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The anti-hypertensive drug reserpine induces neuronal cell death through inhibition of autophagic flux

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ABSTRACT

Reserpine is a well-known medicine for the treatment of hypertension and schizophrenia, but its administration can induce Parkinson's disease (PD)-like symptoms in humans and animals. Reserpine inhibits the vesicular transporter of monoamines and depletes the brain of monoamines such as dopamine. However, the cellular function of reserpine is not fully understood. In this report, we present one possible mechanism by which reserpine may contribute to PD-like symptoms. Reserpine treatment induced the formation of enlarged autophagosomes by inhibiting the autophagic flux and led to accumulation of p62, an autophagy adapter molecule. In particular, reserpine treatment increased the level of α-synuclein protein and led to accumulation of α-synuclein in autophagosomes. Treatment with rapamycin enhanced the effect of reserpine by further increasing the level of α-synuclein and neuronal cell death. *Drosophila* raised on media containing reserpine showed loss of dopaminergic neurons. Furthermore, cotreatment with reserpine and rapamycin aggravated the loss of dopaminergic neurons. Our results suggest that reserpine contributes to the loss of dopaminergic neurons by interfering with autophagic flux.

1. Introduction

Autophagy (specifically, macroautophagy) is an evolutionary conserved catabolic pathway that is responsible for degrading and recycling long-lived proteins and organelles [1]. Autophagy is induced by both extracellular stress conditions (e.g., nutrient starvation, hypoxia, high temperature, and microgravity) and intracellular stress conditions (e.g., damaged organelles) [1,2]. Autophagy is a highly dynamic process. The targeted components are enclosed by the sequestering phagophore (or a sequestering membrane) to form an autophagosome, which fuses with a lysosome to form an autolysosome for degradation via lysosomal hydrolases [3]. Autophagy can be monitored using microscopic and biochemical methods; in particular phosphatidyl ethanolamime (PE)—modified Atg8/LC3 (Atg8-PC/LC3-II) indicates the appearance of autophagosomes, which can be analyzed by fluorescence microscopy or Western blotting [3,4]. The dynamic process of autophagy, such as conversion of autophagosome into autolysosomes, is termed autophagic flux and can be interrupted by lysosomal inhibitors such as Bafilomycin A1, which is therefore a valuable tool for analysis of autophagic flux [5].

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by akinesia, bradykinesia, tremor, rigidity, and postural abnormalities [6]. PD is associated with the selective and progressive loss of dopaminergic neurons and the presence of Lewy body inclusions in neurons of the substantia nigra [7]. α-synuclein is crucial for PD pathogenesis, and inefficient clearance of α-synuclein leads to cellular toxicities [8,9]. Recent studies showed that α-synuclein can be degraded by autophagy as well as by the ubiquitin proteasome system, and α-synuclein protein can be detected in autophagic vesicles [9,10].

Because the molecular mechanism of PD is not clearly understood, PD has been extensively studied using animal and cellular models. Reserpine treatment was one of the earliest animal models for PD research, and the efficacy of L-DOPA, the first-line medicine

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for PD, was verified using reserpine-pretreated mice [11,12]. In addition, chronic administration of reserpine to patients can induce PD-like symptoms such as lethargy, depression, and motor dyskinesia [13,14]. Reserpine is also reported to decrease the level of tyrosine hydroxylase (TH) and the number of TH+ cells in the substantia nigra [15]. Reserpine inhibits the vesicular monoamine transporter (VMAT2), and depletes the brain monoamines such as dopamine by interfering with storage capacity [11]. However, the mechanism by which reserpine induces PD-like symptoms is not fully understood.

In this report we aimed to find autophagy modulating agents and identified reserpine as a negative modulator of autophagy. We found that reserpine treatment inhibited the autophagic flux and increased the level of α-synuclein. We also found that the number of dopaminergic neurons in *Drosophila* was decreased by reserpine treatment. These results suggest that regulation of autophagy might be the additional function of reserpine that induces PD-like symptoms.

2. Materials and methods

2.1. Cell culture and trypan blue assay

HEK293, HCT116, and PC12 cells were grown in Dulbecco's Modified Eagle's medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). A HEK293 stable cell line expressing GFP-LC3 was generated as described previously [16] using a GFP-LC3 plasmid provided by T. Yoshimori [17]. Transfection of HEK293 and PC12 cells was performed using lipofectamine (Invitrogen, Carlsbad, CA, USA). Cell viability was measured using the trypan blue assay. Briefly, cells were seeded in a 24-well plate and then treated with different concentrations of reserpine for the indicated time periods. Viable cells were counted using a Bio-Rad TC10 automated cell counter (Hercules, CA, USA). Reserpin, bafilomycin A1, and rapamycin were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Western blotting

For protein immunoblot analysis, polypeptides in whole cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane filters. Detection was conducted with a 1:2000 or 1:5000 dilution of primary antibody using an enhanced chemiluminescence (ECL) system. Images were acquired using a Chemidoc-it 410 imaging system (UVP, Upland, CA) and LAS4000 system (GE Healthcare, Uppsala, Sweden). The antibody for LC3 was purchased from Novus biological (Littleton, CO, USA), antibodies for AMPK, phospho-AMPK, Erk, and phospho-Erk were from Cell Signaling Technology (Beverly, MA, USA), and the antibody for p62 was from Sigma–Aldrich. The plasmid encoding α-synuclein was purchased from Addgene (Cambridge, MA, USA).

2.3. Immunofluorescence and confocal microscopy

Cells were grown on sterilized glass coverslips. After drug treatment, the cells were fixed with 4% paraformaldehyde. For immunostaining, cells were blocked with 10% goat serum in PBS and stained with a 1:500 dilution of primary antibody in PBS, and then reacted with a 1:1000 dilution of Alexa 488- or Alexa 568-conjugated secondary antibody (Invitrogen). Finally, the slides were washed three times with PBS, stained with DAPI, and mounted in mounting medium (Vector, Burlingame, CA, USA). Images were captured with a Carl Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochem, Germany). GFP-mRFP-LC3 (ptfLC3) constructs were purchased from Addgene.

2.4. Drosophila strains

The tyrosine hydroxylase (TH)-GAL4 fly line was a gift from S. Birman. The UAS-GFP strain was obtained from the Bloomington Stock Center. We crossed these strains to obtain progenies expressing GFP in DA neurons (TH > GFP).

2.5. Quantification of dopaminergic neurons

Male TH > GFP flies were raised on standard fly media containing reserpine and/or rapamycin for 10 days. Fly brains were obtained and fixed with 4% paraformaldehyde as described previously [18]. GFP-positive neurons in dorsolateral region 1 (DL1) clusters from 10 brains of each genotype ($n = 20$) were observed in a blind fashion to eliminate bias using an LSM 700 confocal microscope (Carl Zeiss).

3. Results

3.1. Reserpine induces autophagosome formation

Autophagy is involved in many human diseases such as neurodegenerative diseases and cancers. We screened for novel autophagy-inducing compounds using HEK293 cells stably expressing GFP-LC3 (GFP-LC3 cells). GFP-LC3 cells were treated with the various compounds for 24 h and compounds that induced cytoplasmic punctuates were identified using a fluorescent microscope. This screening revealed that reserpine, a well-known anti-hypertensive and anti-schizophrenia drug, induces autophagosome formation in GFP-LC3 cells. We next treated GFP-LC3 cells with various concentrations of reserpine (0, 1, 2.5, 5, 10, and 20 μM) and examined the cytoplasmic pattern of GFP-LC3 protein. The cytoplasmic punctuates were evident at a concentration of 5 μM reserpine, and treatment with 10 and 20 μM reserpine resulted in enlarged autophagosomes in the cytoplasm (Fig S1A). In particular, we often observed ring-shaped autophagosomes that appeared to include large materials (Fig S1A, 10 μM).

Autophagosomes can be formed either by activation of the autophagy process or by inhibition of autophagic flux. Because we observed enlarged autophagosomes in the cytoplasm, we attempted to determine whether the autophagic process was activated or interrupted. First, we examined the level of the autophagosomal markers LC3-II and p62 by Western blotting. MCF-7 breast cancer cells were treated with various concentrations of reserpine (0, 1, 2.5, 5, 10, and 20 μM) and the expression of LC3 and p62 was examined. The levels of both LC3-II and p62 increased in a dose-dependent manner (Fig S1B). p62 is a useful marker for autophagy and the p62 protein level is generally decreased by autophagy induction, suggesting that autophagic flux was interrupted by reserpine. We examined the level of LC3-II in a time-dependent manner, and found that the level of LC3-II increased after 24 h of reserpine treatment and was maintained at 48 h and 72 h (Fig S1C).

3.2. Reserpine inhibits autophagic flux

Because reserpine increased the level of p62, we hypothesized that reserpine inhibits the autophagic flux. To test our hypothesis, we used a mRFP-GFP-LC3 reporter construct (tfLC3) for autophagic flux whereby autophagosomes appear yellow and autolysosomes appear red [19]. HEK293 cells were transfected with plasmid encoding mRFP-GFP-LC3 and then incubated with reserpine (0, 1, 2.5, 5, 10, and 20 μM). Consistent with previous results, reserpine induced the formation of enlarged vesicles that appeared yellow, indicative of autophagosomes (Fig 1A). In contrast, starvation induced vesicles that were small and red, indicating the formation

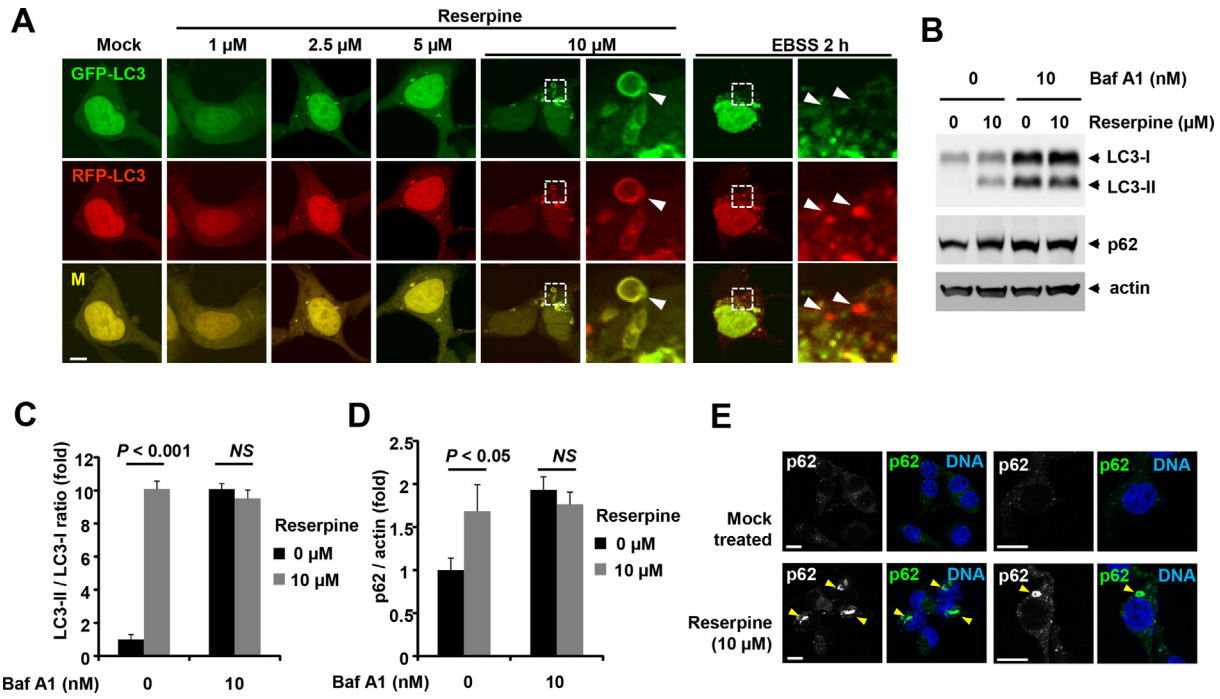

Fig. 1. Reserpine interferes with autophagic flux. A. Reserpine-treated cells did not form autolysosomes. HEK293 cells were transfected with plasmid encoding mRFP-GFP-LC3, and the transfected cells were incubated with the indicated concentration of reserpine for 24 h. Cells were fixed and analyzed with confocal microscopy. As a positive control, the transfected cells were incubated with EBSS for 2 h. B. Reserpine treatment inhibited the autophagic flux. MCF-7 cells were treated with either mock or reserpine in the presence or absence of Bafilomycin A1. C, D. The levels of LC3-II/LC3-I and p62 were analyzed. Experiments were performed in triplicate, and the mean and standard deviations are shown in the graph. E Reserpine induced formation of enlarged autophagosomes in PC12 cells. PC12 cells were treated with 10 μM reserpine for 24 h and the cells were immunostained with anti-p62 antibody. Bars, 10 μM.

of autolysosomes (Fig 1A). These results suggest that reserpine inhibits autophagic flux by interfering with autolysosome formation.

Next, we examined autophagic flux using bafilomycin A1, a lysosomal inhibitor. Reserpine treatment (10 μM) significantly increased the level of LC3-II; however, co-treatment with bafilomycin A1 eliminated the difference in LC3 levels between the control and reserpine treatment (Fig 1B and C). In addition, we examined the level of p62 and found no significant difference with bafilomycin A1 treatment. These results confirmed that reserpine blocks autophagic flux (Fig 1D).

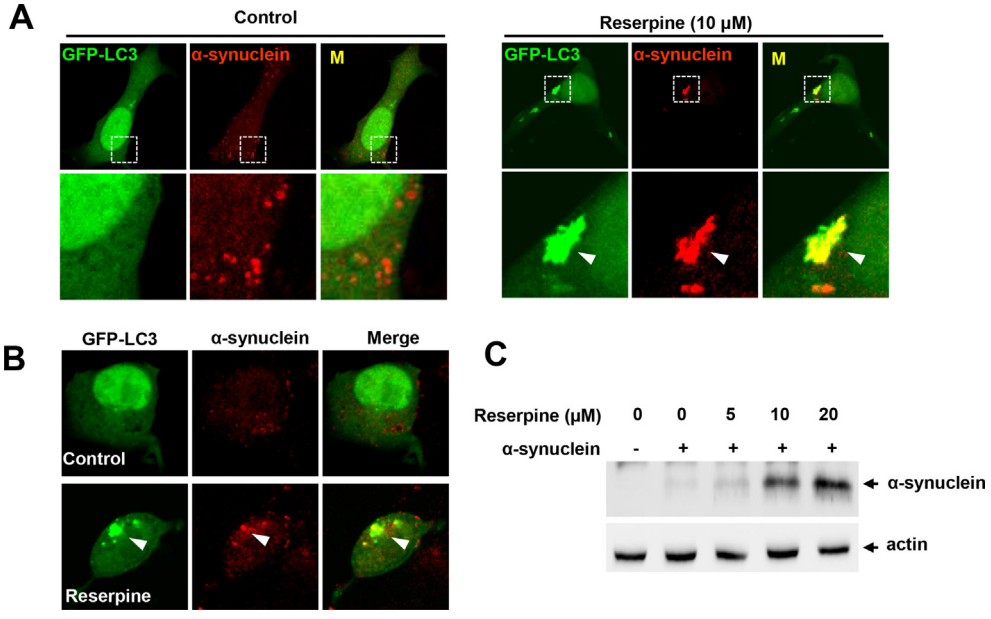

Fig. 2. Reserpine treatment interferes with the degradation of α-synuclein. A. α-Synuclein is colocalized with GFP-LC3 following reserpine treatment. HEK293 cells were transfected with GFP-LC3 plasmid and plasmid encoding His-tagged α-synuclein (His–α-synuclein). Cells were immunostained with anti-His antibody (red). B. α-Synuclein is colocalized with GFP-LC3 following reserpine treatment of PC12 cells. C. The level of α-synuclein protein is increased by reserpine treatment. HEK293 cells were transfected with His–α-synuclein and treated with the indicated concentration of reserpine for 24 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Reserpine induces autophagosome formation in neuronal cells

Because reserpine treatment induces symptoms similar to those of Parkinson's disease, we examined whether reserpine affects neuronal cells. Rat pheochromocytoma PC12 cells were incubated with reserpine and autophagic makers were analyzed by Western blotting. As shown for MCF-7 cells, LC3-II and p62 levels in PC12 cells were increased by reserpine treatment (Fig S2). Because reserpine treatment induces the formation of enlarged autophagosomes in HEK293 cells, we examined whether reserpine has the same effect in neuronal cells. PC12 cells were treated with 10 μM reserpine and the cells were stained with anti-p62 antibody. Whereas control cells showed small p62 spots in the cytoplasm, reserpine treatment induced the formation of enlarged p62-stained vesicles, indicating that reserpine also induces enlarged autophagosomes in neuronal cells (Fig 1E).

3.4. α-synuclein is colocalized with the autophagosome after reserpine treatment

As reserpine is reported to induce Parkinson-like symptoms and α-synuclein is known to be an important player in Parkinson's disease [9,14], we studied whether the localization and expression of α-synuclein is affected by reserpine treatment. First, we examined the colocalization of α-synuclein with autophagosomes. Cells were co-transfected with GFP-LC3 and α-synuclein plasmids, and then treated with reserpine. Whereas α-synuclein was not colocalized with GFP-LC3 in control cells, α-synuclein was aggregated and colocalized with GFP-LC3 spots after reserpine treatment (Fig 2A). We repeated this experiment with PC12 cells and obtained identical results (Fig 2B). Next, we examined the level of α-synuclein protein after reserpine treatment. Cells were transfected with α-synuclein plasmid and then treated with various concentrations of reserpine (0, 5, 10, and 20 μM). Although α-synuclein was hard to detect without reserpine treatment, expression of α-synuclein protein was induced by reserpine in a dose-dependent manner (Fig 2C).

3.5. Reserpine blocks rapamycin-induced autophagy

We examined the effect of co-treatment with rapamycin and reserpine on autophagy. Rapamycin is a well-known inducer of autophagy through inhibition of mTOR. GFP-LC3 cells were treated with reserpine (0, 5, 10, and 20 μM) in the presence or absence of rapamycin. Reserpine and rapamycin cotreatment induced the

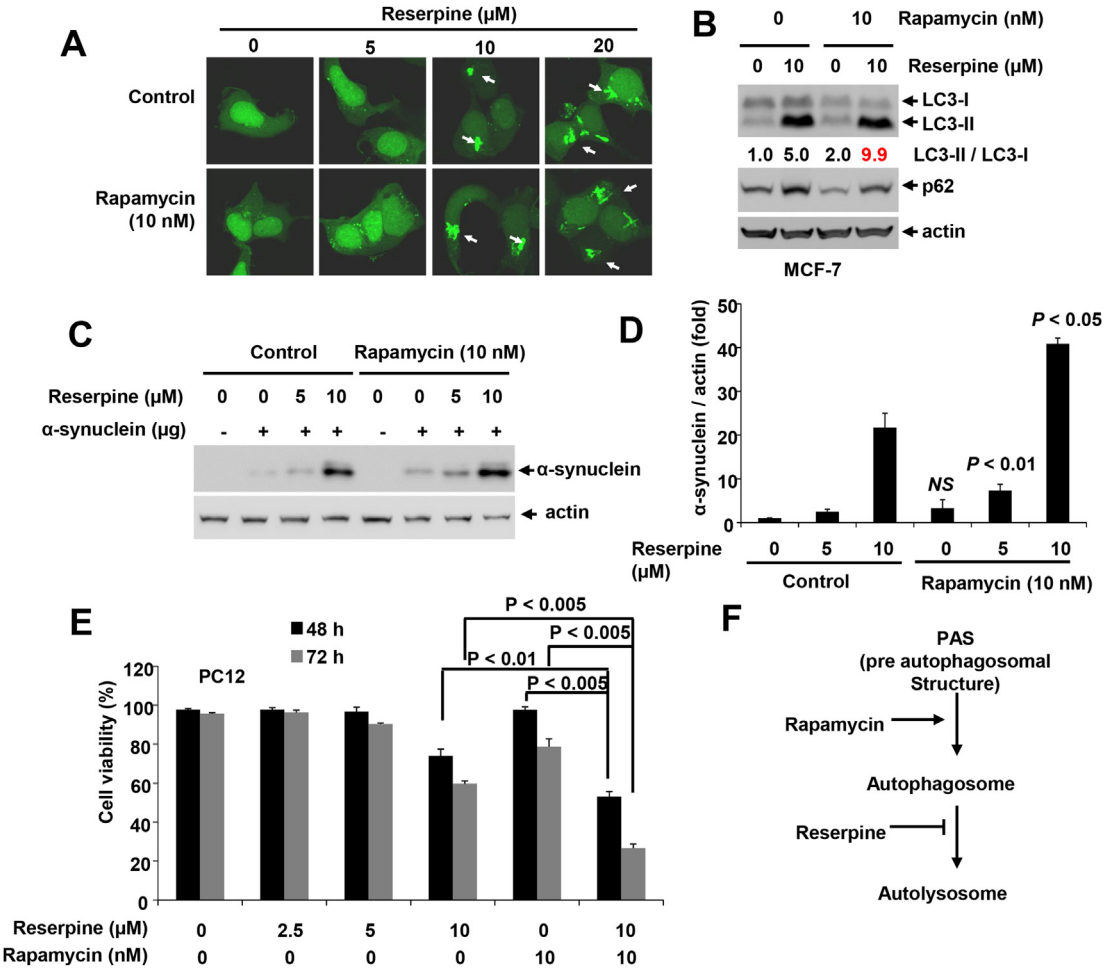

Fig. 3. Reserpine treatment blocked rapamycin-induced autophagic flux. A. Autophagosome formation following cotreatment with reserpine and rapamycin. GFP-LC3 cells were incubated with the indicated concentration of reserpine in the presence or absence of rapamycin (10 nM). B. Treatment with reserpine and rapamycin synergistically increased the ratio of LC3-II to LC3-I. PC12 cells were incubated with the combination of reserpine and rapamycin for 24 h and the cell lysates were subject to Western blotting with the indicated antibodies. C. Cotreatment with rapamycin and reserpine increases the level of α-synuclein. HEK293 cells were transfected with HA–tagged α-synuclein (HA–α-synuclein). Cells were treated with the indicated concentration of reserpine in the presence or absence of rapamycin. D. Rapamycin increased the level of α-synuclein. Experiments were performed in triplicate, and the mean and standard deviations are shown in the graph. E. Treatment with reserpine and rapamycin induces neuronal cell death. PC12 cells were treated with the indicated concentration of reserpine in the presence or absence of rapamycin. Cell viability was measured using the trypan blue assay. Experiments were performed in triplicate, and the mean and standard deviations are shown in the graph. F. A simplified representation of autophagy regulation by reserpine and rapamycin.

formation of enlarged autophagosomes; however, the size of the autophagosomes was not obviously different between reserpine alone and cotreatment (Fig 3A). Next, we examined the level of LC3 and p62 after treatment with reserpine and rapamycin. Rapamycin treatment slightly increased the relative ratio of LC3-II to LC3-I (LC3-II/LC3-I), but incubation with both reserpine and rapamycin increased LC3-II/LC-I to a greater extent (Fig 3B). In addition, reserpine and rapamycin cotreatment increased the level of p62 in PC12 cells, whereas rapamycin alone decreased the level of p62 (Fig 3B). These results suggest that reserpine blocks rapamycin-induced autophagic flux.

3.6. Rapamycin enhances the effect of reserpine

As rapamycin accelerates cellular autophagy and reserpine inhibits the autophagic flux, we assumed that cotreatment with rapamycin and reserpine would exacerbate α-synuclein accumulation and cell death (Fig 3F). HEK293 cells were transfected with plasmid encoding α-synuclein and then treated with reserpine in the presence or absence of rapamycin. As expected, the accumulation of α-synuclein was significantly enhanced by treatment with rapamycin (Fig 3C and D). Next, we examined cell viability after treatment with reserpine and rapamycin. PC12 cells were treated with reserpine in the presence or absence of rapamycin for 48 h and 72 h. Cell viability was decreased by reserpine treatment, and cell death was further increased by cotreatment with rapamycin (Fig 3E).

3.7. Reserpine induces the loss of dopaminergic neurons in Drosophila

As reserpine inhibited the autophagic flux, we examined whether reserpine induces autophagy-related cell death. Cell viability can be affected by autophagy, and both excessive autophagy and autophagic depletion negatively affect cell viability [20,21]. The loss of dopaminergic neurons is one of the major characteristics of early-onset autosomal Parkinsonism, and is also observed in the brains of mutant *Drosophila* [18,22]. We used a transgenic *Drosophila* strain that expresses GFP in dopaminergic neurons, and the flies were raised on medium containing reserpine for 10 days. Flies raised on reserpine-containing medium exhibited a significant decrease in the number of dopaminergic neurons (Fig 4A and B). However, there was no significant difference in dopaminergic neuronal loss between the flies raised on medium containing 1 mM or 5 mM reserpine. These results indicate that reserpine contributes to the loss of dopaminergic neurons. Next, we examined whether cotreatment with reserpine and rapamycin has a synergistic effect on the loss of dopaminergic neurons. Flies raised on medium containing rapamycin, reserpine, or both were analyzed for the number of dopaminergic neurons. Although rapamycin alone did not have any effect on the number of dopaminergic neurons, co-treatment with reserpine and rapamycin significantly decreased the number of dopaminergic neurons (Fig 4C and D).

4. Discussion

Reserpine is an anti-hypertensive drug and anti-psychotic drug [13]. However, administration of reserpine often causes Parkinson-like symptoms in humans and animals by depleting brain monoamines such as dopamine [14,15]. Therefore, reserpine treatment has been used to develop an animal model for PD. α-Synuclein protein plays a crucial role in PD pathogenesis, and recent reports showed that α-synuclein is degraded by the autophagic pathway [9,10]. Thus, impairment of autophagy can led to accumulation of α-synuclein protein, followed by PD or PD-like symptoms. In this

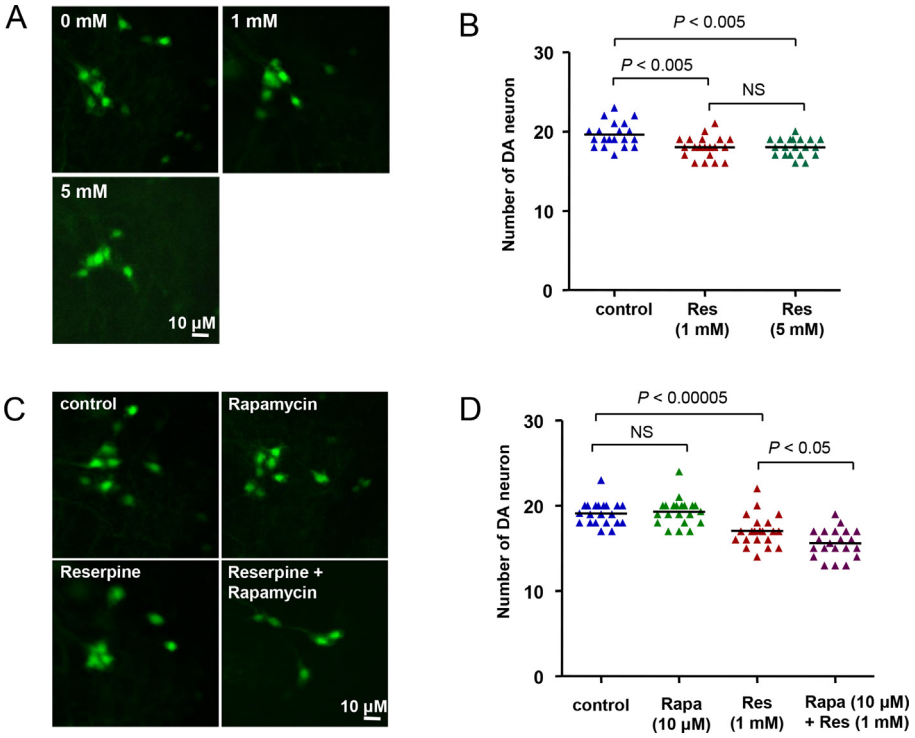

Fig. 4. Reserpine treatment results in the loss of dopaminergic neurons in *Drosophila*. A. Images of dopaminergic neurons from the flies. Twenty flies in each group were raised for 10 days in medium containing the indicated concentration of reserpine. B. Graph showing the number of dopaminergic neurons. The number of GFP-positive dopaminergic neurons was counted and *P* values are shown. C. Synergistic loss of dopaminergic neurons following treatment with reserpine and rapamycin. Twenty flies in each group were raised for 10 days in medium containing rapamycin, reserpine, or both. D. Graph showing the number of dopaminergic neurons in the flies.

report, we demonstrated that reserpine treatment inhibits the autophagic flux and leads to accumulation of α-synuclein protein. Therefore, inhibition of autophagy by reserpine might be a novel cellular mechanism contributing to PD or PD-like symptoms.

More recently, MPTP (1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine) has been preferred for development of an animal model of PD because of its selective effect on the dopaminergic neurons [13]. Upregulation of α-synuclein in the substantia nigra has been reported as a possible mechanism of MPTP neurotoxicity [23]. Although it is well known that reserpine inhibits VMAT2, the effect of reserpine on α-synuclein protein has not previously been studied. Here, we showed that reserpine treatment increases the level of α-synuclein. We also studied cell viability using a PC12 cell model and a *Drosophila* model. Reserpine decreased the viability of PC12 cells and also reduced the number of dopaminergic neurons in *Drosophila*. These results implicate elevation of α-synuclein and induction of neuronal cell death by reserpine as a possible mechanism underlying the PD or PD-like symptoms.

Blocking of autophagic flux can induce extensive accumulation of autophagosomes, which can contribute to cell death. As reserpine induces autophagic flux, we examined the effect of rapamycin, an autophagy activator, on the induction of autophagosomes by reserpine. Observation of GFP-LC3 protein revealed that the size and number of autophagosomes was not reduced by rapamycin treatment (10 nM), suggesting that rapamycin cannot overcome reserpine-mediated inhibition of autophagic flux. Moreover, the ratio of LC-II/LC-I was increased by the cotreatment, indicating further accumulation of autophagosomes. Finally, cell viability was reduced by cotreatment with reserpine and rapamycin. In this report, we focused on the cell viability of neuronal cells, although a similar scheme could be applied to treat cancer cells.

A previous study showed that reserpine decreased the number of dopaminergic neurons in rodents [15]. In this report, we showed that the number of dopaminergic neurons in *Drosophila* was significantly reduced by reserpine treatment. These results indicate that reserpine treatment has a proapoptotic effect and can decrease the number of dopaminergic neurons. In addition, reserpine was identified in a screen for sleep modulators of *Drosophila*, and sleep disorders are reported to be one symptom of PD [24]. Therefore, the *Drosophila* model with reserpine will be a useful model system to study PD. Finally, we demonstrated that reserpine blocked autophagic flux and increased the level of α-synuclein. Recent reports showed that an elevated level of α-synuclein contributes to neuronal cell death. The consequences of reserpine treatment are quite similar to PD pathogenesis in humans [8]. Therefore, our results suggest that the cellular function of reserpine is biologically relevant to the development of animal models of PD.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.145.

Transparency document

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References

[1] B. Levine, D.J. Klionsky, Development by self-digestion: molecular mechanisms and biological functions of autophagy, Dev. Cell. 6 (2004) 463–477.
[2] H.W. Ryu, S.H. Choi, S. Namkoong, I.S. Jang, D.H. Seo, I. Choi, H.S. Kim, J. Park, Simulated microgravity contributes to autophagy induction by regulating AMP-activated protein kinase, DNA Cell. Biol. 33 (2014) 128–135.
[3] D.J. Klionsky, F.C. Abdalla, H. Abeliovich, R.T. Abraham, A. Acevedo-Arozena, K. Adeli, L. Agholme, M. Agnello, P. Agostinis, J.A. Aguirre-Ghiso, H.J. Ahn, O. Ait-Mohamed, S. Ait-Si-Ali, T. Akematsu, S. Akira, H.M. Al-Younes, M.A. Al-Zeer, M.L. Albert, R.L. Albin, J. Alegre-Abarrategui, M.F. Aleo, M. Alirezaei, A. Almasan, M. Almonte-Becerril, A. Amano, R. Amaravadi, S. Amarnath, A.O. Amer, N. Andrieu-Abadie, V. Anantharam, D.K. Ann, S. Anoopkumar-Dukie, H. Aoki, N. Apostolova, G. Arancia, J.P. Aris, K. Asanuma, N.Y. Asare, H. Ashida, V. Askanas, D.S. Askew, P. Auberger, M. Baba, S.K. Backues, E.H. Baehrecke, B.A. Bahr, X.Y. Bai, Y. Bailly, R. Baiocchi, G. Baldini, W. Balduini, A. Ballabio, B.A. Bamber, E.T. Bampton, G. Banhegyi, C.R. Bartholomew, D.C. Bassham, R.C. Bast Jr., H. Batoko, B.H. Bay, I. Beau, D.M. Bechet, T.J. Begley, C. Behl, C. Behrends, S. Bekri, B. Bellaire, L.J. Bendall, L. Benetti, L. Berliocchi, H. Bernardi, F. Bernassola, S. Besteiro, I. Bhatia-Kissova, X. Bi, M. Biard-Piechaczyk, J.S. Blum, L.H. Boise, P. Bonaldo, D.L. Boone, B.C. Bornhauser, K.R. Bortoluci, I. Bossis, F. Bost, J.P. Bourquin, P. Boya, M. Boyer-Guittaut, P.V. Bozhkov, N.R. Brady, C. Brancolini, A. Brech, J.E. Brenman, A. Brennand, E.H. Bresnick, P. Brest, D. Bridges, M.L. Bristol, P.S. Brookes, E.J. Brown, J.H. Brumell, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (2012) 445–544.
[4] H.W. Ryu, W.K. Oh, I.S. Jang, J. Park, Amurensin G induces autophagy and attenuates cellular toxicities in a rotenone model of Parkinson's disease, Biochem. Biophys. Res. Commun. 433 (2013) 121–126.
[5] N. Mizushima, T. Yoshimori, B. Levine, Methods in mammalian autophagy research, Cell 140 (2010) 313–326.
[6] T. Klockgether, Parkinson's disease: clinical aspects, Cell. Tissue Res. 318 (2004) 115–120.
[7] M. Goedert, M.G. Spillantini, K. Del Tredici, H. Braak, 100 years of Lewy pathology, Nat. Rev. Neurol. 9 (2012) 13–24.
[8] M. Bourdenx, E. Bezard, B. Dehay, Lysosomes and alpha-synuclein form a dangerous duet leading to neuronal cell death, Front. Neuroanat. 8 (2014) 83.
[9] T. Vogiatzi, M. Xilouri, K. Vekrellis, L. Stefanis, Wild type alpha-synuclein is degraded by chaperone-mediated autophagy and macroautophagy in neuronal cells, J. Biol. Chem. 283 (2008) 23542–23556.
[10] J.L. Webb, B. Ravikumar, J. Atkins, J.N. Skepper, D.C. Rubinsztein, Alpha-Synuclein is degraded by both autophagy and the proteasome, J. Biol. Chem. 278 (2003) 25009–25013.
[11] S. Duty, P. Jenner, Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease, Br. J. Pharmacol. 164 (2011) 1357–1391.
[12] A. Carlsson, M. Lindqvist, T. Magnusson, 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists, Nature 180 (1957) 1200.
[13] A.H. Leao, A.J. Sarmento-Silva, J.R. Santos, A.M. Ribeiro, R.H. Silva, Molecular, neurochemical and behavioral hallmarks of reserpine as a model for parkinson's disease: new perspectives to a long-standing model, Brain Pathol. (2015), http://dx.doi.org/10.1111/bpa.12253.
[14] V.S. Fernandes, J.R. Santos, A.H. Leao, A.M. Medeiros, T.G. Melo, G.S. Izidio, A. Cabral, R.A. Ribeiro, V.C. Abilio, A.M. Ribeiro, R.H. Silva, Repeated treatment with a low dose of reserpine as a progressive model of Parkinson's disease, Behav. Brain Res. 231 (2012) 154–163.
[15] J.R. Santos, J.A. Cunha, A.L. Dierschnabel, C.L. Campelo, A.H. Leao, A.F. Silva, R.C. Engelberth, G.S. Izidio, J.S. Cavalcante, V.C. Abilio, A.M. Ribeiro, R.H. Silva, Cognitive, motor and tyrosine hydroxylase temporal impairment in a model of parkinsonism induced by reserpine, Behav. Brain Res. 253 (2013) 68–77.
[16] J.H. Yoon, S. Her, M. Kim, I.S. Jang, J. Park, The expression of damage-regulated autophagy modulator 2 (DRAM2) contributes to autophagy induction, Mol. Biol. Rep. 39 (2011) 1087–1093.
[17] Y. Kabeya, N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, E. Kominami, Y. Ohsumi, T. Yoshimori, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing, EMBO J. 19 (2000) 5720–5728.
[18] H. Koh, H. Kim, M.J. Kim, J. Park, H.J. Lee, J. Chung, Silent information regulator 2 (Sir2) and Forkhead box O (FOXO) complement mitochondrial dysfunction and dopaminergic neuron loss in Drosophila PTEN-induced kinase 1 (PINK1) null mutant, J. Biol. Chem. 287 (2012) 12750–12758.
[19] S. Kimura, T. Noda, T. Yoshimori, Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3, Autophagy 3 (2007) 452–460.
[20] Y.H. Yoon, K.S. Cho, J.J. Hwang, S.J. Lee, J.A. Choi, J.Y. Koh, Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells, Invest Ophthalmol. Vis. Sci. 51 (2010) 6030–6037.

[21] R.C. Scott, G. Juhasz, T.P. Neufeld, Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death, Curr. Biol. 17 (2007) 1–11.
[22] J. Park, S.B. Lee, S. Lee, Y. Kim, S. Song, S. Kim, E. Bae, J. Kim, M. Shong, J.M. Kim, J. Chung, Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin, Nature 441 (2006) 1157–1161.
[23] M. Vila, S. Vukosavic, V. Jackson-Lewis, M. Neystat, M. Jakowec, S. Przedborski, Alpha-synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP, J. Neurochem. 74 (2000) 721–729.
[24] A.H. Nall, A. Sehgal, Small-molecule screen in adult Drosophila identifies VMAT as a regulator of sleep, J. Neurosci. 33 (2013) 8534–8540.