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Triggering of inflammasome by impaired autophagy in response to acute experimental Parkinson's disease: involvement of the PI3K/Akt/mTOR pathway

Sabrina Giacoppo, Placido Bramanti and Emanuela Mazzon

Several lines of evidence suggest that the inflammasome activation is involved in the progression of neurodegenerative diseases. However, the relation between Parkinson's disease (PD) and the inflammasome is still unclear. This study was designed to assess the involvement of inflammasome in acute experimental PD. Specifically, acute PD was induced in C57BL/6 mice by an injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). At seven days from MPTP induction, mice were euthanized and the midbrains were sampled to carry out immunohistochemical evaluations and western blot analysis. Our results show the activation of Nod-like receptor-3 inflammasome in acute MPTP mice, as suggested by the increase of nuclear factor-κB expression, which represents the first signal for inflammasome induction. The Nod-like receptor-3 assembly induces the activation of caspase-1, which in turn activates interleukin-1β and interleukin-18 production, as confirmed by our evaluations. A dysregulation of autophagy system was also found in acute MPTP mice by looking at the expression of Beclin-1, LC-3, and Bcl-2, chosen as markers of autophagy. Thus, in an effort to identify the molecular mechanism underlying the well-known crosstalk between autophagy and the inflammasome, we evaluated the involvement of the phosphoinositide-3 kinase/protein kinase-B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, which plays a key role in autophagy. Our results showed a clear upregulation of this signaling after MPTP induction. Taken together, our findings suggest that the triggering of inflammasome could be linked to impaired autophagy because of aberrant upstream activation of the PI3K/Akt/mTOR pathway. Finally, our results propose the inflammasome as a new potential therapeutic target in the management of PD. *NeuroReport* **28:996–1007**

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IRCCS Centro Neurolesi 'Bonino-Pulejo', Messina, Italy

Correspondence to Emanuela Mazzon, PhD, IRCCS Centro Neurolesi 'Bonino-Pulejo', Via Provinciale Palermo, Contrada Casazza, 98124 Messina, Italy
Tel: +39 090 601 28708; fax: +39 090 601 28850;
e-mail: emazzon.irccs@gmail.com

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder with a prevalence increasing from 1% at age 65 years to about 4–5% at age 85 years worldwide [1]. In Italy, about 230 000 individuals with PD have been estimated. PD is typically characterized by the progressive loss of muscle control, compromised balance, bradykinesia, tremors, postural instability, and decline in striatal dopamine levels of the central nervous system, because of the loss of dopaminergic neurons in the substantia nigra pars compacta and the presence of Lewy bodies, consisting of α-synuclein (α-Syn) aggregates [1]. Abnormal protein accumulation is a crucial event of PD, associated with the mitochondrial dysfunction and oxidative stress, and finally with the progressive neuronal cell death. The formation of protein aggregates in PD brains is commonly ascribed to deregulation of the constitutive autophagy system [2]. Autophagy is an intracellular catabolic mechanism responsible for most of the degradation and recycling of cytoplasmic components and intracellular dysfunctional or damaged organelles and their subsequent lysosomal enzyme degradation [3]. Although some early studies recognized the autophagy as a mechanism of cell death, in recent years, it is emerging as a hypothesis as a useful mechanism for defending cells against the toxic effects of protein aggregates and protecting neurons from apoptosis [3]. In addition, autophagy is also implicated in the fine regulation of inflammatory responses [4]. It was found indeed that inhibition of autophagic capacity caused by aging stimulates the activation of inflammasomes. Among the inflammasomes, the best characterized is Nod-like receptor-3 (NLRP3), which represents an essential platform coordinating the inflammatory response [4]. The NLRP3 inflammasome is highly expressed in microglia and is fundamental to the process of neuroinflammation. Increasing evidences strongly suggest an involvement of the NLRP3 inflammasome in the initiation or the progression of neurodegenerative diseases [5]. The inflamamsome can also be activated by an

aberrant accumulation of misfolded proteins. Specifically, NLRP3 activates inflammatory caspases, mostly caspase-1, which cleaves the inactive precursors of interleukin-1β (IL-1β) and IL-18 and stimulates their secretion [4]. Subsequently, these cytokines may amplify inflammatory responses and accelerate the aging process. Although it has been shown that alteration of autophagic flux is involved in PD [6], little is known about the exact role of the inflammasome in its pathophysiology. In this respect, dopamine was found to inhibit NLRP3 activation in both primary microglia and astrocytes through a dopamine D1 receptor–cyclic AMP signaling pathway [7]. Here, we explored the involvement of the phosphatidylinositol-3 kinase (PI3K)/Akt (protein kinase-B)/mammalian target of the rapamycin (mTOR) pathway (PI3K/Akt/mTOR) pathway, which plays a central role in a wide range of fundamental physiological functions, including cell growth, differentiation, survival, protein synthesis, and autophagy [8]. Several lines of evidence suggest that dysregulation in the PI3K/Akt/mTOR pathway is associated with loss of dopaminergic neurons in PD [9]. Recently, gene profiling on dopaminergic neurons from postmortem PD patients showed both upregulation and downregulation of PI3K/Akt/mTOR signaling [10]. The same study also showed an upregulation of genes of the PI3K (classes I–III)/Akt (Akt1, Akt2, and Akt3)/mTOR (mTORC1 and mTORC2) pathway in both non-differentiated and differentiated SH-SY5Y dopaminergic cells treated with 1-methyl-4-phenylpyridinium [10]. In addition, in an animal model of PD, it was shown that dysregulation of the PI3K/Akt/mTOR is linked to autophagy disruption, although the results are controversial [11]. Therefore, we investigated the involvement of inflammasome in an experimental acute model of PD. In addition, we evaluated whether the PI3K/Akt/mTOR pathway could play a role in the underlying relationship between autophagy and the inflammasome.

Materials and methods

Animals

Male C57BL/6 mice (4–5 weeks old, 20–25 g weight) were used for this study. Mice were purchased from Harlan, Milan, Italy and housed in individually ventilated cages and maintained under 12 h light/dark cycles, at 21 ± 1°C and 50–55% humidity, with food and water *ad libitum*.

Ethics statement

All animal care and use was performed in agreement with the European Organization Guidelines for Animal Welfare. The protocol was approved by the Ministry of Health 'General Direction of animal health and veterinary drug' (Authorization 656/2015-PR – D.lgs 26/2014). All efforts were made during experimental procedures to minimize animal suffering and also to reduce the number of animal used.

MPTP-induced acute PD

The MPTP-induced neurotoxicity is the most common experimental PD model used to reproduce the main features of human PD. Acute PD was induced by an intraperitoneal injection of MPTP (Sigma-Aldrich, Milan, Italy), a neurotoxin that causes a selective destruction of dopaminergic neurons of the substantia nigra. In our experimental design, two injections of MPTP (40 mg/kg) were administered 24 h apart to produce acute syndrome.

Experimental design

C57BL/6 mice ($N = 30$) were distributed randomly into the following groups: sham mice ($N = 10$) that received intraperitoneal injections of the MPTP vehicle (saline solution) and acute MPTP mice ($N = 20$) that received two injections of MPTP (40 mg/kg, intraperitoneally dissolved in saline solution) 24 h apart. At the end of the experiment, which occurred at the seventh day from MPTP induction, all animals were euthanized with intraperitoneal injections of Tanax (5 mg/kg body weight). The midbrains were dissected for later analysis. The entire in-vivo experimental design was repeated twice and all results achieved with different techniques were calculated from three independent experiments.

Pole test and body weight variations

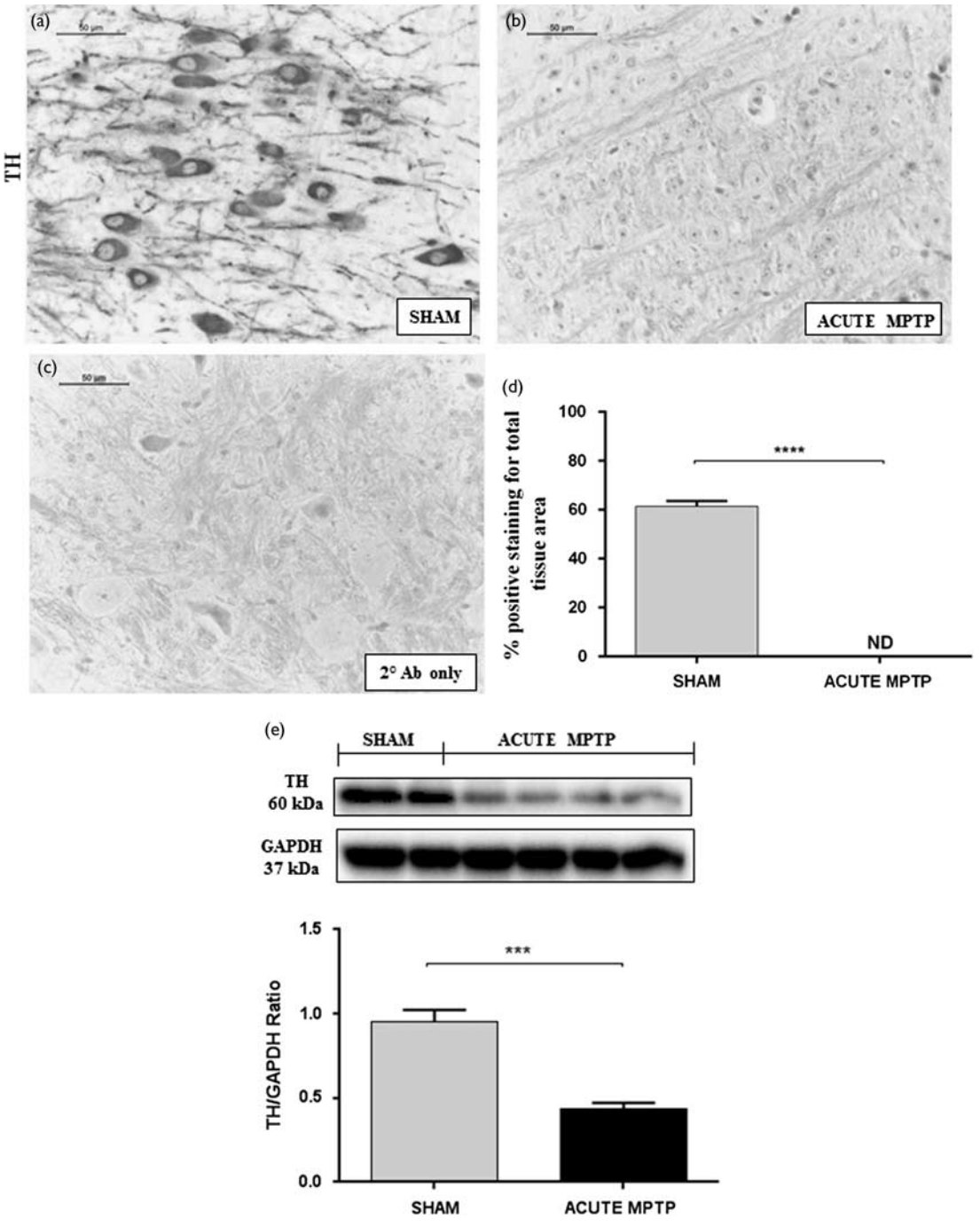
The pole test is commonly used in mice to assess basal ganglia-related movement disorders and in particular bradykinesia during PD. The apparatus consisted of a 55 cm high pole, 5 mm in diameter, wrapped in gauze to prevent slipping and with the base placed in the home cage. The mice were positioned head downward directly (by sliding the fore paws on the pole top while holding the animal by the tail). When placed on the pole, animals orient themselves downward and descend the length of the pole back into the cage. During the behavioral test, animals were timed. The time needed to mice to descend the lenght of the pole until reaching the floor was recorded. Each trial had a cutoff limit of 30 s. The measurements of body weight were taken from the first day of PD-induction (day 0) until the day of sacrifice (day 7) and any loss was annotated as a marker of pathology. The graph shows the daily variation in body weight for each group that has been compared with the day 0 and the values obtained have been expressed as mean ± SEM of all animals for each experimental group.

Immunohistochemistry

The midbrain tissues of all mice were fixed in 10% (w/v) PBS-buffered formaldehyde and 6-μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. Nonspecific adsorption was minimized by incubating sections in 2% (v/v) normal goat serum in PBS for 20 min. Sections were incubated overnight with the following antibodies: anti TH (1 : 500, catalog no. #AB152;

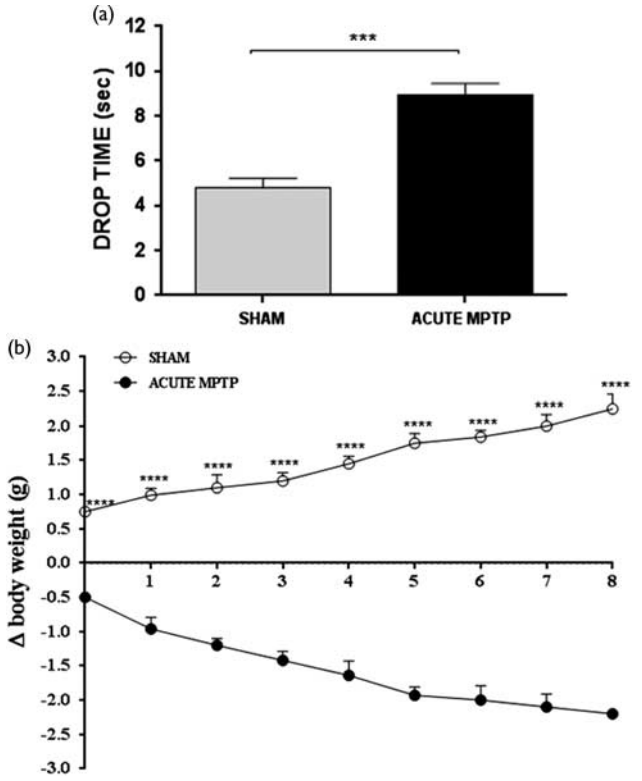
Millipore, Vimodrone, Milan, Italy); anti-LC-3 (1 : 100 in PBS v/v, catalog no. #12741; Cell Signaling Technology, Leiden, The Netherlands); anti-Bax antibody (1 : 100 in PBS v/v, catalog no. #2772; Cell Signaling Technology); anti-NRLP3 (1 : 100 in PBS v/v, catalog no. MAB7578; R&D Systems, Minneapolis, Minnesota, USA); anti-Caspase-1 (1 : 100 in PBS v/v, catalog no. ab108362; Abcam, Cambridge, UK); and anti-IL-18 (1 : 100 in PBS v/v, catalog no. ab191152; Abcam, Cambridge, UK). Endogenous biotin-binding or avidin-binding sites were

Fig. 1



Immunoistochemical analysis for tyrosine hydroxylase (TH). Sham mice showed a positive staining for TH (a). In contrast, a negative staining was observed in acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice (b). Only the secondary antibody in the midbrain section of MPTP mice (c). Densitometric analysis for TH (d). Sham versus acute MPTP, $^{****}P < 0.0001$. Western blot analysis for TH (e). Sham versus acute MPTP, $^{***}P = 0.0002$. ND, no data.

Fig. 2



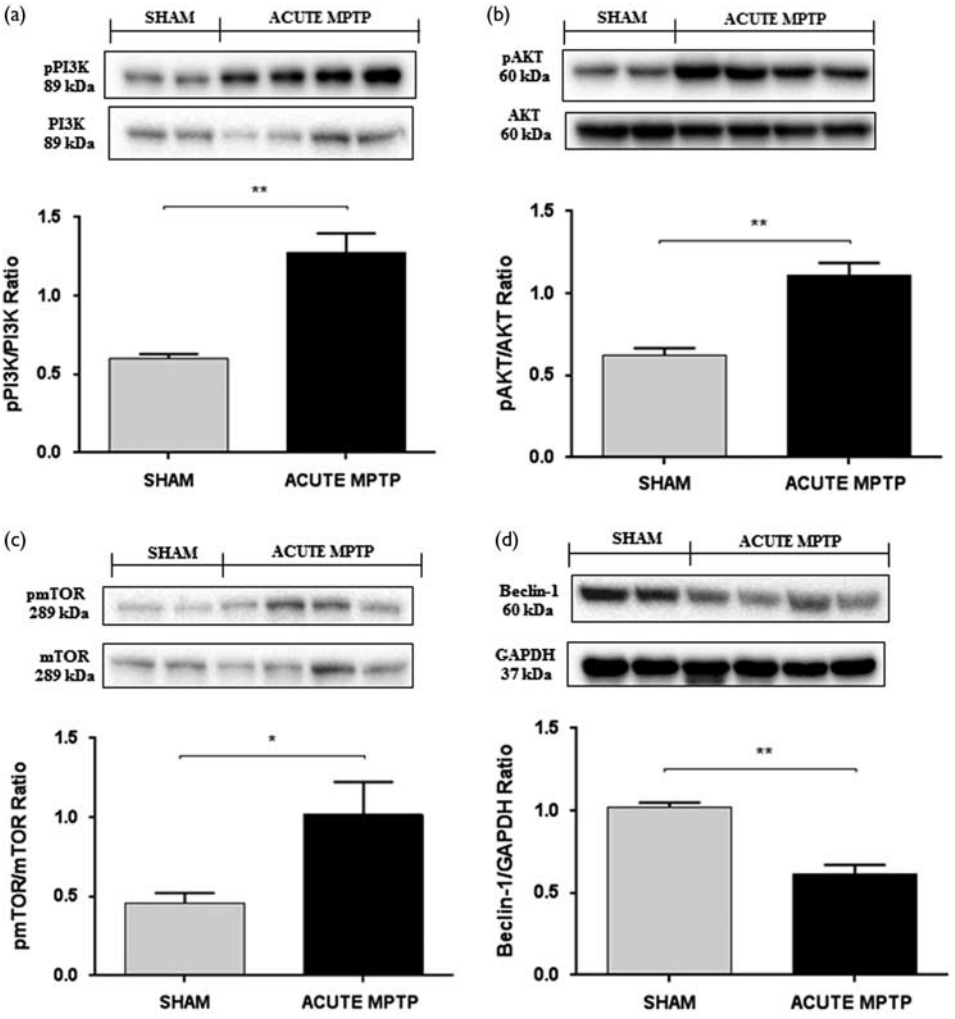
Pole test (a). Sham versus acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), $^{***}P = 0.0006$. Change in body weight in the sham and the acute MPTP group (b). Sham versus acute MPTP, $^{****}P < 0.0001$.

blocked by incubating for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Tissue sections were washed with PBS and incubated with the secondary antibody. Specific labeling was detected using a biotin-conjugated anti-rabbit IgG and avidin–biotin peroxidase complex (Vectastain ABC kit; Vector Laboratories, Burlingame, California, USA). Then, the tissue sections were marked with the DAB peroxidase substrate kit (Vector Laboratories, Burlingame, California, USA) and counterstained with nuclear fast red (Vector Laboratories) (pink background). Then, tissue sections were exposed to either primary or secondary antibody to evaluate antibody specificity. Under these conditions, no positive staining was detected in the tissue sections, indicating that the immunoreactions were positive in all the experiments conducted. Immunohistochemical pictures ($N = 5$ photos from each samples collected from all mice in each experimental group) were acquired using light microscopy; Leica Microsystems, Milan, Italy (LEICA DM 2000 combined with LEICA ICC50 HD camera) and assessed by densitometric analysis using Leica Application Suite V4.2.0 software (Leica Microsystems Srl, Milan, Italy). All negative controls were achieved by incubation with only the secondary antibody in the section of the midbrain, where it was staining positive.

Western blot analysis

All the extraction techniques were performed on ice using ice-cold reagents. Briefly, the tissues of midbrain were homogenized in lysis buffer containing 0.32 M sucrose, 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN_3, 10 mM 2-mercaptoethanol, 50 mM NaF, and protease inhibitor tablets (Roche Applied Science, Monza, Italy), and they were homogenized at the highest setting for 2 min. The homogenates were chilled on ice for 15 min and then centrifuged at 1000*g* for 10 min at 4°C, and the supernatant was collected to estimate the content of cytoplasmic proteins. The pellets were suspended in the supplied complete lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EGTA, and 1 mM EDTA protease inhibitors (Roche Applied Science, Monza, Italy), and were then centrifuged for 30 min at 15.000*g* at 4°C. Then, the supernatant containing the nuclear extract was collected to evaluate the content of nuclear proteins. Supernatants were stored at −80°C until use. The protein concentration in the homogenate was determined using the Bio-Rad Protein Assay (Bio-Rad, Segrate, Italy) using BSA as the standard and 30 μg of cytosol and nuclear extract from each sample was analyzed. Proteins were separated on sodium dodecyl sulfate-polyacrylamide minigels and transferred onto PVDF membranes (Immobilon-P Transfer membrane; Millipore), blocked with PBS containing 5% nonfat dried milk (PBS-milk) for 45 min at room temperature, and subsequently probed at 4°C overnight with specific antibodies, TH (1 : 500, catalog no. #AB152; Millipore); phospho-PI3Kinase (1 : 750, catalog no. #4228; Cell Signaling Technology); PI3Kinase (1 : 1000, catalog no. #4292; Cell Signaling Technology); phospho-Akt (1 : 750, catalog no. #9271; Cell Signaling Technology); Akt (1 : 1000, catalog no. #9272; Cell Signaling Technology); phospho-mTOR (1 : 750; Cell Signaling Technology), mTOR (1 : 1000, catalog no. #2972; Cell Signaling Technology): IkB-α (1 : 500, catalog no. #4814; Cell Signaling Technology); NF-kB (1 : 500, catalog no. #6956; Cell Signaling Technology); Beclin-1 (1 : 750, catalog no. #3738; Cell Signaling Technology); Bcl-2 (1 : 500; catalog no. #3498; Cell Signaling Technology); and IL-1β (1 : 500, catalog no. #12242; Cell Signaling Technology) in 1× PBS, 5% (w/v) nonfat dried milk, 0.1% Tween-20). Then, the membranes were incubated with HRP-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG, or HRP-conjugated anti-rat as the secondary antibody (1 : 2000; Santa Cruz Biotechnology Inc., Dallas, Texas, USA) for 1 h at room temperature. In addition, to ensure that blots were loaded with equal amounts of proteic lysates, they were also incubated with antibody for GAPDH HRP conjugated (1 : 1000, catalog no. #3683; Cell Signaling Technology) and Laminin B1 (1 : 1000, catalog no. #12586; Cell Signaling Technology). The relative expression of protein bands was visualized using an enhanced

Fig. 3



Western blot analysis for phosphoinositide-3 kinase (pPI3K) (a). Sham versus acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), $^{**}P = 0.0017$. Western blot for protein kinase-B (pAKT) (b). Sham versus acute MPTP, $^{**}P = 0.0012$. Western blot for mammalian target of rapamycin (pmTOR) (c). Sham versus acute MPTP, $^{*}P = 0.0236$. Western blot analysis for Beclin-1 (d). Sham versus acute MPTP, $^{**}P = 0.0028$.

chemiluminescence system (Luminata Western HRP Substrates; Millipore, Vimodrone, Milan, Italy) and protein bands were acquired and quantified using the ChemiDoc MP System (Bio-Rad, Segrate, Milan, Italy) and a computer program (ImageJ software, Bethesda, Maryland, USA), respectively. Blots are representative of three separate and reproducible experiments. In detail, a representative blot of samples obtained from two sham mice and four acute MPTP mice is shown and densitometry analysis of all animals is reported. The statistical analysis was carried out on three repeated blots obtained from separate experiments.

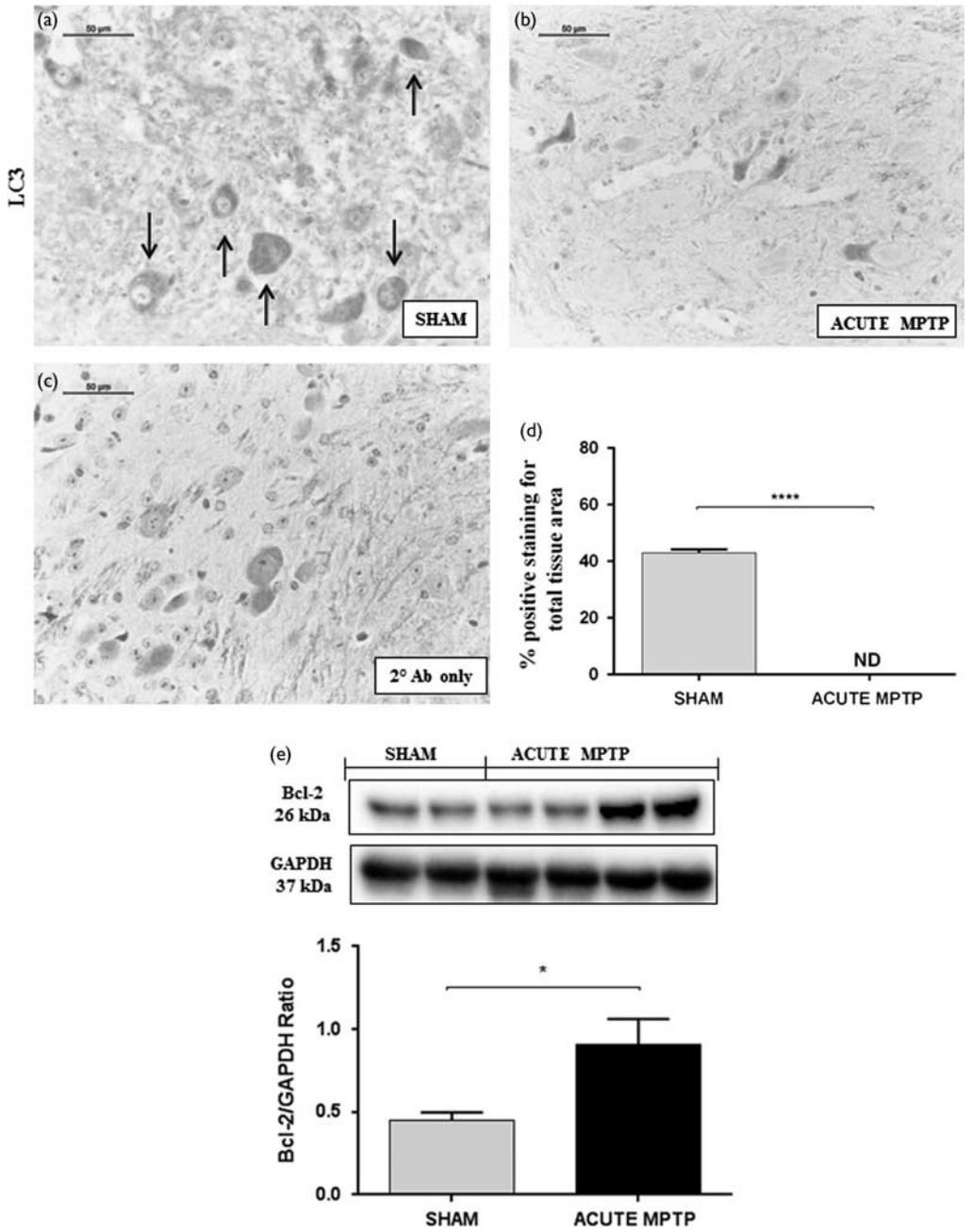
ELISA assay

The levels of IL-1β and IL-18 were measured in serum. Assays were performed using commercial ELISA kits (R&D Systems, Minneapolis, Minnesota, USA). The kits were used according to the manufacturer's protocol. Absorbency was determined using a microplate reader (Microplate Photometer iMARK; Bio-Rad, Segrate, Milan, Italy). The intra-assay coefficient of variations for both assays was less than 10%. The concentration of the cytokines in the tissue was reported as protein in pg/ml. All results were repeated independently at least three times; data are presented as mean ± SEM of three independent experiments.

Statistical evaluation

GraphPad Prism, version 6.0 program (GraphPad Software, La Jolla, California, USA) was used for statistical analysis of the data. The results were statistically analyzed using the Mann–Whitney test for multiple comparisons. A P value less than or equal to 0.05 was considered significant. All values are expressed as mean ± SEM of N experiments. In observations involving immunohistochemistry evaluation, the pictures shown are representative of at least three experiments

Fig. 4



Immunoistochemical analysis for LC-3. A positive staining for LC-3 was observed in sham mice (a, see arrows), whereas a negative tissue localization was found in acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice (b). Only secondary antibody (c). Densitometric analysis for LC-3 (d). Sham versus acute MPTP, ****$P < 0.0001$. Western blot analysis for Bcl-2 (d). Sham versus acute MPTP, *$P = 0.0314$. ND, no data.

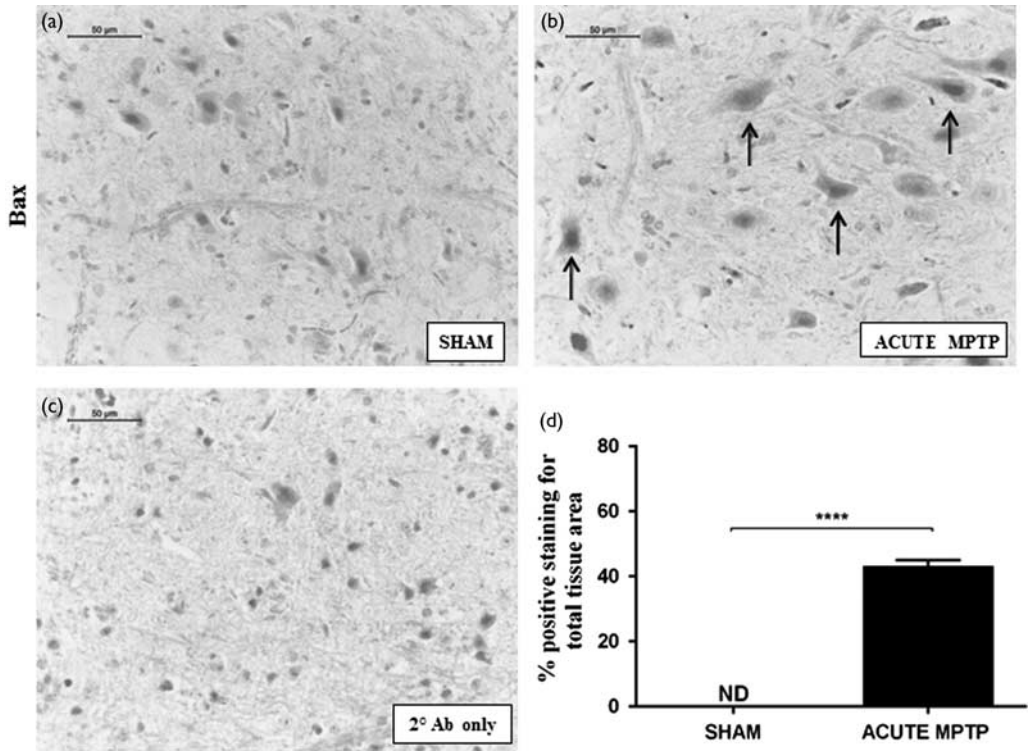
conducted on different days on the tissue sections collected from all the animals in each group.

Results

Evaluation of TH expression and some markers of pathology in acute MPTP mice

The MPTP animal model is a useful model for the study of neurodegeneration in PD as it reproduces clinical, biochemical, and neuropathological changes similar to those observed in human PD. One of the first signs of MPTP-induced neurotoxicity is the neuronal damage in the substantia nigra, which causes a decrease in tyrosine hydroxylase (TH). Immunohistochemical analysis carried out in midbrain sections showed a positive staining for TH in sham mice (Fig. 1a). Conversely, a negative staining for TH was observed in acute MPTP mice

Fig. 5



Immunoistochemical analysis for Bax. Sham mice showed a negative staining for Bax (a), whereas acute MPTP mice showed an enhanced Bax expression (b, see arrows). Only secondary antibody (c). Densitometric analysis for Bax (d); Sham versus acute MPTP, ****$P < 0.0001$. ND, no data.

(Fig. 1b, $P < 0.0001$, only the secondary antibody in Fig. 1c, densitometric analysis in Fig. 1d). The enhanced expression level of TH was further confirmed by western blot analysis ($P = 0.0002$; Fig. 1e). In parallel, the deterioration in TH levels coincides with motor deficit and bradykinesia, assessed as a parameter of disease. Indeed, acute MPTP mice subjected to a pole test showed reluctance and disorientation when placed head-up on top of the pole, also showing motor incoordination and bradykinesia (drop time: mean 8.96 ± 0.114) compared with sham mice (drop time: mean 4.78 ± 0.321) ($P = 0.0006$; Fig. 2a). Animals were also evaluated every day for any weight variations. As expected, after acute MPTP induction, significant body weight loss was observed in the acute MPTP group ($\Delta = -2.2 \pm 1.025$), whereas a normal increase in body weight was detected in sham group ($\Delta = +2.5 \pm 0.987$) ($P < 0.0001$; Fig. 2b).

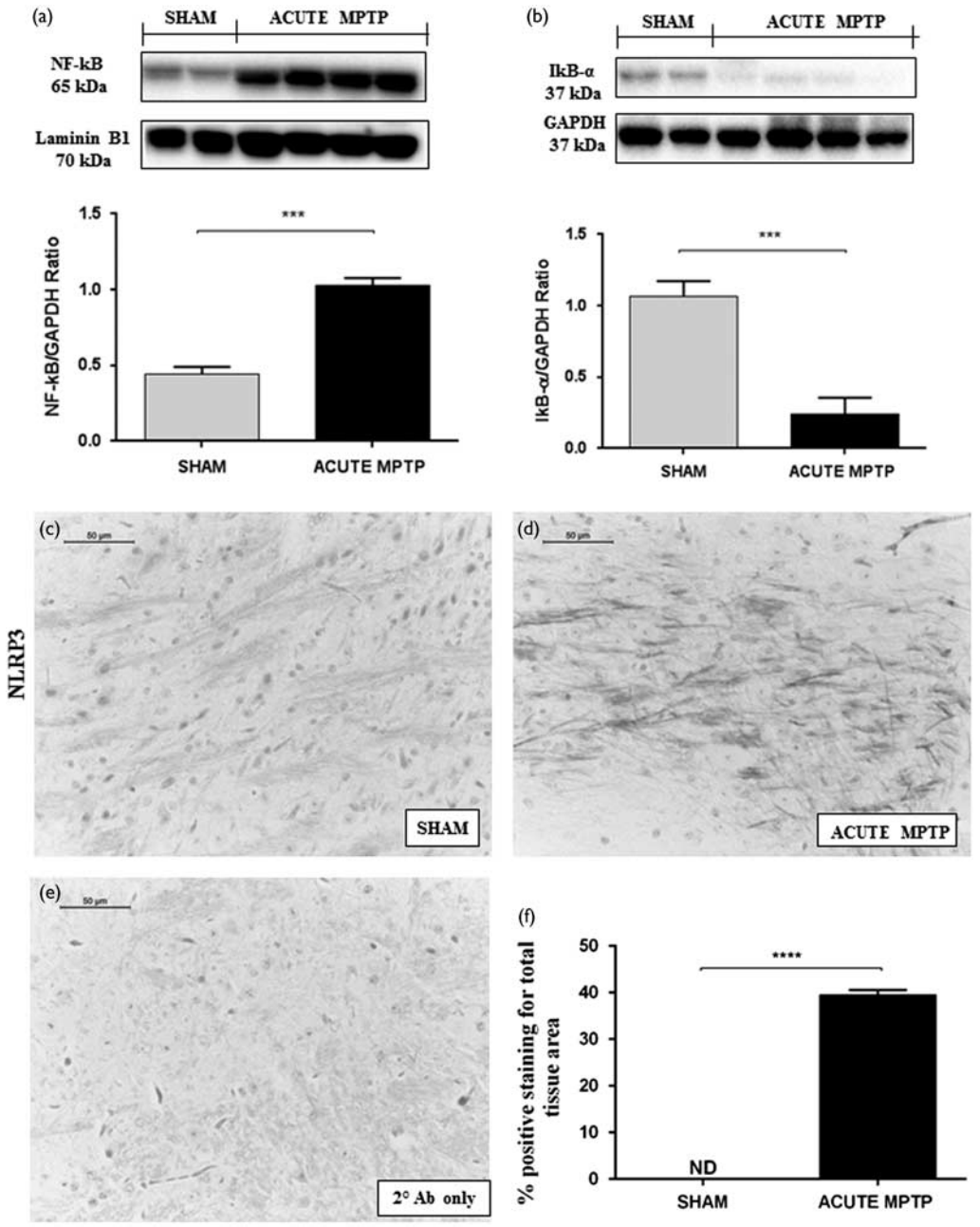
Involvement of the PI3K/Akt/mTOR pathway in acute MPTP mice

Western blot analysis was performed in order to investigate the modulation of the PI3K/Akt/mTOR signaling pathway at 7 days after acute MPTP induction in mice midbrains. Specifically, we focused on the phosphorylation status of PI3K/Akt/mTOR as its activation is mediated by phosphorylation of the proteins involved. Our results showed an upregulation of the PI3K/Akt/mTOR pathway in acute MPTP mice. Specifically, an enhanced expression of pPI3K ($P = 0.0017$; Fig. 3a), pAKT ($P = 0.0012$; Fig. 3b), and pmTOR ($P = 0.0236$; Fig. 3c) was observed in midbrain tissues taken from acute MPTP mice compared with sham ones. No differences were found between sham and acute MPTP groups for non-phosphorylated PI3K, Akt, and mTOR proteins.

Dysregulation of autophagy in acute MPTP mice

The autophagy is a catabolic process for the autophagosomic-lysosomal degradation of proteins activated mainly in response to nutrient deprivation, but it has also been associated with neurodegenerative diseases. To investigate the involvement of autophagy in a PD model, we evaluated the expression of Beclin-1 and LC-3. Particularly, by western blot analysis, we found a decreased expression of Beclin-1 in acute MPTP mice compared with sham animals ($P = 0.0028$; Fig. 3d). In addition, immunohistochemical evaluation showed a positive staining for LC-3 in sham mice (Fig. 4a) and, in contrast, a negative staining in acute MPTP mice (Fig. 4b, $P < 0.0001$, only the secondary antibody in Fig. 4c; see densitometric analysis Fig. 4d). We also evaluated the expression of Bcl-2 in its capacity to act as an inhibitor of autophagy by binding Beclin-1. Sham mice showed a basal expression of Bcl-2, appreciably

Fig. 6



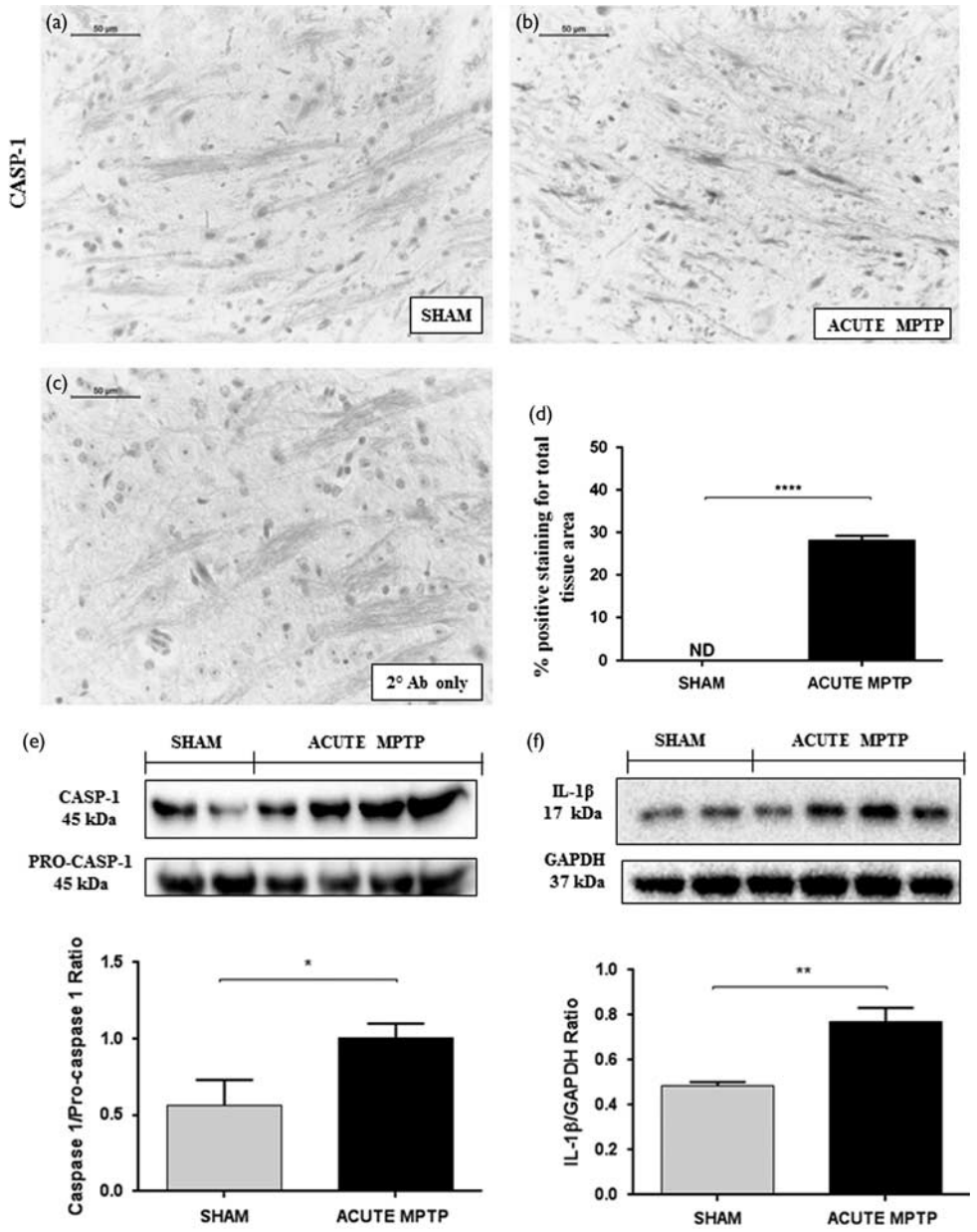
Western blot for NF-κB (a). Sham versus acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), ***$P = 0.0001$. Western blot for IκB-α (b). Sham versus acute MPTP, ***$P = 0.0010$. Immunohistochemical analysis for NLRP3. Sham mice showed a negative staining for NLRP3 (c). In contrast, immunopositivity for NLRP3 was observed in acute MPTP mice (d). Only secondary antibody (e). Densitometric analysis for NLRP3 (f); Sham versus acute MPTP, ****$P < 0.0001$. ND, no data.

increased in the samples from acute MPTP mice ($P = 0.0314$; Fig. 4e).

Crosstalk between autophagy and apoptosis in acute MPTP mice

As is known, deregulation of autophagy leads eventually to cell death of the midbrain dopaminergic neurons. Immunohistochemical evaluation showed the appearance of protein effectors of mitochondrial apoptosis, such as proapoptotic Bax protein. As expected, we found a completely negative staining for Bax in sham animals (Fig. 5a). In contrast, acute MPTP mice showed Bax upregulation (Fig. 5b, $P < 0.0001$, only the secondary antibody in Fig. 5c, densitometric analysis in Fig. 5d).

Fig. 7



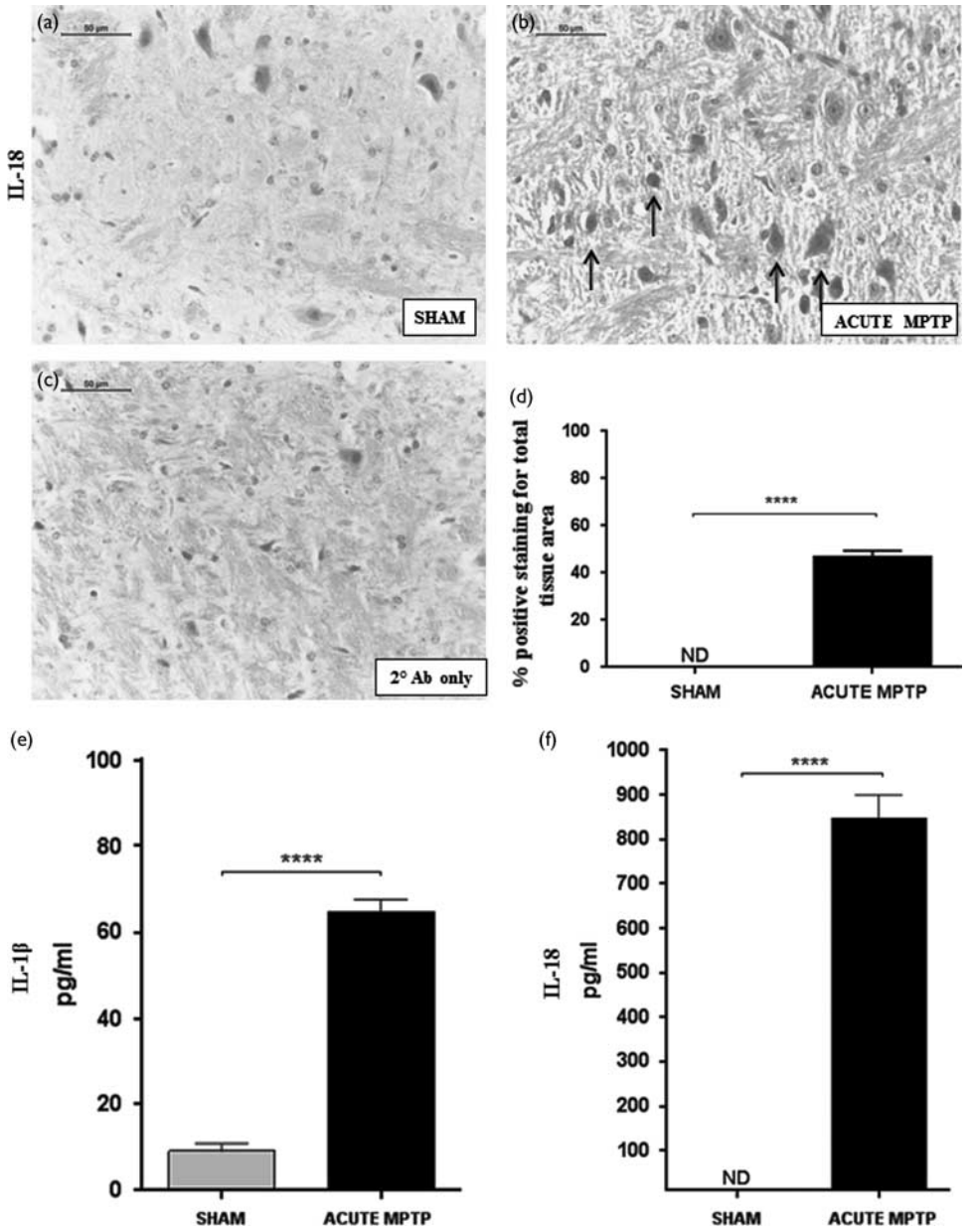
Immunohistochemical analysis for caspase-1. Sham mice showed a negative staining for caspase-1 (a). In contrast, immunopositivity for caspase-1 was observed in acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice (b). Only secondary antibody (c). Densitometric analysis for caspase-1 (d); Sham versus acute MPTP, $^{****}P < 0.0001$. Western blot for caspase-1 (e). Sham versus acute MPTP, $^{*}P = 0.0117$. Western blot for IL-1β (f). Sham versus acute MPTP, $^{**}P = 0.0083$. ND, no data.

Inflammasome activation in acute MPTP mice

The neuronal activation of the NLRP3 inflammasome was investigated in acute experimental PD. Thus, we evaluated the expression of NF-κB, recognized as the first signal in inflammasome activation. NF-κB is a dimeric transcription factor present in the cytoplasm of cells in an inactive form because of its association with a class of inhibitory proteins called IκBs. Following different stimulus effects, IκB undergoes phosphorylation and is subsequently degraded, allowing NF-κB to translocate into the nucleus and induce gene expression. By western blot analysis, we found an increase in nuclear NF-kB after acute MPTP induction ($P = 0.0001$; Fig. 6a) and at the same time a decrease in cytoplasmic IκB-α expression ($P = 0.0010$; Fig. 6b).

In addition, we investigated NLRP3 expression. A negative staining for NLRP3 was observed in sham

Fig. 8



Immunohistochemical analysis for interleukin-18 (IL-18). Sham mice showed a negative staining for IL-18 (a). In contrast, immunopositivity for IL-18 was observed in acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mice (b, see arrows). Only secondary antibody (c). Densitometric analysis for IL-18 (d). Sham versus acute MPTP, $^{****}P < 0.0001$. ELISA assay for IL-1β (e); Sham versus acute MPTP, $^{****}P < 0.0001$. ELISA assay for IL-18 (f); Sham versus acute MPTP, $^{****}P < 0.0001$. ND, no data.

animals (Fig. 6c). In contrast, we found a positive staining in acute MPTP mice (Fig. 6d, $P < 0.0001$, only the secondary antibody in Fig. 6e, densitometric analysis in Fig. 6f). NLRP3, in turn, binds its adaptor protein PYCARD to assembly the inflammasome complex, which recruits caspase-1. Thus, we evaluated the caspase-1 expression at 7 days from acute MPTP induction. By immunohistochemical analysis, no positive staining for caspase-1 was observed in sham mice (Fig. 7a), whereas an intense positive staining in acute MPTP mice (Fig. 7b, $P < 0.0001$, only secondary antibody in Fig. 7c, densitometric analysis in Fig. 7d) was found. The increase in caspase-1 expression after MPTP induction was also confirmed by western blot analysis ($P = 0.0117$; Fig. 7e). Moreover, caspase-1 promotes the cleavage of the proinflammatory cytokines such as IL-1β and IL-18. Western blot analysis showed a level basal of IL-1β expression in sham animals. In contrast, increased

expression of IL-1β was observed in samples collected from acute MPTP mice ($P=0.0083$; Fig. 7f). In addition, a negative staining for IL-18 was also found in sham mice (Fig. 8a), whereas acute MPTP mice showed a marked positive staining for IL-18 (Fig. 8b, $P< 0.0001$, only the secondary antibody in Fig. 8c, densitometric analysis in Fig. 8d). Finally, the increased levels of these proinflammatory cytokines were also confirmed in the serum of acute MPTP mice by the ELISA assay ($P< 0.0001$; Fig. 8e and f).

Discussion

Neurodegenerative diseases such as Alzheimer's disease and PD share a common cellular and molecular pathogenetic mechanism involving aberrant misfolded protein and aggregation [12]. In this context, autophagy, a lysosomal degradation pathway, represents a major method for removal of aggregated cellular proteins and dysfunctional organelles [12]. However, autophagy declines with age, leading to deficient elimination of abnormal protein aggregated and thus stimulating cellular stress, failure, and cell death [13]. Although recent studies suggest a link between autophagy and the inflammasome activation [4,14,15], little is known about the exact role of the inflammasome in PD pathogenesis. Previous studies have shown that several cellular signaling pathways, including the PI3K/AKT/mTOR pathway, play a crucial role in the process of autophagy and consequently in the clearance of protein aggregates in neurodegeneration [16]. The dysregulation of PI3K/Akt/mTOR also seems to be involved in the loss of dopaminergic neurons in PD [9]. Data from microarray-based gene expression profiling on human dopaminergic neurons showed both upregulation and downregulation of genes of the PI3K/Akt/mTOR signaling pathway in PD patients [17]. At the same time, in experimental models of PD, it was established that dysregulation of the PI3K/Akt/mTOR is linked to autophagy disruption, but the results remain conflicting [8,11]. Indeed, it was proven that autophagy may or not be correlated to the PI3K/Akt/mTOR pathway alteration depending on the disease and the duration of the disorder [18]. Here, using an acute model of PD, we found a clear upregulation of the PI3K/Akt/mTOR pathway in midbrain tissues following MPTP induction as proven by enhanced phosphorylation of PI3K, Akt, and mTOR in acute MPTP mice compared with the sham group. mTOR regulates the translational rate and the initiation step in several cellular processes during protein synthesis and, therefore, and it is considered the critical downstream target of the PI3K/Akt pathway. In addition, mTOR was recognized to act as a negative regulator of autophagy, by inhibiting the autophagy-related proteins, required for the autophagosome complex formation [19]. To analyze the involvement of autophagy, we chose to examine the expression of Beclin-1 and LC-3, two key proteins involved in autophagy, that have detected previously in dopaminergic neurons of the substantia nigra in a mouse model of PD [20]. Here, we have found that MPTP induction reduces the expression of Beclin-1 as well as of LC-3-II in acute MPTP mice compared with sham ones. In parallel, we evaluated the expression of Bcl-2, which not only functions as an antiapoptotic protein but also as an antiautophagy protein through its inhibitory interaction with Beclin-1 [21]. Our data showed an increased expression of Bcl-2 in acute MPTP mice (Fig. 2d) that could explain the inhibition of the autophagic system as a consequence of the Beclin-1/Bcl-2 complex formation. In addition, as reported by Zhang *et al.* [22], there is a correlation between Beclin-1, whose overexpression can reduce the abnormal accumulation of α-Syn, and the expression and TH, the main regulator and rate-limiting enzyme of dopamine synthesis, whose decrease is believed to trigger PD. Specifically, the activation of Beclin-1-dependent autophagy may prevent the loss of TH-positive neurons in PD [22]. Immunohistochemistry and western blotting were used to detect alterations in the expression of TH, showing a decrease in TH expression in general dissection of midbrain from acute MPTP mice compared with sham ones, probably because of dysregulation of autophagic process. In parallel, the deterioration in TH levels coincides with motor deficit and bradykinesia, assessed as parameters of disease. Indeed, acute MPTP mice subjected to the pole test showed reluctance and disorientation when placed head-up on top of the pole, also showing motor incoordination and bradykinesia compared with sham mice.

Therefore, in light of the finding of recent researches, as well as our results, we suggest autophagy as a defensive and protective mechanism of cells not only in counteracting protein aggregates formation but also in protecting neurons from apoptosis. Our results indeed showed that after MPTP administration, the autophagy deregulation leads to an increased expression of protein effectors of mitochondrial apoptosis, such as proapoptotic Bax protein.

In the last decades, the role of autophagy in the regulation of inflammatory response was reported [4]. Particularly, an aberrant accumulation of misfolded proteins leads to inflammasome activation [23]. A recent study of 2016 showed that α-Syn activates NLRP3 inflammasome, leading to microglia-mediated neuroinflammation and dopaminergic neuronal degeneration [24]. Accordingly, our results confirmed that neuronal activation of NLRP3 inflammasome is involved in PD progression.

To verify the NLRP3 inflammasome trigger, we examined NF-κB signaling, considered the first signal that induces the transcription of inflammasome components [25]. This is a necessary step as NLRP3 levels in resting cells are not enough for its activation [25]. NF-κB is generally inactive in the cytoplasm because of the inhibition of IκB-α. In response to a wide range of stimuli,

IκB-α is phosphorylated by the enzyme IκB-α kinase so that NF-κB is free to translocate into the nucleus and to promote the expression of inflammatory mediators. Our results showed that MPTP exposure led to upregulation of NF-κB and consequently to downregulation of IκB-α. As a consequence, we observed NLRP3 inflammasome activation in the midbrain sections of acute MPTP mice, proven by increased levels of NLRP3, which is considered the factor limiting the inflammasome assembly [25]. After assembly, NLRP3 is bound to apoptosis associated speck-like protein, an adaptor protein containing a caspase recruitment domain, and they induce caspase-1 activation [26]. Accordingly, our evaluations showed an increased expression of caspase-1 in acute MPTP mice compared with sham ones. As a consequence, caspase-1 promotes the secretion of proinflammatory cytokines including IL-1β and IL-18.

Our results confirmed an increase in IL-1β expression as well as of IL-18 in the midbrain sections of acute MPTP mice compared with sham ones that were found to exacerbate inflammation in PD [27]. The enhanced levels of IL-1β and IL-18 were further confirmed in the serum of acute MPTP mice with ELISA assays. Therefore, it is presumable that the increased level of proinflammatory cytokines could aggravate inflammatory response and accelerate the aging process, leading eventually to neuronal cell death in PD.

Conclusion

Our results suggest that in an experimental acute model of PD, the dysregulation of autophagy system exposes cells to NLRP3 inflammasome activation may involve the PI3K/Akt/mTOR pathway. Moreover, the activation of inflammasome through secretion of proinflammatory mediators is believed to amplify neuroinflammation, which plays a key role in PD. The importance of our results could be correlated with the discovery of new pharmacological strategies targeting on molecular mechanism underlying the link between autophagy and the inflammasome that could be useful for the treatment of PD.

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Conflicts of interest

There are no conflicts of interest.

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