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A Cell Protective Mechanism in a Murine Model of Parkinson's Disease

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Abstract: Parkinson's disease is characterized by progressive degeneration of the dopaminergic (DA) neurons in the substantia nigra (SN). However, the mechanism underlying DA cell death remains unclear. While apoptotic cell death has been implicated in DA cell death in Parkinson's disease, autophagy - a regulated cellular process for degrading intracellular proteins and organelles under stress - in Parkinson's disease is not known. Here, we report evidence of autophagy in DA neurons of the SN in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease. Mice were treated with saline or MPTP (40 mg/kg) and sacrificed after 7 days, and brain tissue samples were processed for immunohistochemistry using a tyrosine hydroxylase (TH) antibody to reveal the SN area. TH-positive tissues were then processed for transmission electron microscopy and examined for ultrastructural evidence of autophagy. MPTP treatment induced significant morphological changes that closely resembled the autophagic process. This autophagic degeneration was observed in ~35% of DA neurons in MPTP mice. We conclude that at the ultrastructural level MPTP treatment produced a prominent autophagic process in DA neurons. The identification of autophagy in the MPTP model of Parkinson's disease may provide an insight into the mechanism of cell protection and may lead to a novel therapeutic strategy in Parkinson's disease.

Key Words: Ramadan, fasting, diabetes, hypertension, stroke

Introduction

Parkinson's disease is a neurodegenerative disease characterized by a progressive loss of the dopaminergic neurons (DA) in the substantia nigra pars compacta. The interest in studying cell loss and the mechanism of cell death is evident from the enormous number of papers about Parkinson's disease in the literature. For the most part, cell death in Parkinson's disease is defined in terms of apoptosis. Apoptosis is defined as an active, physiological cascade of events, characterized by cell shrinkage due to dehydration, nuclear chromatin condensation and fragmentation, and membrane blebbing. Necrosis as the other type of cell death in Parkinson's disease is defined as a passive, degenerative pathway characterized by cell and mitochondrial swelling, metabolic collapse, flocculation of chromatin, rupture of the nuclear and cytoplasmic membrane and dispersal of cell contents (1). The other cell death pathway (2), autophagy, is not very clear in neurodegenerative disease.

At present, the most effective experimental model of Parkinson's disease is the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment of animals. In 1982, a number of young Californian drug-addicts developed a severe Parkinson's-like syndrome after injecting a potent pethidine derivative contaminated with MPTP (3,4). Exposure to MPTP produced symptoms of bradykinesia and rigidity almost identical to those of idiopathic Parkinson's disease, and in one patient these were shown to be related to a severe loss of DA neurons of the substantia nigra (SN) (3).

Morphological assessment of different modes of cell death, apoptosis, necrosis or autophagic degeneration, can contribute significantly to understanding this neuronal loss. Electron microscopy should be considered the gold standard for cell death observation (5).

Autophagy is responsible for the degradation of normal proteins involved in cellular remodeling found during metamorphosis, aging and differentiation, as well as for the digestion and removal of abnormal proteins that would otherwise accumulate following toxin exposure, cancer or degenerative disease (6,7). Autophagy is correlated with the formation of autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles (with double membranes), membranous whorls and multivesicular bodies, as well as the engulfment of entire organelles (i.e. mitochondria), which can be seen at the ultrastructural level (8). A number of studies have demonstrated the presence of autophagy in the brain in Huntington's disease (9). Similarly, autophagic degeneration was observed in Alzheimer's disease (10). In 1997, in Parkinson's disease patients, an ultrastructural examination revealed characteristics of apoptosis and autophagic degeneration in melanized neurons of the SN (11). The results suggest that even at the final stage of the disease, the dopaminergic neurons are undergoing an active process of cell death.

Thus, we designed the following study to test the evidence for autophagy in DA neurons of SN in the MPTP model of Parkinson's disease.

Materials and Methods

Drug treatments: Twelve male mice weighing 20-24 g were divided into 2 groups and treated with a single injection of saline or MPTP (40 mg/kg) i.p.

Tyrosine hydroxylase (TH) immunostaining: One week after the treatment, the mice were transcardially perfused with phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde. Brains were serially sectioned from roughly the beginning to the end of the SN area, with the help of a mouse brain atlas, on a cryomicrotome at 40 µm. Free floating sections were incubated overnight at 4 °C with a monoclonal anti-TH antibody (1:800) (Sigma) to reveal the SN cells, and developed with an avidin-biotin-peroxidase method.

Ultrastructural Analysis: For electron microscopy, after TH staining and identifying the SN area, free floating sections from the MPTP or saline treated group were used. Tissue pieces 40 µm thick were first immersed in 2.5% glutaraldehyde in 0.1 phosphate buffer (pH 7.2) postfixed in 1% osmium tetroxide in 0.1 phosphate buffer (pH 7.4) and then dehydrated in graded ethanol series and flat embedded in Araldite. On the Araldite block surface, only the TH immunostained area (SN) was cut, and the other area (unstained) was left out by trimming. Ultrathin sections (40-60 nm thick) taken at the level of the brown (immunostained) SN area were placed on grids (200 mesh) and double stained with uranyl acetate and lead citrate. The grids containing the sections were observed under a Carl Zeiss EM-900 electron microscope, and photomicrographs were taken.

Semiquantitative morphological analysis of autophagic structure: Whole sections on grids from each block of brain were examined with an electron microscope, and the total number of cells and the number of cells with an autophagic structure were analyzed. Autophagic structure was defined with a double membrane as described before (8). The percentages of cells with an autophagic structure were calculated in the total number of neurons from the SN area. The difference between the groups was examined by Student's t test, and P values less than 0.05 were considered significant.

Results

MPTP induced a loss of DA in the SN (Figure 1). At the ultrastructural level, MPTP treatment induced significant morphological changes, which closely resembled the autophagic process, and C-shaped multivesicular bodies (Figure 2). The percentage of cells with an autophagic structure was ~35% of the total number of neurons from the SN area. In autophagic degenerating neuron cytoplasm, a double membrane structure with lysosome–like vacuoles was observed. Numerous vacuoles containing cytoplasmic fragments were present in the cytoplasm. The number of vacuoles containing materials was very high in the DA neurons of MPTP-treated mice. Features of autophagy were occasionally observed in < 3% of DA neurons of saline-treated mice. The difference between the groups was significant ($p < 0.05$).

Discussion

In the present study, we observed autophagic vacuoles in the SN neurons of MPTP-treated mice. Autophagy may utilize some common regulatory mechanism in these cells. Furthermore, the onset of autophagy can precede that of

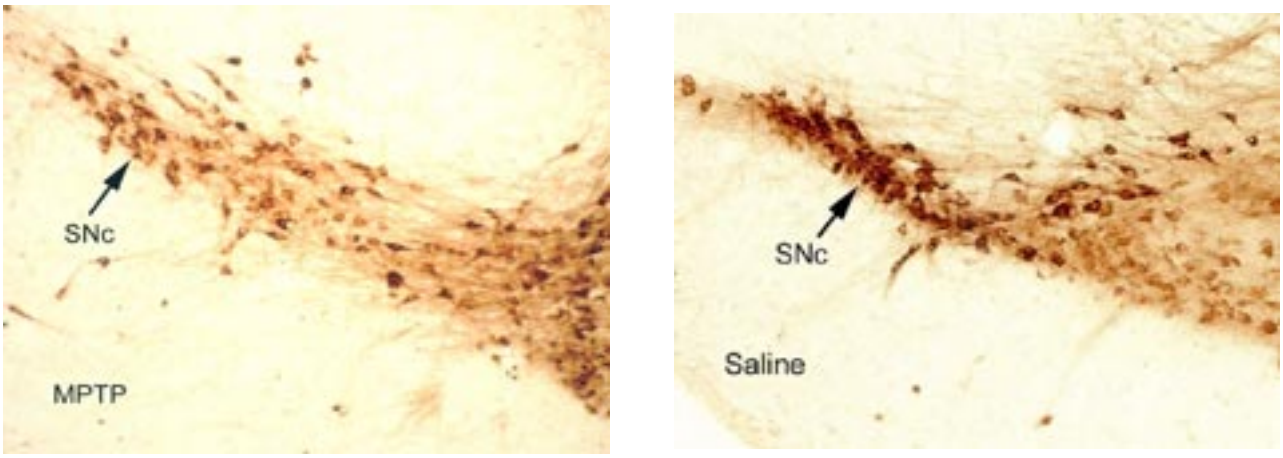

Figure 1. MPTP-induced loss of dopaminergic neurons in substantia nigra. Dopaminergic cells stained with antibodies for tyrosine hydroxylase in the substantia nigra of MPTP and saline treated mice 7 days after treatment. The number of neurons is less in the MPTP treated group than in control. SNc: Substantia Nigra compacta (x50).

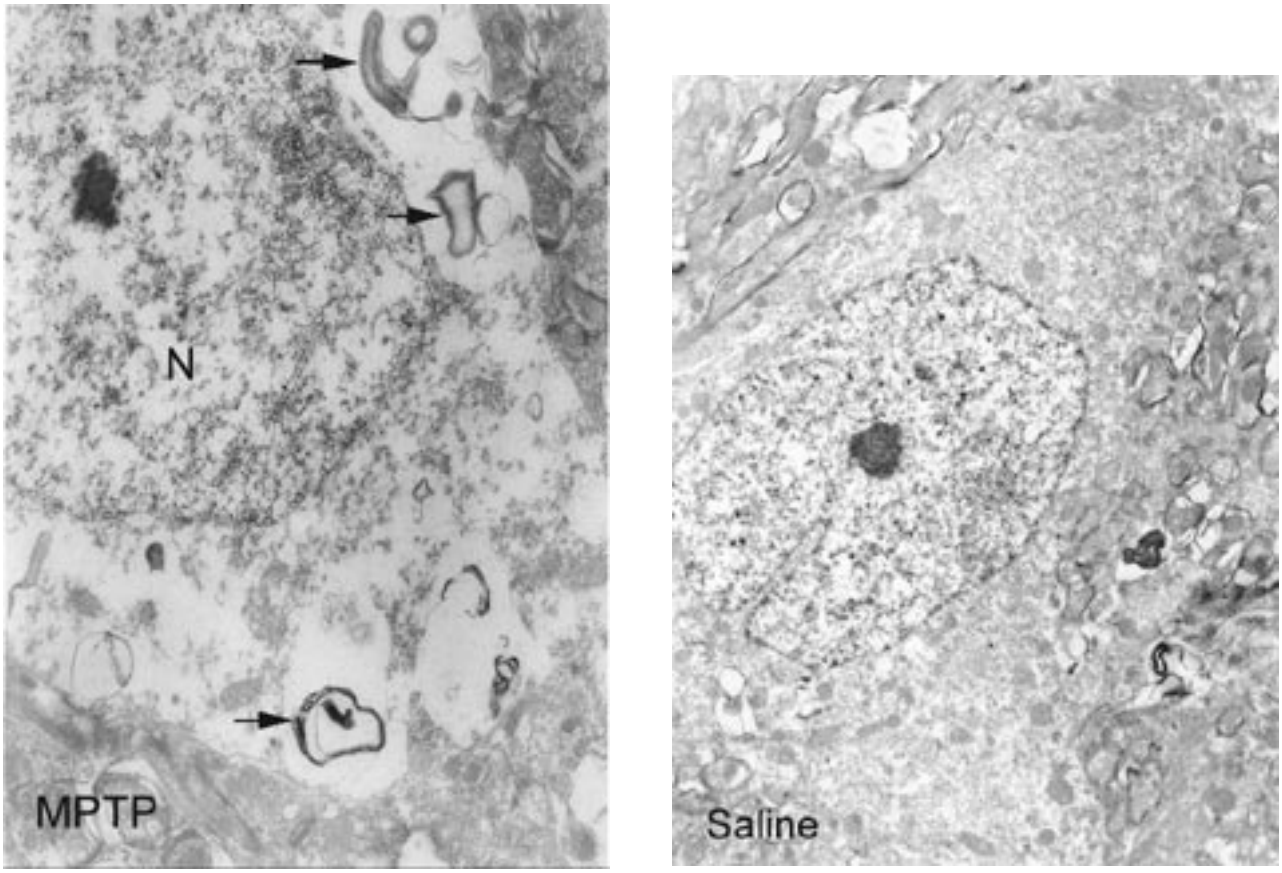

Figure 2. Electron microscopic photomicrographs showing autophagic structures and C-shaped multivesicular bodies (arrows) in MPTP treated dopaminergic neurons. N: nucleus. Tissue samples were taken from the MPTP and saline treated groups (x5000).

apoptosis and, according to at least one report, delays apoptosis (12,13). In newborn rat hepatocytes, the inhibition of cyclic AMP produces glycogen autophagy (14). It has been strongly suggested that MPTP administration produces a cyclic AMP decrease in SN cells (15). Under nutrient-deficient conditions, the yeast *S. cerevisiae* sequesters its own cytoplasmic components into vacuoles in the form of "autophagic bodies", and spherical structures completely enclosed in a double membrane were found near the vacuoles of protease-deficient mutant cells when the cells were shifted to nutrient-starvation media (5). In addition, the induction of increased intracellular levels of lysosomal proteinases and their mRNA by treatment with a combination of hormones showed C-shaped multivesicular bodies and the formation of autophagic vacuoles (16).

Autophagy may be a degrading process for intracellular proteins via ubiquitin-mediated pathways (17). It can be speculated that Lewy bodies in neurodegenerative diseases may be produced by the autophagic process (18). Nowadays, intracellular protein accumulations in neurodegenerative diseases are becoming very important (19-21). The relation between intracellular protein accumulations and the autophagic process needs to be studied further.

A time course study of DA neuronal death caused by MPTP showed that the active phase of degeneration began at 12 h postinjection and continued for up to 4 days. During this period, there was a greater decrease in TH-defined neurons than in Nissl-stained neurons, suggesting that MPTP can cause a loss in TH without necessarily destroying the neuron. Cell death occurs in 2-4 days after MPTP treatment, and, at the seventh day postinjection, TH immunostaining can be used to accurately determine the number of living neurons in the SN (22). Based on this report, we suggest that a defense or protective mechanism can be seen after the injection of MPTP, because the cells observed on the seventh day were alive with an autophagic structure.

Our data showed that autophagy was stimulated by MPTP treatment. Autophagy may reduce the toxic effect of MPTP on neuronal cells in SN. The blockade of TH+ cell death may be related to the blockade of the cell death pathway by producing autophagic granules. The mechanisms of autophagy in the cell death pathway are unclear. If there is any relation between the blockade of cell death and autophagy, we may speculate that autophagic structures play a role in blocking the cell death pathway. If so, it can be also speculated that defects in autophagy have serious consequences, and that these can be linked to neurodegenerative disease. In addition, it may be of some value in the treatment of Parkinson's disease or in reducing the risk of neurodegeneration.

These results raise the possibility that the existing autophagy in neurons in the MPTP model of Parkinson's disease may provide an important clue as to the neurodegenerative mechanism. The possibility of producing an autophagic structure may protect the cells, and defects or insufficiencies in this process may lead the cells to die in neurodegenerative diseases.

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