; Mizuno *et al.*, 1987), which does not seem to occur during 6-OHDA-induced toxicity (Kang *et al.*, 2012). The importance of basal autophagy and neuronal degeneration was demonstrated by elegant studies where impairment in basal autophagy in the nervous system leads to neurodegeneration accompanied by polyubiquitinated protein aggregation in cytoplasmic inclusion bodies (Hara *et al.*, 2006; Komatsu *et al.*, 2006).

Autophagy acts as a protective mechanism promoting cell survival in response to a variety of stressors, and only in very few cases, it participates as a programmed cell death mechanism (Shen *et al.*, 2012). Contradictory findings have been reported on the role of autophagy-dependent cell death and survival mechanisms in dopaminergic cell death induced by MPP+ (Chu *et al.*, 2007; Dehay *et al.*, 2010). A protective role for autophagy in paraquat, rotenone, and 6-OHDA has been postulated in most studies so far (Dadakhujaev *et al.*, 2010; Dehay *et al.*, 2010; Gonzalez-Polo *et al.*, 2007, 2009; Niso-Santano *et al.*, 2011; Pan *et al.*, 2009; Xiong *et al.*, 2011). However, contradictory results have suggested that autophagic cell death participates in MPP+/MPTP toxicity based on the inhibitory and stimulatory effects of bafylomycin (Rodriguez-Blanco *et al.*, 2012) and rapamycin (Chu *et al.*, 2007), respectively. 6-OHDA-induced dopaminergic cell death was also associated with autophagic cell death by the inhibitory effect of 3-MA.

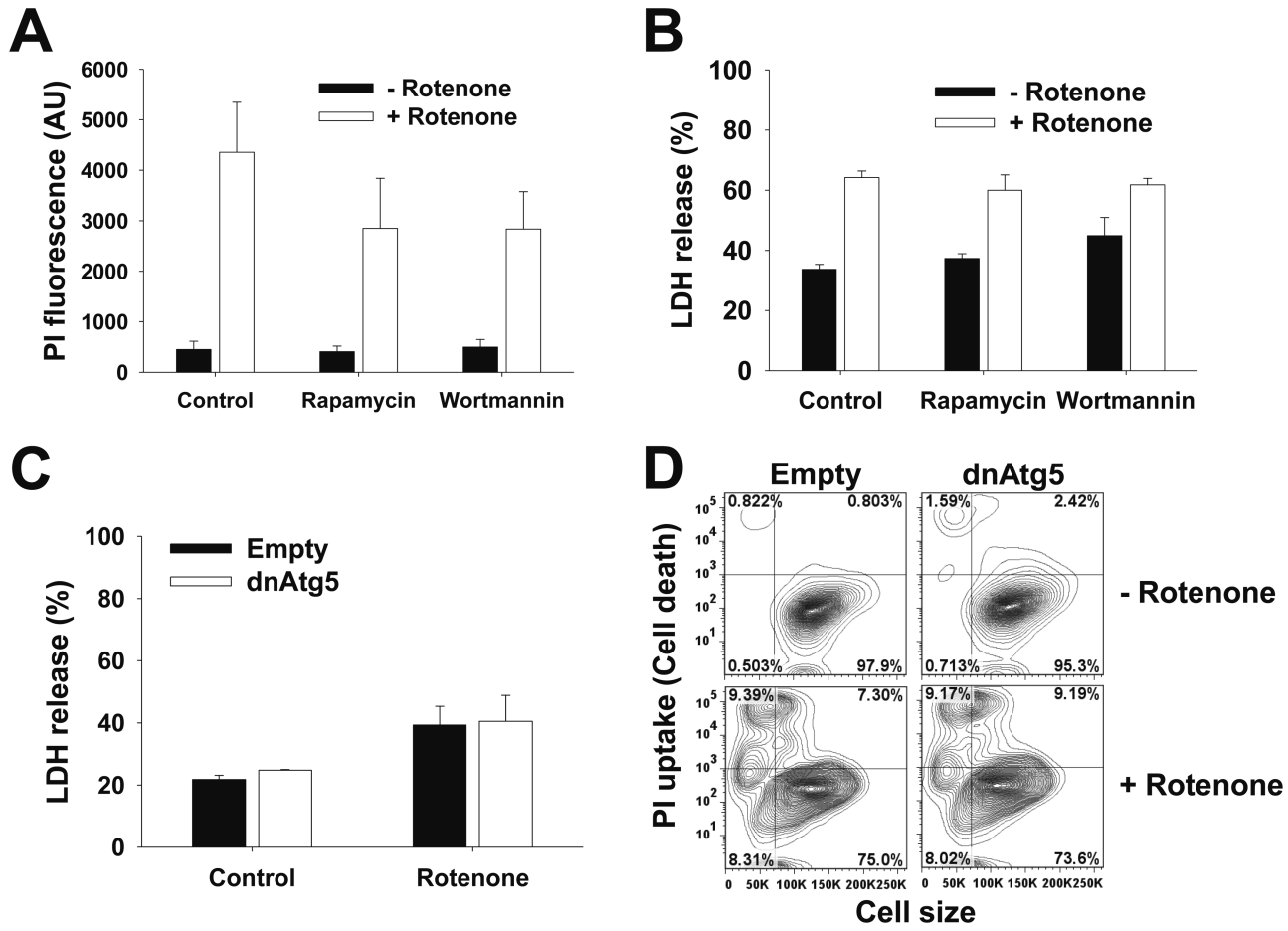

FIG. 5. Rotenone and 6-OHDA toxicity is not affected by pharmacological modulators of autophagy, or impairment of Atg5-dependent pathway. In (A, B, E, and F), cells were subjected to 1 h pretreatment with rapamycin (5μM) or wortmannin (200nM). In (C, D, G, and H), cells were infected with Ad-Empty or Ad-dnAtg5 at 1.5 MOI for 24 h. Then, cells were exposed to rotenone (4μM) (A–D) or 6-OHDA (50μM) (E–H) for 48 h. Loss of cell viability or plasma membrane integrity was determined by measurement of released LDH activity (B and C, and F and G) or by PI uptake analysis with flow cytometry (A, D, E, and H) as explained in Figure 3. D and H, Contour plots represent changes in PI uptake simultaneously compared with cell shrinkage as a marker for apoptotic volume decrease and are representative of 3 independent experiments. Data in bar graphs represent percentage of released LDH activity and are means ± SEM of 3 independent experiments. Abbreviations: 6-OHDA, 6-hydroxydopamine; dnAtg5, dominant negative form of Atg5; LDH, lactate dehydrogenase; MOI, multiplicity of infection; PI, propidium iodide.

We found that rapamycin stimulated MPP^+ toxicity, which suggests a role of autophagic cell death. However, wortmannin, considered an inhibitor of autophagosome formation, had a similar stimulatory effect. In contrast, paraquat toxicity was only shown to be reduced by rapamycin, but wortmannin had no effect on paraquat-induced cell death, whereas neither 6-OHDA nor rotenone were shown to be modulated by these agents. As summarized in Supplementary Table 1 and Figure 7, both rapamycin and PI3K inhibitors wortmannin, LY294002, and 3-MA, widely used to modulate autophagy, have a variety of secondary/nonautophagy-related effects. Interestingly, rapamycin has been shown to protect against the accumulation of aggregated proteins independently of autophagy by reducing protein synthesis (King *et al.*, 2008). Thus, we consider that conclusions based solely on pharmacological regulation of autophagy might be misleading.

To date, a wide variety of signaling molecules have been shown to regulate autophagy (He and Klionsky, 2009; Yang and Klionsky, 2010). Yeast genetic studies identified Atg proteins as core components of the molecular machinery involved in the formation of autophagosomes (Mizushima *et al.*, 2011). The Atg12-Atg5 and Atg8 (LC3)-phosphatidylethanolamine (PE) ubiquitin-like conjugation systems function at the late stages of autophagosome formation (Fig. 7). LC3 and Atg5 are not required for the initiation of autophagy but mediate phagophore expansion and autophagosome formation. An Atg5/Atg7-independent macroautophagy process induced by etoposide and starvation has been recently reported, which seem to depend on Atg1 (Ulk1) and Atg6 (beclin 1) signaling. Conversely, Ulk1/2- and beclin-independent autophagy pathways seem to require Atg5 signaling (Cheong *et al.*, 2011; Grishchuk *et al.*, 2011; Seo *et al.*, 2011; Smith *et al.*, 2010). Atg5 knockdown was

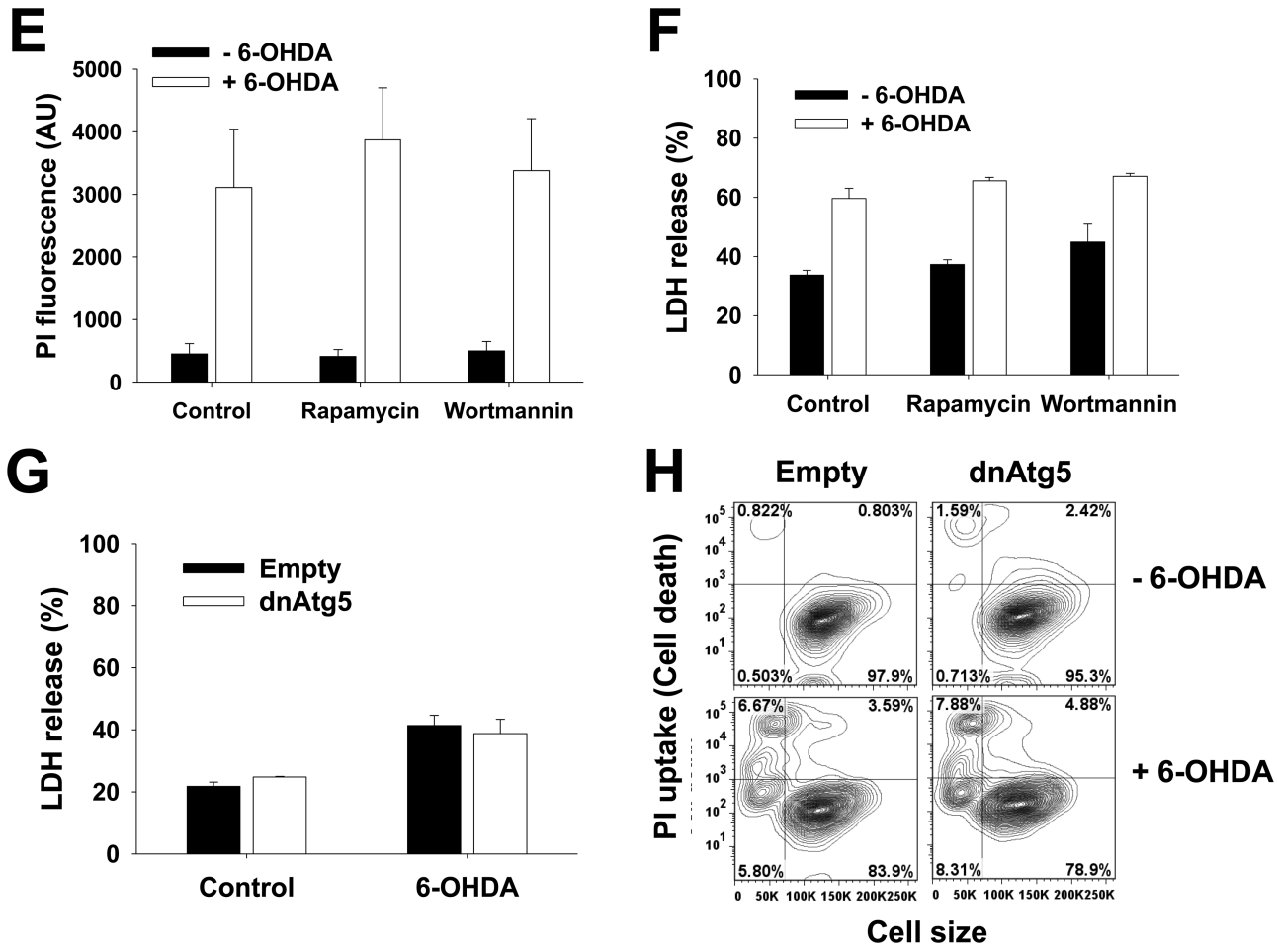

FIG. 5. Continued

reported to prevent the protective effects of rapamycin against rotenone-induced cell death (Pan *et al.*, 2009). In contrast, Atg7, Atg5, and LC3, but not beclin 1, knockdown were shown to protect SH-SY5Y cells against MPP^+ (Zhu *et al.*, 2007). In contrast, in our results, we demonstrated that dnAtg5 significantly enhanced the toxicity of paraquat and MPP^+ but not that of rotenone. In addition, dnAtg5 had no effect on 6-OHDA toxicity. The difference between this and the studies described above might relate to the experimental approach used. siRNA-mediated knockdown of Atg5 might only partially reduce its expression levels (Ahmadi *et al.*, 2003; Zhu *et al.*, 2007), and it has been shown that low levels of Atg5 expression are sufficient to maintain autophagy (Hosokawa *et al.*, 2007). Thus, a better approach may be the use of the dn (K130R) version used in our study (Yang *et al.*, 2010).

The degradation of the autophagosome cargo is executed inside the lysosomal compartment by the activity of a number of hydrolases (Kaminskyy and Zhivotovsky, 2012), which maintain cellular homeostasis by recycling cellular content (Turk and Turk, 2009). Cathepsins B, L, and D are the most abundant lysosomal proteases (Kaminskyy and Zhivotovsky, 2012), and despite their ubiquitous expression, combined deficiency of cathepsins B and L induces neurodegeneration (Felbor *et al.*, 2002). Using an inhibitor of cathepsins B/L/S, we demonstrated the correlation between inhibition of autophagy and lysosomal hydrolase activity and increased cell death induced by paraquat and MPP^+. Inhibition of cathepsins L and D has been previously reported to decrease 6-OHDA toxicity (Lee *et al.*, 2007; Xiang *et al.*, 2011), whereas we observed no effect of cathepsin inhibition in rotenone- or 6-OHDA-induced cell death.

A number of studies have demonstrated the role of caspase-dependent apoptosis in dopaminergic cell death induced by parkinsonian toxins (Kermer *et al.*, 2004; Mattson, 2006; Okouchi *et al.*, 2007). However, with the discovery of autophagy and the establishment of the concept of autophagic cell death, many reports have rushed to suggest that cell death induced by parkinsonian mimetics might involve caspase-independent pathways, particularly autophagic cell death (Chu *et al.*, 2005, 2007; Han *et al.*, 2003; Harbison *et al.*, 2011; Li *et al.*, 2011; Ramachandiran *et al.*, 2007; Rodriguez-Blanco *et al.*, 2012). We observed that inhibition of caspases protects against the toxicity induced by paraquat, MPP^+, rotenone, and 6-OHDA.

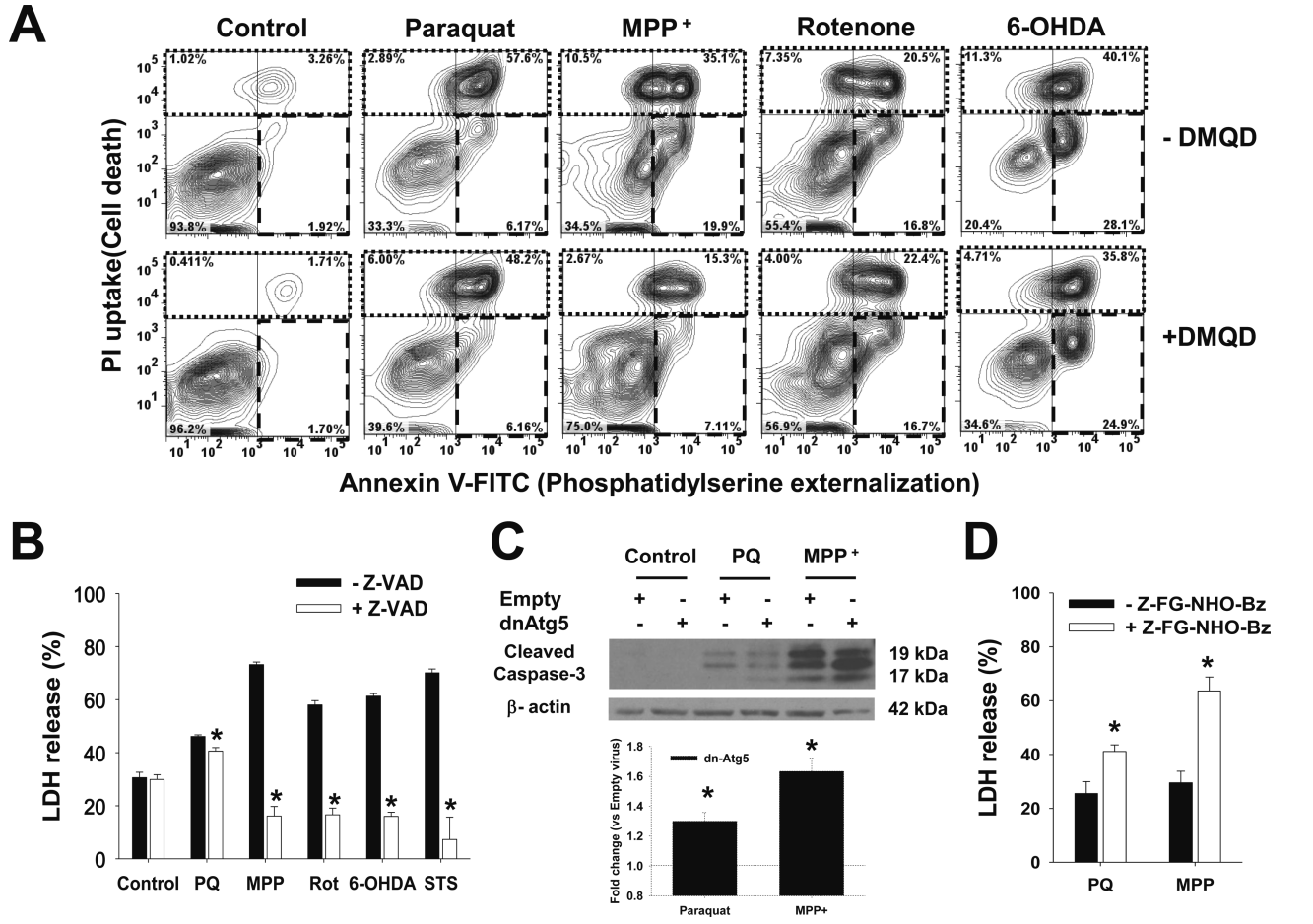

FIG. 6. Caspases mediate cell death induced by the PQ, MPP+, rotenone, and 6-OHDA, whereas lysosomal hydrolase activity only regulates PQ and MPP+ toxicity. Cells were pretreated with the caspase-3 inhibitor Ac-DMQD-CHO (A), the pan-caspase inhibitor Z-VAD-FMK (B), or the cathepsin inhibitor Z-FG-NHO-Bz (D) at 50μM for 1 h. In (C), cells were infected with Ad-Empty or Ad-dnAtg5 at 1.5 MOI for 24 h. Then, cells were treated with PQ (0.5mM), MPP+ (2.5mM), rotenone (Rot, 4μM), 6-OHDA (50μM), or STS (20nM) for 48 h. A, Apoptosis was measured by Annexin V-FITC/PI staining. In contour plots, early apoptosis is identified as an increase in the number of Annexin V (+)/PI (−) cells (lower right quadrant, broken line region). Late-apoptotic and necrotic cells are identified as Annexin V (+)/PI (+) (upper left quadrant, dotted line region). Plots are representative of 3 independent experiments. In (B and D), cell death was determined by measuring released LDH activity, and data represent percentage of released LDH and are means ± SEM of 3 independent experiments. *$p < .05$, significant difference with respect to the corresponding neurotoxin treatments in the absence of caspase or cathepsin inhibitors. C, Representative WB analysis of caspase-3 activation. Inset bar graph represents the densitometric analysis of cleaved caspase-3/β-actin normalized with respect to the corresponding Empty virus + drug treatment values. Abbreviations: 6-OHDA, 6-hydroxydopamine; dnAtg5, dominant negative form of Atg5; LDH, lactate dehydrogenase; MOI, multiplicity of infection; MPP+, 1-methyl-4-phenylpyridinium; PI, propidium iodide; PQ, paraquat; STS, staurosporine; WB, Western immunoblotting.

Interestingly, only caspase-3 was shown to play a role in MPP+ toxicity. We also report that impairment of Atg5-mediated autophagy enhances the cleavage/activity of caspase-3 induced by MPP+. These findings agree with a number of studies that have indeed shown that inhibition of autophagy stimulates caspase-dependent cell death (Boya *et al.*, 2005; Eisenberg-Lerner *et al.*, 2009; Hou *et al.*, 2010; Loos *et al.*, 2011).

An interesting observation is that although Atg5-dependent autophagy and lysosomal hydrolase activity protect against MPP+-induced cell death, the toxicity induced by rotenone was not affected by these mechanisms. Rotenone and MPP+ are complex I inhibitors whose toxic effect were initially largely referred to be mediated by similar mechanisms. Interestingly, recent reports have demonstrated that rotenone and MPP+ actually exert distinct effects on alterations in cellular metabolism and activation of signaling cascades, supporting the idea that their toxicity is mediated by distinct mechanisms (Giordano *et al.*, 2012; Song *et al.*, 2012; Yacoubian *et al.*, 2010).

All together, our results demonstrated that paraquat, MPP+, and rotenone impair autophagic flux and that 6-OHDA is the only parkinsonian toxin capable of increasing autophagic rate. Furthermore, we demonstrated, for the first time, a protective role of Atg5-dependent autophagy in paraquat and MPP+ toxicity, which correlates with the activity of cathepsins. In contrast, neither pharmacological modulation of autophagy nor impairment of Atg5-dependent signaling seem to affect rotenone or 6-OHDA toxicity. We also conclude that findings based only on the pharmacological regulation of autophagy might be misleading based on the reported secondary/nonautophagy-related effects of agents such as rapamycin, and PI3K inhibitors

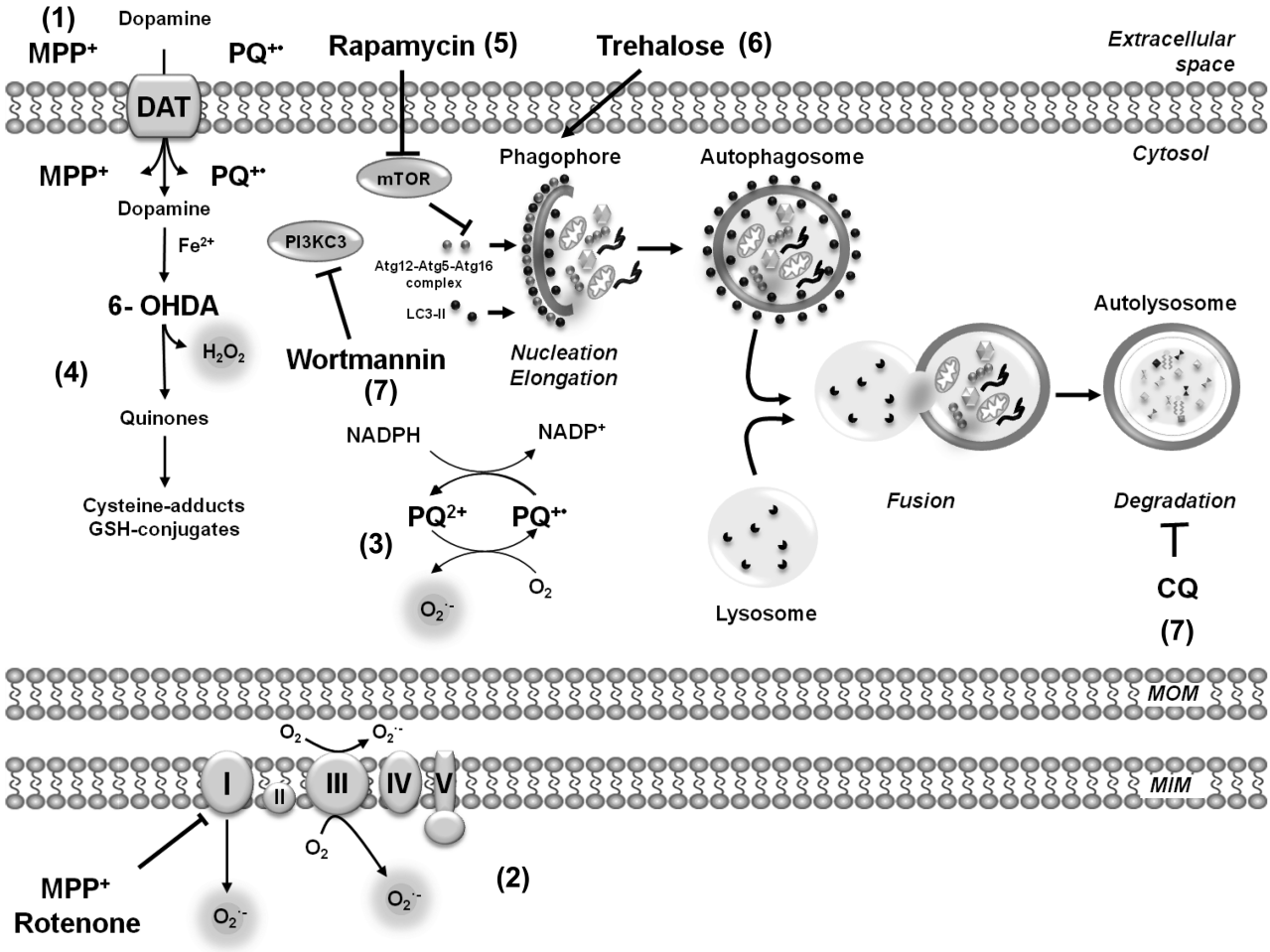

FIG. 7. Putative targets for neurotoxicants and pharmacological modulators of autophagy. The parkinsonian mimetics PQ, the mitochondrial complex I inhibitors MPP^+ and rotenone, and the dopamine analog 6-OHDA are used *in vitro* and *in vivo* as experimental PD models. (1) DAT have been shown to uptake MPP^+ and 6-OHDA, whereas DAT-mediated PQ uptake is still controversial. (2) MPP^+ and rotenone are widely used inhibitors of complex I. However, recent evidence suggests that their toxic effects might be mediated by distinct processes and/or that complex I-independent mechanisms might also be involved. (3) PQ has been proposed to generate superoxide anion ($O_2^{\bullet-}$) formation both in the cytosol and in the mitochondrial matrix. (4) 6-OHDA toxicity has been shown to be mediated by reactive oxygen species (ROS) formation via a nonenzymatic autooxidation process. 6-OHDA autooxidation also generates quinones, which have the capacity to react with thiol-containing molecules (cysteine and glutathione [GSH]). (5) Rapamycin is a well-established stimulator of autophagy via the inhibition of the serine/threonine kinase mTOR, whereas trehalose (6) acts as an mTOR-independent autophagy activator. (7) The formation of autophagosome precursors requires class III PI3Ks and is prevented by PI3K inhibitors such as wortmannin. However, as summarized in Supplementary Table 1, these pharmacological modulators have a number of secondary/nonautophagy-related effects; thus, conclusions based solely on pharmacological regulation of autophagy might be misleading. (8) The lysosomotropic agent, CQ, inhibits the acid-dependent degradation of the autolysosome content without affecting autophagosome-lysosome fusion, which results in the accumulation of autophagolysosomes that cannot be cleared. Abbreviations: 6-OHDA, 6-hydroxydopamine; CQ, chloroquine; DAT, dopamine transporters; MPP^+, 1-methyl-4-phenylpyridinium; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PQ, paraquat.

(Supplementary Table 1). Finally, we observed that cell death induced by parkinsonian mimetics is largely mediated by caspase-dependent apoptosis, discarding the role of autophagic cell death in this process.

SUPPLEMENTARY DATA

Supplementary data (references Chen and Wang [2001], El-Kholy *et al.* [2003], Ethier and Madison [2002], Ferby *et al.* [1996], Foster and Fingar [2010], Gong *et al.* [2012], Halvorsen *et al.* [2002], Hazeki *et al.* [2006], Hsuan *et al.* [2006], Hou *et al.* [2012], King *et al.* [2001], Klionsky *et al.* [2012], Kofman *et al.* [2012], Lan *et al.* [2012], Malagelada *et al.* [2010], Mnich *et al.* [2010], Niso-Santano *et al.* [2006], Nakanishi *et al.* [1992], Renna *et al.* [2010], Sagi *et al.* [2007], Salinas *et al.* [2003], Sun *et al.* [2004], Tsai *et al.* [2012], Wang *et al.* [2010], and Wu *et al.* [2009] are cited in Supplementary Table 1) are available online at http://toxsci.oxfordjournals.org/.

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